H-02 Constructed Wetland Studies

Amphibians and Plants

FY-2010 ANNUAL REPORT

Savannah River Ecology Laboratory
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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 January 2010 to February 2011. 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. Thirteen species of amphibians have colonized the H-02 wetland complex since its construction. Those species differ in their feeding habits and duration of the larval period. To investigate whether species differences in life history affects their sensitivity to metals we examined the effects of Cu exposure on three species of amphibians: southern leopard frogs (*Rana sphenocephala*), southern toads (*Bufo terrestris*), and eastern narrowmouth toads (*Gastrophryne carolinensis*). For all three species we used an exposure period that extends from fertilization to metamorphosis. We conducted all

studies in both a controlled laboratory setting with precise biologically relevant Cu treatments and in the wetlands where larvae were exposed to a water chemistry gradient under ambient field conditions. We observed substantial species-level variation in sensitivity to contaminants at both the egg and larval stages.

Our vegetation sampling recorded 23 total species (or species groups) of vascular aquatic plants in the H-02 treatment cells – 15 in 2008 and 2009 and 19 in 2010. Four species or species groups, *Cynodon dactylon, Hyrdocotyle ranunculoides*, the combined *Lemna minor/Spirodela polyrrhiza*, and the planted *Schoenoplectus californicus* were ubiquitous, occurring in 18 or more plots. Five new species were recorded in 2010: *Hydrocotyle umbellata* (manyflower marsh-pennywort), *Ludwigia repens* (creeping primrose-willow), *Murdannia keisak* (wartremoving herb), *Rubus* sp. (blackberry) and *Typha latifolia* (broadleaf cattail). 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 & Stacey Lance

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 January 2010 to February 2011, 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 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-10 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-10 and early FY-11 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 increases in Cu and Zn levels and 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 – EXPERIMENTAL ASSESSMENT OF AMPHIBIAN USE OF THE H-02 WETLANDS

Stacey Lance, R. Wesley Flynn, Matt Erickson, Tracey Tuberville, Brian Metts, and David Scott

INTRODUCTION

Exposure to environmental contaminants is one of many documented reasons for the amphibian population declines occurring globally (Sparling et al. 2001; Davidson et al 2002; Lannoo 2005). Amphibians are especially sensitive to environmental contaminants due to their highly permeable skin, unshelled eggs, and exposure to terrestrial and aquatic environments at different life stages (Alford and Richards, 1999; Blaustein et al., 2003). In fact amphibians are often considered important bioindicators and environmental sentinels (Blaustein and Wake, 1995). Amphibians are affected by a wide array of contaminants that may occur globally or locally including organics such as pesticides, herbicides, fungicides, and fertilizers and inorganics, such as metals and metalloids (Blaustein et al. 2003). Many of these contaminants can be found in high concentrations in the shallow, lentic or ephemeral wetlands that amphibians tend to breed and develop in (Mann and Bidwell, 2001). Many studies have examined the impact of organic contaminants on amphibians and it is clear that they can have lethal or sub-lethal effects (reviewed in Boone and Bridges 2003; Sparling 2010). However, substantially less is known concerning the effects metals on amphibian populations (Hopkins and Rowe 2010).

The heavy metal copper (Cu) is omnipresent in the environment and occurs naturally in rock, soil, water, sediment, and, at low levels, air. Cu is an essential element crucial for many biochemical pathways (Halliwell and Gutteridge 1984; O'Dell 1990), but can be toxic at concentrations only slightly higher than the normal physiological range (Herkovits and Helguero, 1998). In aquatic systems, levels of Cu are artificially elevated due to anthropogenic activities, such as discharges from industries or domestic wastewater treatments, urban or agricultural runoff, and mining operations. Because Cu does not break down in the environment aquatic organisms can experience both acute and chronic exposure. Copper is known to negatively affect neuro-endocrine processes in fish (reviewed in Handy 2003) and to impair osmoregulation in fish and invertebrates (reviewed in Grosell et al. 2002 and Grosell et al. 2007). In addition, copper can have negative effects on growth and survival of numerous fish species (reviewed by Clearwater et al. 2002). Far less research has examined the impact of Cu on amphibians; however, the existing studies demonstrate a wide array of effects including mortality (Luo et al. 1993; Bridges et al. 2002; Baud and Beck 2005; Chen et al. 2007; Herkovits and Helguero, 1998), embryonic deformities (Luo et al. 1993; Buchwalter et al. 1996; Haywood et al. 2004; Chen et al. 2007), delayed metamorphosis (Parris and Baud, 2004; Chen et al. 2007), reduced body size (Haywood et al. 1993; Lande and Guttman 1973; Chen et al. 2007), and aberrant behavior (Redick and La Point, 2004). These studies have provided insight into the potential effects of Cu, however, they represent only four different species. From the reviews on aquatic invertebrates and fish (Brix et al. 2001; Grosell et al. 2002, 2007) it is evident that species vary greatly in their sensitivity to Cu exposure. Thus there remains a need to examine additional species of amphibians that occur locally on the SRS.

In addition to studying Cu effects on more species, it is critical to also examine chronic exposure throughout egg and larval development. Most of the above studies examining Cu effects only examined acute toxicity, typically 96 hours to seven days. Short-term exposure studies may seriously under-estimate the effects of Cu and other contaminants on survival. For example, when American toads (*Bufo americanus*) were exposed to cadmium (Cd) survival was nearly 100% during the first four days, but by day 60, survival had dropped to only 22% in the highest Cd treatments (James and Little, 2003). Similarly, northern leopard frogs (*Rana pipiens*) chronically exposed to copper survived well (>96%) during the early developmental stages (Gosner stage 19-25), but less than 10% of tadpoles in the high Cu treatment survived to metamorphosis (Chen et al. 2007). Furthermore, concentrations that are sublethal during acute exposure may be lethal during chronic exposure (Bridges 2000). Given the suspected role of contaminant exposure on amphibian population declines (Sparling et al. 2001; Davidson et al 2002; Davidson 2004; Lannoo 2005), it is important to examine the full range of ways exposure can negatively impact a population. Thus it is critical to chronically expose amphibians and to measure both lethal and sublethal effects.

Examining multiple response variables is important for assessing how environmental contaminants affect a population. It is clear that contaminants can kill animals directly, but exposure can also impair reproduction, reduce growth rates, disrupt normal development, or increase susceptibility to disease (Carey and Bryant, 1995). For amphibians in particular there are numerous attributes known to influence survival and reproductive success. For example, size at, timing of, and survival to metamorphosis all influence recruitment into the adult population (Scott, 1994) and thus these attributes can be used to assess the effects of biotic and abiotic stressors on amphibians (Smith, 1987; Semlitsh et al., 1988; Bridges, 2000). Larger size at metamorphosis can have a number of benefits including greater overwintering success, survival to first reproduction, and earlier reproduction (Smith, 1987; Semlitsh et al., 1998). Additionally, larger females can carry more eggs, and larger males can access more females during breeding, leading to increased reproductive success (Berven, 1982). Delayed metamorphosis, or reduced size at metamorphosis can impact demographic processes of a population, potentially leading to declines or even local extirpations (Bridges, 2000). For amphibians breeding in temporary ponds, delayed metamorphosis can result in death due to pond desiccation and limit juvenile recruitment into a population. When average age at reproduction is increased, population growth rates decrease (Stearns, 1992). A shift towards later reproduction in an amphibian community can alter the demographic structure of a population resulting in a gradual decline in size over time. For amphibians then it may be especially important to examine sublethal effects of chronic contaminant exposure in addition to the more traditional lethal effects of acute exposure.

Our objectives in FY-2010 and early FY-11 were to examine the effects of Cu exposure on *Rana sphenocephala*, *Gastrophryne carolinensis*, and *Bufo terrestris*. Our objectives included 1) investigating species that inhabit the H-02 wetlands but on which no previous Cu studies have been done, 2) examining both acute and chronic Cu exposure, 3) examining lethal and sublethal effects, and 4) measuring the bioaccumulation of Cu.

METHODS

Field Capture – 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. 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 (Rainbow Bay and other isolated 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 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.

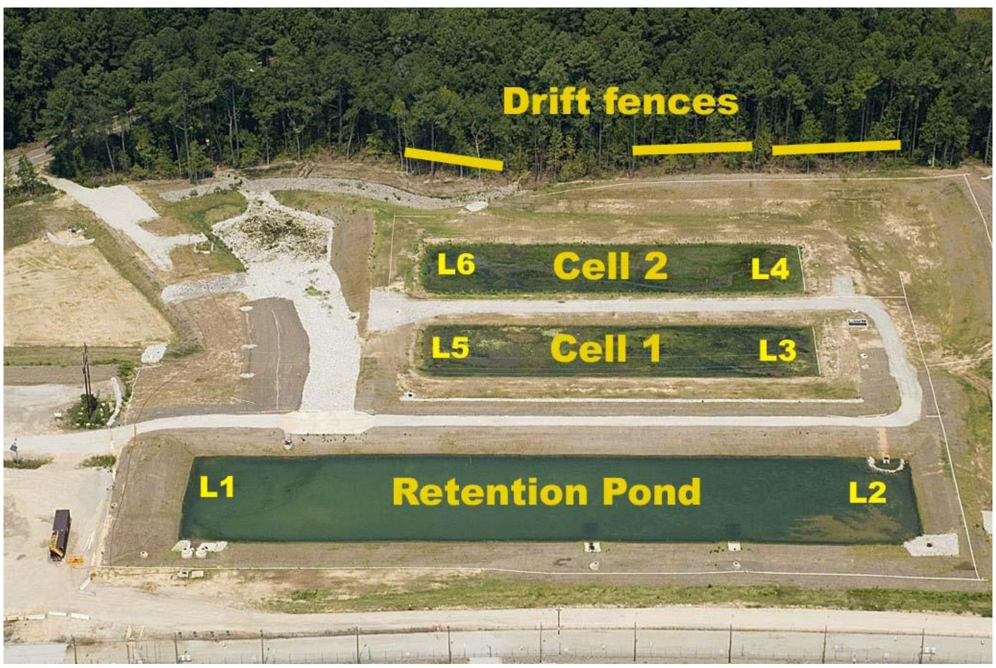

Figure II-1. Drift fence/pitfall trap and egg/larval field experiment locations within the H-02 wetland system. Locations L1 & L2 (retention pond), L3 & L4 (influent end of wetland cells), and L5 & L6 (effluent end of wetland cells) represent a gradient of water chemistry along which the “bucket studies”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

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 (*B. terrestris*) 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 (*G. carolinensis*) 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 (*R. sphenocephala*) 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.

Laboratory and field studies on the effects of trace metals:

We are conducting laboratory and field experiments to test the effects that trace metals have on three common amphibian species. In the laboratory studies described below, eggs and larvae were reared in SREL's greenhouse and/or 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. Experiments in FY-09 documented that egg and early hatchling survival of *R. sphenocephala* was reduced at higher Cu concentrations (50, 100 and 150 ppb). In FY-10 we conducted additional field and laboratory experiments on larval *R. sphenocephala*, as well as eggs and larvae of *B. terrestris* and *G. carolinensis*. Our specific methods vary slightly across the three species but our general methods are described here.

Egg Trials

Our egg trials are conducted in the SREL greenhouse and/or Animal Care Facility. Our objectives were primarily to identify the Cu concentration at which egg development was severely impaired. We either collected fresh egg masses from the field (*R. sphenocephala*) or collected and bred pairs of animals in the lab and used the resulting eggs (*B. terrestris, G. carolinensis*). We carried out all egg trials to Gosner stage 24-25, when larvae are free swimming and feeding. For all species, we placed a sample of eggs from each female in 0.5-L containers with 400 ml synthetic water and Cu solution. Since it was difficult to count and remove a specific number of eggs without damaging them, we took a portion of each egg mass containing 15-30 eggs and counted them after they were placed in treatment. We used different treatments of Cu concentration ranging from 0 to 150 ppb Cu. We placed the containers on a table in a randomized fashion and inspected them daily to note and remove any nonviable/dead individuals.

Larval Trials

For the larval trials we used a subset of the survivors from the egg trials. We reared individual larvae in 1-L containers (~800 ml of solution) at the same Cu concentration they experienced during the egg trial. We placed all containers on tables and inspected them daily. Every week we fed them a size-adjusted food ration and changed the water. To track growth, every week we weighed a random subsample of 6 individuals from each treatment and weighed all tadpoles. When individuals died we documented their mass, presence or absence of malformations, and date of mortality. For individuals that survived to metamorphosis we measured mass, snout-vent length, and noted when front legs appeared and when the tail was resorbed. Malformations were present only in tadpoles and included edema and scoliosis of the tail. When tadpoles died in treatment or were collected during field trials we stored them in whirl-pacs® at -20 ^{o}C for metals analysis. For metamorphs we used immersion in 3% tricaine methanesulfonate (MS-222) as a method of euthanasia. For 29 of the *R. sphenocephala* metamorphs we collected part of the liver for gene expression studies (see “In Progress” below).

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*).

In our in situ field experiments, we reared eggs in 12-L floating buckets at each of six locations (influent and effluent ends of the retention pond and two wetland cells) in the H-02 system to evaluate responses along the water chemistry gradient (Figure II-1). We constructed bucket enclosures by drilling

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.

three 5-cm holes around the top of the bucket and covering the holes with aquarium silicone and mesh screen to allow ambient water flow through the bucket and colonization by algae. The buckets were cured and then submerged in water for 24 hrs to allow irritants to leach from the silicone. We fitted the buckets with square flotation collars made of Styrofoam to keep experimental animals suspended in the water column. To exclude potential predators, we covered the tops of buckets with a fine mesh (Figure II-2).

Females were treated as an independent variable in this study, and tadpoles from each clutch were reared in separate buckets. Thus, at each of the six locations we had four buckets (one per female) and we placed 15 tadpoles into each bucket. Larvae were supplementally fed weekly. An exact count of tadpoles was made once a week. In order to examine uptake of metals over time, we collected and analyzed serial samples of tadpoles for Cu and Zn concentrations. We based the number of tadpoles collected during the final sampling effort on density. Since the serial samples previously collected were too small to be analyzed for metals independently, we took more samples from high-density buckets. Since some buckets had low numbers of survivors, we took fewer samples to ensure some tadpoles reached metamorphosis. Tadpoles were collected upon emergence of the front legs and metamorphosis was completed in the laboratory. We placed pre-metamorphic tadpoles in 1-L containers with a paper towel moistened with water from their respective locations in the wetland.

Metals Analysis

We freeze-dried biological samples collected for metal analysis and then homogenized each sample in a coffee grinder. When possible we used individual samples, but in instances of small sample mass (e.g., small tadpoles or metamorphs) we combined samples with similar history. We digested a subsample of each homogenized sample (~250 mg) in 10 ml of trace metal grade nitric acid (70% HNO3) using microwave digestion (MarsExpress, CEM Corp., Matthews, NC). After HNO3 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).

RESULTS

Laboratory and field studies on the effects of trace metals:

In general we have observed significant egg mortality at higher Cu concentrations for the three species tested to date (Table 1). Species appear to differ in their sensitivity to Cu treatments (Figure II-3), with *G carolinensis* most sensitive (highest egg mortality at low Cu levels*), B. terrestris* intermediate, and *R. sphenocephala* least sensitive. In addition, in several trials, eggs from different females varied in survivorship (across species; Table 1), and in one trial for *Bufo* the source location of the female (captured at the H-02 site or at a reference location) affected egg response. These results suggest that females may also differ in sensitivity to Cu, which provides the basis for adaptation or acclimation to elevated metal levels to occur over time.

To date we have analyzed the results of the larval experiments for *Rana* and *Bufo* (Table 1). *Bufo* larvae are more susceptible than *Rana* to increased Cu concentrations, although there also appears to be a trend for increased larval mortality even for *Rana* at higher Cu levels (100 ppm; Figure II-4). Comparison of the total body burdens of Cu in laboratory trials versus field experiments indicates that the lab experiments appear to bracket the Cu concentrations in the H-02 system (Figure II-4).

Table II-1. Results of analysis of variance (ANOVA) for effects of copper on egg and larval survival for three amphibian species.

Species	Source (effect)	DF	F-value	P-value
<u>Egg survival</u>				
R. sphenocephala				
Trial 1	Cu	4	73.3	<0.0001
	Female	3	2.04	0.1162
Trial 2	Cu	4	41.8	<0.0001
	Female	2	17.7	<0.0001
B. terrestris				
Trial 1	Cu	4	82.3	<0.0001
	Location	1	107.7	<0.0001
	Female	6	4.9	<0.0002
Trial 2	Cu	5	7.7	<0.0001
	Female	3	15.1	<0.0001
G. carolinensis				
	Cu	7	335.8	<0.0001
	Female	3	0.9	0.435
<u>Larval survival</u>				
R. sphenocephala				
	Cu	3	3.0	0.09
	Female	3	9.7	0.0036
B. terrestris				
	Cu	5	30.7	<0.0001
	Female	3	3.1	0.0610

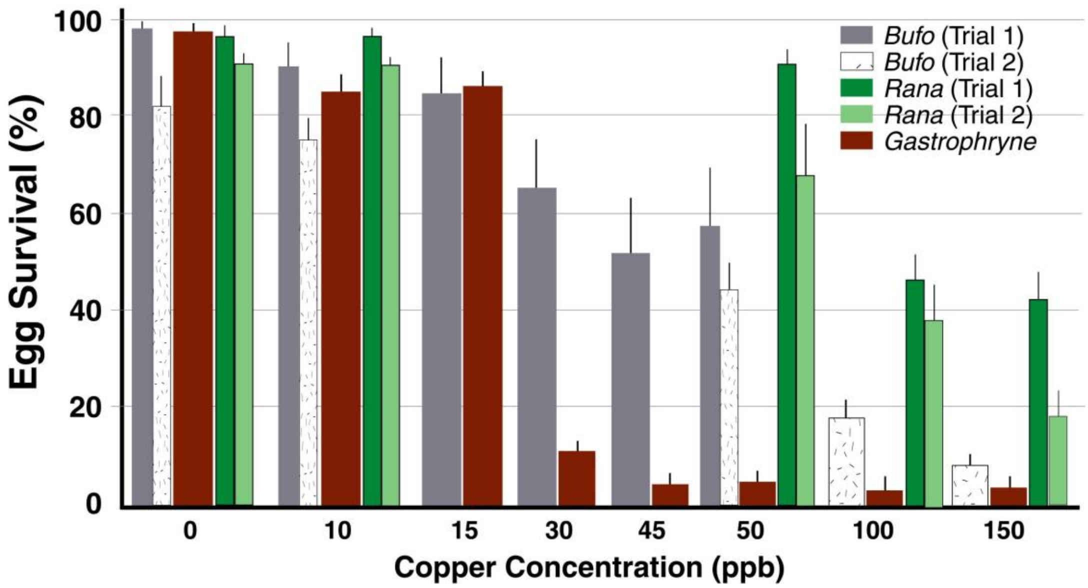

Figure II-3. Combined egg survival for three amphibian species across a range (0-150 ppm) of copper concentrations. All trials were conducted with 30-50 eggs in 400-ml containers. .

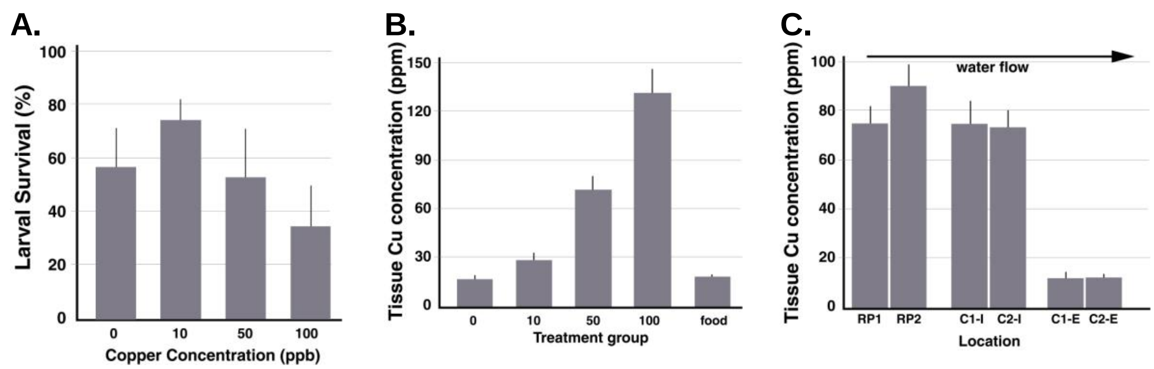

Figure II-4. A. Larval survival of *R. sphenocephala* tadpoles reared at four Cu concentrations; B. Final body burdens of copper in metamorphs from the larval treatment groups; C. Final body burdens of copper in metamorphs from larvae reared across the water quality gradient in the H-02 wetland system (RP = retention pond locations, C-I = influent ends of wetland cells, C-E = effluent ends of wetland cells).

Field Capture:

We continue to capture multiple amphibian and reptile species at both H-02 and Rainbow Bay reference site. No new amphibian species occurrences have been documented at either site and leopard frogs, green frogs, and bull frogs remain the most common species. However a new reptile occurrence was documented when for a brief period a juvenile alligator used the H-02 wetland complex. We still have not recorded a single species of salamander at the H-02 wetlands. In the winter of 2010 we counted more than 200 southern leopard frog egg masses in the H-02 complex. Though we do not know what percent of these eggs survived it is clear that leopard frogs are using it as a breeding ground. At Rainbow Bay the marbled salamander remained the dominant species. In spring of 2010 over 10,000 metamorphs were captured and marked leaving the wetland. In addition, mole salamanders successfully reproduced at Rainbow Bay for the first time in several years.

In Progress:

Continuation: We are currently working on three manuscripts detailing our studies on the three species. In addition we are working on genetic analyses of the southern leopard frogs. We created cDNA libraries from the 29 liver samples we collected from metamorphs. Those samples have been fully prepared and sent out to a core facility to be sequenced using the Illumina platform. These data will allow us to directly compare the gene expression profiles of individuals, thereby comparing the activity of 1000's of genes at once. These profiles will indicate which sets of genes are up or down regulated in response to contaminant exposure that in turn provide insight into what biological pathways are impacted (such as oxidative stress, apoptosis, DNA repair, oncogene regulation).

New Experiments: Based on our results of examining the effects of Cu on the three species of amphibians we are designing and conducting new experiments. To date our laboratory experiments have only examined the effects of Cu from the aqueous environment. In the field larvae are exposed to Cu in the aqueous environment and also additional, more concentrated Cu levels in their food. In addition, larvae in the H-02 system are exposed to Zn in the water and food. Zn has been shown to ameliorate the toxic effects of Cu in some aquatic organisms. We are just now completing a pilot study examining the combined effects of exposure to Cu and Zn in southern leopard frog eggs. We have also fully designed a large-scale experiment for southern toads. That experiment will examine the effects of both Cu and Zn and also compare Cu exposure from water and food. In addition to field and laboratory studies we will include mesocosm studies. Mesocosms will allow us to examine density, competition, and simultaneous aqueous and food exposure to metals in a more realistic environment. Our study on southern toads will start in March 2011 and be carried through into the next cycle of funding. We will also incorporate gene expression studies into this project.

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. The results of our egg and larvae trials clearly indicate that Cu can negatively affect survival. Most importantly the three species responded quite differently. It is vital to further investigate these and other species to accurately assess the effects. In addition, we have seen that the collection location of the female impacts our results. This could indicate that the longer females inhabit the H-02 complex the lower their reproductive capacity will be and/or that over generations it may become more of a sink. Alternatively, over many generations animals may become better adapted to the conditions. Future studies are needed to evaluate this risk.

Our results also clearly indicate how effective the H-02 wetlands are at removing Cu and Zn from the water. Our *in situ* studies show that animals reared in the effluent ends of the wetland cells accumulate significantly less metal than those in the influent ends or the retention pond.

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 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.

BROADER IMPACTS

In 2010 we presented our research on the H-02 wetland amphibian studies at three scientific conferences. In March we presented at the meeting of the Southeastern Chapter of the Society for Environmental Toxicology and Chemistry held at the University of Georgia. In July we presented at the annual meeting of the American Genetic Association held at the University of Hawaii, Hilo. In November we presented at the 8th Annual Ecological Genomics Symposium. All three presentations included young scientists gaining research experience after earning their undergraduate degree. Matt Erickson has now started a MS degree program at Georgia Southern University. In addition, two undergraduate students (Clemson University and Allen University--both women, one from an underrepresented group) assisted with the research in summer of 2010. We also will be presenting our work this spring at the 2011 Georgia Water Resources Conference and have had a paper "Effects of Copper on Amphibians Inhabiting a Constructed Wetland on the Savannah River Site" accepted for the proceedings of the conference. Finally, this winter we hosted a reporter and film crew from the *USA Today* and brought them to the H-02 wetland complex and discussed our research.

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

Rebecca R. Sharitz, Linda Lee, & Paul Stankus

INTRODUCTION

Natural wetlands typically 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 *Schoenoplectus californicus* (California giant bulrush), a species that had been successfully established several years earlier in the A-01 wetlands. As the H-02 wetlands mature, the complexity of the vegetation community might increase as additional native species become established. Alternatively, the vigorous spread of the giant bulrush plantings may reduce the species richness within the wetland cells. In addition, 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 wetland 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; and 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 to determine the success of the bulrush plantings and to assess 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 and FY-10 we re-sampled these plots to track development of the plant community over time, such as establishment of additional plant species through dispersal 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 August 2008. Twelve 5-m^2 circular plots were systematically placed across each wetland cell 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 August 2008, August 2009, and early September 2010, to quantify plant species presence, surface coverage of each species, and stem density of *S. californicus* (giant bulrush). 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 *Lemna minor* and *Spirodela polyrrhiza*, the duckweed species, into one group as they usually occurred together and it was not possible to estimate their separate coverages. (Hereafter in this report they will be treated as a single species.) We did not attempt to determine cover values of submerged vegetation (e.g., *Utricularia* spp. [bladderworts], algae) as surface vegetation obscured the presence of these species. Coverage estimates were not used for the bulrush plantings; in order to gain a better estimate of structural complexity, and to monitor success of the plantings, we counted the number of bulrush stems in each plot.

RESULTS

Species occurrence – We recorded 23 total species of vascular aquatic plants in the H-02 treatment cells, 15 in 2008 and 2009 and 19 in 2010. Four species, *Cynodon dactylon* (Bermudagrass), *Hydrocotyle ranunculoides* (floating marsh-pennywort), *Lemna minor* and *Spirodela polyrrhiza* (the duckweeds), and the planted *S. californicus* (giant bulrush) were ubiquitous, occurring in 18 or more plots each year. Nine species were relatively uncommon, occurring in five or fewer plots each year, and the rest were intermediate in abundance. Five new species were recorded in 2010: *Hydrocotyle umbellata* (manyflower marsh-pennywort), *Ludwigia repens* (creeping primrose-willow), *Murdannia keisak* (wartremoving herb), *Rubus* sp. (blackberry) and *Typha latifolia* (broadleaf cattail). 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 and 2010.

Vegetation cover – Average cover estimates per species ranged from less than 1% to greater than 20% in 2008, and to greater than 25% in 2009 and 2010 (Fig. III-1). *Limnobium spongia* (American spongeplant) and *Lemna minor/Spirodela polyrrhiza* (the duckweeds) were major components in all three years. Coverage of *Azolla caroliniana* (mosquito fern) has increased since 2008, while *Sagittaria filiformis* (threadleaf arrowhead), *Hydrocotyle ranunculoides* (floating marsh-pennywort) and *Cynodon dactylon* (Bermudagrass) have decreased each year. Species of algae covered much of the water surface of both cells in 2008 and 2009, but could not be quantified. However, casual field observations suggest that coverage of algae was reduced in 2010. *Pistia stratiotes* (water lettuce), a highly invasive species that was reported in one of the wetland cells in 2008 and 2009, was not found in the 2010 survey.

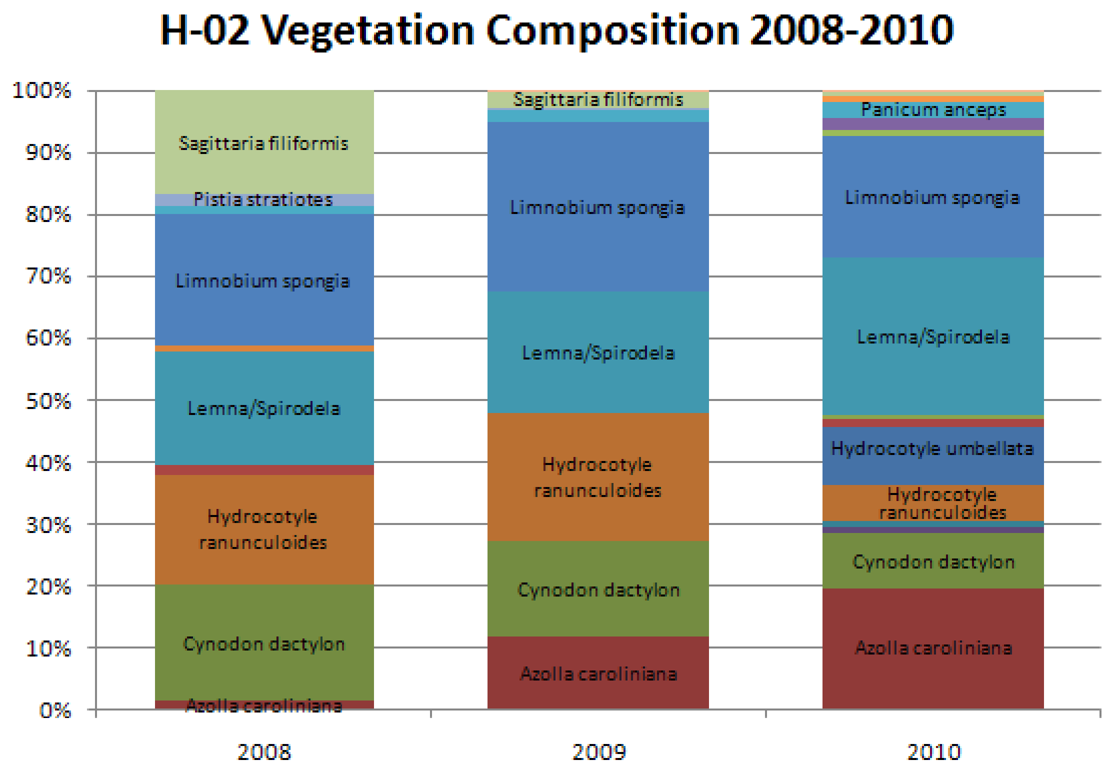

Fig. III-1. Average percent cover of naturally established plant species in the H-02 constructed wetlands in 2008, 2009 and 2010, not including the planted *Schoenoplectus californicus.*

Stem density of *Schoenoplectus californicus* **–** The planted giant bulrush occurred in all sample plots during all three years although there was great variation among plots (Fig III-2). Densities ranged from 8-137 stems/m^2 in 2008, to 17-85 stems/m^2 in 2009, and 8-55 stems/m^2 in 2010. The average density was somewhat lower in 2010 than in 2009 (Fig III-3) but remains higher than in 2008. Many of the stems were in dense thickets, and were commonly leaning or blown over.

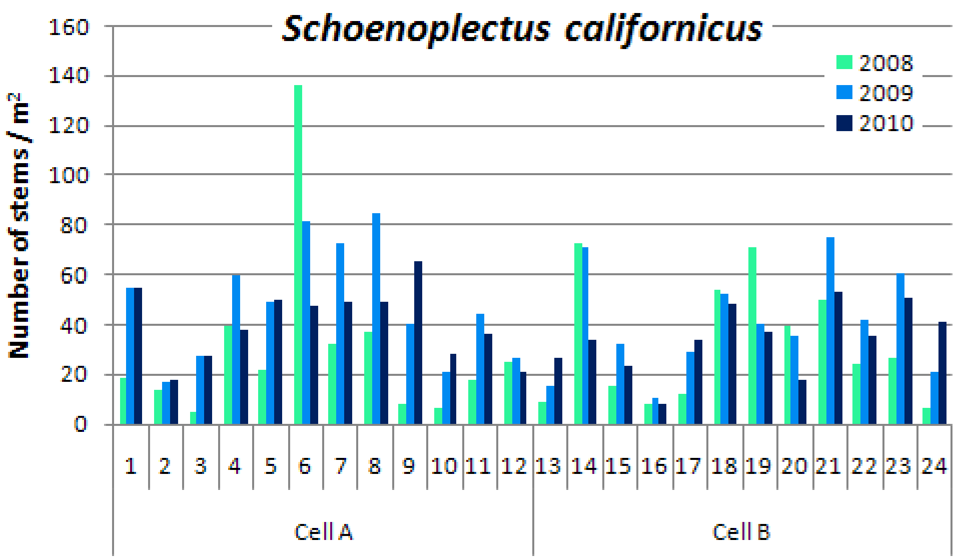

Fig. III-2. Stem density of the planted giant bulrush, *Schoenoplectus californicus*, by plot within each wetland cell in 2008, 2009 and 2010

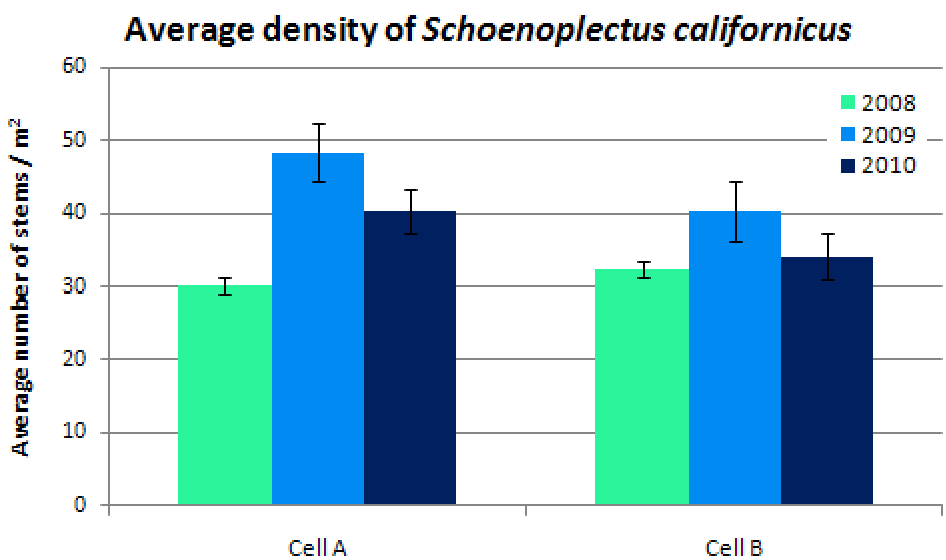

FIG. III-3. Average density of *Schoenoplectus californicus* in each cell of the H-02 wetlands in 2008, 2009 and 2010. Error bars are standard deviations.

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. The planted *Schoenplectus californicus* (giant bulrush) remains the dominant species although several floating aquatics, especially *Limnobium spongia* (spongeplant), *Lemna minor and Spirodela polyrrhiza* (the duckweeds), and *Azolla caroliniana* (mosquito fern) have increased in coverage. Mosquito fern is of particular interest because it is a nitrogen-fixer. Coverage of this species has increased steadily from a small presence in 2008 to nearly 20% of total vegetation cover in 2010. Densities of the planted giant bulrush varied greatly among our sample plots in both wetland cells, but the variability declined in 2010, indicating a more even distribution of stems.

Though *Schoenoplectus californicus* was the only species actively planted in the wetlands, at least 23 additional species have been recorded. Most species found in the cells are typical native species of local wetlands; however, two of the plants previously found in the H-02 wetlands, *Alternanthera philoxeroides* (alligatorweed) and *Pistia stratiotes* (water lettuce), are on the South Carolina Department of Natural Resources Aquatic Nuisance Species list (Table III-1). In 2010, alligatorweed was found in only one sample plot in each of the wetland cells, with a cover value of less than 10%. This species has been reported in other wetland and aquatic sites on the SRS over the years (e.g., Batson et al.1985).

Water lettuce, a highly invasive species new to the SRS, was abundant in one of the wetland cells in 2008. Efforts to eradicate it appear to have been successful. Cover declined in 2009, and in 2010 no individuals were found. Water lettuce is not cold-tolerant, therefore manual removal efforts may have been aided by the unusually cold winter of 2009-2010. Inadvertent introduction of potentially undesirable species is a common problem associated with obtaining plant material for wetland construction or restoration (Maki & Galatowitsch 2004).

Table III-1. Plant species that are potentially invasive, exotic, or new to Aiken County or the SRS that have been 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[5]	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; [5]potentially eradicated from the H-02 wetlands by 2010

*Not reported in Batson et al. 1985

Cynodon dactylon (Bermudagrass), found in 19 of the 24 sample plots, is considered a weedy invasive in South Carolina although it has been previously reported on the SRS (Table III-1). Both *Limnobium spongia* (spongeplant) and *Hydrocotyle ranunculoides* (floating marsh-pennywort) are 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 bulrushes may have been acquired from a nursery where these other species are common, and seeds or other propagules of these plants may have been present in the sediment surrounding their roots.

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 *Phragmites australis*, the invasive common reed, which consequently has negative effects on other frog species – Clarkson & Devos 1986), we are unaware of any negative effects of the H-02 non-native 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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