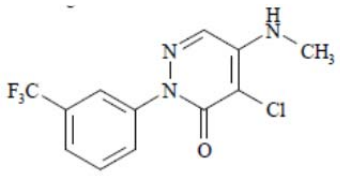
Ruben Koch
SWS 6905 Project
December 1, 2011

INFLUENCE OF EXPOSURE DURATION AND RECOVERY ON THE TOXICITY OF NORFLURAZON TO *L. MINOR*

Introduction

Norflurazon is a pre-emergence herbicide used to control annual, broadleaf, and grass weeds registered since 1974. This herbicide must be incorporated into the soil either through rainfall or irrigation. It is used to control crabgrass spp., barnyardgrass, foxtail spp., and spikerush, as well as many broadleaf weeds including prickly sida, purslane, Russian thistle, and shepherd's purse. The herbicide is used on many different crops (e.g. citrus and corn) in addition to right-of-ways (United States Environmental Protection Agency Washington). Furthermore, norflurazon may be adsorbed by soil colloids, which lowers its mobility in organic clay soils (Essington 2004). Norflurazon readily leaches in soils with low organic content and has much potential to contaminate surface water through runoff (Wilson et al 2006).

Norflurazon (4-chloro-5-methylamino-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H-pyridazionone) is classified in the



fluorinated pyridazinone chemical family. It blocks carotenoid biosynthesis by blocking the enzyme phytoene desaturace (Vencill 2002). Carotenoids serve as photoprotective agents that prevent damage to the organism due to absorption of excess energy by chlorophyll molecules (Vencill 2002). If the transfer of energy does not occur, it can react with molecular oxygen forming singlet oxygen which is extremely reactive. Consequently, singlet oxygen further reacts with and destroys

Ruben Koch
SWS 6905 Project
December 1, 2011

carotenoids and membrane lipids (Taiz et al 2002). The carotenoids provide protection from these deleterious reactions by quenching, the rapid state of dissipation of potential energy stored in chlorophyll caused by photochemistry.

Duckweed (*Lemma minor* L) is a single leaf common aquatic macrophyte that proliferates in fresh waters from tropical to temperate zones (7 to 30 C°) and a pH ranging from 4.5 to 8.5 (Mohlenbrock 2011). Duckweed reproduces rapidly either sexually or asexually doubling every 16 to 48 hours depending on environmental conditions (Wang 1989). Duckweed is also a source of food, shelter, and shade for fish and invertebrates. Duckweed, as well as other aquatic plants, can be a nontarget plant unintentionally affected by norflurazon. *Lemna* species are often used as a surrogate for other aquatic macrophytes in environmental risk assessment due to its relative sensitivity, ease of culturing, and small lab footprint.

Numerous studies have addressed issues like phytodegradation of norflurazon (Massad et al 2004), runoff losses of norflurazon (Southwick et al 1993), reduction of norflurazon leaching in a sandy soil (Chandran et al 1999), toxicity of norflurazon to the aquatic macrophyte *Vallisneria Americana* (Wilson 2007), and adsorption and deactivation of norflurazon by activated charcoal (Lamoreaux et al 1989). No data was available to characterize the toxicity of norflurazon to *Lemna minor* and the potential recovery once norflurazon exposure ceased. This study evaluated the toxicity of norflurazon to *Lemna minor*. It also characterized the recovery of effected *Lemna minor* populations once norflurazon was removed.

Ruben Koch
SWS 6905 Project
December 1, 2011

Material and Methods

Toxicity characterization

A range-finding study was first conducted to determine the effective concentration range for norflurazon toxicity. This range-finding study evaluated five different concentrations of norflurazon and one control (0, 10, 50, 100, 500, and 1000 ug/L). Results from this study were used to define the concentration range in subsequent studies.

Lemna minor L. plants were grown in covered petri-dishes containing 150 mL of 10% Hoagland's solution (Hothem et al., 2003) made using reconstituted moderately hard water (MHW). All plants materials and assays were grown under sterile conditions at the Indian River Research and Education Center (UF/IFAS IRREC, Fort Pierce). The commercial formulation of norflurazon, Solicam® DF, was used for all assays. This formulation contains 78.6% norflurazon and 21.4% inert ingredients.

Exposure solution preparation

Ten milligrams of Solicam were dissolved in 1 liter of MHW to prepare the stock solution, described in (Table 1). Five replicates of *L. minor* were exposed to the following concentration of norflurazon: 0, 10, 25, 50, 100, 250, and 500u g/L for a period of 10 days. Each autoclaved petri-dish (Pyrex 90x50 N. 3140) received 100 mL of treatment solution

Table .1 Stock solution dosing concentrations for norflurazon and media condition data

Temp. C°	pH	EC
25	7.5	60.5

Treatment N10 (ppb)	Treatment N10 (mg/L)	Stock Solution MHW ml	Dosing of Solicam ml
0	0	100	0
10	0.01	99.8728	0.1272
25	0.025	99.6819	0.3181
50	0.050	99.3639	0.6361
100	0.1	98.7277	1.2723
250	0.25	96.8193	3.1807
500	0.5	93.6386	6.3614

Ruben Koch
SWS 6905 Project
December 1, 2011

(Table 1). Nine to 12 fronds of *Lemna minor L*. were added into each dish. All vessels were marked accordingly and transferred to the tissue culture room and laid out in a rectangular, completely randomized pattern under cool white, fluorescent grow-lights (F40 T12/cm plus, four 40 watt bulbs) set 24 cm above the bottom surface. All racks were lined with aluminum foil for maximum light reflectance (Fig. 1). Light intensity ranged from 90 to 125 (µmols) and for the photoperiod was 16 h light: 8 h dark. Each dish was covered with an additional petri-dish and sealed with Paraffin tape to prevent environmental contamination during the trial. Concentrations of norflurazon were confirmed by extracting two separate 500 mL aliquots of stock solution for each treatment concentration described in (Table 1). Norflurazon concentrations were confirmed by analysis using a Varian CP-3800 gas chromatograph equipped with dual thermionic selective detectors. Norflurazon recoveries from the treatment solutions were greater than 95% indicating a high degree of accuracy and precision in preparing the treatment solutions.

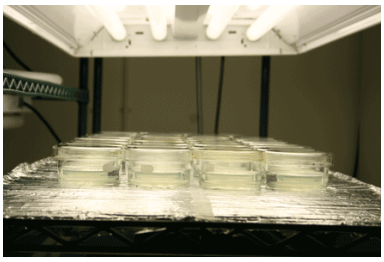


Figure 1. 48 X 72 inch exposure table lower surface: under-laid with aluminum foil. Petri dishes were randomly placed under the lights.

Measurements of Toxicity

Total numbers of symptomatic and asymptomatic fronds were recorded every other day for the duration of the exposure trial. Photographs were also taken using a Canon 420B SLR camera with EFS 18-150 mm lens for one representative replicate on the same days. The symptomatic fronds were identified based on a distinguishable pattern of chlorosis due to chlorophyll degradation. Symptomatic plants exhibited a well-defined

Ruben Koch
SWS 6905 Project
December 1, 2011

linear-margin between chlorotic and non-chlorotic tissue across the leaves, as opposed to overall general chlorosis (Figures 2 A, B). The growing media was never changed or altered during the exposure period. Growth rates for exposure and recovery periods were calculated as:

$$R = \frac{F_b - F_a}{Interval}$$

where the recovery (R) is the number of fronds produced during the interval period, F_b is the number of fronds at the end of the interval, F_a is the number of fronds at the beginning of the interval, and $Interval$ is the number of days comprising the interval.

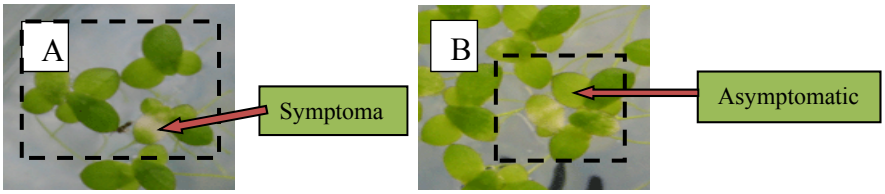


Figure 2 A and B Exposure & symptomatic & asymptomatic *Lemna minor* L. exposed to norflurazon. (A) Norflurazon treatment 25ppb after 48 hour exposure, (B) 240 hours from first recovery: previous 25 ppb exposure.

Recovery characterization

Upon completion of the exposure toxicity trial, 10 fronds were removed from each replicate, cleaned by immersion of the fronds in a 400 mL flask containing 250 mL of nanopure water for 30 sec. This process was repeated for a total of three separate immersions for each individual replicate, followed by transfer into another clean and sterile covered petri-dish containing 100 mL of the 10% Hoagland's nutrient media made with moderately hard water. The replicates were placed back under the same lights in a completely randomized order as discussed earlier.

Ruben Koch
SWS 6905 Project
December 1, 2011

A total of three separate transfers into new clean stock solution were performed during the recovery trial. Ten fronds (±1) initially were removed at the end of each recovery trial period, and placed in clean media in order to replenish nutrient supplies and to provide dilution of possible remnants of norflurazon exudates from the plants. The three recovery intervals were 10, 11, and 5 days respectively. Total numbers of symptomatic and asymptomatic fronds were recorded as follows: every second day for the first transfer, every fourth day for the second transfer, and only once after five days for the third and final transfer. Photographs were also taken as previously described. Symptomatic fronds were identified using the same criteria previously described, and the stock solution was never changed or altered during each trial period. At the end of the exposure trail and the second and third recovery trials, 15 to 20 fronds were loaded into the mouth of a 15 mL vial filled with Nano-pure water (Fig. 3) and photosystem II electron transport efficiency (Fv/Fm) was measured using an OS5p Multi-Mode Chlorophyll Fluorometer.

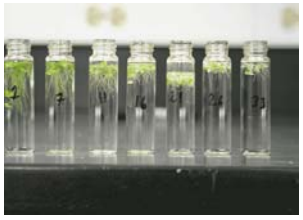


Figure 3, 15 ml vial containing 15 to 20 fronds, for Fv/Fm test: Fm - is maximal fluorescence measured during the first saturation pulse after dark adaption; Fv/Fm - This ratio is an estimate of the maximum portion of absorbed quanta used in PSII reaction centers; Fv - variable fluorescence.

Statistical Analysis:

All data were analyzed using IBM© SPSS© Statistics (version 19) software. Analysis of variance (one-way ANOVA) was conducted with Tukey's HSD ($p < 0.05$) to determine differences between treatment means for the five replicate measurements for each endpoint measured.

Ruben Koch
SWS 6905 Project
December 1, 2011

Growth rates were not consistently affected during the first 96 h of exposure (Fig 6). However, significant reductions in growth rate were observed at concentrations ≥ 25 ug/L after 144 h exposure, ≥ 50 µg/L after 192 h exposure, and ≥ 25 µg/L after 240 h exposure. Following 144 h exposure, growth rates were reduced by 72 % at the 25-500 ug/L treatments, respectively. Reductions ranged from 32% - 91% and 41% - 99% for the 192h and 240h exposure periods respectively.

Results

Lemna minor L. was exposed to norflurazon for a period of 10 days at the six different concentrations and control, followed by 26 days for the recovery assay. Significant effects ($p < 0.05$) were observed as early as 48 h for the 500 µg/L treatment (Fig 4). Treatment effects became more pronounced as the exposure period increased, with the greatest difference occurring at 192 and 240 h. Following 192 and 240 h exposure, total frond counts were statistically lower at norflurazon concentrations of 25 µg/L and higher. These reductions were (192) 21%, 34%, 58%, 71%, and 74% (240) 30%, 46%, 70%, 83% and 86% for the 25, 50, 100, 250, and 500 µg/L treatments, respectively.

In contrast with the frond counts, the occurrence of symptomatic (chlorotic) fronds was noted as early as 48h for the 25 µg/L treatment (Fig 5). All norflurazon treatments (except 10 µg/L after 48 h exposure) increased the incidence of symptomatic tissue. Symptomatic tissues occurred in 30-39%, 51-65%, 70-79%, 79-91%, and 80-98% of the fronds after 48, 96, 144, 192, and 240 h exposure, respectively.

Ruben Koch
SWS 6905 Project
December 1, 2011

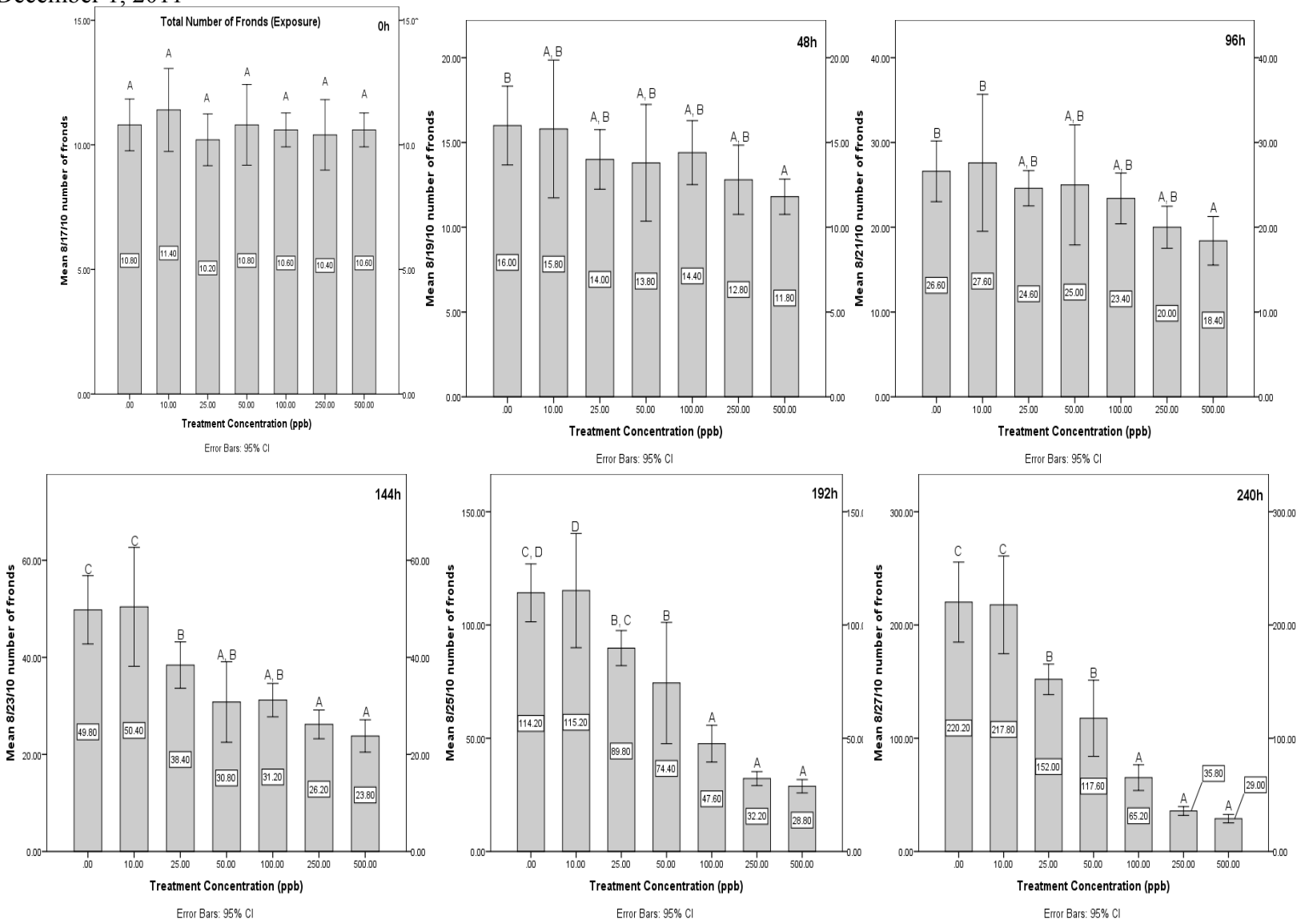


Figure 4: Total mean number of fronds during exposure of Duckweed (*Lemna minor L.* to norflurazon. Each of the six graphs represents a different time period (0, 48, 96, 144, 192, and 240 in hours of exposure, respectively) when the fronds were counted. Each bar in the graph is denoted by a letter; values with the same letter are not significantly different at ($p<0.05$). The square within each bar states the numeric mean.

Ruben Koch
SWS 6905 Project
December 1, 2011

At the end of the exposure trial, chlorophyll fluorescence Fv/Fm measurements were taken for each replicate. No significant differences were observed between the control and the 10 µg/L treatment, with mean Fv/Fm values ranging from 0.7995 to 0.802. However, the destruction of chlorophyll in the 25-500 µg/L treatment prevented measurable levels of chlorophyll fluorescence (Fig 7).

In order to observe possible latent effects and to characterize potential recovery, representative plants from each replicate were transferred to fresh media and allowed to grow for an additional period. This recovery study was divided into three separate growing time intervals. The first interval was 10 days with measurements taken every 48 hours (Fig. 8). This period includes recovery measurements through 240 hours.

Growth rates for plants exposed to 10-100 µg/L were initially similar to the control after 48 hours. However, following 96 hours recovery, significant reductions in frond production rate were evident though 240 hours recovery. These significant reductions were observed for plants exposed to norflurazon concentrations greater than or equal to 10-25 µg/L. Reductions in frond production rates were dose-dependent.

Ruben Koch
SWS 6905 Project
December 1, 2011

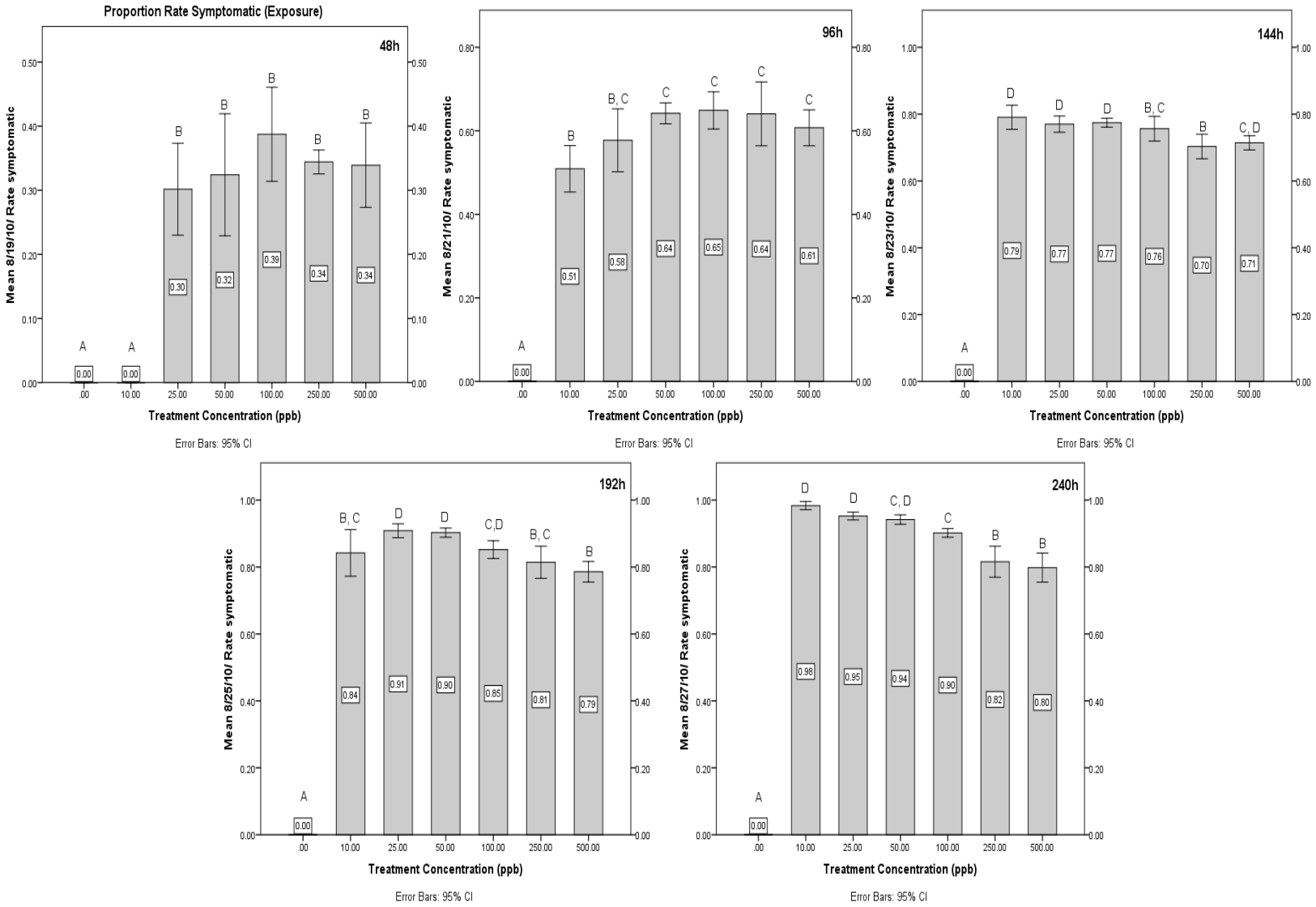


Figure 5: Proportion of symptomatic plants during exposure to norflurazon. Each bar in the graph is denoted by a letter; values with the same letter are not significantly different at ($p<0.05$). Proportion calculation (48h-0h/48h). The square within each bar states the numeric mean.

Ruben Koch
SWS 6905 Project
December 1, 2011

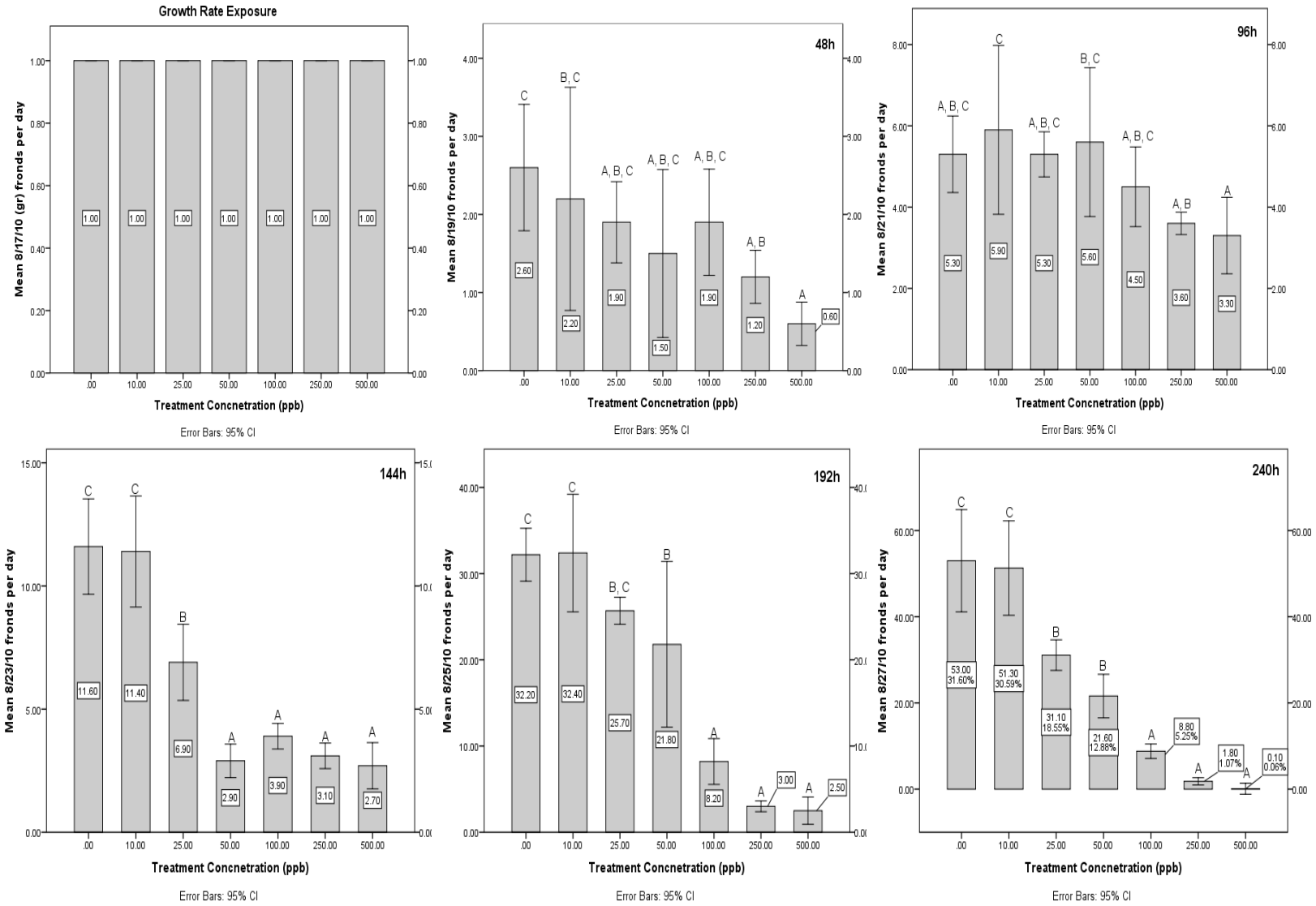


Figure 6: *L. Minor* growth rate during exposure to norflurazon. Each bar in the graph is denoted by a letter; values with the same letter are not significantly different at ($p<0.05$). The square within each bar states the numeric mean. Note; at the 240h a proportional percentage among the Treatments was added.

Ruben Koch
SWS 6905 Project
December 1, 2011

The second recovery stage was conducted for 11 additional days, with counts taken every 4th day (96 hours) following the first count at 70 h. This period followed recovery from 240 to 504 hours of the recovery phase (Fig 9). Frond production rates were similar across all treatments after 72 (312) hours. However, significant reductions in growth rates for the plant exposed to 500 µg/L norflurazon were observed after 168 (408 h total recovery interval) and 264 (504 h total recovery interval) hours. During this period, frond production rates of plants exposed to the 10-250 µg/L concentrations had recovered to control levels.

The third recovery interval lasted for 5 days with the final count occurring at the end of the 5th day (120 hours/624 hours for entire recovery period) (Fig. 10). No significant differences were observed for any of the treatments after 120 hours, indicating that plants had recovered from the previous norflurazon exposure. In addition, no significant differences were observed for (Fv/Fm range: 0.79 – 0.81) measurements taken at the end of the last two recovery intervals (504 and 624 h total) (data not shown).

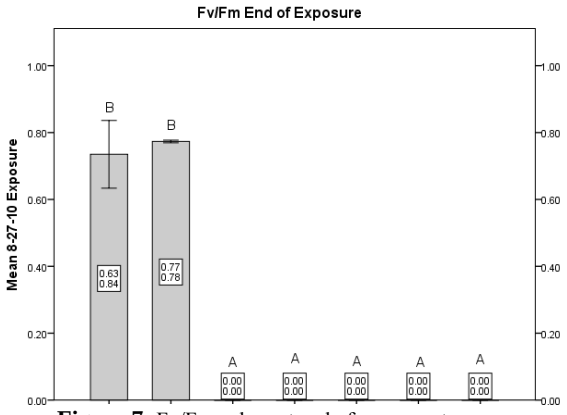


Figure 7: Fv/Fm values at end of exposure to norflurazon. The square within each bar states the numeric the upper and lower mean standard deviation.

Ruben Koch
SWS 6905 Project
December 1, 2011

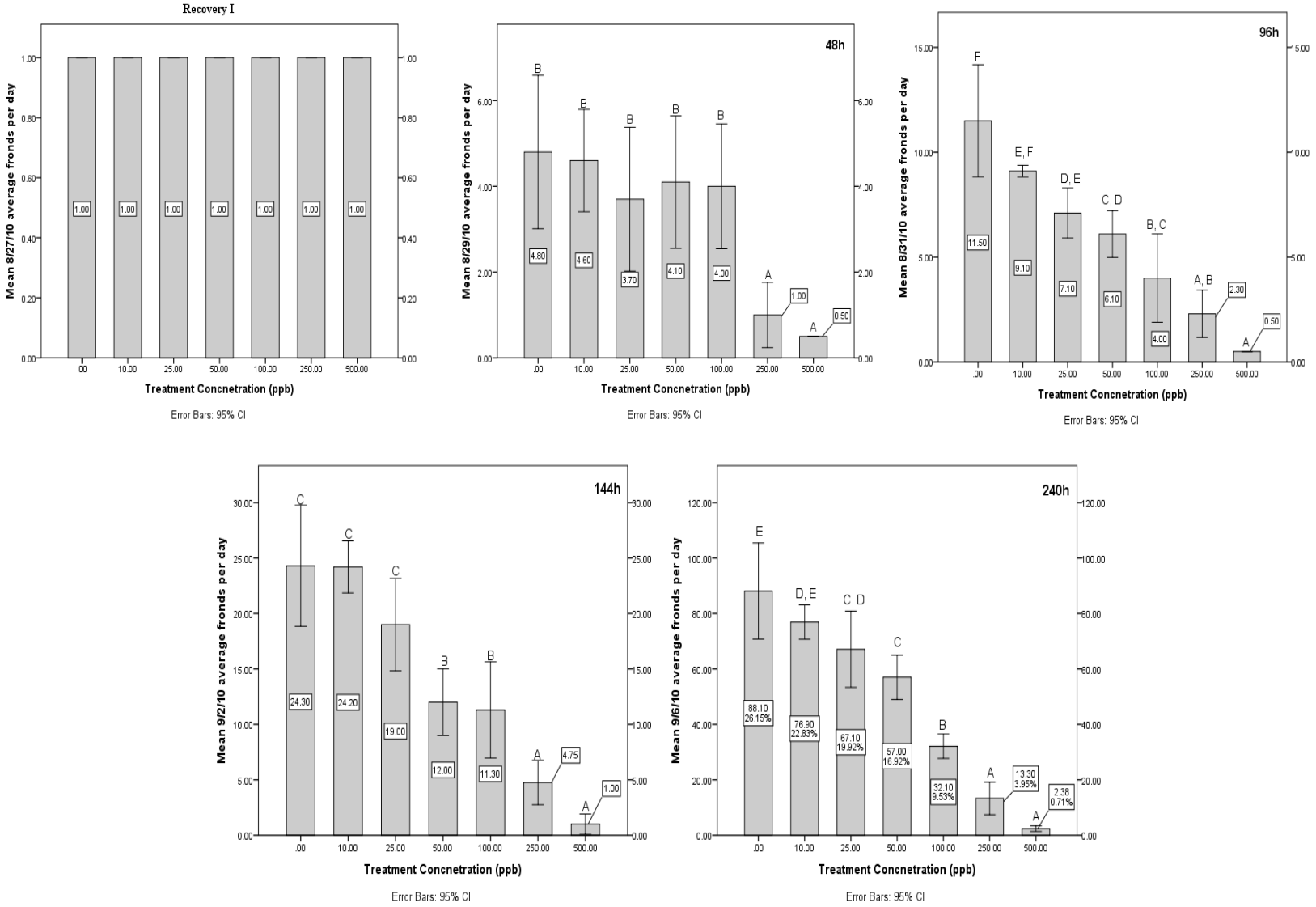


Figure 8: Mean Growth rate during 1st recovery period (0 -240 h). Each bar in the graph is denoted by a letter; values with the same letter are not significantly different at ($p<0.05$).

Ruben Koch
SWS 6905 Project
December 1, 2011

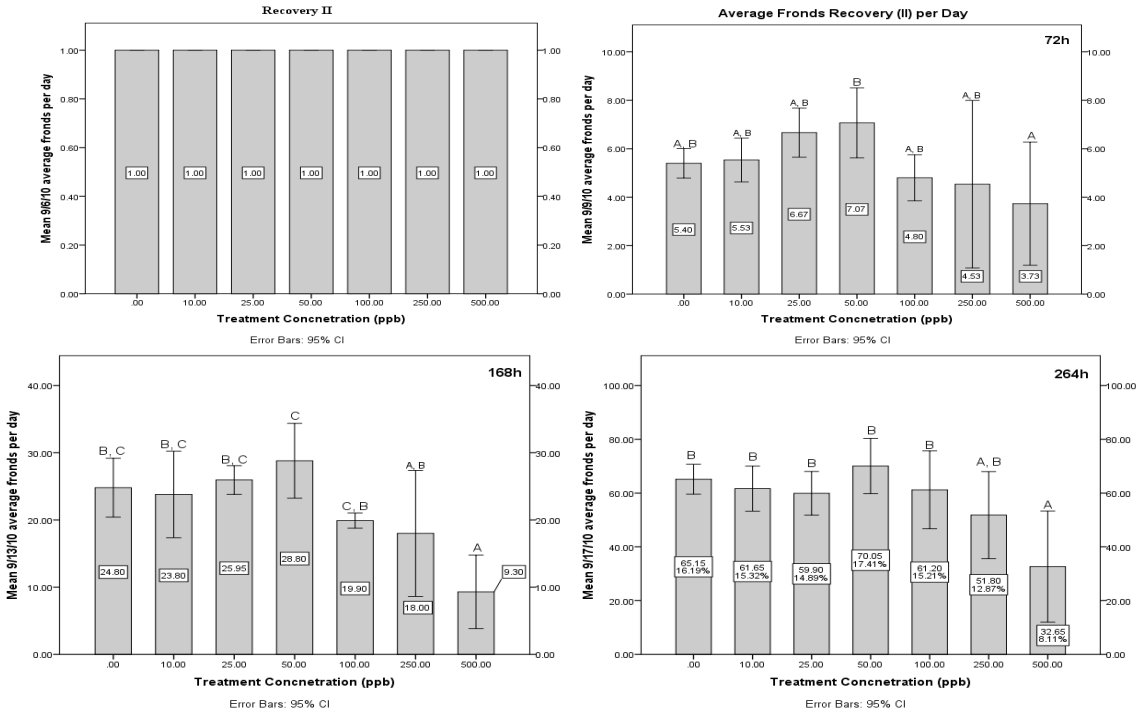


Figure 9: Mean growth rate during 2nd recovery period (240 – 504 h). Each bar in the graph is denoted by a letter; values with the same letter are not significantly different at ($p<0.05$).

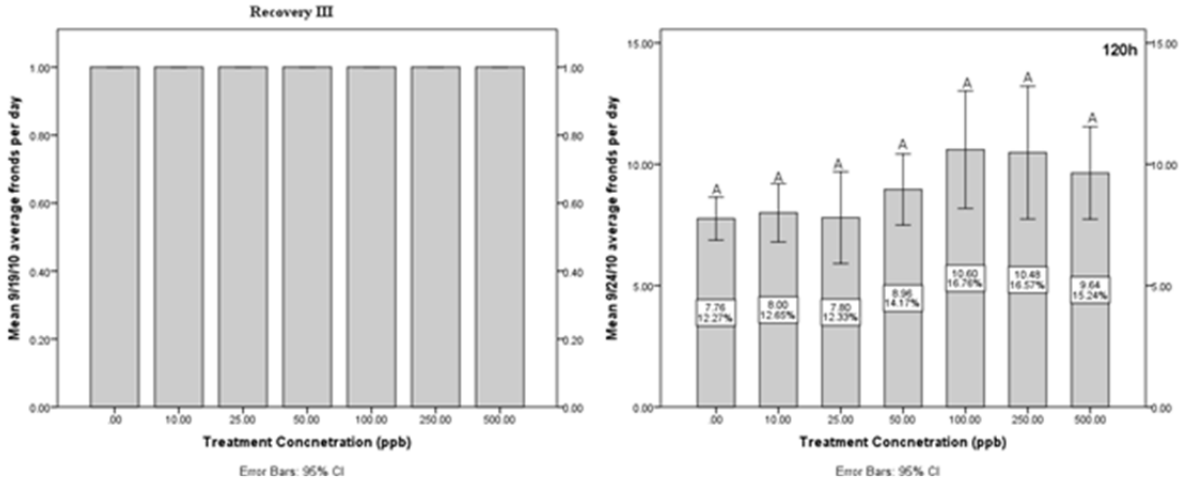


Figure 10: Mean growth rate during 3rd recovery period (504 – 624 h). Each bar in the graph is denoted by a letter; values with the same letter are not significantly different at ($p<0.05$).

Ruben Koch
SWS 6905 Project
December 1, 2011

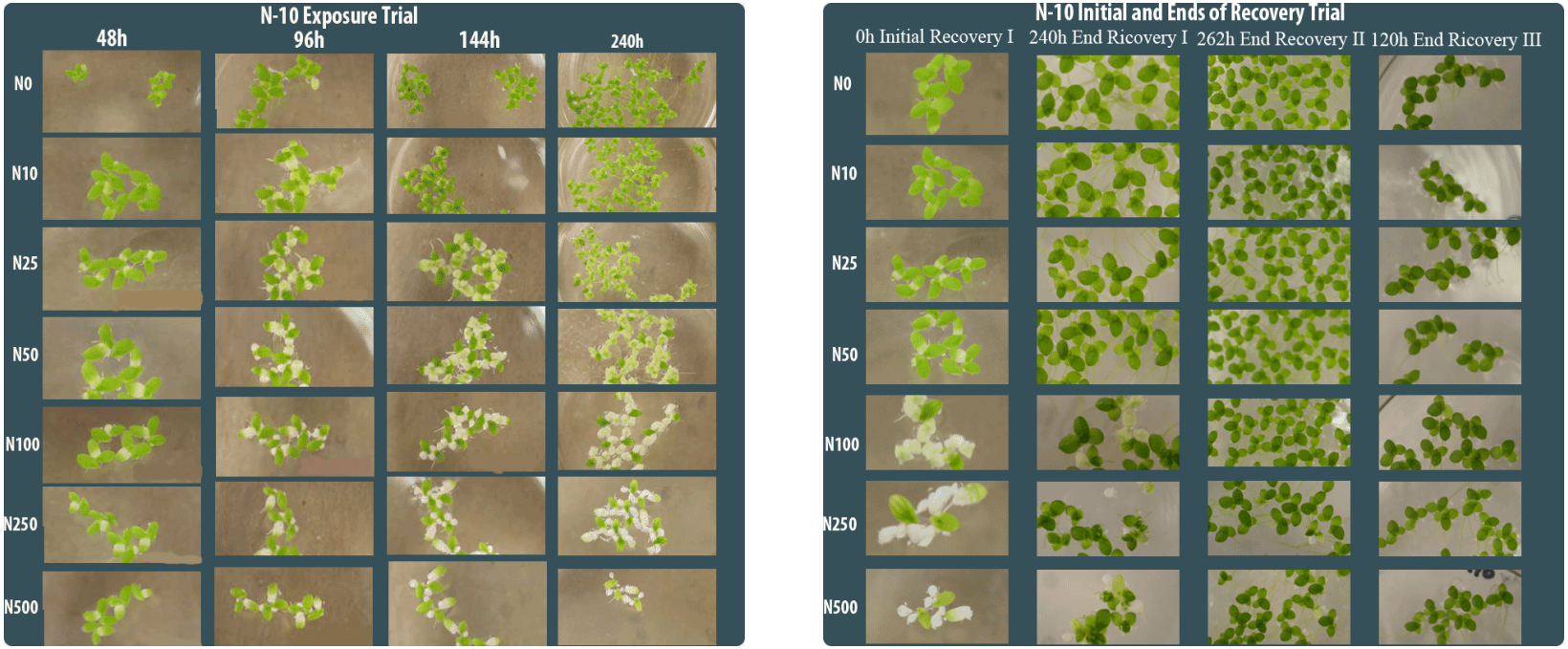


Figure 11: Example of norflurazon effects on L. Minor during exposure and recovery period.

Ruben Koch
SWS 6905 Project
December 1, 2011

Conclusion

This experiment demonstrated that norflurazon significantly reduces frond production in a dose-dependent fashion, with significant reductions occurring as early as after 48 hours of exposure to concentrations greater than 25 µg/L (Fig 11). Toxic effects were readily apparent in affected leaves. Following exposure, results indicate that duckweed has the capacity to recover from injury. Onset of recovery was directly proportional to the concentration of norflurazon to which plants were exposed. Injury did not appear to cause phenotypic mutations of the progeny, indicating non-lasting effects. However, additional studies would be useful for determining possible effects on internal biochemical markers such as protein content and quality. Finally, these experiments were conducted in a controlled environment under sterile conditions. These results may not be directly representative of actual effects in field situations. However, they do provide a useful starting point for characterizing the toxicity of norflurazon to *L. minor*, and for characterizing recovery potential following exposure. Results should also be useful for conducting ecological risk assessments focused on norflurazon.

Ruben Koch
SWS 6905 Project
December 1, 2011

Works Cited

Breitenbach, J., Zhu, C. and Sandmann, G. "Bleaching Herbicide Norflurazon Inhibits Phytoene Desaturase by Competition with the Cofactors." *J. Agric. Food Chem.* (2001): 5270-5272.

Chandran, R.S. and Singh, M. *Reduction of Norflurazon Leaching in a Sandy Soil by Adjuvants*. Bulletin of Environmental Contamination and Toxicology. Lake Alfred, FL, USA: Springer-Verlag New York Inc., 1999.

Essington, Michael E. *Soil and Water Chemistry An Integrative Approach*. 2. Boca Raton: CRC Press LLc, 2000 N.W., 2004.

Frankart, C., Eullaffroy, P.and Vernet, G. "Comparative effects of four herbicides on non-photochemical." *Environmental and Experimental Botany* 49 (2003): 159-168. ELSEVIER. 21 September 2002. <www.elsevier.com/locate/envexpbot>.

Hothem, Seth, Marley, Karen A. and Larson, Richard A. "Photochemistry in Hoagland's Nutrient Solution." *Journal of Plant Nutrition* (2003): 845-854. 12 November 2011. <http://dx.doi.org/10.1081/PLN-120018569>.

Kegley, S.E., Hill, B.R., Orme S., and Choi A.H. "Norflurazon." 2010. *The Pesticide Action Network (PAN) Pesticide Database.* 12 May 2011. <http://www.pesticideinfo.org/Detail_Chemical.jsp?Rec_Id=PC36413#Water>.

Lamoreaux, Robert J., Corbin, Virginia L., and Johl, Balraj S. "Adsorption and Deactivation of Norflurazon by Activated Charcoal." 1989. Weed Technology, Vol. 3, No.2.

Massad, W., Criado, S., Bertolotti, S., Pajares, A., Gianotti, J., Escalada, J.P., Amat-Guerri, F., and Garcia, N.A. *Photodegradation of the herbicide Norflurazon sensitised by Riboflavin. A kinetic and mechanistic study*. Rio Cuarto, Argentina; Rio Gallegos, Argentina; and Madrid, Spain.: Elsevier Ltd., 2004.

Mohlenbrock, Robert H. "PLANTS Profile -Lemna minor L. Common Duckweed." 1995. *USDA - Natural Resources Conservation Service.* 12 May 2011. <http://plants.usda.gov/java/profile?symbol=LEMI3>.

Sandman, G., Bramley, P.M., and Boger, P. *The Inhibitory Mode of Action of the Pyridazinone Herbicide Norflurazon on a Cell-Free Carotenogenic Enzyme System*. Konstanz, West Germany and Surrey, England: Academic Press, Inc., 1980.

Schuler, L.J. and Rand, G.M. "Aquatic Risk Assessment of Herbicides in Freshwater Ecosystems." *Arch Environ Contam Toxicol* (2007): 54:571–583. 2 November 2011.

Southwick, L.M., Willis, G.H. and Bengtson, R.L. *Runoff Losses of Norflurazon: Effect of Runoff Timing*. Baton Rouge, Louisiana.: American Chemical Society, 1993.

Taiz, Eduado Zeiger and Lincoln. *Plant Phisiology Third Edition*. Sunderland, MA: Sinauer Associates, Inc., 2002.

United States Environmental Protection Agency Washington, D.C. 20460. "Reregistration Eligibility Decision (RED) Documents." 31 May 2002. *U.S ENVIRONMENTAL PROTECTION AGENCY.* 12 May 2011. <http://www.epa.gov/oppsrrd1/REDs/norflurazon_tred.pdf>.

Vencill, W.K. "Herbicide handbook." *Weed Science Society of America* (2002).

Ruben Koch
SWS 6905 Project
December 1, 2011

Voigt, C., Oster, U., Bornke, F., Jahns, P., Dietz, K., Leister, D. and Kleine, T. "In Depthe analysis of the distinctive effects of norflurazon implies that tetrapyrrole biosynthesis, organellar gene expression and ABA cooperate in the GUN-type of plastid signalling." *Physiologia Plantarum* 138 (2009): 503-519.

Wang, W. "Literature Review on Duckweed Toxicity Testing." *Academic Press, Inc.* (1990).

Wilkinson, Robert E. "Carotenogenic Inhibation by Norflurazon in Wheat." *PESTICIDE BIOCHEMISTRY AND PHYSIOLOGY 23,* (1985): 370-377.

Wilson, Chris. "Aquatic Toxicology Notes: Predicting the Fate and Effects of Aquatic and Ditchbank Herbicides." *University of Florida- IFAS Extension* (2006).

Wilson, P.Chris, Boman, Brian and Foos, Jane F. "Norflurazon and Simazine Losses in Surface Runoff Water From Flatwoods Citrus Production Areas." *Springer Science+Business Media, LLC 2007* (2007): 78:341-344.

Wilson, P.Chris, Wilson, Sandra B., and Haunert, Daniel. "Toxicity of the Norflurazon to the Aquatic Macrophyte Vallisneria Americana (MICHX.)." *Journal of Toxicology and Environmental Health* (2006): 1167-1179.