H-02 CONSTRUCTED WETLAND STUDIES

AMPHIBIANS AND PLANTS

FY-2009 ANNUAL REPORT

Savannah River Ecology Laboratory
University of Georgia
Drawer E
Aiken, SC 29802

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SREL Contacts: David Scott (scott@srel.edu)
Tracey Tuberville (Tuberville@srel.edu)

TABLE OF CONTENTS

Figures

EXECUTIVE SUMMARY

The Savannah River Ecology Laboratory (SREL) initiated amphibian and vegetation surveys at the H-02 constructed treatment wetlands in May 2008. The ecological research conducted by SREL at the site focuses primarily on four questions related to these treatment wetlands: 1) Over time, what amphibians, reptiles, and plants have become established in the wetlands? 2) Is there any evidence that elevated trace metal levels (e.g., copper and zinc) in the wetlands affect amphibian reproductive and recruitment success? 3) How do the amphibian diversity and numbers compare to other, more natural, wetlands? 4) As the constructed wetlands age, how will the amphibian community respond?

This report summarizes our amphibian and vegetation sampling at the H-02 treatment wetlands from May 2008 to November 2009. We used permanent plots established for vegetation monitoring and aquatic trapping to characterize biota of the treatment wetlands. Adjacent drift fence arrays with pitfall traps were used to estimate breeding amphibian numbers and juvenile production. Biotic samples were collected to determine copper and zinc burdens, and field and laboratory experiments were employed to assess metal effects. To date we have recorded 2,972 captures of 27 amphibian and reptile species at the H-02 treatment wetlands, including successful production of juveniles by a total of 10 amphibian species. This level of reproductive success was higher than at the natural wetland reference site, which has a short hydroperiod and has dried early in each of the last two years. However, both juvenile and adult amphibians have elevated Cu and Zn levels at the H-02 site compared to reference wetlands; laboratory and field experiments are ongoing to better understand potential biological effects these trace metals may have on

common amphibian species. Our vegetation sampling recorded 22 total species (or species groups) of vascular aquatic plants in the H-02 treatment cells – 18 in FY-08 and 16 in FY-09. Four species or species groups, *Cynodon dactylon, Hyrdocotyle ranunculoides*, the combined *Lemna minor/Spirodela polyrrhiza*, and the planted *Schoenoplectus californicus* were ubiquitous, occurring in 20 or more plots (>83%) both years. Vegetation will continue to be monitored and compared from year to year; these data can also be compared with similar created wetland systems (e.g., the A-01 system) and natural wetlands on the SRS.

CHAPTER I. INTRODUCTION AND OVERVIEW

David Scott, Rebecca R. Sharitz, & Tracey Tuberville

The Savannah River Ecology Laboratory (SREL) initiated ecological studies related to the operation of the H-02 constructed wetlands in May 2008. Constructed wetlands are one method to treat and improve water quality at regulated outfalls on the Savannah River Site (SRS; Bach et al. 2008). Trace metals such as copper, lead, and zinc are removed by adsorption to organic matter and clay particles, and sulfate-reducing bacteria enable the precipitation of metal ions in the anaerobic soils (Nelson et al. 2006). As constructed treatment wetlands age they may become more efficient at removing metals, and may serve the dual functions of both improving water quality and providing wildlife habitat.

The Southeast is home to a rich diversity of pond-breeding amphibians (frogs, toads, and salamanders) that rely on wetlands of various types—including constructed wetlands—as breeding habitats. The basic ecology of many of these species has been studied by SREL on the SRS for many years (e.g., Semlitsch et al. 1996), and in recent years SREL's focus has shifted to more applied studies such as effects of coal combustion waste on focal amphibian species (Rowe et al. 2001, Snodgrass et al. 2004, Peterson et al. 2009) and the recovery of local amphibian populations after the construction and start-up of the DWPF facility (Pechmann et al. 2001). Our current amphibian studies at the H-02 wetlands are a continuation of our interest in how human activities and industrial processes may affect amphibian populations, and these studies dovetail nicely with the core principles of the SRS as a National Environmental Research Park (NERP).

The H-02 wetlands were designed to comply with regulatory guidelines for process and storm water discharge from H-Area facilities, but they may also provide wildlife benefits. The wetlands are still in their early phase of establishment, and exhibit large fluctuations in several water chemistry parameters (Bach et al. 2008). By observing and experimentally assessing the response of amphibians to pH, Cu, and Zn levels in the H-02 wetlands under both field and more controlled laboratory settings, we will better understand the suitability of the H-02 created wetlands for wildlife habitat, especially amphibians.

Chapter II of this report contains the results of SREL's amphibian and reptile sampling at the H-02 treatment wetlands from May 2008 to November 2009, with comparative data from the reference site, Rainbow Bay. In addition to monitoring amphibian and reptile use of the wetlands, we collected data on metal burdens of amphibians inhabiting the wetlands, and we conducted preliminary field and laboratory tests on effects of Cu concentration on amphibian development in three species (the southern toad, *Bufo terrestris*, the eastern narrowmouth toad, *Gastrophryne carolinensis*, and the southern leopard frog, *Rana sphenocephala*).

Chapter III of this report describes the results of our vegetation monitoring and assessment of changes in the plant community. One objective of the amphibian studies at the H-02 constructed wetlands is to understand the relationship between amphibian community reproductive success and the changes in wetland vegetation structure over time. In FY-08 we conducted baseline measures of vegetation in the H-02 wetlands, established 24 permanent plots, and used standard metrics such as plant density, percent cover, and species richness to characterize the plant community in the constructed wetland cells. In FY-09 we re-sampled these plots to determine changes associated with the establishment of additional plant species or the loss of species as a result of competition with the giant bulrush (*Schoenoplectus californicus*).

In summary, our observations and experiments performed in FY-09 in the H-02 wetlands will help quantify the suitability of these wetlands for amphibians. Our research continues to focus on four questions related to these treatment wetlands: 1) Over time, what amphibians, reptiles, and plants have become established in the wetlands? 2) Is there any evidence that elevated metals levels in the wetlands (e.g., Cu and Zn) affect amphibian reproductive and recruitment success? 3) How do the amphibian diversity and numbers compare to other, more natural, wetlands? 4) As the constructed wetlands age, how will changes in vegetation composition and structure affect the amphibian community?

We propose to continue studies on wetland plant community development and on focal amphibian species to better understand any water quality or contaminant thresholds that may that may negatively affect local populations. Because body burdens of trace elements acquired during the aquatic larval phase are retained through metamorphosis, these metals may be transferred from the wetland system (where they were acquired) into terrestrial food webs.

This study will ultimately give us an understanding of how amphibians are an important pathway in trace element accumulation and elimination, and the extent to which they transfer metals from the H-02 wetlands to terrestrial food webs.

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CHAPTER II – AMPHIBIAN AND REPTILE USE OF THE H-02 CONSTRUCTED WETLANDS

David Scott, Tracey Tuberville, Matt Erickson, and Brian Metts

INTRODUCTION

Amphibians have been the subjects of numerous ecotoxicology studies (reviewed in Linder et al. 2003 and Sparling et al. 2000), many of which have demonstrated adverse effects of trace metals on amphibian growth and survival (Rowe et al. 2002; Snodgrass et al. 2003, 2004). Amphibians are highly sensitive to environmental contaminants for several reasons. In particular the combination of permeable skin (used for both gas exchange and osmoregulation) and potential exposure in both aquatic and terrestrial habitats puts them at high risk. Exposure to trace metals can have a range of effects including adverse physiological changes (Hopkins et al. 1998), decreased survivorship of larvae (Baud & Beck 2005; Horne & Dunson 1995; Rowe et al. 2001; Roe et al. 2006), increased time to metamorphosis (James et al. 2005; Roe et al. 2006), and decreased size at metamorphosis (Peterson et al. 2009). Effects on larval traits have significant implications because they also affect adult fitness traits such as age at first reproduction, survival, and fecundity (Semlitsch et al. 1988; Scott 1994). In our H-02 study we are monitoring the amphibians that use the wetlands, and experimentally examining the effects of trace metal exposure on common southeastern species of amphibians with varied life histories, ecologies, and exposure risks, including the southern toad, *Bufo terrestris*, the eastern narrowmouth toad, *Gastrophryne carolinensis*, and the southern leopard frog, *Rana sphenocephala*.

Copper (Cu) concentrations in the H-02 system can vary spatially throughout the system, ranging as high as 31-37 ppb in the influent in summer months to 7 ppb in the effluent exiting the treatment wetlands (G. Mills and N. Etheridge, *unpublished data*). Levels in portions of the retention pond have reached 340-590 ppb (Bach et al. 2008). These concentrations may be of concern for normal amphibian development. While some species have only shown short-term (acute) toxicity effects at Cu concentrations above those observed in the H-02 constructed treatment cells [e.g., a 96-hr LC_{50} for the boreal toad (*Bufo boreas*) of 0.12 mg/L, or 120ppb (Dwyer et al. 2005); a 24-hr LC_{50} for the Argentine toad (*Bufo arenarum*) of 0.085 mg/L (85 ppb; Herkovits & Helguero 1998)], other species have been strongly affected by much lower chronic exposures [e.g., 41% reduced survival of spring peeper tadpoles (*Pseudacris crucifer*) at Cu concentrations of 5.5 μg/L (5.5 ppb; Baud & Beck 2005)]. Under laboratory conditions, low Cu has not been shown to directly affect larval survival or growth but has been shown to significantly increase the larval period (and therefore duration of exposure), which may increase actual mortality under field conditions. For example, the gray treefrog (*Hyla chrysoscelis*) had an increased larval period at Cu levels of 3.18 μg/L (Parris & Baud 2004). It is important to examine effects throughout the full larval period, as the most ecologically relevant impacts may not occur until late in the larval period (Snodgrass et al.

2005).

The potential toxicity of various metal species to amphibians and the concentrations at which effects are observed is a complex phenomenon that is influenced by pH, dissolved organic carbon (DOC) levels, the presence of other metal ions and/or acid-volatile sulfides (AVS), as well as the developmental stage and species of amphibian (Horne & Dunson 1995). For example, Cu may inhibit larval growth at concentrations of 60 µg/L in one species but not another, eggs may show no effect even at 150 µg/L Cu, and effects may be reduced by the presence of either Zn or DOC or by a lowered pH. This is a key point for the H-02 wetlands—which have high levels of Zn and DOC, and elevated pH— the bioavailability and toxicity of Cu is not necessarily proportional to its total concentration, but may be influenced by other components of the system.

METHODS

Field Methods – Drift-fence/pitfall trapping (Dodd & Scott 1994), aquatic trapping, and call surveys are standard techniques to monitor amphibians – we are using all three to assess amphibian use of the H-02 wetland cells. In mid-AUG 2008 we installed three partial drift fences (20 m in length per fence) along the north edge of the H-02 constructed wetlands to capture immigrating adults and emigrating juveniles (see description of drift fences in the Rainbow Bay sampling section). Captured animals are released on the opposite side of the fence to continue their movement. Data on species richness and juvenile recruitment are being used to compare amphibian success in the H-02 wetlands to their success in reference wetlands on the SRS in order to assess the performance of the constructed wetlands as suitable aquatic breeding habitat. Some juveniles and adults are also being collected for metal analyses (see methods below).

We supplemented the drift fence technique with aquatic trapping (i.e., using minnow traps) to assess larval species richness, numbers, and health, and to collect aquatic larvae and adults for metal analysis. In AUG 2008 we established 24 permanent trap locations (12 per wetland cell) to facilitate vegetation sampling (see Chapter III) and allow comparison of successional changes in vegetation with amphibian population changes. Each aquatic trapping session (1/mo in JUN, JUL, and AUG) consisted of 24 traps set for four days/three nights in the two constructed treatment wetlands, with 8-10 additional traps placed in the retention pond. Traps were checked daily and species, number of individuals, and life stage were recorded.

Study Species – For our experimental studies to date we have chosen species that are well suited to manipulative investigations of potential biological effects of trace metals. 1) The southern toad is found throughout much of the Southeast and is one of the most common species that breeds in the H-02 aquatic habitats. The southern toad is a habitat generalist, breeds in a wide variety of aquatic habitats (lakes, ponds, streams, ephemeral wetlands, floodplain pools), and has aquatic larvae with a relatively short larval period (30-55 d); thus its aquatic exposure to elevated Cu and Zn is relatively brief. Additionally, this species has been the subject of several other studies of metal effects, particularly arsenic and selenium. 2) The eastern narrowmouth toad is a small southeastern species that occurs at H-02 and that also has a short larval period (30-70 days). However, unlike the southern toad, the larvae of narrowmouth toads filter-feed plankton from the water column (Pechmann 1994), and thus have a very different food source (and possibly contaminant exposure pathway). 3) The southern leopard frog is another common species that also has been well-studied for many contaminants other than Cu and Zn. In contrast to many amphibians that have a relatively narrow breeding period, the leopard frog has both fall (Sep-Oct) and late winter (Feb-Mar) breeding periods (Caldwell 1986). The two breeding pulses create larval cohorts that may differ in their larval periods, and thus the length of exposure to metals—some may develop in 3-4 mo, while others may take > 6-7 mo to metamorphose. 4) Two other species, the bullfrog (*Rana catesbeiana*) and the green frog (*Rana clamitans*), are more aquatic as adults than the toads and leopard frog, and larvae may remain in the ponds for extended periods. Thus, these two species may have both prolonged larval and adult exposure to trace metals.

Metal Experiments Methods – In all our experiments to date, amphibian eggs and larvae have been exposed to varied levels of metals either in the field or in controlled laboratory experiments. In our *in situ* field experiments, eggs were placed in 12-L floating buckets at each of the six locations in the H-02 system (Fig. II-1 & Fig. II-2). Bucket enclosures were constructed by drilling three 5-cm holes around the top of the bucket and covering the holes with mesh screen to allow ambient water flow through the bucket and colonization by algae. Screen was attached with silicone, and the buckets were cured and then rinsed for

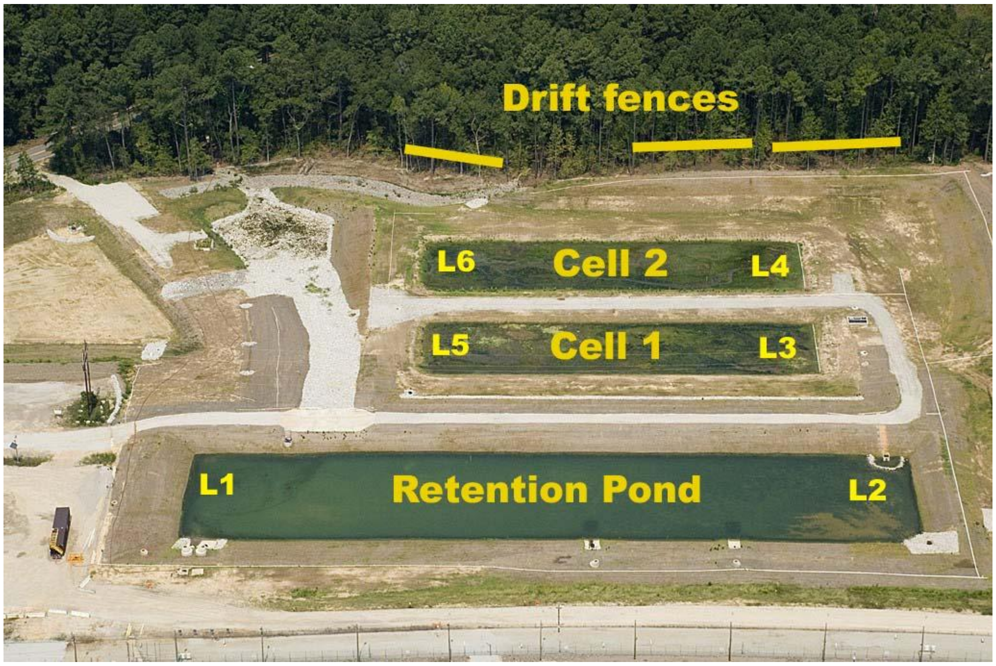

Figure II-1. Drift fence/pitfall trap and egg/larval field experiment locations within the H-02 wetland system. Locations L1 & L2 (retention pond), L3 & L4 (influent end of wetland cells), and L5 & L6 (effluent end of wetland cells) represent a gradient of water chemistry along which the “bucket studies” have been conducted. Highest pH, Cu, and Zn levels occur in the retention pond, and progressively lessen throughout the treatment wetlands.

24 hrs to allow irritants to leach from the silicone. Each bucket was seated into a square Styrofoam flotation collar, which kept experimental animals suspended in the water column. The tops of buckets were also covered with a fine mesh to exclude potential predators. Once egg development was completed the same buckets were used for the larval study. Due to the densities used in each bucket (10-15 hatchlings per bucket), larvae were supplementally fed weekly. Periphitometers were constructed from 15 x 20 cm acrylic for use in quantifying algal food resources and associated metal concentrations.

In the laboratory studies described below, eggs and larvae were reared in

SREL's greenhouse and Animal Care Facility. In these studies we precisely control water chemistry variables by using a standard mix of synthetic dilution water for toxicity tests using freshwater organisms (USEPA 2002) — 48 mg/L $NaHCO_2$, 30 mg/L $CaSO_4$, 30 mg/L $MgSO_4$, and 2 mg/L KCl added to deionized MILLI-Q® water, plus appropriate levels of Cu.

Figure II-2. Floating bucket experiments used to examine the success of amphibian eggs and larvae in the H-02 system. Experiments to date have investigated the success of three species – narrowmouth toads, southern toads and southern leopard frogs – along the water chemistry gradient with the H-02 system. From left to right above – retention pond, wetland cell effluent end, and wetland cell influent end.

H-02 Field Experiments — The H-02 treatment complex consists of the retention pond, which receives the Tritium Facility effluent, and two constructed wetland cells. Water in the retention pond has the highest levels of Cu, Zn, and pH, and after a residence time of several days water exiting the wetland cells has lower levels of these parameters (Bach et al. 2008, G. Mills *unpublished data*). Our field experiments are conducted along this water chemistry gradient, which means that several factors vary simultaneously. In one study (narrowmouth toad), an additional site (Flamingo Bay) on the SRS was used as a control pond for the H-02 trials. Details regarding the design of our field experiments to date are presented below.

- *#1 Preliminary Trials and Observations* – From FEB-MAY 2009 we monitored breeding events in the wetlands and conducted pilot field experiments with two species (southern leopard frog and southern toad). We discovered *R. sphenocephala* egg masses (N=6) on FEB 11 in both wetland cells, with many additional masses laid thru early MAR. On FEB 19 we counted 17 fresh masses in Cell 1 and 30 in Cell 2 – adult males called in both these wetlands. On MAR 5 we discovered 15 egg masses laid in the retention pond one or two days earlier. Breeding by *R. sphenocephala* also occurred at one of our reference ponds, Ellenton Bay, during this period.

  We began an *R. sphenocephala* egg-hatching trial on MAR 18 2009, using buckets at locations 1-6; we used eggs from four "clean" females from Ellenton Bay, 50-75 eggs per bucket, with all females represented at all locations (N=24 buckets). After hatching 8-10 larvae were left in buckets, and after one month survivors were collected for metal analyses.

  A similar pilot study of metal uptake was conducted for *B. terrestris*. On APR 3, 2009, eggs (N=30-50) from four clutches were placed in buckets at L1, L2, and L6. After hatching, 10 larvae were kept in each bucket for up to 1-mo and survivors collected for metal analyses.

- *#2 Narrowmouth Toad* – On JUN 1 2009, we collected adult *G. carolinensis* from a reference site (Linda's Pond) near Rainbow Bay. We bred adults in aquatic tanks, and allowed eggs to develop and hatch in place; we did not attempt to separate eggs from each female, and the tadpoles represented a mix of 8-10 clutches. On JUN 11 we placed newly hatched tadpoles in bucket arrays (four per location) at the influent and effluent ends of the retention pond (L1 and L2, Fig. II-1) and at the ends of each wetland cell L3-L6, Fig. II-1). Two bucket arrays (N=8) were also placed in Flamingo Bay on JUN 20 as a control.

Ten *G. carolinensis* tadpoles were placed in each bucket and fed Reptomin® sticks (estimated 1/tadpole) once a week. Buckets were inspected five days a week beginning JUN 18 2009, and the number of tadpoles observed was recorded. Inspections also included looking for aquatic invertebrates that may have colonized buckets and major developmental events such as emergence of forelegs and hindlegs. On JUN 23 2009, new sets of two buckets each were placed at the influent and effluent ends of the retention pond and of Cell 1. On JUN 24 2009, periphitometers were placed at H-02 and Flamingo Bay and a max/min thermometer was place at the influent end of the retention pond. Sub-samples of tadpoles were collected on JUN 26, JUL 4, and JUL 13 to be used in metals analysis. The experiment ended JUL 13 2009.

- *#3 Southern Leopard Frog* – From SEP 20-23, 2009, many *R. sphenocephala* bred at the H-02 site. We collected fresh egg masses from four females on SEP 23, hatched eggs over several days, and began another field trial on OCT 1. Females were treated as an independent variable in this study, and clutches were partitioned separately at each location. Fifteen *R. sphenocephala* tadpoles from each clutch were placed in a bucket at locations 1-6 (N=4 buckets per location). Larvae were supplementally fed a mixture designed specifically for tadpoles that ensures the micronutrients necessary for metamorphosis. An exact count of tadpoles was made once a week. On Nov 5 and Dec 4, two tadpoles were collected from each bucket to be used for metals analysis. This trial is ongoing.

H-02 Laboratory Experiments — Simultaneous variation in multiple water chemistry parameters in the field adds another layer of complexity to interpreting the results. By manipulating just one or two factors under controlled laboratory conditions, we hope to identify the most important variables affecting amphibian aquatic success in the H-02 system. Amphibian eggs were placed in 0.5-L containers with 400 ml of the synthetic water and Cu solution. To date we have conducted the lab studies on one species, the southern leopard frog (*R. sphenocephala*). Additional studies on other species are planned to try to tease apart the complex interactions of pH, Cu, and Zn levels.

- Our first egg study on *R. sphenocephala* was conducted in the SREL greenhouse. Eggs from three H-02 females were collected on Sep 21 2009. In this initial study, our objectives were primarily to assess the effects of pH on egg development and identify the Cu concentration at which egg development was severely impaired. We placed 15-30 eggs of each *R. sphenocephala* female in 400-ml containers of each Cu (0, 10, 50, 100, 150 ppb) and pH (7.5, 9.0) treatment, replicated twice, for a total of 60 containers. Eggs were allowed to hatch and larvae develop to the feeding stage. The trial began SEP 23 2009 and ended SEP 29 2009. During inspections, mortality, behavior, and malformations were recorded.

- In the second study we raised eggs from four female *R. sphenocephala* to the feeding stage in five copper treatments in the SREL Animal Care Facility. Egg masses were collected from a reference site (Ellenton Bay) on OCT 14, 2009. A sample (~30 eggs) from each clutch was collected for genetic analysis. We placed 10-20 eggs from each female in each of five Cu concentrations (control, 10, 50, 100, and 150 ppb), replicated four times for a total of 80 containers. Containers were randomized on a table and inspected daily. The trial began OCT 16 and ended OCT 27; during inspections, mortality, behavior, and malformations were recorded.
- In a study of the effects of copper concentration on larval growth and survival, we placed individual larvae from four females in 1-L containers (~800 ml of solution) at four copper concentrations – 0, 10, 50, and 100 ppb (13 reps per treatment, N=208 containers). Tadpoles are fed a size-adjusted food ration and water is changed weekly. The trial was started in early NOV 2009, and is currently ongoing.

Metals Analysis – Biological samples collected for metal analysis were freeze-dried and ground with a coffee grinder to homogenize the sample—when possible individual samples were used, but in instances of small sample mass (e.g., small tadpoles or metamorphs) samples with similar history were combined. We digested a subsample of each homogenized sample (~250 mg) in 10 ml of trace metal grade nitric acid (70% HNO_3) using microwave digestion (MarsExpress, CEM Corp., Matthews, NC). After HNO_3 microwave digestion, we brought samples to a final volume of 15 ml with 18 MΏ deionized water. We used inductively coupled plasma mass spectrometry (ICP-MS) (Perkin Elmer, Norwalk, CT) to determine concentrations of Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Lead (Pb), Mercury (Hg), Nickel (Ni), Selenium (Se), Strontium (Sr), Vanadium (V), and Zinc (Zn) in our samples as well as in certified reference material (TORT-2 and LUTS-1; National Research Council of Canada, Ottawa, Canada).

The Rainbow Bay Reference Site – The amphibian community has been monitored for 31 years at Rainbow Bay (RB) in Barnwell County on the SRS, approximately 3 km from the H-02 wetlands. Rainbow Bay is a relatively undisturbed freshwater wetland known as a Carolina bay (Sharitz 2003). Carolina bays are natural elliptical depressions that vary in size (long axis extremes from 50 m to 8 km; Sharitz and Gibbons 1982) and in the degree to which they retain water.

Rainbow Bay differs from the H-02 constructed wetlands in many respects – it does not have stream water input, and thus is filled by rainfall; it is a temporary pond with a surface area of approximately 1 ha and a maximum water depth of 1.4 m. Although it was once an herbaceous wetland dominated by rush *(Juncus repens),* spike-rush *(Eleocharis sp.),* bulrush *(Scirpus cyperinus),* panic grass *(Panicum verrucosum),* and knotweed *(Polygonum sp.)*, several prolonged drought periods have resulted in a more closed canopy wetland dominated by sweetgum *(Liquidambar styraciflua),* swamp tupelo *(Nyssa biflora),* and red maple *(Acer rubrum).* This study site is not unusual in any obvious manner relative to other amphibian breeding ponds of its size in this region (Sharitz 2003), except for its relatively undisturbed condition and protected status during the last 59 years. The number of species of amphibians (27) we have observed in our study at RB is representative of the diversity found in southern regions of the U. S. For example, Dodd (1992) collected 16 species of amphibians during a six-year study at a small pond in the north Florida sandhills. The similarity that RB has with the H-02 wetlands that make RB of value as a reference site is that both sites are currently fish-free and in close proximity (< 3 km). Because all permanent water habitats on the SRS contain fish, and fish have such a strong impact on amphibian species success, long-term data from a fish-free reference site are critical.

Drift Fence Sampling -- We have sampled the amphibians migrating to and from RB using a terrestrial drift fence with pitfall traps. The pond was encircled by a drift fence of aluminum flashing (440 m long, 50 cm high, buried 10-15 cm deep in the ground) in SEP 1978. Pitfall traps (40-L buckets) were buried inside and outside the fence flush to the ground and next to the fence at 10-m intervals. These traps have been checked daily from 21 SEP 1978 through NOV 2009. For many species, this sampling technique has provided a nearly complete annual census of the number of breeding adults and of juvenile recruitment, and thus a thorough understanding of "natural" amphibian population dynamics.

Results

H-02 wetland captures – We recorded 2972 captures of 27 amphibian and reptile species at the H-02 treatment wetlands from Jun 2008 through Nov 2009 (Table II-1), including 10 new species captured in FY-09 that were not documented in FY-08. As expected, amphibians occurred in far greater abundance than reptiles, due to their reliance on aquatic habitats for breeding and larval development. Compared to FY-08, however, more new reptile species (7) were documented than amphibians (3) – again in FY-09, no salamanders were captured at the H-02 wetlands, indicating that few if any have colonized. Of the 13 frog and toad species captured, the most common were the three ranid frog species: bullfrogs, southern leopard frogs, and green frogs. Similar to FY-08, in FY-09 nine species of frogs and toads produced juveniles in the wetlands (Table II-1).

Metal (Cu and Zn) concentrations in biota – We conducted metal analyses for three amphibian species from the H-02 system across multiple life stages, as well as samples of potential food resources and comparison samples from reference sites (Table II-2). These data are somewhat preliminary, as numerous collected samples still remain to be analyzed. Nonetheless, several trends are apparent. First, Cu and Zn levels from animals collected in the H-02 system are elevated compared to animals from reference sites. Averaged across species, Cu and Zn levels in juvenile, sub-adult, and adult amphibians from reference sites averaged 7.3 and 70.9 ppm respectively, compared to 65.0 and 205.4 ppm in metamorphosed amphibians at the H-02 site. Second, larval body burdens of

TABLE II-1. The 27 species of amphibian and reptiles captured at the H-02 constructed wetlands from MAY 2008 – SEP 2009. Monthly aquatic sampling was conducted in summer months in FY-08 and FY-09 using minnow traps at 24 systematically chosen sample locations; terrestrial sampling was conducted using three 15-m drift fences with pitfall traps on the north side of the wetlands.

Species	Capture Method	Adults	Larvae/Immature (Juveniles)*
REPTILES:			
Lizards			
Anolis carolinensis	fence	2	3
Eumeces fasciatus	fence	3	1#
Eumeces laticeps	fence	3	0
Sceloperus undulatus	fence	4	1#
Scincella laterale	fence	5	0
Turtles			
Sternotherus odoratus	fence	2	0
Trachemys scripta	fence	0	2
Snakes			
Cemophora coccinea	fence	2	0
Diadophis punctatus	fence	0	1#
Nerodia fasciata	aquatic trap	0	1#
Storeria occipitomaculata	fence	2	0
Tantilla coronata	fence	10	1
Thamnophis sirtalis	fence	1	3#
Virginia valeriae	fence	1	0
AMPHIBIANS:			
Frogs & Toads			
Acris gryllus	aquatic trap	3	2 (0)
Bufo terrestris	fence	47	35 (293)#
Eurycea cirrigera	fence	1	0 (0)
Gastrophryne carolinensis	fence	153	13 (95)#
Hyla chrysoscelis	fence	0	0 (..1)#
Hyla cinerea	fence & aquatic trap	5	6/3 (45)#
Hyla gratiosa	fence	0	5 (3)#
Hyla squirella	fence	0	0 (3)#
Pseudacris crucifer	fence	2	0 (0)
Rana clamitans	fence & aquatic trap	2	116/112 (506)#
Rana catesbeiana	fence & aquatic trap	6	78/93 (213)#
Rana sphenocephala	fence & aquatic trap	102	86/184 (706)#
Scaphiopus holbrookii	fence	8	0 (0)
TOTALS (all species)		364	341/402 (1865)

** The "Larvae" category indicates captures of amphibian tadpoles; "Immature" represents amphibian or reptile individuals that were too young to sex accurately; "Juveniles" are amphibians that were identified as having recently metamorphosed from the wetlands based on their size and/or the presence of a small tail nub.*
 # indicates species that produced juveniles in FY-09

Cu in the H-02 system appear to be elevated well above Cu levels in potential food items, although this trend is not as consistent in Zn. Third, larvae appear to lose much (but not all) of their Cu and Zn body burden at metamorphosis, and there may be species differences in metal retention at metamorphosis (e.g., juvenile green frogs appear to have much higher levels of Cu than juvenile leopard frogs, but the trend is reversed for levels of Zn).

TABLE II-2. Copper (Cu) and zinc (Zn) concentrations in samples from the H-02 site and reference locations. H-02 and reference sites highlighted separately.

Site	Location*	Species#	Stage	Cu (ppm) Mean ± 1 SD	Zn (ppm) Mean ± 1 SD
H-02	RP	BUTE	Larval	329.9 ± 66.2	700.4 ± 104.9
H-02	WET	BUTE	Larval	359.9	661.8
H-02	Fence	BUTE	Juvenile	20.8 ± 5.8	113.6 ± 6.8
H-02	Fence	BUTE	Sub-adult	19.6	113.4
H-02	RP	RASP	Larval	351.8 ± 123.5	917.9 ± 278.4
H-02	WET	RASP	Larval	481.1 ± 10.6	721.7 ± 55.2
H-02	Fence	RASP	Juvenile	37.3 ± 28.1	529.1 ± 526.8
H-02	Fence	RACL	Juvenile	182.4 ± 73.5	65.6 ± 9.6
H-02	RP	Algae	--	186.0	696.0
H-02	WET	Algae	--	27.6 ± 11.6	113.2 ± 40.4
Ellenton	BAY	BUTE	Juvenile	9.8	74.6
Ellenton	BAY	PSCR	Juvenile	6.1	62.5
Ellenton	BAY	RASP	Juvenile	3.0	63.2
Rainbow	BAY	RACL	Sub-adult	8.9	78.9
Linda’s	BAY	SCHO	Juvenile	8.5 ± 1.0	75.5 ± 8.0

* Location—samples taken from terrestrial animals captured at drift fences (Fence) or from aquatic animals captured in funnel traps or dip-netted in constructed wetland cells (WET), the retention pond (RP), or natural bay reference sites (BAY)
# Species—BUTE=*Bufo terrestris*, RASP=*Rana sphenocephala*, RACL=*Rana clamitans*, PSCR=*Pseudacris crucifer*, SCHO=*Scaphiopus holbrookii*

Preliminary results for field and lab experiments –

- *Field Experiment #1* – Egg development for both *R. sphenocephala* and *B. terrestris* in the experiments, wetlands, and retention pond appeared to be normal. We observed no difference in hatching among locations for either species. Larval samples collected for future metal analyses are currently stored at -20 °C.
- *Field Experiment #2 – Narrowmouth Toad.* Survival of *G. carolinensis* larvae in all treatments was poor, including the control treatment at Flamingo Bay. All larvae in the retention pond locations (L1 & L2) died within the first week; within 10 days, most larvae at the influent end of the wetland cells (L3 & L4) were also dead. After 12 days only a small number of larvae still survived in the H-02 treatments, all in Cell 2 at L6. No tadpoles at H-02 survived to metamorphosis. In contrast, although survival was also poor at Flamingo Bay, mortality was neither as swift nor dramatic, and 8.9% (5 of 56; 24 of the original 80 larvae were collected for metal analysis) survived to metamorphosis.
- *Field Experiment #3 – Southern Leopard Frog.* Egg survival of *R. sphenocephala* was reduced at higher copper concentrations (50, 100 and 150 ppb) in both egg trials (Figure II-3, Figure II-4). In the ongoing larval study that accompanies the egg trials, after 2 mo the observed mortality is

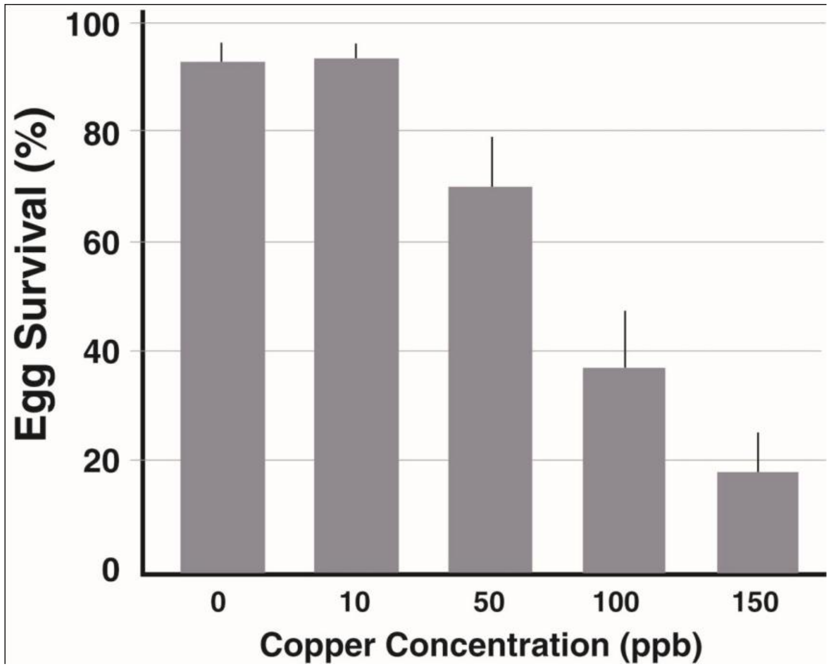

Figure II-3. Egg survival of *Rana sphenocephala* at five copper concentrations; Trial #1 in the SREL greenhouse.

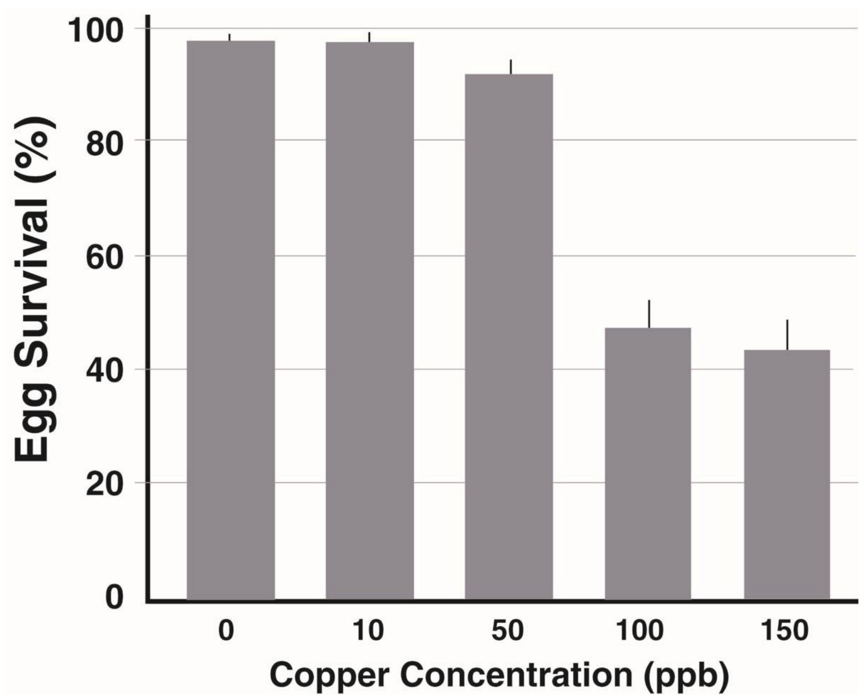

Figure II-4. Egg survival of *Rana sphenocephala* at five copper concentrations; Trial #2 in the Animal Care Facility.

11% in the control treatment, 2% in 10 ppb, 11% in 50 ppb, and 19% in 100 ppb.

Rainbow Bay Comparison – From 2006-2008 regional rainfall was 2.8 to 9.6 in (7.1 – 24.4 cm) below average. Reduced rainfall continued from Jan-Jun 2009, with the 6-mo total for the period 2.86 in (7.26 cm) below average. The continued drought resulted in another relatively dry year at the reference site, Rainbow Bay. The bay first began filling on Mar 2, 2009, and dried on May 1; maximum water level was 39 cm. The short hydroperiod (61 days) precluded successful metamorphosis by almost all species. The exception was the eastern spadefoot toad, *Scaphiopus holbrookii*, which bred at the bay on Apr 3. However, in spite of a very large breeding population (675 females), only 462 juveniles developed quickly enough to metamorphose and exit the bay before it dried.

Discussion and Conclusions

Since the H-02 wetlands serve as an attractant to many species of nearby amphibians, it is important that the water chemistry is not lethal to eggs or larvae and does not affect the reproductive capabilities of individuals or population health as a whole; i.e., the site is not an “ecological trap” or population sink (Rowe et al. 2001). Frog and toad species tend to accumulate high concentrations of many trace elements compared to other aquatic organisms because larvae feed on material that may be mixed with fine sediments. Our results to date indicate that many amphibian species are attracted to the H-02 wetland complex, and 10 species have successfully produced juveniles. Our studies also indicate that these species are accumulating elevated levels of trace metals during the larval period compared to amphibians at reference sites. Pilot experiments that examined the biological effects of Cu and Zn on eggs and larvae have produced mixed results. It is important that we continue to couple experimental studies with our field observations to determine which species may be the most sensitive to trace metals and which portions of the system (if any) may be detrimental to amphibians.

The bioaccumulation of trace metals by amphibians is governed by numerous factors, including the properties of the specific metal ions, conditions in the aquatic environment, and the ecology and life stage of the amphibian species (Roe et al. 2005). Tadpoles graze on submerged surfaces, including sediments, which may result in relatively high bioaccumulation of metals compared to other organisms (Unrine et al. 2007). The duration of the larval period, both within and among species, also influences contaminant levels and effects (Snodgrass et al. 2004, Snodgrass et al. 2005). Previous studies (e.g., Snodgrass et al. 2003) also have documented that the body burdens of metals accumulated by aquatic larvae may be altered dramatically at metamorphosis because of the massive physiological and morphological changes that occur during this period. For example, in bullfrogs the morphological reconstruction during metamorphosis resulted in the virtual elimination of some trace elements accumulated during the larval period (e.g., aluminum, arsenic), whereas other metals (e.g., selenium, strontium) were retained in metamorph tissue (Snodgrass et al. 2003). Our preliminary results on Cu and Zn levels in H-02 amphibians are consistent with these previous findings; i.e., the metal ion, amphibian species, and amphibian life stage all appear to influence metal concentrations. Additional studies should enable us to determine why some species (e.g., narrowmouth toads) may be more sensitive to metals, and whether the transport of Cu and Zn to the terrestrial environment is of concern.

Based on the species diversity and production of juveniles, the H-02 wetland system appears to provide quality amphibian habitat. Future sampling at the H-02 wetlands will reveal whether the permanent-pond amphibian community remains stable, or whether it becomes dominated by fewer species due to either biological or chemical interactions. The consistency of successful reproduction at the H-02 wetlands (coupled with the lack of juvenile recruitment by all species except *S. holbrookii* at Rainbow Bay) demonstrates, once again, the importance of hydroperiod to amphibian communities (Pechmann et al. 1989). Rapid pond drying often causes complete reproductive failure in amphibian species, and early pond drying is a fairly common event at RB that represents a pervasive risk (e.g., Taylor at al. 2006). Hydroperiod variation in natural wetlands promotes a diverse community of amphibians, but the production of juveniles is episodic, with large numbers of metamorphs being produced when the rare combination of favorable conditions occurs. The stable hydroperiod afforded by the H-02 system may result in a less diverse community, but one with more predictable juvenile recruitment. As noted in our FY-08 report, it will be of interest to examine whether changes in the types of predators or the levels of metals in the H-02 system will affect the amphibian diversity as the system ages and becomes more established.

Planned studies in FY-10 – In FY-10 we plan to continue the drift fence and aquatic trap sampling, and expand our laboratory and field experiments to additional species. The apparent sensitivity of *G. carolinensis* to H-02 water chemistry points to the need for additional work on this species, as well as expanding to include other species such as green frogs and bullfrogs that have longer larval periods as well as more exposure to metals in the aquatic habitat as adults. We will continue to test for location effects (which correspond to water quality parameters) on growth and stage-specific survival, and will collect additional subsamples for metals and genetic analyses. We will also collect and analyze metals in the organic film that is found on sediment and plant surfaces, and which is a major component of tadpole food.

In addition to the potential effects of water chemistry on life history traits such as growth and survival, there are concerns that contaminant-induced genetic changes may negatively impact both individuals (through increased mutation rates) and populations (through loss of genetic diversity). In one such study of fish along a metal (cadmium and Cu) gradient, metal contamination had significant genetic effects at both the individual and population levels (Bourret et al. 2008). Bioaccumulated Cu in that study ranged from 20-185 µg/g dry weight; in our analyses of Cu levels in amphibians from H-02 we have found much higher Cu concentrations in larvae of two species (*B. terrestris,* 283-377 µg/g, *R. sphenocephala*, 242-489 µg/g) and terrestrial juveniles of a third (*R. clamitans*, 130-234 µg/g). For FY-10 we propose to include a genotoxicology component to our research. We will develop species-specific microsatellite genetic markers for one species (either *B. terrestris* or *R. sphenocephala*) as the tool to assess genetic effects. We will directly assess germline mutation rates as well as levels of population genetic variation. From these assays we will be able to correlate contaminant levels with germline mutation rates and assess the impacts of contaminant exposure on the population genetics. Together, these genetic endpoints along with the life history endpoints will allow us to infer whether contaminant exposure causes genetic damage and whether this damage is likely to have long-term population-level consequences.

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CHAPTER III – VEGETATION COMMUNITY OF THE H-02 WETLANDS: IMPORTANCE TO AMPHIBIANS

Rebecca R. Sharitz, Paul Stankus, & Linda Lee

INTRODUCTION

In general, natural wetlands tend to have greater vegetation species richness and cover than created wetlands. In many cases, created wetlands may be planted with one or more species to establish early vegetation cover, as was the case with the H-02 wetlands. These wetland cells were planted in FY-07 with the giant bulrush (*Schoenoplectus californicus*), a species that had been successfully established several years earlier in the A-01 wetlands. As the H-02 wetlands mature and undergo natural vegetation succession, the complexity of the giant bulrush community may increase as additional native species become established. Alternatively, the vigorous spread of *S. californicus* plantings may reduce the species richness within the wetland cells. As the wetlands age, the levels of dissolved organic carbon (DOC) also may become more similar to natural wetlands. The higher, perhaps more stable, levels of DOC and organic matter in an older constructed wetland may translate to greater contaminant removal efficiency, and a more suitable wildlife habitat.

Even in an engineered aquatic system dominated by one plant species such as the giant bulrush, the structural complexity of the habitat can be an important component of amphibian success. Many amphibian species require variety in vegetative structure for egg laying. Once hatched, many amphibian larvae need vegetation as cover to hide from predators. In addition, shading provided by vegetation may be important in maintaining appropriate thermal conditions for both larval and adult amphibians. Also, as noted above, the DOC associated with a well-established plant community will reduce metal bioavailability.

In FY-08 we conducted baseline measures of vegetation in the H-02 wetlands, both from the standpoint of the success of the bulrush plantings and overall plant cover and diversity. We established permanent plots and used standard metrics such as plant density, percent cover, and species richness to characterize the plant community. In FY-09 we re-sampled these plots to determine changes associated with the establishment of additional plant species or the loss of species as a result of competition with the giant bulrush. Over time the giant bulrush is expected to remain the dominant species in the constructed facility, but it is not known if the presence of native species will increase or decrease. Vegetation will continue to be sampled and compared from year to year; these data can also be compared with similar created wetland systems (e.g., the A-01 system) and natural wetlands on the SRS.

METHODS

The H-02 wetland cell grid – A vegetation/amphibian sampling grid was established in the wetlands in AUG 2008. Twelve 5-m^2 circular plots were systematically placed across each wetland cell (total N=24) at approximate 30-m (length) by 10-m (width) intervals. Metal-free 75-cm PVC pipe was used to establish permanent mid-points of each plot. These plots were the focal points of both the vegetation sampling and amphibian trapping.

Vegetation sampling – We sampled the vegetation in the H-02 wetlands in AUG 2008 and AUG 2009, to quantify plant species presence, surface coverage of each species, and stem density of the giant bulrush (*S. californicus*). We measured 1.25 m from the mid-point of each plot and circumscribed the 5-m^2 area to be sampled. Within each plot we identified each species, and visually estimated its coverage in seven cover class categories: 1 (<1% coverage), 2 (1-<10%), 3 (10-<25%), 4 (25-<50%), 5 (50-<75%), 6 (75-<95%), and 7 (95-<100%). We combined the duckweed species, *Lemna minor* and *Spirodela polyrrhiza*, into one group as they usually occurred together and it was not possible to estimate their coverages individually. We also counted the number of giant bulrush stems within each plot as a measure of stem density and an estimate of structural complexity.

RESULTS

Species occurrence – We recorded 22 total species (or species groups) of vascular aquatic plants in the H-02 treatment cells, 18 in 2008 and 16 in 2009. Four species or species groups, *Cynodon dactylon, Hyrdocotyle ranunculoides*, the combined *Lemna minor/Spirodela polyrrhiza*, and the planted *S. californicus* were ubiquitous, occurring in 20 or more plots (>83%) across the two wetland cells both years. Eight species were relatively uncommon, occurring in five or fewer plots each year, and the rest were intermediate in abundance. In addition, eight species were found in one of the wetland cells but not in both in 2008; this declined to five species in 2009. Two of these species are considered to be potentially problematic, *Pistia stratiotes* and *Alternanthera philoxeriodes*, found only in Cell 2 in 2009.

Vegetation cover – Average cover estimates per species ranged from less than 1% to greater than 25% in 2008 and greater than 35% in 2009 (Fig. III-1). Species with the greatest cover in both cells included *Cynodon dactylon*, *Hydrocotyle ranunculoides, Lemna minor/Spirodela polyrrhiza*, and *Limnobium spongia* (Fig. III-2). *Sagittaria filiformis,* which was abundant in 2008, declined

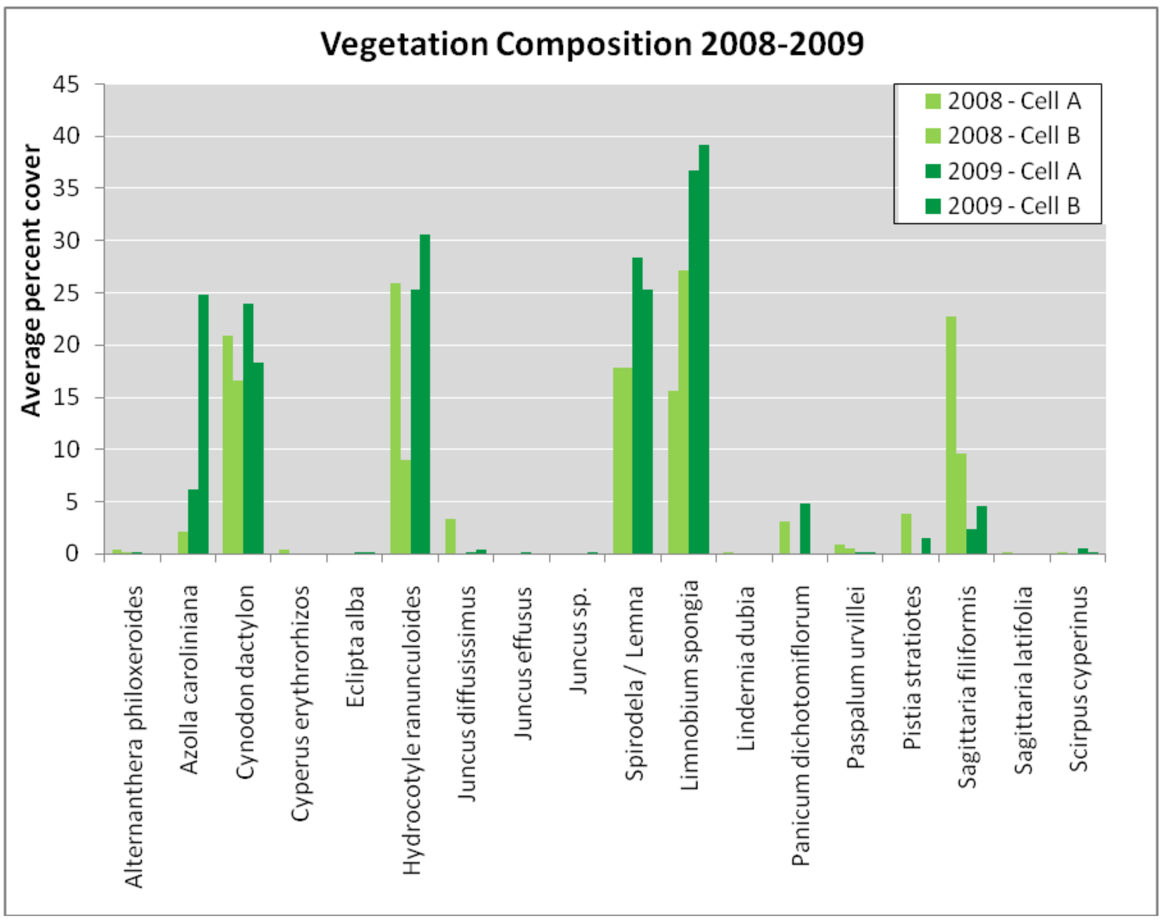
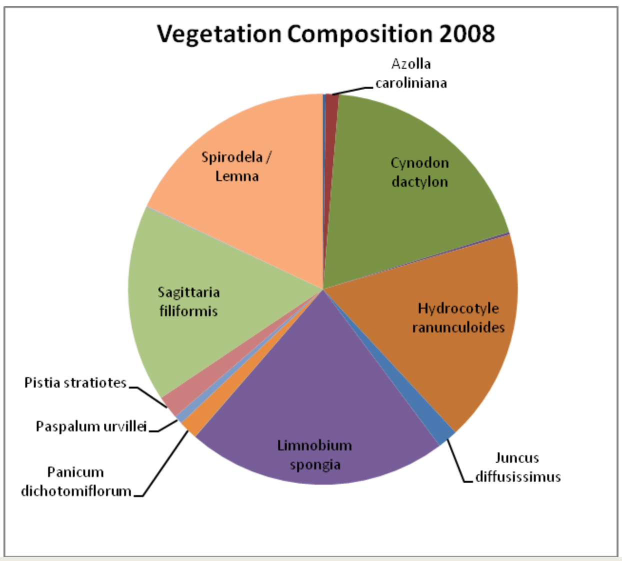


Fig. III-1. Average percent cover of naturally established plant species in the H-02 constructed wetlands in 2008 and 2009, not including the planted *Schoenoplectus californicus.*

substantially in 2009, and *Azolla caroliniana* increased greatly in 2009, especially in Cell 2. Species of algae covered much of the water surface of both cells in both years, but could not be quantified. In addition, submerged floating stems of *Utricularia* sp. were occasionally noted, but percent cover could not be determined. Relative abundance of three of the four dominant species in 2008 remained similar in 2009, however, there was an increase in *Azolla caroliniana* and a decline in *Sagittaria filiformis* (Fig. III-2).



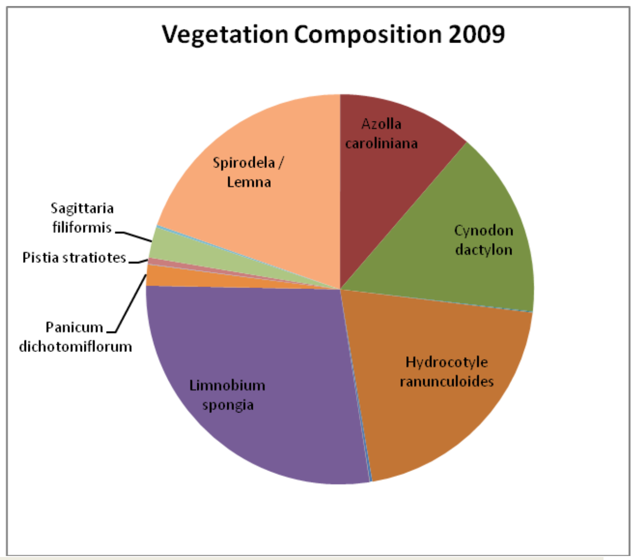


Fig. III-2. Relative percent cover of dominant plant species in both H-02 wetland cells combined, exclusive of *Schoenoplectus californicus*.

Stem density of *Schoenoplectus californicus* – The giant bulrush occurred in all sample plots during both years although there was great variation among plots (Fig III-3). Densities ranged from 4-137 stems/m^2 in 2008 to 11-85 stems/m^2 in 2009, and generally increased between 2008 and 2009, with a few exceptions. Average density for each of the cells was 30-32 stems/m^2 in 2008, increasing to 40-48 stems/m^2 in 2009 (Fig. III-4). By 2009, many of the stems were in dense thickets, and were commonly leaning.

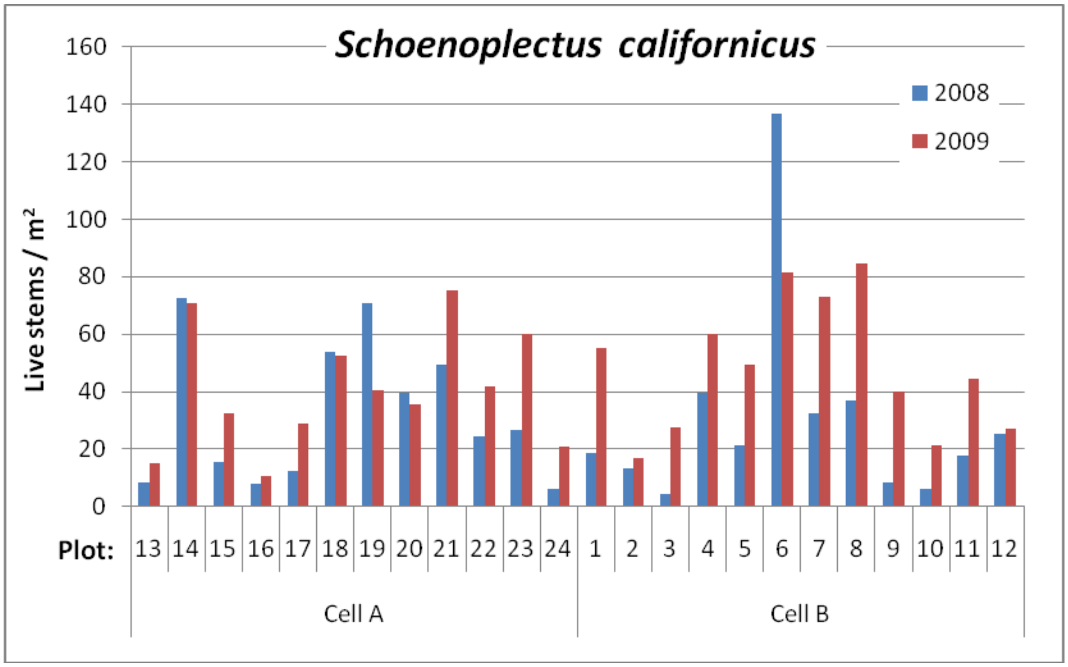


Fig. III-3. Stem density of the planted giant bulrush, *Schoenoplectus californicus*, by plot within each wetland cell in 2008 and 2009

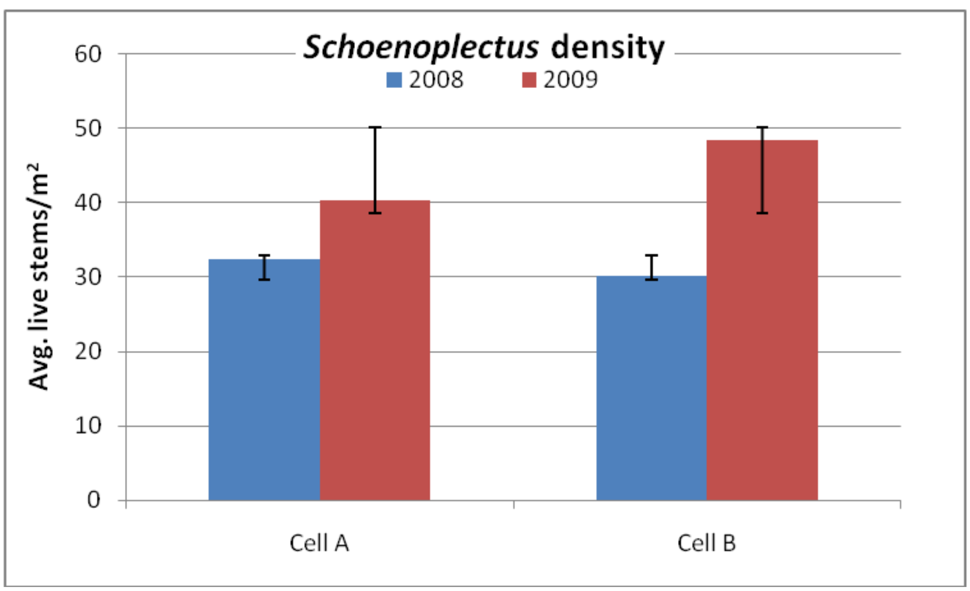


FIG. III-4. Average density of *Schoenoplectus californicus* in each cell of the H-02 wetlands in 2008 and 2009. Error bars are standard deviations.

DISCUSSION AND CONCLUSIONS

The dominant plant species were generally similar between the two cells of the H-02 wetland complex; most differences in species composition reflected differences in the less abundant species. Most of the colonizing plants are typical wetland species of the region that may have been dispersed into the wetland cells by windblown seed or by other vectors such as waterfowl. Additional wetland plants may continue to become established in these wetlands as they mature, although it is likely that the dense growth of the giant bulrush (*S. californicus*) will restrict their spread in deeper water areas. The species composition and the habitat structure provided by these species should support populations of pond-breeding amphibians if other habitat conditions, such as water chemistry, are suitable.

The number of species in the wetland cells is also comparable with that found in small natural wetlands of the region. Recent studies of Carolina bays on the SRS reported plant species richness values between 15-32 (Mulhouse et al. 2005, De Steven & Toner, 2004), although as many as 58 species have been reported in other SRS bays (Kirkman & Sharitz 1994), especially those with highly variable hydroperiods.

Densities of the planted giant bulrush varied greatly among our sample plots in both wetland cells. In a more intensive study of the planting success of this species, substantially higher stem densities were found (E. Nelson, SRNL, *personal communication*, 2008). These differences likely reflect the different locations of the sample plots. Whereas our plots were placed in both edge and center locations in the cells, Nelson's study was designed to determine establishment success and focused on more interior areas of the wetland cells.

It is likely that propagules of some of the plant species that have colonized the wetlands were present in the sediment surrounding the roots of the planted *S. californicus*. Such inadvertent introduction of non-native or potentially undesirable species is a common problem associated with obtaining plant material for wetland construction or restoration (Maki & Galatowitsch 2004). Two of the plants in the H-02 wetlands, *Alternanthera philoxeroides* and *Pistia stratiotes*, are on the South Carolina Department of Natural Resources Aquatic Nuisance Species list (Table III-1). Only small amounts of *A. philoxeroides* were found in the wetland cells, and this species has been reported in other wetland and aquatic sites on the SRS over the years (e.g., Batson et al.1985). However, *P. stratiotes* is a new and highly invasive species that was abundant in one of the wetland cells in 2008 but not the other. In addition, one of the more common plants in both wetland cells, *Cynodon dactylon*, is considered a weedy invasive in South Carolina although it has been previously reported on the SRS (Table III-1). *Limnobium spongia* and *Hydrocotyle ranunculoides* are both new to Aiken County, according to the South Carolina Plant Atlas, and were not reported in the previous plant survey of the SRS (Batson et al. 1985). The plants of *S. californicus*, which does not naturally occur in South Carolina, may have been acquired from southern sites (e.g., Florida) where these other species also occur.

Table III-1. Plant species that are potentially invasive, exotic, or new to Aiken County or the SRS that were found in the H-02 wetland cells.

Species	New to Aiken County[1]	New to SRS[2]	Native, Introduced or Invasive[3]	Illegal in SC[4]
Alternanthera philoxeroides	No	No	Invasive	Yes
Azolla caroliniana	Yes	No	Native	No
Cynodon dactlyon	No	No	Invasive	No
Hydrocotyle ranunculoides	Yes	Yes?*	Native	No
"*Lemna minor* " (= *L. perpusilla*)	Yes?	No	Native	No
Limnobium spongia	Yes	Yes	Native	No
Paspalum urvillei	No	No	Invasive	No
Pistia stratiotes	Yes	Yes	Native	Yes
Sagittaria filiformis (= *S. subulata* var. *gracillima*)	Yes	No	Native	No
Schoenplectus californicus	Yes	Yes	Introduced	No

Sources: [1]South Carolina Plant Atlas; [2]Batson et al. 1985; [3]USDA Plants Database; [4]SC Illegal Aquatic Plant List

*Not reported in Batson et al. 1985

Efforts have been made to remove *P. stratiotes* from the H-02 wetland cell in which it occurs (E. Nelson, *personal communication*). This species reproduces rapidly by vegetative offshoots formed on short, brittle stolons, but it seldom flowers and produces seed, so it may be possible to control this species by continued manual removal. In addition, it is not cold-tolerant, so winter temperatures may also cause a decline in its abundance. The reduced amount of *P. stratiotes* in 2009 reflects the ongoing physical efforts to remove the plants.

The presence of these non-native species demonstrates one of the persistent problems associated with using non-local plant materials in wetland construction or restoration. In an examination of aquatic plant materials ordered from vendors in 17 states, Maki & Galatowitsch (2004) reported that 93% of the orders contained a plant or animal species not specifically requested. In wetland construction or restoration efforts, there is often an initial period of invasion by undesirable species. Typically, if proper hydrologic conditions are imposed, such invasions are temporary (Mitsch & Gosselink 2000), although selective removal may be necessary in the beginning.

Although there are reports of invasive plant species negatively affecting amphibians either directly or indirectly (e.g., a preference by bullfrogs for habitats with the invasive common reed, *Phragmites australis*, which consequently has negative effects on other frog species – Clarkson & Devos 1986), we are unaware of any negative effects of the H-02 invasive plant species on amphibians in a flowing water system such as the H-02 wetlands. Some aquatic invasive plants may increase transpiration and decrease hydroperiod (Zedler & Kercher 2004) in some wetlands, however, and shortened hydroperiods have the potential to impact the amphibian community.

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