EDWARDS AQUIFER
HABITAT CONSERVATION PLAN (HCP)
2013 APPLIED RESEARCH

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1.0 EDWARDS AQUIFER HABITAT CONSERVATION PLAN (HCP) APPLIED RESEARCH (2013)

1.1 Executive Summary

The Edwards Aquifer Habitat Conservation Plan (HCP) is founded on long-term biological goals for the covered species that inhabit the Comal and San Marcos springs/river ecosystems. To support the long-term biological goals, flow management objectives (flow regimes) were established that are presumed to be protective of the threatened and endangered species in these systems. The low-flow conditions (discharge and extended durations) incorporated in the HCP flow regime and projected to occur during severe drought have occurred very infrequently (or not at all) during the historical record. Consequently, complete testing of ecological response(s) to these conditions in the wild is unlikely. Therefore, testing of simulated conditions in laboratory and/or field environments is mandatory to address HCP unknowns.

Section 1.1.1 of the HCP describes the two-phased approach incorporated in the program (EARIP 2011). Phase I involves implementing a package of minimization and mitigation measures, as well as springflow protection measures quickly upon issuance of the Incidental Take Permit (ITP). These measures are presently being conducted to provide protection for the species covered by the ITP and their associated ecosystems. As several uncertainties were documented during the development of Phase I, an Adaptive Management Plan (AMP) is concurrently in place for the HCP. The AMP will be guided by information from monitoring data, applied research activities, evaluations of technical and engineering alternatives, improved groundwater models, and developed ecological models (EARIP 2011). The aforementioned activities will all take place during Phase I and assist the Implementing Committee (IC) with decisions regarding appropriate modifications, if any are needed, to the program. Phase II will begin no later than Year 8 of the ITP and if necessary, may require additional measures to achieve the springflows to meet the biological goals of the HCP.

The HCP lays out the path forward for answering key questions and filling in data gaps to test Phase I assumptions and ultimately assist with Phase II decisions. As described in section 6.3.4.2 of the HCP, the focus of Phase I research is on the fountain darter relative to the Comal system as well as the Comal Springs riffle beetle (EARIP 2011). To accommodate scheduling and funding, Phase I applied research was divided into three tiers (Tier A, B, and C) with Tier A focusing on habitat requirements and responses. Subsequently, Tier B will focus on direct impacts to the species from low-flow, while Tier C will come later and build upon tiers A and B to address the implications of timing, frequency, and duration of multiple events as well as assist in validation of ecological models where applicable.

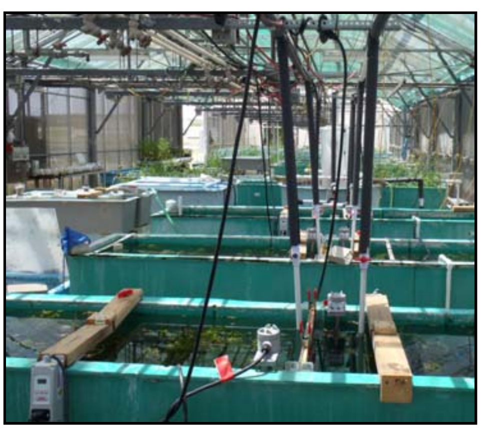
The applied research focus in 2013 was addressing several key questions surrounding physical habitat and food source responses related to the fountain darter (*Etheostoma fonticola*; *hereafter*, fountain darter). Valuable information has been acquired on both response of aquatic vegetation and food source relative to changes in flow and water quality conditions.

The four 2013 HCP applied research studies conducted and discussed in this report include the following:

- ***Field vs. Laboratory Study (Section 2).*** Laboratory vs. field comparison of aquatic vegetation in the Comal ecosystem.
- ***Vegetation Tolerance Studies (Section 3).*** Low-flow threshold evaluation of aquatic vegetation.
- ***pH Drift Study (Section 4).*** Evaluate the effects of bicarbonate (HCO3-) utilization by select aquatic vegetation types present in the Comal and San Marcos springs systems.
- ***Food Source Study (Section 5).*** Low-flow food source threshold study.

Sections 2 through 5 of this report document the immense amount of work that was conducted and analyzed relative to 2013 HCP applied research. Section 6 of this report delves deeper into lessons learned, outlines potential HCP ecological model application, and provides recommendations for future HCP applied research. Important discoveries that could well be substantive contributions to the HCP moving forward are highlighted below.

The ***Field vs. Laboratory Study*** was conducted simultaneously in the Old Channel of the Comal River and the U.S. Fish and Wildlife Service San Marcos Aquatic Resource Center (ARC) greenhouse. This study provided a wealth of information regarding the simulation of field parameters in a laboratory environment as well as the difference between response patterns or trends versus actual results. Although there were statistically significant differences in actual results (some quite large), the overall trends or patterns in results were similar between the laboratory and field. These patterns are encouraging and suggest the laboratory data can be used to project the direction of response of varying environmental conditions with some level of confidence.



This is critical, and very much applicable to the HCP ecological model, since some factors simply cannot be manipulated easily nor tested in the field. For example, sedimentation and herbivory in the field, undoubtedly, attributed to differences in growth between the laboratory and field plants. The statistically significant differences between laboratory and field results is concerning and highlights the uncertainty of using laboratory results solely to make management decisions or to populate ecological models. Overall, data from this study supports the intuitive conclusion that, where possible, field data should be used to project plant growth response.

The field vs. laboratory study was not designed to provide direct input into the HCP ecological model, but rather to test theories behind applied research to better inform decision making and model formulation as the HCP marches on. The take home message is that both laboratory- and field-generated data are valuable, but should be used with caution in ecological models attempting to simulate actual conditions in the wild. As such, it is recommended that ranges of results be used to populate the HCP ecological model rather than specific numbers.

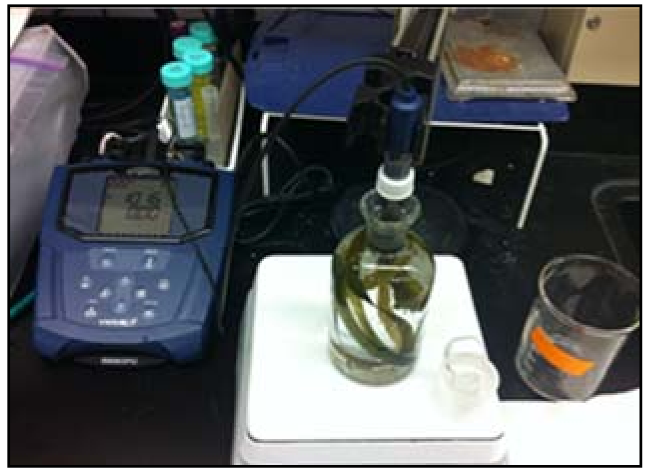
The ***Vegetation Tolerance Studies*** were conducted in both the ARC greenhouse and adjacent pond. The laboratory studies indicated that the four species tested (*Ludwigia repens*, *Riccia fluitans*, *Cabomba caroliniana*, and *Vallisneria* spp.) would likely survive short-term exposure to warm-water conditions with relatively low free carbon dioxide (CO2). This would suggest that if brief periods of low-flow conditions occur, these aquatic plant species would survive exposure up to 34 degrees Celsius (°C) for a period of a few weeks under similar ecological conditions. While there are clearly limits to what the plants can survive (most died at 37 °C), these species appear resilient to short-term perturbations.

The outcome of both the greenhouse and pond experiments suggest that the overall habitat that the aquatic plants of the Comal and San Marcos rivers provide to the listed species would be unlikely to completely disappear with limited temporal exposure to extreme low-flow conditions. With that said, two overarching cautions are in order: (1) data apply only to short-term exposures to the warm, low CO2 growth conditions and assumes the plants remain in the water (not left exposed by low water levels), and (2) data suggest the plants can survive these conditions when all other growth conditions are optimal. The plants were capable of surviving the *physiological challenge* of high temperature and low CO2 as established in the greenhouse and pond, but it may not mean that they would as easily survive the *ecological challenge* that would accompany such conditions in a field setting.

It was also encouraging that *Ludwigia repens* (hereafter, *Ludwigia*), which is a plant of known importance to the fountain darter and a target for extensive habitat restoration (photo of *Ludwigia* grown and used for restoration in Landa Lake above), was one of these plants. This was a major HCP unknown and directly affected the development of the long-term biological goals for the fountain darter. On a less positive note, the build-up of different types and levels of algae over the course of both the laboratory and pond experiments raises questions and concerns as to the potential affect algae will have on aquatic plants in the wild during low-flow conditions.

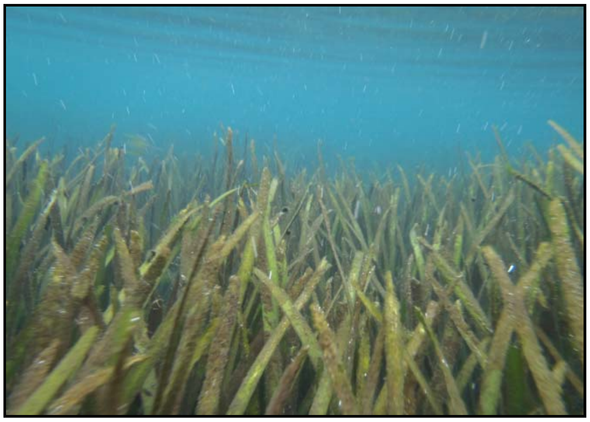
Finally, another observation of the pond study goes beyond the effect of plant survival and/or growth in an outdoor limited or no-flow environment. The water-quality parameters experienced in both the pond pre-trial and formal experiment have the potential to directly impact fountain darters (pictured on the bottom of Landa Lake to the right) and other fishes. The experiment was conducted under hot, summertime conditions in a black-lined pond with water temperatures maxing out above 35 °C. The critical thermal maximum (CTM) for the fountain darter is approximately 34.8 ˚C (Brandt et al. 1993)

with reproduction and larval survival concerns at water temperatures much cooler than that. As such, fountain darters would be unlikely to live for an extended period of time in an environment (no flow, elevated summer air temperatures) like the ones tested in the pond studies. High temperatures and no-flow conditions also resulted in low dissolved oxygen measurements in the mornings near the end of the pond experiment. Reduced dissolved oxygen conditions are stressful to most fishes, and can be lethal if they fall too low. As such, dissolved oxygen concentrations similar to those observed in this experiment may have detrimental effects to fountain darter survival when flows are severely reduced and water temperatures greater than 30 ˚C are observed in the wild.



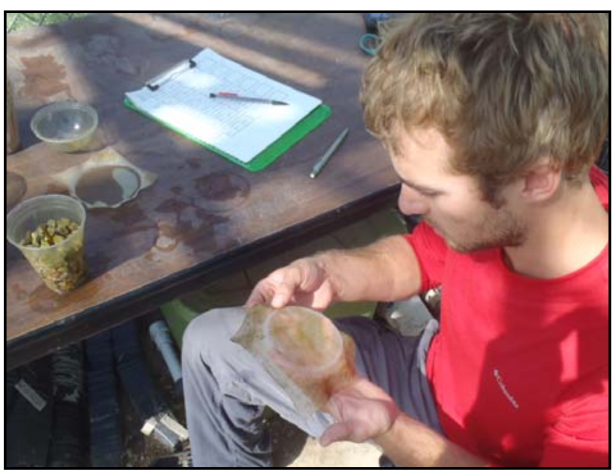
The ***pH Drift Study*** was conducted at the Center for Reservoir and Aquatic Systems Research facility at Baylor University. The results are the most straightforward of any of the 2013 applied research efforts, with data directly establishing the bicarbonate (HCO_3^-) utilization potential of six species of aquatic plants within the Comal/San Marcos River systems. Two native species with relatively high utilization as fountain darter habitat (*Riccia fluitans* [hereafter, *Riccia*], *Cabomba caroliniana* [hereafter, *Cabomba*]) are shown to not utilize HCO_3^- under field conditions. Furthermore, neither species shows evidence of developing the capacity to utilize HCO_3^- under CO_2-stress conditions. Therefore, these species seem to have elevated risk of being lost from these river systems if flow conditions change and result in lower flows, longer water residence time, lower flushing rates, higher pH, and significantly lower CO_2 availability.

Sagittaria platyphylla (hereafter, *Sagittaria*) also was not able to utilize HCO_3^- during the time period tested. However, unlike *Cabomba* and *Riccia*, *Sagittaria* is capable of forming emergent leaves and avoiding CO_2 limitation by obtaining the gas from the atmosphere instead of water. Therefore, at least in shallow waters (<0.5m), *Sagittaria* may not be lost from this system. Both *Hygrophila polysperma* (hereafter, *Hygrophila*) and *Ludwigia* develop the ability to utilize HCO_3^- when growing under CO_2 stressed conditions. Additionally, since both species are capable of forming emergent leaves, they can also alleviate CO_2 stress by sending out emergent stems and leaves. This is an important finding with respect to *Ludwigia* as (1) it supports the continued use of this species for ongoing restoration efforts in both systems, and (2) it allows for a re-evaluation of the necessity of having the nonnative *Hygrophila* included in the long-term biological goals for the fountain darter.



Finally, the *Vallisneria* species growing in the Comal River is a strong HCO3- user. As such, it is likely that this species will survive in the San Marcos and Comal rivers under extreme low-flow conditions. A key HCP unknown was whether the expanses of *Vallisneria* in Landa Lake (pictured to the left) might crash under elevated water temperatures, which would cause massive vegetation decay and subsequent high levels of oxygen demand in the water column. Coupled with the vegetation tolerance studies, this study has alleviated this concern at least up to ≈35 °C.

The ***Food Source Study*** was conducted at the ARC and established a critical thermal maximum (CTM) of 37.89 °C for *Hyalella azteca* (an amphipod used as a surrogate for fountain darter food) taken directly from the Comal River. It is noteworthy that the mean *Hyalella azteca* CTM (37.89 °C) exceeds the fountain darter CTM (34.8 °C) reported by Brandt et al (1993), suggesting that if environmental conditions caused water temperatures to increase, the CTM of the fountain darter would occur before that of their food source. However, CTM is only a single factor experiment, and thus this data only provides a glimpse of how *Hyalella azteca* or the fountain darter may respond to increasing temperatures under extreme drought.



In both the laboratory and pond experiments, cooler temperatures and the presence of *Riccia* both significantly improved the survival of *Hyalella azteca* which is encouraging considering the aforementioned results regarding tolerance of aquatic vegetation. The laboratory and pond studies also documented that when confronted with multiple stressors, *Hyalella azteca* death occurs at considerably cooler water temperatures than the CTM. The interpretation of the laboratory and pond food source experiments, in conjunction with vegetation availability, suggests *Hyalella azteca* would likely cease to be a food source before their CTM temperatures occur in the wild.

HCP Ecological Model Parameterization

As mentioned, the Field vs. Laboratory Study was not designed to provide direct input into the HCP ecological model, but rather to test theories behind applied research to better inform decision making and model formulation. The Vegetation Tolerance studies, pH Drift study, and Food Source studies provide several results for consideration in HCP Ecological model parameterization. These apply to the aquatic vegetation model, fountain darter model and possibly to water quality model validation at low flows as follows:

Aquatic Vegetation Model(s)

- Upper temperature threshold ranges (<35 °C) for plant survival for three species tested (*Cabomba*, *Vallisneria*, *Riccia*).
- Upper temperature threshold ranges (≈37 to 40 °C) for *Ludwigia* survival.
- Lower CO_2 threshold ranges for plant survival and continued growth for all species tested (<5 mg/L).
- Relative growth rates for species tested relative to temperature and CO_2 changes
    - Water temperature - 22 °C, 28 °C, 34 °C, 37 °C
    - CO_2 - <5 mg/L, 9-12 mg/L, 30-40 mg/L
- Preliminary data on algal growth in temperature and CO_2 treatments.
- HCO_3^- use inputs for six species prevalent in the Comal and San Marcos systems (*Cabomba*, *Vallisneria*, *Riccia, Hygrophila, Ludwigia, Sagittaria*)

Fountain Darter Model

- Food source input (using *Hyalella azteca*)
    - Water temperature thresholds
    - *Riccia* benefit

Water Quality Model

- Water temperature and dissolved oxygen data collected in pond studies may be applicable for water quality model refinement or validation under extremely low-flow or no-flow conditions in the summer.

Although the aforementioned parameters are put forward for consideration, it is emphasized that specific use of any of the 2013 applied research will be determined by the HCP ecosystem modeling team with guidance from the HCP Science Committee.

Recommendations for Future Applied Research

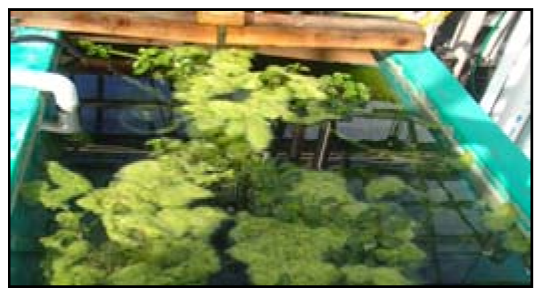
The Field vs. Laboratory Study was successful in highlighting the uncertainty surrounding using laboratory or field results solely to make management decisions or to populate ecological models. As such, no further comparison studies are recommended at this time. Should certain plant species, environmental parameters, or spatial locations relative to aquatic vegetation and plant growth turn out to be highly sensitive in the ecological model(s), then future investigation may be warranted. With information on six key aquatic plant species in the Comal and San Marcos rivers added to the known HCO_3^- use patterns of Texas wild rice and *Hydrilla verticillata*, no additional pH drift studies are recommended at this time.

Based on the 2013 HCP applied research findings from the Vegetation Tolerance studies and Food Source studies, the following five Applied Research recommendations are presented.

1) ***Ludwigia* growth field study:** The apparent suitability of *Ludwigia* to substitute for *Hygrophila* with regard to providing fountain darter habitat is encouraging. However, a further investigation of *Ludwigia* growth under varying environmental conditions in the Comal River (Landa Lake, Old Channel, and New Channel) and in the San Marcos River (upstream and lower sections) is warranted. While the 2013 greenhouse and pond studies strongly support the use of *Ludwigia*, the results of the laboratory vs. field experiment cautions against too much extrapolation.

2) **Plant competition study:** An *in situ* plant competition study using *Ludwigia* and two nonnative species (*Hygrophila* and *Hydrilla verticillata*) is also recommended. Previous research has shown that under relatively stagnant flow conditions, *Hygrophila* strongly outcompeted *Ludwigia.* However, it would be very useful to have data under more reasonable field conditions. In addition, studying the competitive ability of both of these species vs. *Hydrilla verticillata* is warranted for understanding the San Marcos River plant dynamics.

3) **Algae dynamics study:** Based on the laboratory and field experiments, in conjunction with the annual build-up of algae in portions of the San Marcos and Comal rivers, an applied research study directed at understanding the effect of water quality on algal growth, as well as the effect of algal growth on the survival of aquatic vegetation is recommended.



4) **Food source temperature refinement study:** The objective of this study is to evaluate the temperature range between 28 °C and 34 °C to more accurately determine a threshold temperature for amphipods. This is important in that, at 28 °C, fountain darters can exist just fine and reproduce to a limited degree, but near 34 °C their reproduction shuts off and survival becomes tenuous. If food really should become limiting at 29 °C rather than 33.5 °C, there is the potential for this parameter to be extremely important. However, if the threshold is truly more near 34 °C, then direct temperature impacts to fountain darters would likely trump any food source response in the ecological model.

5) **Food source response to plant species:** The objective of this investigation is to evaluate whether *Vallisneria* and *Ludwigia* provide similar benefits to amphipod survival to what was experienced with *Riccia*. The reason for this investigation is that *Riccia* is likely the first plant species to be eliminated from the system during low flows while *Vallisneria* and *Ludwigia* should persist.

It is emphasized upfront that several cautions are highlighted and discussed throughout this report. It is imperative that these be carefully reviewed and recognized prior to interpretation of the 2013 HCP applied research results.

In conclusion, the 2013 HCP applied research has provided a wealth of information to assist the HCP process particularly with (1) HCP ecological model parameterization, (2) future HCP applied research, and (3) re-evaluation of HCP long-term biological goals. The complexity of the interactions are immense but will continue to be sorted out over time with the assistance of the HCP ecological model(s), more-refined applied research, and continued bio-monitoring. Applied research activities conducted in 2013 helped break down and clarify the picture in many instances, while continuing to emphasize the importance of the HCP applied research efforts and ecological modeling moving forward.

1.2 Acknowledgments

The project team would like to acknowledge the U.S. Fish and Wildlife Service San Marcos Aquatic Resource Center (ARC) scientists and staff. In particular, we thank Dr. Ken Ostrand, Dr. Tom Brandt, Dr. Jeff Hutchinson, and Mr. Randy Gibson for all their guidance, assistance, patience and cooperation during this whirlwind of 2013 HCP applied research activity. As described throughout this report, the majority of laboratory and pond research was conducted at the ARC facilities in San Marcos, Texas. We would also like to thank the HCP Science Committee for their timely input regarding approaches and methods for research activities. Finally, a special "thank you" is extended to Dr. Floyd Weckerly for lending his ear and guidance regarding experimental design and statistical interpretation.

2.0 FIELD VS. LABORATORY STUDY

2.1 Introduction

Aquatic plants are an integral part of most freshwater aquatic ecosystems. Aquatic vegetation provides valuable ecological functions, including sediment stabilization (Barko and James 1998, Sand-Jensen 1998), nutrient cycling (Barko and James 1998), primary production (Sand-Jensen et al. 1982), and metazoan habitat for foraging and predator avoidance (Rozas and Odum 1988). Understanding the dynamics between aquatic plants and the local habitat and fauna is an important step in understanding the ecosystem processes of an aquatic system and vital in the case of this HCP.

Typically, the least-contested type of scientific research pertinent to applied management decisions are comprehensive studies in the wild over long periods of time, encompassing a wide range of changes in the environmental variables of interest with recurrences of a wide range of events for repeatability. Unfortunately, this rarely occurs in the scientific world for a number of factors including cost, time, infrequency of extreme events, etc. For instance, the best way to study the effect of low-flow on aquatic plants in the Comal or San Marcos systems is to track the condition of aquatic plants as total system discharge decreases in those two systems repeatedly over time. On a positive note, the HCP bio-monitoring (conducted over the past decade and ongoing) has developed an extensive baseline on the condition of aquatic plants in these systems and has, in fact, captured the response of several above- and below-average flow condition events. Unfortunately, or fortunately from a water resources standpoint, extreme low-flow conditions necessary to truly test aquatic plant thresholds have not been experienced in either the Comal or San Marcos systems. As mentioned, this is a universal shortcoming and not just specific to this HCP.

To compensate for such shortcomings, researchers employ specifically designed field experiments or investigations conducted within a laboratory setting to predict plant response to changes in the natural environment. However, with respect to aquatic plants, it is infrequent in the literature where direct comparisons between laboratory and field experiments, or either of these vs. long-term observational data, have been attempted. In most cases, it is not practical to test laboratory studies directly to field experiments because of cost, time, and differences in the level of control of environmental variables and outside influences/stressors. As such, laboratory studies are frequently used as surrogates to inform decision making. However, when laboratory experiments are the sole source of input for applied management decisions, a large measure of uncertainty can be introduced and, if so, must be acknowledged. For example, in a comparison of field-generated data from four continents and over 1,600 plant species using a similar measure of temperature sensitivity, it was shown that warming experiments considerably under-predicted plant responses when compared to long-term observations (Wolkovich et al. 2012). That observation in part led the authors of that *Nature* journal article to conclude, "Our results introduce uncertainty into ecosystem models that are informed solely by experiments and suggest that responses to climate change that are predicted using such models should be re-evaluated."

During HCP development, a topic frequently debated by stakeholders and scientists alike was the magnitude of applied research that would be needed to address key data gaps specific to the HCP, and where and how that research should be conducted. This led to multiple discussions regarding *in situ* experimental channels, off-site experimental channels, off-site ponds, raceways, laboratory settings, and combinations thereof. It is not the intent of this report to answer that debate but rather to highlight lessons learned from this study relative to present and future HCP applied research activities.

The objective of the Laboratory vs. Field Study was to compare aquatic vegetation growth over time when conducted simultaneously in laboratory and *in-situ* experiments held at similar flow and water quality conditions. The null hypothesis is that, when held under similar physiochemical conditions, similar aquatic vegetation growth will be experienced between the laboratory and field treatments. To test this hypothesis, the growth response of three aquatic plant species growing under similar physiochemical and flow conditions was evaluated in a research greenhouse as well as in a river system where all three species are present. The purpose of this study was to investigate if plant growth and/or directional trends would be similar between the laboratory and field treatments. The basic intent is to document sources and levels of uncertainty from laboratory and field experiments to help guide future design of HCP applied research. A second benefit of comparative investigations is that guidance may be gleaned on how laboratory and/or field experiments should be interpreted relative to conditions likely to be experienced in the wild. This latter topic will be useful to guide HCP ecological model development.

2.2 Data Review and Available Literature

Ecological studies are often carried out through field experimentation. This is due in part to the scope and scale of what is being studied and to the fact that many environmental variables and interactions are too complex to be incorporated in a laboratory setting. Field experiments provide a direct and naturalistic method for assessing natural phenomena, but come with the disadvantage of decreased experimental control, and typically the expenditure of more time and money relative to laboratory investigations. Additionally, experimental outcomes in the field may vary annually, seasonally, or by location, with quantification of single interacting factors often proving to be quite difficult (Diamond 1983). It can also be problematic to try to generalize results from field studies to systems outside of those that were directly studied (Wilson 2009).

The benefit of conducting experiments in a laboratory setting is that variables can be controlled. This provides a more precise approach to measuring the effect of a single treatment, but often it can be difficult to address all of the variables that may be at play under natural conditions. Although disparities often exist between laboratory and field experiments, hypothesis testing via these different methodologies can provide more robust conclusions (Diamond 1983). With that said, a great deal of time was not spent on combing the available literature for how successful direct laboratory vs. field comparisons have been as this was not the main point of the investigation. This investigation was conducted in response to concerns posed by stakeholders regarding applied research that surfaced during the HCP process and ranged the full spectrum. On one extreme, it was often stated that laboratory experiments are not transferable at all to what is happening in the wild and, thus, why waste money on them? On the other extreme, it was just as frequently argued that all answers need to be obtained from laboratory studies because variables can be better controlled and manipulated. There is a level of truth to both statements, but neither answers the question in total, nor does this comparison.

The following data sources were reviewed to ensure the study was designed to meet the intent of the HCP and be applicable to the Comal and San Marcos ecosystems:

- Edwards Aquifer Recovery Implementation Program (EARIP) Habitat Conservation Plan (EARIP 2011)
- Historical Comal system aquatic vegetation maps (surveys from late 1990s—Baylor University; 2000 to 2012—BIO-WEST 2001–2013a; 2009—River Systems Institute)
- Historical San Marcos system aquatic vegetation maps (Surveys from 2000 to 2012; BIO-WEST 2001–2013b; 2009—River Systems Institute)
- Aquatic vegetation species' lists for the Old Channel of the Comal River (HCP sponsored full-system mapping of aquatic vegetation; BIO-WEST 2013 draft data)
- Life history information of common aquatic vegetation in the Comal and San Marcos ecosystems

From this review, a study plan was prepared and presented to the HCP Science Committee in February 2013, and was subsequently approved.

2.3 Materials and Methods

In spring 2013 simultaneous experiments were embarked upon to determine how aquatic plants would respond to two flow regimes when cultivated under greenhouse conditions vs. *in situ* conditions. The *in situ* study location is a 200-meter-long (m) section of the Old Channel of the Comal River (just downstream of Elizabeth Street). The laboratory portion was carried out in the 1,200 m^2 research greenhouse located at the ARC in San Marcos.

2.3.1 Pre-study Activities

The first step was to develop a holding container that would support 30 potted aquatic plants (10 per species) and withstand a month-long trial in the wild. After a thorough search of available equipment and containers, and a series of internal team meetings regarding design, the team designed and constructed a Mobile Underwater Plant Propagation Tray (MUPPT) (Figure 1).

Figure 1. Mobile Underwater Plant Propagation Tray (MUPPT) prototype.

The MUPPT is constructed of steel and covered in a nontoxic, polyurea-blend, spray-on coating to prevent rust. It is 1.3 m long by 1.0 m wide and supports four hard-plastic removal trays that hold 12 quart pots each. Each of the four trays is secured by two vertical bars with stainless steel clips to secure not only the trays but each individual pot as well. The design incorporates a slanted front panel to allow water movement through the plant leaf matter while protecting the substrate and root stock from scour. Transport handles on the front and rear can also be used to secure the MUPPT in place on the bottom and/or to each other.

The MUPPT prototype was subsequently tested with plants in both the ARC ponds and within the Old Channel of the Comal River, before full production was initiated. For this study, six MUPPTs were used in the field component in the Old Channel.

Concurrent with MUPPT testing, fragments of two species, *Ludwigia* and *Cabomba caroliniana* (hereafter, *Cabomba*), along with rosettes of *Sagittaria platyphylla* (hereafter, *Sagittaria*), were collected from mother colonies in the Old Channel upstream of the field study location. Plant material collected from the river was brought back to the ARC and treated with a 10-percent potassium permanganate ($KmNO_4$) solution for 15 minutes to prevent the spread of introduced organisms. Plants were propagated in 10.16-centimeter (cm) diameter by 10.16-cm-tall black nursery “quart” pots filled with 900 milliliters (mL) of sandy clay loam soil. Parent material was measured and cut into 8-cm-long apical fragments. Two apical fragments were planted per pot. Single rosettes of *Sagittaria* were planted into each pot with a total of 100 pots per species produced. These potted plants were placed into quarantine troughs in the ARC greenhouse under 30-percent shade for grow-out and monitoring for nuisance aquatic species. The plants were allowed to grow for a period of 7 weeks to ensure they were well established.

Prior to the formal experiment, preliminary data were collected to inform the study design in both the field and laboratory settings. Discrete water velocity measurements were made across a diverse range of vegetated and open-water habitats (based on location, depth and vegetation type) within the 200-m study reach of the Old Channel during normal flow conditions. These measurements were reviewed to establish an appropriate set of flow conditions that could be tested with experimental plants in the field, as well as simulated at the ARC for testing in the laboratory. Field treatments were characterized based on velocity, depth, and photosynthetically active radiation (PAR) so that these conditions could be matched in the ARC tanks. Average CO2 and water temperature in °C were measured within the study reach so that these parameters could also be simulated at the ARC.

Early in the study design phase, it was determined that a low-flow and minimal-flow treatment would be established to examine the effects of low-to-no-flow conditions on aquatic plants in the spring/river systems. These two flow regimes were considered based on the desire not to use exorbitant amounts of water at the ARC and the better consistency of lower-flow conditions in a flowing channel. Typically, the open-channel flow conditions in the Old Channel were higher and more variable than those at near-shore areas because of the dynamic nature of the channel including vegetation, debris, channel width, sinuosity, etc. Therefore, treatment locations nearer to the shore and in more protected areas were selected for the study (Figure 2). Based on these factors, it was determined that two velocity regimes would be appropriate for testing. The first is a low-flow treatment (≈0.15–0.25 feet/second [ft/sec]) and the second is a minimal-flow treatment (<0.05 ft/sec).

Figure 2. **Mobile Underwater Plant Propagation Tray (MUPPT) in the Old Channel of the Comal River.**

2.3.2 Greenhouse Experiment Setup

The laboratory portion of the study was conducted in the ARC greenhouse. The greenhouse is a 1,200-square-meter glass-and-fencing enclosure with a 30-percent shade covering. The greenhouse is supplied with Edwards Aquifer well water, which has water chemistry similar to that of Comal spring water flowing through the Old Channel (see Section 2.4). To mimic water temperature and CO2 conditions found in the Old Channel, water was supplied to the experimental troughs via a reservoir containing a heater and a custom-built degassing chamber (Figure 3). Three 950-L fiberglass tanks (Living Stream Model MT-1024, Frigid Units Incorporated, Toledo, Ohio) located in the ARC greenhouse were divided into six independent tanks and used as treatment tanks (Figure 3). When divided in half, the overall dimension of each Living Stream was similar in size to the MUPPTs used in the field. Tanks were plumbed to receive water from a 560-liter (L) circular reservoir tank. Incoming source well water from the Edwards aquifer was passed through a degassing tower 1-m-long and 10.2 cm in diameter, filled with plastic bio-filter media, to allow dissolved CO2 levels to be manipulated so that they were similar to average CO2 levels measured at the field site.

Figure 3. Reservoir Tank with CO2 degasser (left) and living stream divided in two right).

Each of the six treatment tanks were randomly selected to receive one of the two velocity regimes. Three Smart-Pond submersible pumps (Geo global partners, West Palm Beach, Florida) rated at 2,000 gallons per hour were used to provide velocities within the three low-flow treatment (0.15–0.25 ft/sec) tanks. These treatment tanks were set up for circular flow.

Incoming reservoir water supplied to the three minimal-flow treatment tanks was used to maintain water velocities of <0.05 ft/sec. Figure 4 shows the random layout of low-flow (LF) and minimal-flow (MF) treatments in the living streams at the ARC.

2.3.3 Field Experiment Setup

Six sites within the Old Channel study reach were selected for the field treatments (Field) based on velocities and PAR rates previously measured. The three minimal-flow treatment sites had an average velocity of ≈ 0.03 ft/sec, while the three low-flow treatment sites had an average velocity of ≈0.19 ft/sec. Sites were attempted to be chosen with a similar noon PAR rate as the research greenhouse. One MUPPT was placed in each of the six field treatment sites in the Old Channel

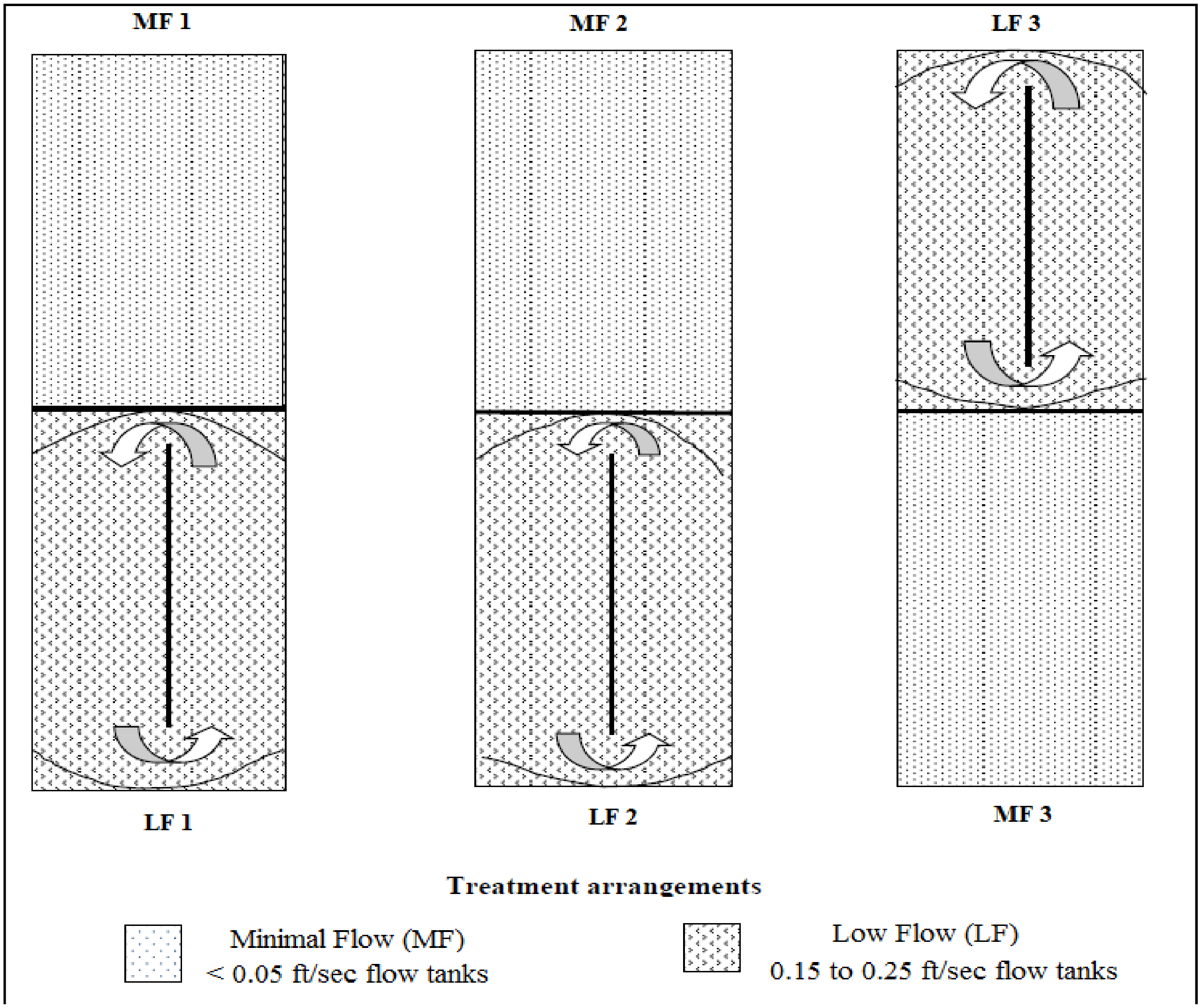


Figure 4. Random Treatment arrangements in living streams at San Marcos Aquatic Resource Center (ARC).

(Figure 5). At the beginning of the experiment, plants were assigned to a treatment using a randomized block design within each MUPPT. The randomized block design was recommended by the HCP Science Committee during the February discussion to address concerns regarding potential point velocity differences that may be experienced at the upstream edge and downstream edge of the MUPPT as plants grew over the course of the study. Initial total stem length was measured and recorded.

Prior to study implementation, 10 plants of each of the species that had been potted and grown, along with the plants utilized for treatments in this study, were harvested for initial biomass measurements (Figure 6). Plants to be placed in the field were transported via sealed coolers to their respective treatments. The MUPPTs were adjusted so that plant canopy was no greater than 30.5 cm below the surface of the water so that conditions were similar to that of the laboratory tanks. Plants were allowed to remain in their treatments for a period of 28 days (April 8 through May 6).

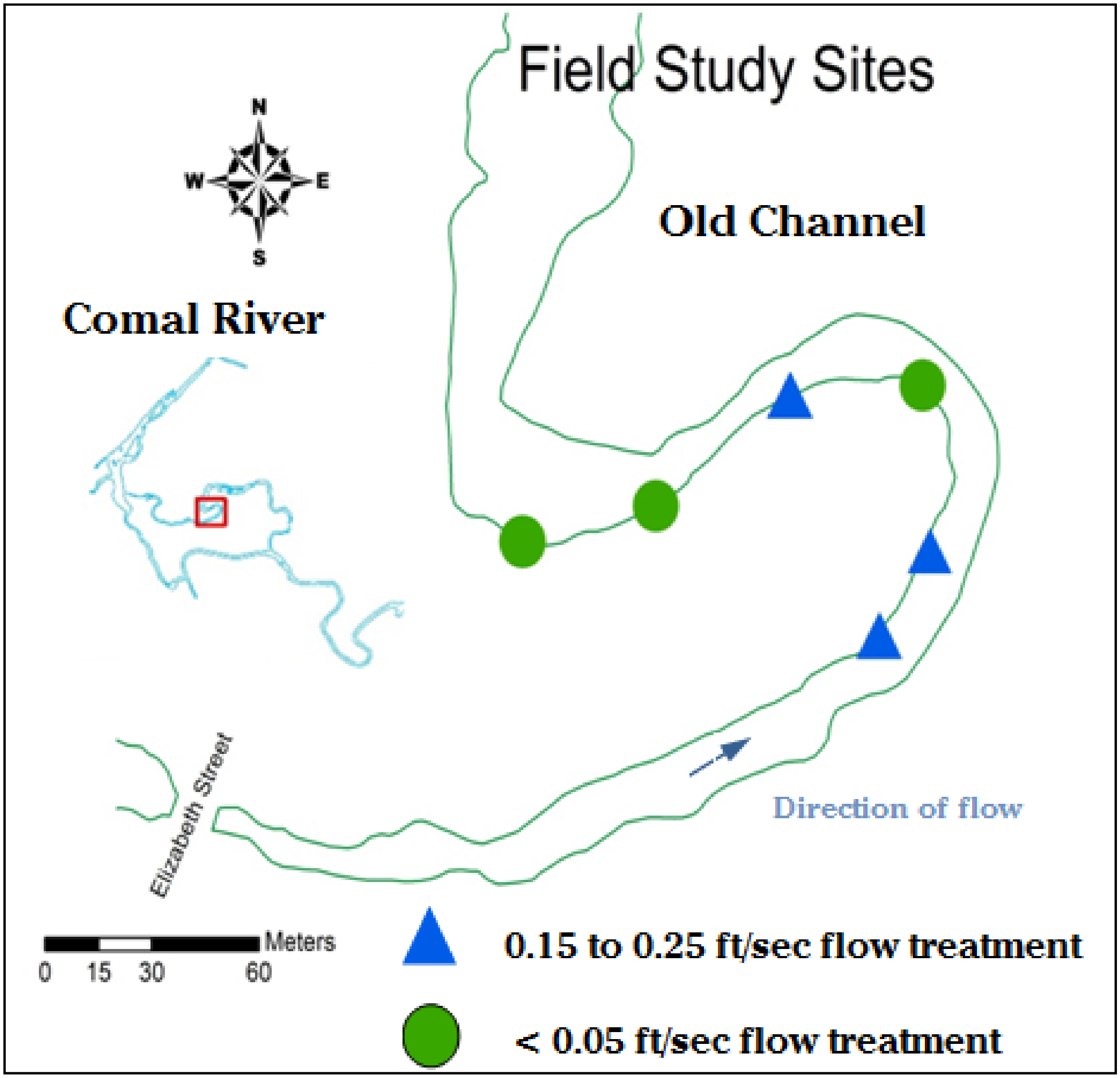


Figure 5. **Locations of field treatments in the Old Channel of the Comal River below Elizabeth Street. Green circles represent minimal-flow (MF) treatments (<0.05 ft/sec) and blue triangles represent low-flow (LF) treatments (0.15–0.25 ft/sec) sites. Treatment sites are numbered upstream to downstream, with river flowing in a northerly direction in this reach.**

Figure 6. **Harvesting of plants and separation of rooted vs. leaf material.**

Water temperature and dissolved CO2 were measured three times per week at three locations within the Old Channel study reach and within each of the six laboratory treatment tanks. Dissolved oxygen, temperature, and pH were measured using a YSI multi-parameter sonde with a pro series handheld data unit (YSI Inc., Yellow Springs, Ohio). Dissolved CO2 was measured using the Oxyguard portable CO2 Analyzer (Oxyguard International AS, Berkerød, Denmark). Point velocity and PAR were measured at plant canopy height, in five randomly selected points within each treatment. Point velocity (ft/s) was measured using a Hach FH950 flow meter and adjustable wading rod. The PAR was measured with the MQ-200 Quantum meter (Apogee Inc., Logan, Utah). If changes were noted in the average CO2 in the field, appropriate adjustments were made in the water supply reservoir tank. Increasing or decreasing flow through the reservoir tank manipulated CO2. In addition, one HOBO® TidbiT v2 water temperature data loggers (thermistor) was attached to each of the six MUPPTs and placed in each of the six laboratory treatment tanks as well as the reservoir tank. Thermistors were set to record every 15 minutes and were left in place for the duration of the study.

In order to examine the potential difference in additional water chemistry parameters between the greenhouse and field study location, pH, ALK, nitrate, nitrite, total nitrogen, and total phosphorus were collected at the start and at the conclusion of the study in the laboratory water supply reservoir tank and at one central location in the Old Channel study reach. Grab water samples were collected in pre-prepared sample bottles provided by the analytical laboratory. Once collected, samples were immediately transferred to the analytical laboratory for analysis.

At the end of the 28-day study period, final stem number and total stem length measurements were recorded. Plants were harvested at the end of the trial to assess biomass. Standard brown paper lunch bags were dried for 24 hours at 60 °C then weighed on an electronic balance to obtain a tare weight to the nearest 0.001 gram (g). Above-ground growth (shoots) and below-ground growth (roots) were divided to be analyzed separately. Shoots were clipped at the soil surface, and the divided plants were then rinsed and placed into separate, tared, labeled paper bags. Bagged plants were transported to Baylor University where they were dried for approximately 96 hours at 60 °C and weighed to the nearest 0.001g.

2.3.4 Experimental Design

The experiment was established as a 2 X 2 factorial design. The two principal factors were *location* (laboratory vs. field) and *flow* (minimal vs. low-flow velocities) (Table 1). Each factor combination was replicated three times. In the field three MUPPTs were used for each flow level with each MUPPT containing 10 individual potted plants of each species. In the laboratory, three experimental tanks were established for each flow condition with each tank also containing 10 potted plants of each of the three species. Although each MUPPT or tank contained 10 individual potted plants per species, for statistical analysis the MUPPT or tank were the "experimental unit" because pots within a single MUPPT or tank cannot receive different treatments.

Table 1. Illustration of the 2 X 2 factorial design of the experiment.

FLOW	LOCATION	
	Laboratory	Field
Minimal	3 tanks	3 MUPPTs[a]
Low	3 tanks	3 MUPPTs

[a] MUPPT=Mobile Underwater Plant Propagation Tray.

2.3.5 Response Variables

Survival

The total survival for each species for each MUPPT or tank was computed simply as the total number of surviving plants of each species.

Relative Growth Rate (RGR)

Plant relative growth rate (RGR) is a measure of growth over a specified time period relative to the current plant size and has units of [mass / (mass x time)]. In this study RGR was estimated on a per-day basis and mass (in g) on a dry mass basis (RGR units are $g\ g^{-1}d^{-1}$ or more simply, d^{-1}). Conceptually, this is a measure of proportional change in mass per day. The RGR for each surviving planted pot was calculated from the final biomass in the pot relative to the average biomass of the plants of that species harvested at the beginning of the experimental growth period.

The RGR for each pot was estimated as:

$$RGR=(\ln W_f - \ln W_i)/(\Delta\ time)$$

Where:
W_f=dry biomass of plant in a pot at end of growth period
W_i=average dry biomass of plants harvested at the beginning of the growth period
Δ time=growth period (days)

Note that RGRs for each pot could be positive (increase in biomass) or negative (decrease in biomass) relative to the initial plants. An average RGR for each species in each MUPPT (field) or tank (lab) was computed from the RGRs of surviving pots of that species in that experimental unit.

Change in Number of Leaves/Stems

The change (increase or decrease) in total number of leaves (*Sagittaria*) or stems (*Ludwigia, Cabomba*) was computed for each surviving plant by subtracting the average number of leaves/stems of the initial plants from the number in each pot at the end of the experimental growth period. The per-tank average was computed as the mean for all surviving pots in each MUPPT or laboratory tank. A change in the total number of leaves or stems reflects the plants basal area growth.

Change in Total Leaf/Stem Length

The change (increase or decrease) in total combined length of all leaves (*Sagittaria*) or stems (*Ludwigia, Cabomba*) was computed for each surviving plant by subtracting the average of the initial plants from the number in each pot at the end of the experimental growth period. The per-tank average was computed as the mean for all surviving pots in each MUPPT or laboratory tank. A change in the total combined leaf or stem length is a measure of total energy investment in surface area for capturing light.

Maximum Length

The final maximum length for each pot was determined as the length of the longest leaf or stem in the pot at the end of the experimental growth period. The per-tank average was computed as the mean for all surviving pots in each MUPPT or laboratory tank.

Aboveground:Belowground Ratio (AGBG)

The aboveground:belowground ratio (AGBG) of surviving plants was computed as the ratio of dry biomass aboveground (leaves, stems) to belowground (roots, rhizomes). The per-tank average was computed as the mean for all surviving pots in each MUPPT or laboratory tank. Shifts in AGBG ratio arise from differences in allocation of biomass to roots (nutrient absorption and anchorage) and shoot (light harvesting and photosynthetic potential). Historically, shifts in allocation patterns are regarded as plant optimization for current growth conditions (e.g., increase in roots when nutrients are in short supply, or increase in shoots when light is limited), although recent analyses caution against simplistic interpretations of these ratios (Reich 2002).

2.3.6 Statistical Analysis

Statistical analyses were made using Two Way ANOVA which allows the simultaneous analysis of each major factor (Zar 2010). This analysis has the benefit of allowing estimates of the *interaction* between the two factors to be evaluated. An interaction between the factors indicates that the response of a plant to changes in one factor (e.g., flow) depends on the level of the other factor (e.g., lab or field). For clarity, graphical representations of some of the possible interaction responses of two variables are shown in Figure 7.

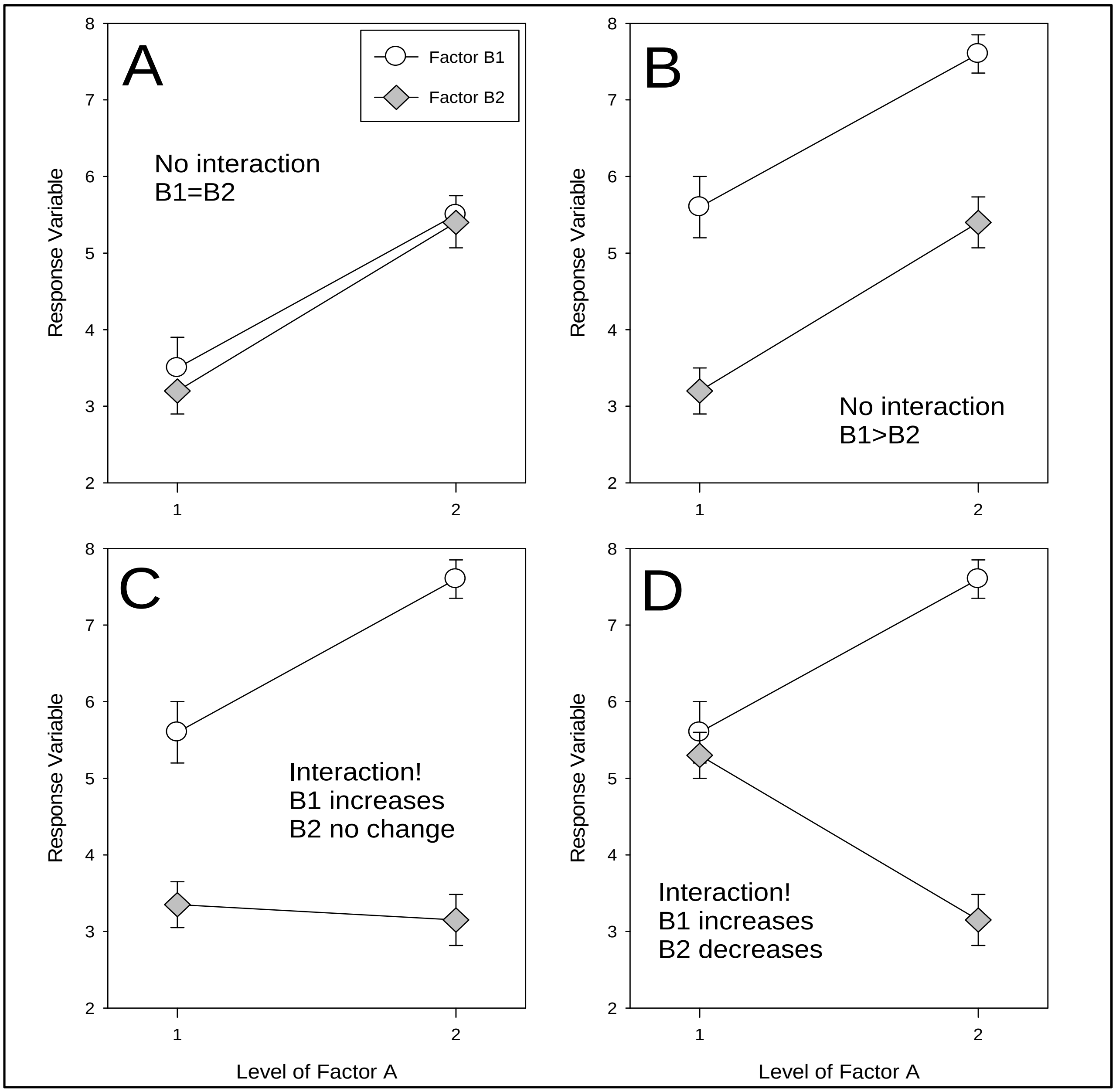


Figure 7. Graphical representation of some common outcomes of a 2 X 2 factorial analysis. (A) Factors do not interact and are not statistically different from each other, (B) factors do not interact, but factors differ significantly from each other, (C and D) two possible interactions among factors.

The lack of significant interaction between the factors indicates that the *pattern* of response of the factors is consistent, although the absolute response levels may differ (Figure 7A and B). Figure 7A shows a situation where factors do not interact and the response to the factors is not different, while Figure 7B shows a situation without interaction (*pattern* is the same) but where the responses are statistically different in absolute value. Figure 7C and D show two possible situations where interaction between the two factors is significant.

In interpreting the results of a Two Way ANOVA, first the interaction factor must be evaluated. If the interaction term is not significant, the overall significance of the major factors provided by the Two Way ANOVA can be interpreted normally (e.g., overall factor A or factor B is or is not significant). However, if the interaction is significant, this indicates that the response of factor A (e.g., flow) depends on the level of factor B (e.g., lab or field). Hence, when the interaction is significant, the response of each factor must be evaluated separately for each level of the other factor (e.g., *t*-tests comparing A1 vs. A2 at each level of B and vice versa).

2.4 Results

The experiment was initiated on Monday, April 8, with the transfer of plants from the ARC acclimation areas to the MUPPTs in the Old Channel and treatment tanks in the greenhouse. Figure 8 shows the placement of plants into the MUPPTs in the Old Channel while Figure 9 shows both the minimal-flow and low-flow treatments at the ARC.

Table 2 presents the results of the water chemistry analysis of samples collected from the laboratory water supply reservoir tank and at a central field location in the study reach of the Old Channel at the start and conclusion of the experiment. Results for both water sources for these parameters were very similar. No manipulation of any of these parameters was attempted during the course of the study. In addition to the basic water chemistry analysis of grab water samples, water temperature and water column CO2 concentration were measured three times per week in both the Old Channel and water supply reservoir tank at the ARC. Thermistors recorded temperature at 15-minute intervals within each of the six laboratory treatment tanks and at each of the six MUPPT field locations. Table 3 shows the comparison of the 15-minute thermistor data between treatments, including the reservoir tank, with a visual representation of the daily means shown in Figure 10.

A comparison of daily mean temperature values indicates temperatures in the field and laboratory low-flow treatments are not significantly different (two-sample *t*-test, t=1.3498, df=114.5, p=0.1797). However, daily mean temperatures of field and laboratory minimal-flow treatments are significantly different (t=5.9699, df=119.094, p<0.001) by about 1 degree (23.2114 field vs. 22.21266 lab, 95% confidence interval [CI] of difference in means=0.667-1.33) (Figure 10). Although statistically significant, the small differences in water temperatures in this study were not the likely explanation for substantial differences in plant growth. Figure 11 displays the measured CO2 in the Old Channel and ARC reservoir tank over the course of the study period. A two-sample *t*-test of the CO2 data showed no statistical difference between the Old Channel and ARC reservoir tank (t=-0.8269, df 21.438, p=0.4174; mean field=9.53; mean lab=10.08).

Figure 8. Random placement of plants into the MUPPT in the Old Channel, Comal River.

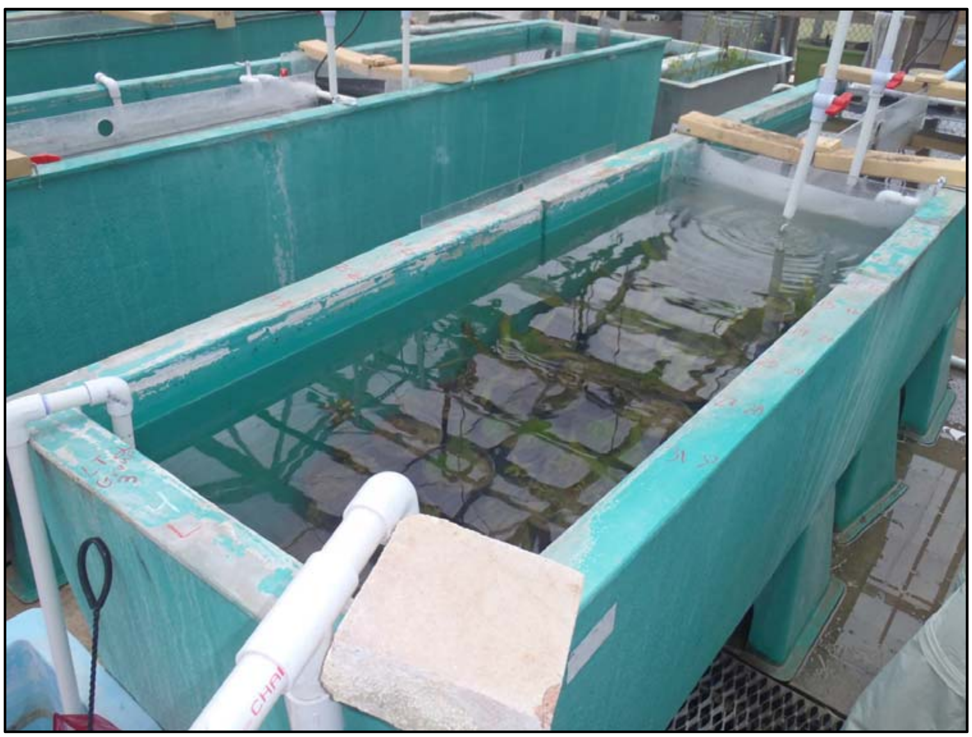


Figure 9. Plants in minimal-flow treatment (foreground) and in the low-flow circulation treatment (background) at the start of the study.

Table 2. **Basic water chemistry parameters in San Marcos Aquatic Resource Center (ARC) reservoir tank and Old Channel, Comal River.**

PARAMETERS	UNITS	ARC (RESERVOIR)		OLD CHANNEL (COMAL RIVER)	
		4/9/2013	5/6/2013	4/9/2013	5/6/2013
pH	pH units	7.8	7.9	7.8	7.6
Alkalinity, total	mg/L[a]	260	260	230	240
Kjeldahl-N	mg/L	0.434	0.278	0.161	0.404
Nitrate-N/IC	mg/L	1.67	1.6	1.72	1.67
Nitrite-N/IC	mg/L	<0.02	<0.02	<0.02	<0..02
Total nitrogen	mg/L	2.11	1.88	1.88	2.08
Total phosphorus	mg/L	0.0619	<0.02	<0.02	<0.02

[a] mg/L=milligrams per liter.

Table 3. **Water temperature data collected via thermistors in each treatment and the San Marcos Aquatic Resource Center (ARC) reservoir tank.**

THERMISTOR ID	COMPONENT	TREATMENT[a]	WATER TEMPERATURE (°C) minimum	average	maximum
1	LAB (tanks moving away from reservoir)	LF 1	21.44	23.66	25.44
2		MF 1	19.59	23.10	25.03
3		LF 2	21.52	23.62	25.49
4		MF 2	20.87	23.06	24.92
5		MF 3	21.19	23.32	25.17
6		LF 3	21.16	23.60	25.58
13	Reservoir	Reservoir	21.92	23.72	25.01
7	FIELD (upstream to downstream)	LF 1	22.46	23.81	25.24
8		LF 2	22.43	23.77	25.18
9		MF 1	22.30	23.73	25.10
10		LF 3	22.51	23.70	24.79
11		MF 2	22.25	23.74	25.23
12		MF 3	22.43	23.74	25.07

[a] LF=low flow, MF=minimal flow.

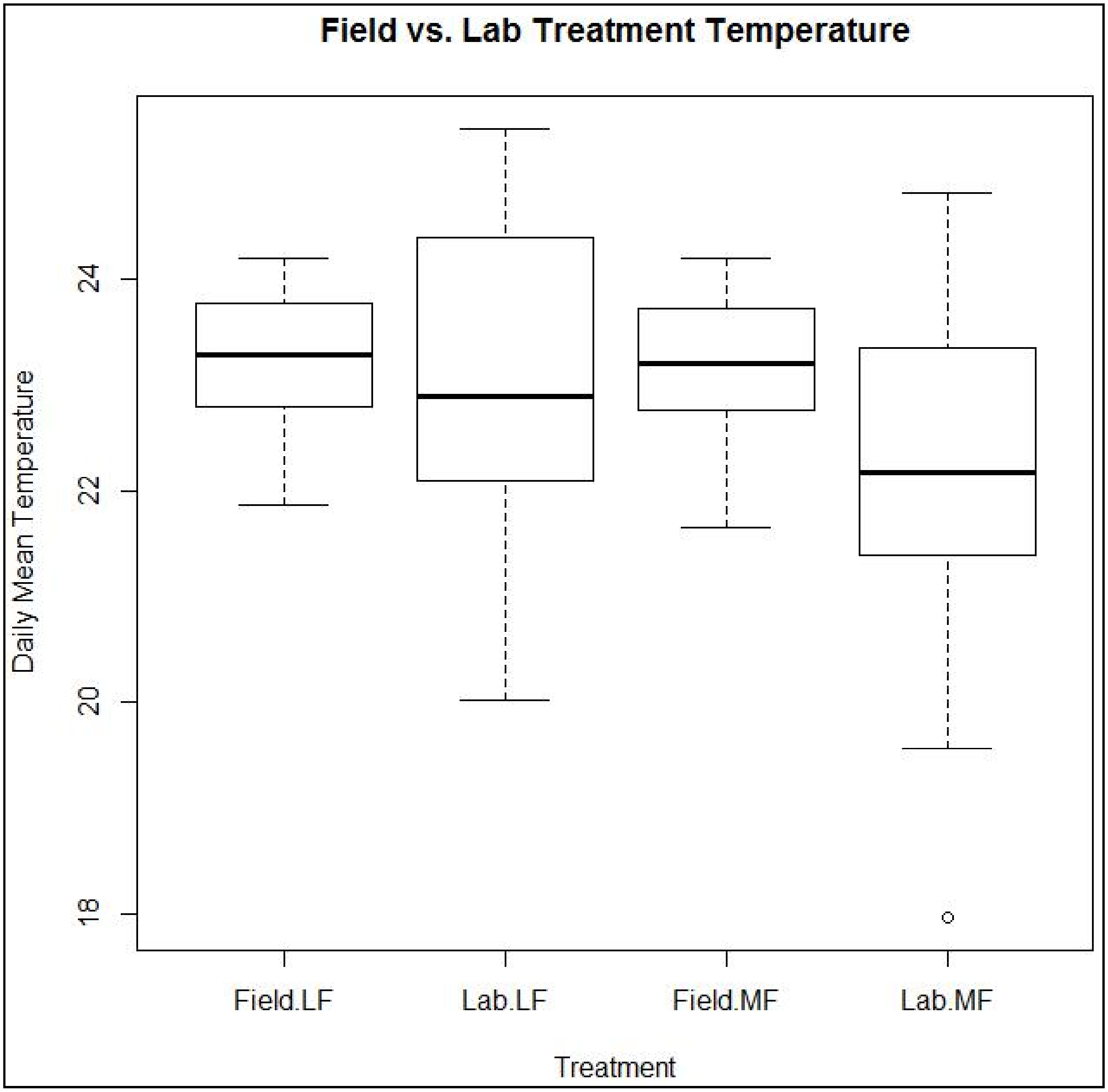


Figure 10. **Box-whisker plots of water temperature results between treatments. The bold horizontal line represents the median of the data. The box is bounded by the first and third quartiles (representative of the middle 50% of the data). Dashed vertical lines, or “whiskers”, extend from the box 1.5 times the interquartile range (~ 2 standard deviations). LF=low flow, MF=minimal flow.**

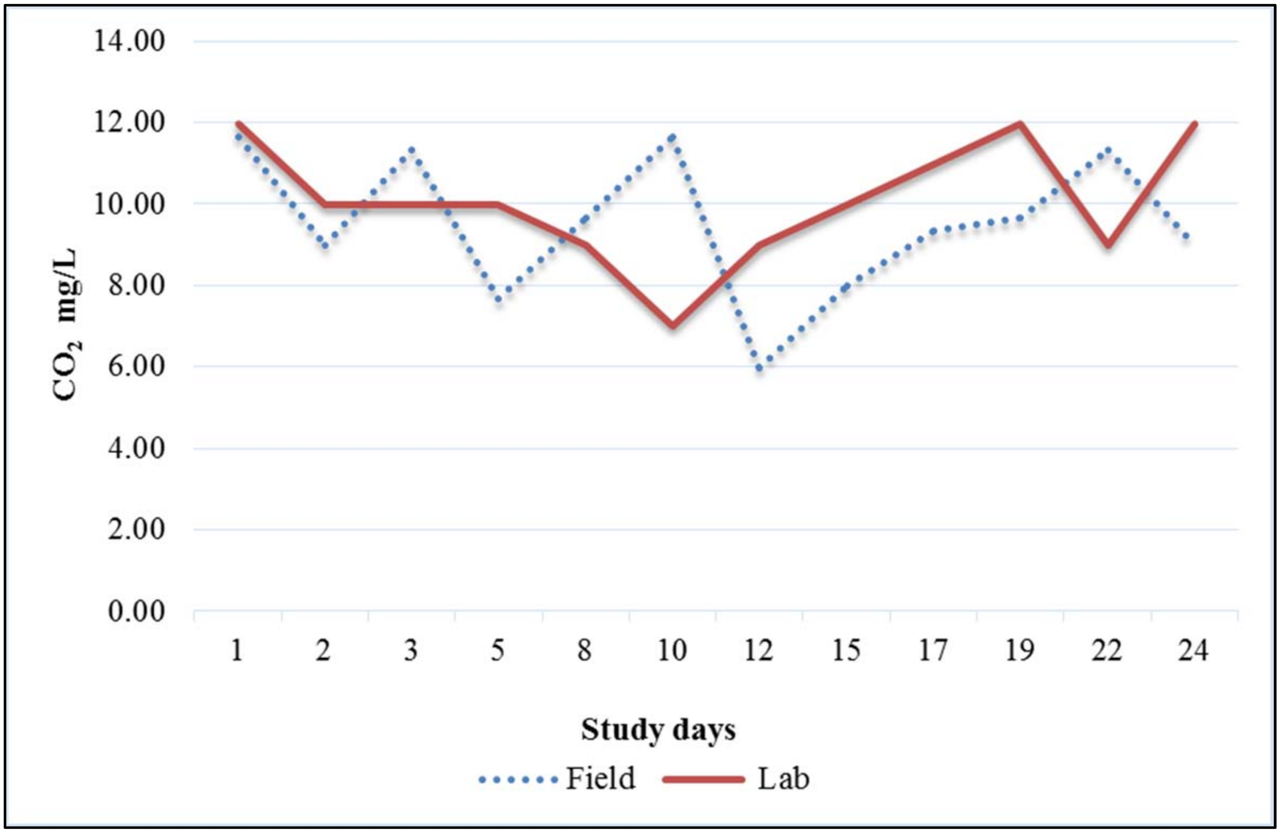


Figure 11. Dissolved carbon dioxide (CO2) concentration over time for both the Old Channel study reach and San Marcos Aquatic Resource Center (ARC) reservoir tank.

Figure 12 and Table 4 shows the point velocities measured over time within each treatment (tank or MUPPT). As expected, there was more variability in water velocities experienced by the study plants in the field treatments than those in the laboratory. The upstream-most field site, Field LF1 in the low-flow treatment (0.15 to 0.25 ft/sec), experienced the highest point velocities, while Field LF2 experienced the greatest variability of all the low-flow treatments. Field sites MF1 and MF3 were slightly higher than all other treatments in the minimal-flow treatment (Figure 12, bottom), yet the means were near the treatment goal of 0.05 ft/sec or below (Table 4). Field MF3 (minimal-flow treatment) was located on the corner of a bend, and it became evident during the study that upstream vegetation and debris caused shifting velocities at this location. However, even with that added variability, overall there was an extremely low velocity range for the minimal-flow treatments. Point velocities between similar treatments across experiments were tested using Wilcoxon rank-sum tests. A Wilcoxon rank sum test showed that combined field units vs. combined laboratory units were significantly different for both low-flow ($W=1136.5$, $p=0.002727$) and minimal-flow ($W=1275.5$, $p=<0.001$) treatments. Ultimately, the project team determined these differences (Table 4, Figure 12) were not large enough to warrant the elimination of any treatment units from subsequent plant growth analysis.

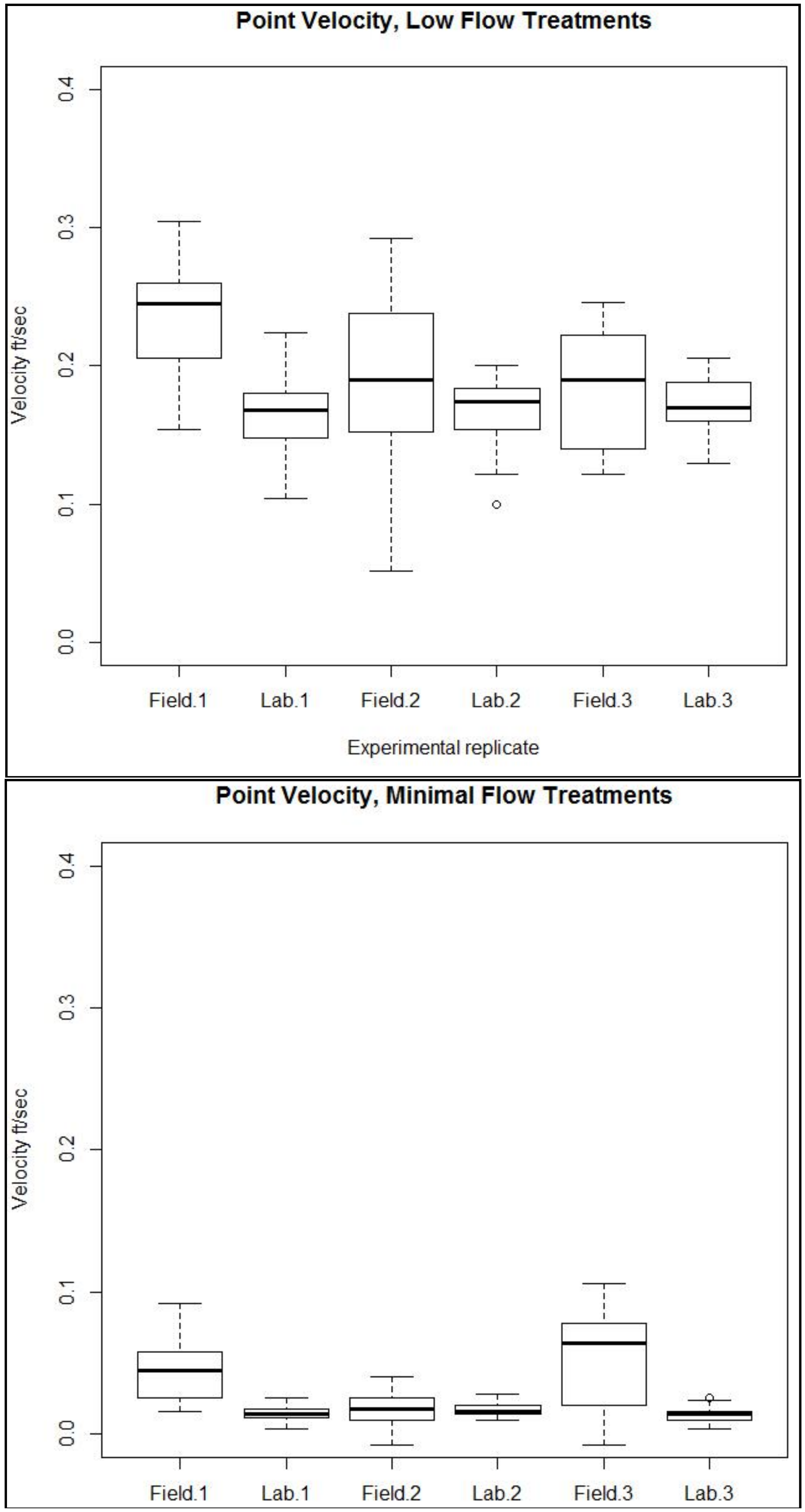


Figure 12. **Box-whisker plots of point velocity measurements in 0.15 to 0.25 ft/sec treatments (top) and <0.05 ft/sec treatments (bottom).**

Table 4. **Summary of point velocity measurements in 0.15 to 0.25 treatments (low-flow) and <0.05 treatments (minimal-flow). Measurements in feet per second (ft/sec).**

POINT VELOCITY MEASUREMENTS (FT/SEC) IN LOW-FLOW TREATMENTS (0.15 TO 0.25 FT/SEC)						
	FIELD			LAB		
	LF1	LF2	LF3	LF1	LF2	LF3
Maximum	0.30	0.29	0.25	0.22	0.20	0.22
Minimum	0.15	0.12	0.12	0.10	0.10	0.10
Mean	0.24	0.20	0.19	0.17	0.17	0.17
Standard deviation	0.05	0.05	0.04	0.03	0.03	0.03
Range	0.15	0.17	0.12	0.12	0.10	0.12
POINT VELOCITY MEASUREMENTS (FT/SEC) IN MINIMAL-FLOW TREATMENTS (>0.05 FT/SEC)						
	FIELD			LAB		
	MF1	MF2	MF3	MF1	MF2	MF3
Maximum	0.09	0.04	0.11	0.03	0.03	0.03
Minimum	0.02	-0.01	-0.01	0.00	0.01	0.00
Mean	0.05	0.02	0.05	0.01	0.02	0.01
Standard deviation	0.02	0.01	0.03	0.01	0.01	0.01
Range	0.08	0.05	0.11	0.02	0.02	0.02

Figure 13 provides a summary of the PAR measurements taken over the course of the study. This chart must be interpreted with caution because the plants at the ARC received fairly constant sunlight conditions throughout the day, whereas the Old Channel lighting was primarily dictated by the time of day of sampling. As such, to the degree possible, PAR measurements were taken during the same late morning to early afternoon window each time. The upstream-most site (Field LF1) in the Old Channel had the least amount of canopy cover and being on the north-south straightaway and eastern bank, clearly received the most sunlight during the measurement period. Light availability and potential implications are discussed in Section 2.5.

The experimental response of *Sagittaria* (Figure 14), *Ludwigia* (Figure 15), and *Cabomba* (Figure 16) are shown for each of the measured variables. These figures show MUPPT/tank totals (survival) or average per-pot values of surviving pots (all other parameters).

Figure 17 and Table 5 shows the results of the Two Way ANOVA for each species for each of the six reported parameters. Examination of Table 5 and Figure 17 leads to three key generalizations:

1. Few instances of factor interaction, indicating that the *pattern* of response (increase/decrease/no change) in response to flow levels is similar between the laboratory and the field.

2. General lack of flow response (except in two cases of interaction). That is, the two flow regimes examined were not sufficiently different to result in vegetative responses over the course of these experiments.

3. Numerous instances where the location factor (lab vs. field) is significant, indicating that the absolute value of the response variables differ between the laboratory and field (although the *pattern* of response usually did not).

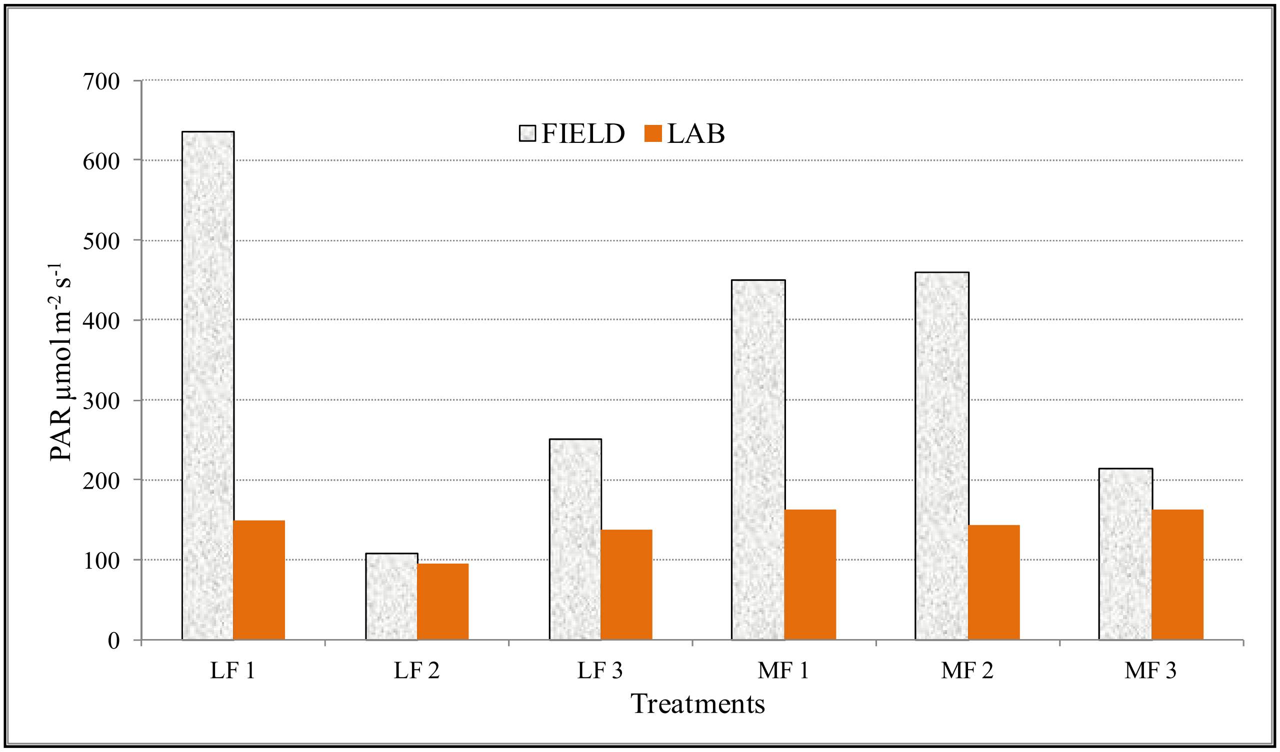


Figure 13. **Average photosynthetically active radiation (PAR) readings per treatment (LF=low flow, MF=minimal flow).**

The survival, RGR, and change in leaf number of *Sagittaria* did not differ between laboratory and field or between low- and minimal-flow treatments. Maximum leaf length was moderately (but significantly) higher in laboratory cultures than in the field (the lab maximum leaf length of 20–25 cm was some 20% higher than for plants grown in the field). The change in total leaf length was twice as high in the laboratory vs. the field (an average of 162 cm in the lab vs. 83.5 cm in the field). For *Sagittaria*, a significant interaction existed for AGBG. This interaction was driven by the higher values of the laboratory minimal-flow treatment, where the AGBG ratio of 1.89 was considerably higher than all other treatments (1.40–1.42, see Table 5).

Ludwigia survived well at all locations. There was 100 percent survival in the laboratory while two plants in each of two MUPPTs in the field died (Figure 15). However, there was no factor interaction and significant difference of either location or flow for survival (Table 5, Figure 17). *Ludwigia* showed a significant interaction for RGR (p=0.03, Table 5). This interaction was driven by the very low RGR for the field minimal-flow MUPPTs, two of which had negative RGRs (plant biomass at the end of the experimental growth was lower than at the beginning). During the course of the experiment, fine sediment deposition on leaves of MUPPT plants was quite evident in all field treatments but this was more prevalent for the minimal-flow treatments. Due to the controlled environment, sedimentation was not evident in any of the laboratory treatments. Although plants were carefully cleaned three times per week, it is speculated that fine sediment accumulation on leaves of *Ludwigia* (which grows better in swifter moving water) caused the reduced RGR relative to *Cabomba* and *Sagittaria* (which often thrive in habitats with lower velocity). *Ludwigia* showed significantly higher growth in stem number, stem maximum length, and growth in total stem lengths

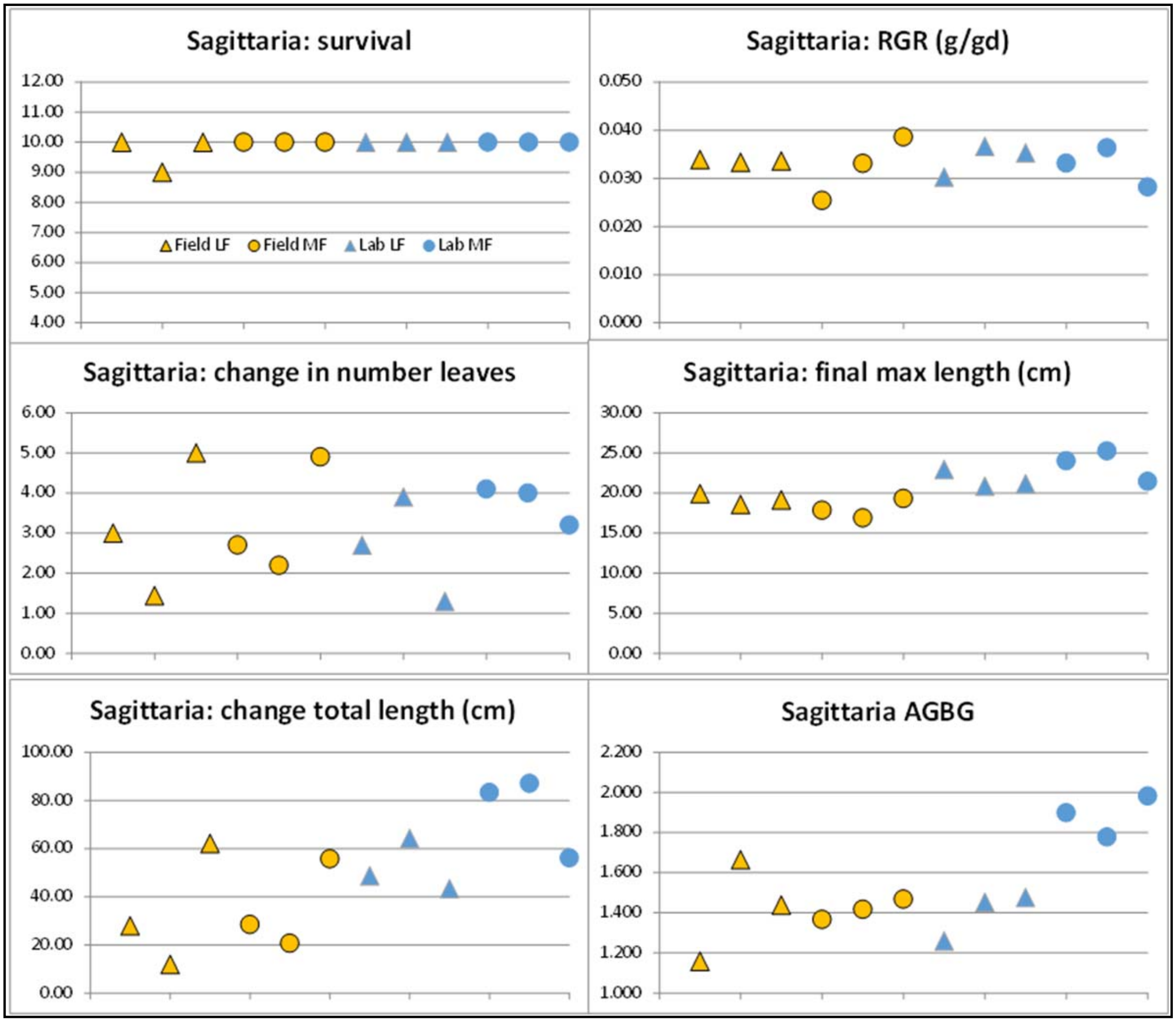


Figure 14. ***Sagittaria platyphylla*** **survival, relative growth rate, and various growth parameters. Data shown are totals (survival) or average per-pot data for surviving plants (remainder of parameters) in each of the trays (MUPPTs) or tanks.**

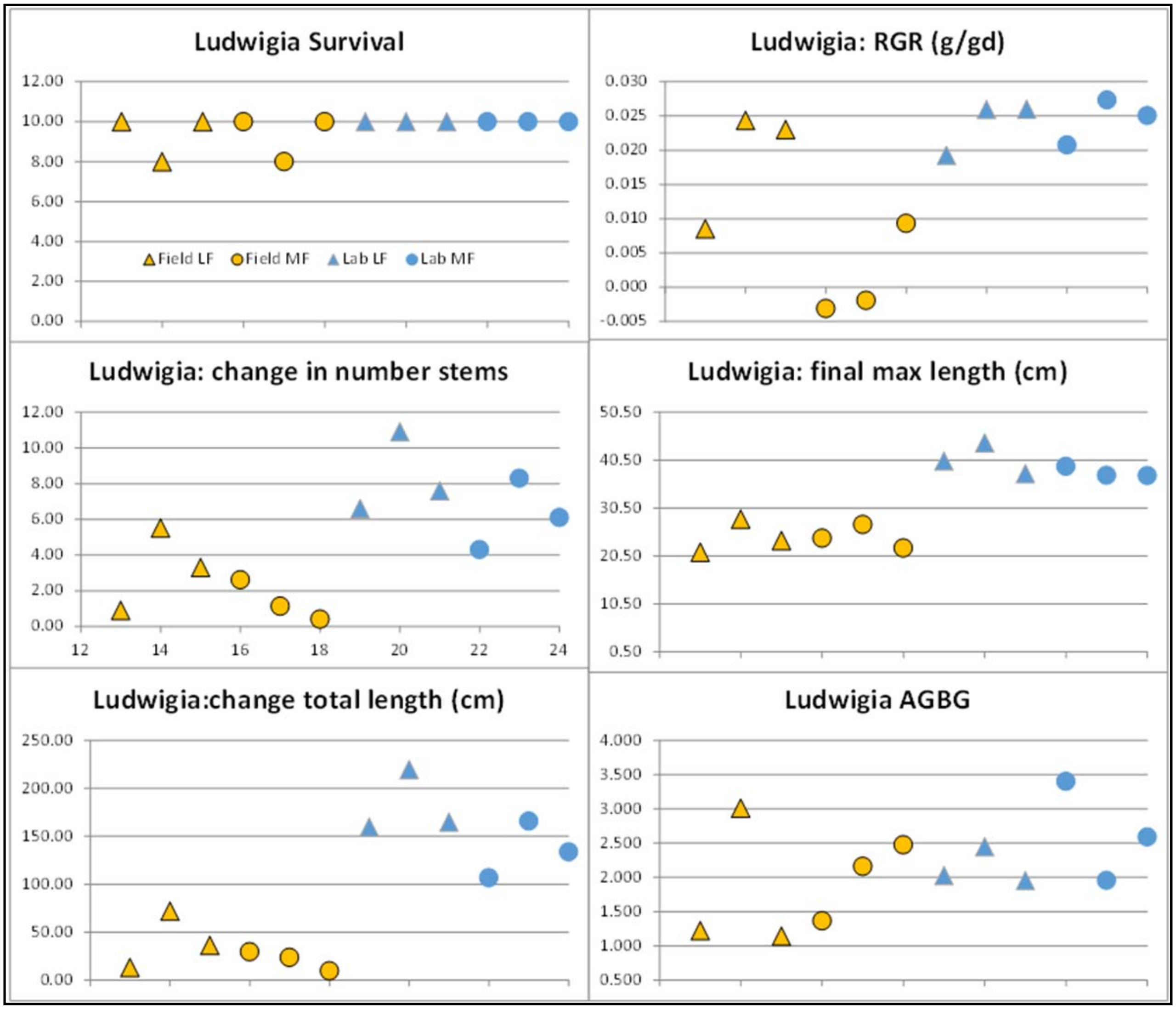


Figure 15. ***Ludwigia repens*** **survival, relative growth rate, and various growth parameters. Data shown are totals (survival) or average per-pot data for surviving plants (remainder of parameters) in each of the trays (MUPPTs) or tanks.**

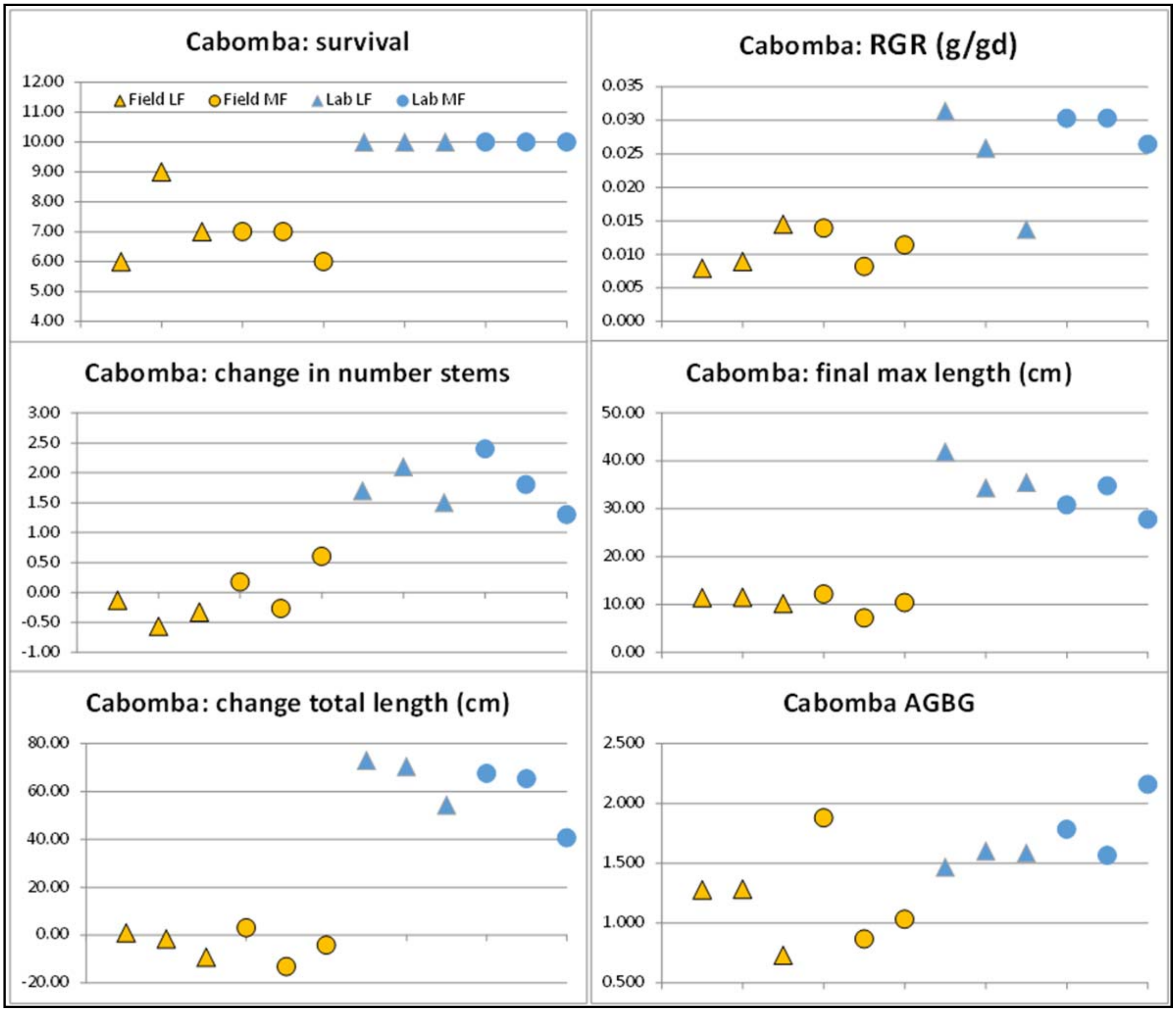


Figure 16. ***Cabomba caroliniana* survival, relative growth rate, and various growth parameters. Data shown are totals (survival) or average per-pot data for surviving plants (remainder of parameters) in each of the trays (MUPPTs) or tanks.**

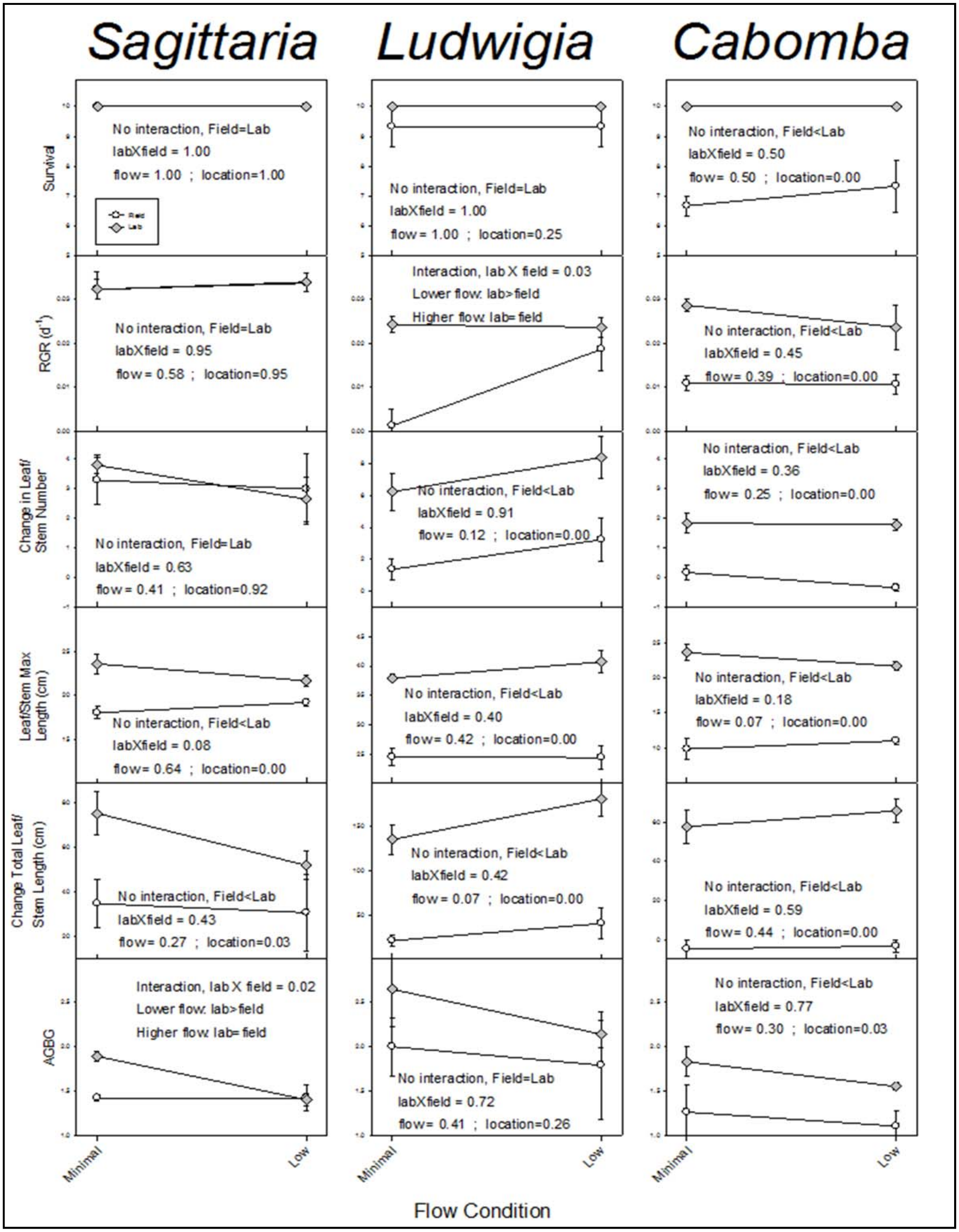


Figure 17. **Interaction graphs (plotted values are means ± SE, n=3) for the results of the Two Way ANOVA.**

Table 5. Mean ± SE or key parameters and p values for results of Two Way ANOVA (flow X location). Values based on averages for the three tanks (lab) or three MUPPTs (field) for each of the four location X flow combinations. Where the interaction term (Loc X Flow) is not significant (p>0.05), a single overall significance of each factor is reported. If the interaction term is significant, the response of each factor depends on the level of the other factor, so the significance of flow for each location and the significance of location for each flow condition is reported (ns=not significant, *=p<0.05).

SPECIES	PARAMETER	FIELD LOW-FLOW	FIELD MIN-FLOW	LAB LOW-FLOW	LAB MIN-FLOW	LOC X FLOW	FLOW	LOCATION
Sagittaria	Survival	10±0	10±0	10±0	10±0	1.00	1.00	1.00
	RGR[a]	0.0337±0.0003	0.0323±0.0041	0.0340±0.0021	0.0323±0.0023	0.95	0.58	0.95
	Change leaf #	2.97±1.18	3.27±0.83	2.63±0.75	3.77±0.28	0.63	0.41	0.92
	Leaf max length	19.20±0.39	18.02±0.70	21.65±0.66	23.57±1.12	0.08	0.64	**0.00**
	Change leaf total length	30.88±17.27	34.97±10.64	52.13±6.27	75.48±9.75	0.43	0.27	**0.03**
	AGBG[b]	1.42±0.15	1.42±0.03	1.40±0.07	1.89±0.06	**0.02**	Lab, * Field, ns	Low, * High, ns
Ludwigia	Survival	9.33±0.67	9.33±0.67	10.0±0	10.0±0	1.00	1.00	0.25
	RGR	0.0187±0.0048	0.0013±0.0038	0.0237±0.0023	0.0243±0.0018	**0.03**	Lab, ns Field, *	Low, * High, ns
	Change stem #	3.23±1.33	1.38±0.65	8.37±1.30	6.23±1.16	0.91	0.12	**0.00**
	Stem max length	24.40± 2.02	24.47±1.42	40.68±1.87	37.92±0.64	0.40	0.42	**0.00**
	Change stem total length	40.41±17.09	20.85±5.88	181.30±19.11	135.35±17.07	0.42	0.07	**0.00**
	AGBG	1.79±0.61	2.00±0.33	2.14±0.15	2.65±0.42	0.72	0.41	0.26
Cabomba	Survival	7.33±0.88	6.67±0.33	10±0	10±0	0.50	0.50	**0.00**
	RGR	0.0107±0.0022	0.0110±0.0017	0.0237±0.0050	0.0287±0.0013	0.45	0.39	**0.00**
	Change stem #	-0.34±0.13	0.17±0.25	1.77±0.18	1.83±0.32	0.36	0.25	**0.00**
	Stem max length	11.02±0.44	9.87±1.46	37.25±2.35	31.08±2.03	0.18	0.07	**0.00**
	Change stem total length	-3.30±3.06	-4.82±4.67	65.93±5.83	57.82±8.63	0.59	0.44	**0.00**
	AGBG	1.10±0.18	1.26±0.31	1.55±0.04	1.83±0.17	0.77	0.30	**0.03**

[a] RGR=relative growth rate.
[b] AGBG=aboveground:belowground ratio.

in the laboratory compared to the field. It is possible that the more protected environment in the laboratory allowed for longer stems. This phenomenon was also observed by Bormann (2012). Herbivory in the wild also may be an influencing factor on growth of *Ludwigia* in the field treatments, especially when considering the growth parameters measured in this study, including stem length, stem number, and aboveground biomass.

Cabomba survival and growth showed no significant interactions between location and flow. This plant showed a complete absence of significance to the flow factor and a consistent impact of the location factor with all factors showing significantly higher rates in the laboratory vs. the field (Table 5, Figure 17). Survival in the laboratory was 100 percent while all field MUPPTs showed mortality (1-4 of the initial 10 plants died). As mentioned previously with regard to *Ludwigia*, herbivory may be an important factor influencing the growth of *Cabomba* in the field treatments. Specific to *Cabomba*, turtles were visually observed feeding on these plants in several of the

MUPPTs. *Cabomba* RGR was positive in both the laboratory and the field, but was 3 times higher in the laboratory than the field. In contrast, the number of *Cabomba* stems and total length of stems *declined* in the field during the experimental growth period potentially a result of herbivory.

In Figure 17, values are based on averages for the three tanks (lab) or three MUPPTs (field) for each of the four location X flow treatment combinations. Where the interaction term (Loc X Flow) is not significant ($p>0.05$), a single overall significance of each factor is reported. If the interaction term is significant, the response of each factor depends on the level of the other factor so the significance of flow for each location (minimal flow and low flow) is reported.

2.5 Discussion

Although a high level of attention was given to keeping the laboratory conditions as similar to the field as possible, some parameters were simply not simulated equally or attempted for simulation during this study. Water temperature, CO2, and point velocity measurements were fairly well controlled, but slight differences may still have attributed to some of the differences noted in the experiment. Factors that likely played a more prominent role in the results include the higher daily shading due to riparian canopies in the field, fine sediment deposition on plants in the Old Channel, and herbivory. The first was acknowledged before the experiment, and sites were attempted to be placed in more open areas with available sunlight. However, the Old Channel has its constraints relative to open areas with the appropriate flow conditions. It is also unknown how important a factor this was as the PAR measurements (with the caveats about measurement times previously discussed) were consistently higher in field treatments than reported in the laboratory treatments.

The level of sedimentation on the MUPPT plants that occurred in the field treatments was not anticipated prior to the study. As previously noted, shoreline areas with low velocities were used for MUPPT placement. Some of these areas had no vegetation present to start with, or they required the removal of *Nuphar* to make them suitable for MUPPT placement. As such, monitoring of these shoreline areas for sediment deposition on plants over the years has not occurred. This was a valuable lesson learned regarding potential restoration areas and suitability of plants for upcoming restoration activities in this stretch of the Old Channel. A second impact of the sedimentation that may have actually led to reduced plant growth, including shorter lengths or number of stems, is the cleaning that was conducted every other day over the course of the study to keep the sediment off. Every attempt was made to clean in a non-disturbing way, but it is possible that leaves were inadvertently damaged or plants stressed during the cleanings. Cleaning material off of plant leaves in the laboratory treatments occurred as well due to the settling of particulate matter, but on a much less frequent basis.

Finally, the level of herbivory that occurred in these shoreline habitats was not anticipated. Herbivory was noted within treatments of all three aquatic plant species. Specifically, caterpillars (*Paraponyx* sp.) were observed on *Sagittaria*, and turtles were seen in and around the MUPPTs and on occasion visually observed eating *Cabomba*. Although not herbivory, a deer walked across one MUPPT (Field site MF1), completely destroying several of the pots and plants.

The factors described above, which potentially influenced the results, are complexities of the natural environment that are difficult to simulate in a laboratory environment. The overall lack of interaction between flow and location suggests that the overall *pattern* observed in the laboratory should reflect the *pattern* in the field. However, the differences in actual results between the laboratory and field is concerning. Ultimately, this study supports the intuitive conclusion that, where possible, field data should be used to project plant growth response.

Please refer to Section 6 for a more detailed discussion regarding lessons learned, HCP ecological model application, and recommended future applied research relative to the 2013 HCP field vs. laboratory study described in this section.

3.0 VEGETATION TOLERANCE STUDIES

3.1 Introduction

The Comal and San Marcos springs/rivers support a unique assemblage of submerged aquatic vegetation. Aquatic plants in these systems are adapted to the specific water quality and quantity conditions issuing from the Edwards Aquifer at both springs locations (Comal and San Marcos). Although an abundance of information is available on species types and areal coverage in these systems, limited information is available relative to how aquatic plants within these systems react to extreme water quality conditions. As several endangered aquatic animal species rely heavily on the Comal and San Marcos springs/river plant communities, it is imperative to determine how the existing aquatic plant species and communities might react to low spring flow conditions described within the flow management objectives in the HCP. The expectation is that extreme water quality conditions will likely be exhibited in the wild for short durations of time when evaluated in the context of the low end spectrum of the HCP flow management objectives.

The vegetation tolerance studies conducted in 2013 involved both a laboratory and pond component, each attempting to address concerns regarding plant response to minimal to no springflow and resulting water quality conditions. All vegetation tolerance studies were conducted at the ARC.

The objective of the low-flow threshold of native aquatic vegetation laboratory study (Laboratory Experiment) was to evaluate the effects of elevated water temperatures in combination with low CO2 levels and minimal flow on selected aquatic plants. The project team hypothesis was that extreme water temperatures coupled with low CO2 levels and minimal flow would negatively impact aquatic plant growth potentially leading to plant death.

The objective of the low-flow threshold of native aquatic vegetation pond study (Pond Experiment) was to evaluate the effects that loss of water flow in an outdoor environment would have on standard water quality parameters and survival and growth of native plant species. It is hypothesized that loss of flow into the experimental water body will result in significant changes in water quality parameters, leading to slower growth or reversal of growth leading to reduced survival of these plant species.

3.2 Data Review and Available Literature

Water and sediment temperatures play an important role in the global distribution and community structure of aquatic plants by affecting their physiology, growth rates, dormancy periods and reproductive traits (Sculthorpe 1967, Welch 1952). The effect of water temperatures on aquatic plant growth has been well studied. A majority of aquatic plant species are known to persist between 10 and 45 ˚C, though temperature preferences for individual species vary seasonally and geographically (Madsen and Adams 1988). Some species are able to persist under warm water conditions formed artificially (Grace and Tilly 1976). Many species exhibit differing life cycle habits in response to these differences. The invasive hydrophyte *Hydrilla verticillata* is known to increase tuber production at warmer water temperatures (McFarland and Barko 1999). In general, the peak biomass of aquatic plants occurs during warmer seasons when shoot elongation and branching is at maximum potential. Results of Barko and Smart (1981) showed increasing shoot length in *Myriophyllum spicatum* and *Hydrilla verticillata* with increasing temperatures. Optimal growth for *Potamogeton* (Stuckinia) *pectinatus* occurs between 23 and 30˚C (Spencer 1986), and many seagrasses are known to increase their growth rates in response to warm temperatures (Bulthuis 1987). Photosynthetic rates are known to increase with increasing temperatures in *Cabomba caroliniana* (Bultemeir 2008). In *Vallisneria americana*, cooler water temperatures (10–14˚C) are thought to induce winter bud formation, while sexual reproduction most often occurs in waters above 20˚C (McFarland and Shafer 2008). However, the southern ecotypes of *Vallisneria americana* typically never produce winter buds. Other responses of aquatic plants to increased temperatures include heterophylly and production of immature shoots (Arber 1920, Bostrack and Millington 1962, Kane and Albert 1982).

Conversely, growth of some non-vascular plants seems to be positively influenced by cooler temperatures. Sand-Jensen and Riis (1997) found the moss *Sphagnum subsecundum* had a faster growth rate in deep, cooler waters than in shallow, warmer waters, and Kelly and Whitton (1987) showed growth rates of *Rhynchostegium riparioides* peaked in spring and autumn. While optimal growth of aquatic bryophytes may occur at cooler water temperatures, survival of certain species to increased water temperatures is possible (Carballiera et al. 1998).

While temperature is a key factor in the ecology of aquatic plants, other factors are also important. Studies have documented the importance of biological interactions between macrophyte species (Titus and Stephens 1983, Doyle et al. 2003), physical factors (Barko et al. 1984, Barko and Smart 1986, Madsen et al. 2001) and chemical properties of the environment (Titus et al. 1990, Pagano and Titus 2004, Engelhardt 2006, Bailey 2012) on macrophyte growth and distribution.

While many factors affect distribution and abundance of aquatic macrophytes, these plants also contribute to the aquatic environment in which they are found. They are important for many reasons including purifying water, recycling nutrients, providing refugia for zooplankton, providing cover for invertebrates, providing cover for fish, providing a food source, affecting flow patterns, and creating discrete habitat as physical structure in the water column (Cowx and Welcomme 1998). Aquatic macrophytes present within the San Marcos and Comal River systems have been documented by multiple ecological studies in the region (Lemke 1989, USFWS 1996, Poole and Bowles 1999, Owens et al. 2001, BIO-WEST 2002–2013a,b). Three common rooted species providing fountain darter habitat in these systems include eelgrass (*Vallisneria* spp.; hereafter, *Vallisneria*), *Cabomba*, and *Ludwigia*. All three are an important part of the aquatic macrophyte community in the San Marcos and Comal rivers.

Cabomba is a submerged aquatic plant that grows in stagnant to slow-flowing freshwater, and spreads primarily by stem fragments. *Cabomba* prefers a warm, humid climate with a temperature range of 13–27 °C and can grow well in turbid water (WSDE 2013). *Ludwigia* can be found growing under a wide range of conditions including streams, ponds, wetlands and drainage ditches. It as an amphibious plant that produces both submerged and emergent portions, and can grow in wet terrestrial habitats (Godfrey and Wooten 1981). *Vallisneria* is a submerged, rosette-forming, native aquatic plant that occurs in streams, lakes, and brackish water habitats. Since it is considered an important aquatic plant for wildlife, much work has been done studying responses of *Vallisneria* to environmental factors such as light availability and salinity changes. Total biomass production of *Vallisneria*, *Elodea canadensis*, and *Potamogeton nodosus* has been shown to increase with both increasing light and increasing temperature to at least 28 °C (Barko et al. 1984), whereas Boustany et al. (2010) found salinity directly impacted growth of *Vallisneria*, but that light effects were less direct.

3.3 Laboratory Experiment

The objective of the Laboratory Experiment was to evaluate the effects of elevated water temperatures in combination with low CO2 levels and minimal flow on selected aquatic plants. The experimental design consisted of a series of three experiments (two pre-trial studies and the subsequent formal laboratory experiment) conducted in the 1,200-square-meter research greenhouse located at the ARC. Section 3.3.1 describes the methods and results of the pre-trial studies, while Section 3.3.2 thru Section 3.3.4 describes the formal Laboratory experiment methods, results and discussion, respectively.

3.3.1 Pre-trial Studies

The purpose of the pre-trial studies was to investigate the general influence of CO2 and water temperature on growth of the aquatic plant *Ludwigia* and the liverwort *Riccia* across several different treatments. This exercise, although limited in scope, helped to guide further development of the main vegetation tolerance laboratory experiment. The first pre-trial evaluation was qualitative in nature and did not account for emergence of vegetation during the investigation. The second pre-trial was more quantitative in nature and designed to investigate how *Ludwigia* would react if forced to grow entirely under submersed conditions.

Pre-trial 1

For the first pre-trial, *Ludwigia* and *Riccia* were selected for study, as both species provide important habitat for the fountain darter in these systems. Five potted and established plants were selected from a stock of *Ludwigia* originally collected from the Comal River and propagated in the ARC greenhouse. These plants had been potted in quart-sized nursery pots 10.16 cm in diameter by 10.16 cm tall. They were allowed to establish under greenhouse conditions for approximately 2 weeks. *Riccia* was selected from established, quarantined, parent material held at the ARC for experimental use.

Experimental equipment set up consisted of five 18.93 L glass aquarium tanks with bottom-draining stand pipes arranged over a fiberglass sump tank allowing for easy drainage (Figure 18). Three of the tanks were plumbed to receive reservoir water from a round, 681-L fiberglass tank with a water recirculation system and degassing tower to provide low CO2 levels. The reservoir tank had been circulating for several weeks prior to ensure CO2 levels were stable and at the desired concentration. As presented in Table 6, one tank was plumbed to receive Edwards Aquifer well water, considered the control tank, and one tank received water added to it manually. Heating was provided by 300- or 400-watt, consumer-grade submersible aquarium heaters. Flow into tanks was adjusted to approximately 0.95 L per minute (L/m) for tanks 1, 2, and 3. Tank 4 received a higher flow of 1.9 L/m flow in order to maintain water temperature.

Figure 18. Pre-trial 1 equipment setup, including a five-tank array plumbed with various CO2 concentration and water temperature treatments.

Initially, all tanks were filled with Edwards Aquifer well water. One pot of *Ludwigia* and three experimental cups of *Riccia* were placed into each glass aquarium. The experimental cups for *Riccia* consisted of parfait cups with clear plastic dome tops (Figure 19), mesh screen covering the top opening, and small holes punched into the cup to allow through-flow. After a 24-hour acclimation period, each tank was adjusted toward its respective treatment description reaching its

Table 6. Description of Pre-Trial 1 treatment tanks.

TANK	DESCRIPTION
1	Reservoir water with heater set to 29 degrees Celsius (ºC).
2	Reservoir water with heater set at 29 ºC for 11 days, then adjusted to 40 ºC for remainder of trial.
3	Reservoir water with no heater.
4	Well water with no heater (control tank).
5	Filled and manually topped-off with reservoir water. No flow, no heater.

Figure 19. *Riccia fluitans* in an experimental cup.

target treatment at the end of the next 24-hour period. Water temperature and dissolved CO2 were measured twice a day, morning and evening, for a total duration of 18 days in all five tanks and the water supply reservoir. Temperature measurements were made using a YSI pro-series multi-probe sonde (YSI Inc., Yellow Springs, Ohio) and CO2 concentrations were measured using an Oxyguard portable CO2 Analyzer (Oxyguard International AS, Berkerød, Denmark).

Overall, physiochemical parameters of each treatment tank remained relatively constant for the duration of the 18-day study. Dissolved CO2 remained low (<5 milligrams per L [mg/L]) in all tanks receiving reservoir water, whereas high CO2 levels (25–40 mg/L) occurred in Tank 4. A detailed description of physiochemical parameters is not presented here, but is available in the project notebooks submitted to the Edwards Aquifer Authority (EAA) for the vegetation tolerance project. Initially, the *Ludwigia* plants were observed to be similar in size and robust in growth with no emergent tips, and the *Riccia* was robust with bright green coloration. Slight algal growth was noted to be present on all the *Ludwigia* plants when they were placed into the treatments. At day 7, *Ludwigia* in all tanks was noted as having emergent tips, and *Riccia* was noted as emerging from the tops of several experimental cups. By day 10, excessive algal growth was noted in tanks 4 and 5, while tanks 1, 2, and 3 exhibited minimal-to-no algal growth. On day 11, the temperature was adjusted to 40 °C in Tank 2. At day 14, *Ludwigia* in Tank 2 was covered with crystalized calcium and the leaves of the plant were pale and transparent, yet new growth was emerging from the base of the plant. By day 18 (the final day of the pre-trial), heavy algae build up was present in Tank 4. Also on day 18, *Ludwigia* in tanks 1 and 3 were healthy with robust emergent tips growing out of the top of the tanks, while Tank 5 had sparse emergent growth. At the study conclusion, most of the leaves of *Ludwigia* in Tank 2 had senesced yet new growth continued to emerge from the base of the plant. Similarly, the *Riccia* in Tank 2 was pale gray in color and obviously in poor health by the conclusion of the pre-trial.

In summary, *Ludwigia* was able to survive across all treatments. Qualitatively, tanks 1 and 3 were observed to have mostly emergent growth. Tanks 4 and 5 were observed to have less emergent growth but were characterized as healthy. *Ludwigia* in Tank 2 was regarded as alive due to new growth emerging from the bottom of the plant stems, although all top growth had senesced or was otherwise not living. *Riccia* was able to survive across treatments 1, 3, 4, and 5 but did not survive in Tank 2.

Pre-trial 2

Based on pre-trial 1, a second trial was conducted using the same plants (*Ludwigia* and *Riccia*). The experimental equipment set up was the same as pre-trial 1 (Figure 18) with the exceptions that all tanks were covered with plexiglass tops to prevent vegetation becoming emergent, and pre-study plant preparation for *Ludwigia* was slightly different. Table 7 lists the pre-trial 2 treatments that were identical in nature to pre-trial 1. Two 8-cm fragments of *Ludwigia* collected from the New Channel of the Comal River were planted in 6.35-cm-long by 6.35-cm-wide and 5.08-cm-deep nursery containers filled with clay loam soil. A total of 20 pots of *Ludwigia* were planted and allowed to establish for 1 week. *Riccia* was again selected from established, quarantined, parent material held at the ARC for experimental use.

Table 7. Description of Pre-Trial 2 treatments.

TANK	DESCRIPTION
1	Reservoir Water with heater set to 29 degrees Celsius (ºC).
2	Reservoir water with heater set at 29 ºC for 3 days, then raised to 40 ºC for remainder of trial.
3	Reservoir water with no heater.
4	Well water with no heater (control tank).
5	Filled and manually topped off with reservoir water. No flow, no heater.

Initially, all tanks were filled with Edwards Aquifer well water. Three randomly selected pots of *Ludwigia* were placed into each treatment tank. Prior to placement, all *Ludwigia* plants were measured and total stem length from soil level to apical tip was recorded for each pot to document an initial growth measurement (initial [i] growth). Clumps of *Riccia* approximately 8 g wet weight (initial [i] weight) were placed in experimental cups and three cups were added to each treatment tank. After a 24-hour acclimation period, each tank was adjusted toward its respective treatment description reaching its target treatment by the end of the following 24-hour period. Water temperature, dissolved CO2, dissolved oxygen (DO), and pH were measured twice a day (morning and evening) for a total duration of 8 days in all five tanks, and the water supply reservoir using the same equipment that was used during pre-trial 1.

At the end of the study, total stem length of *Ludwigia* plants were measured from soil level to apical tip to document the final growth measurement (f growth). All *Riccia* plants were weighed to document the final wet weight in g (f weight). To determine added growth or added wet weight initial measurements (i growth or i weight) were subtracted from final measurements (f growth or f weight).

Similar to the first pre-trial, physiochemical parameters of treatment tanks remained relatively constant during the 8-day second pre-trial. Dissolved CO2 remained low (<5 mg/L) in all tanks receiving reservoir water, whereas high CO2 levels (25–35 mg/L) occurred in Tank 4. As stated for pre-trial 1, a detailed description of physiochemical parameters is not presented herein, but is available in the project notebooks submitted to EAA for the vegetation tolerance project. Initially, the *Ludwigia* plants were observed to be similar in size and robust in growth with no emergent tips. The *Riccia* was robust with bright-green coloration. By day 4, the *Ludwigia* plants in Tank 2 (29 °C; raised to °40 C, low CO2) were noted to have senesced leaves with little green growth. The *Riccia* plants were still green in color. At day 4, the *Ludwigia* plants in tanks 1, 3, 4, and 5 were healthy and putting on new growth, and the *Riccia* plants were also green. It should be noted that algal growth was also evident in Tank 4 (well water with high CO2). By day 7, both *Ludwigia* and *Riccia* in tanks 1, 3, 4, and 5 were alive with heavy algae growth in Tank 4. *Ludwigia* and *Riccia* plants in Tank 2 were dead.

Plants in tanks 1, 3, 4, and 5 were able to maintain or add additional growth across the study period. Of those treatments, the *Ludwigia* plants in Tank 4 (control) grew the most (55.5-cm growth), while plants in Tank 1 grew the least (20.5-cm growth). All plants in Tank 2 did not survive to final harvest. Since *Ludwigia* and *Riccia* plants in other tanks subjected to low CO2 treatments and cooler water temperatures survived it was concluded that the temperature in Tank 2 was likely the key factor leading to the plants' death. Additionally, plant maturity and establishment could also play a role in survivability under the pre-trial conditions since the older *Ludwigia* plants used in the first pre-trial were able to survive the 40 °C treatment.

3.3.2 Formal Laboratory Experiment

Based on the data review and literature search as well as the pre-trial studies, the formal laboratory experiment was designed and conducted at the ARC. For the formal laboratory experiment, *Vallisneria*, *Cabomba*, *Ludwigia*, and *Riccia* were selected. These plant species were chosen for this study because of their importance as habitat for the fountain darter, and because they make up a large portion of the aquatic plant community in the Comal River. Parent plant material was collected from the Comal River system. *Ludwigia* and *Cabomba* was sourced from parent stands located in the New Channel of the Comal River while *Vallisneria* plants were sourced from Landa Lake. *Riccia* material was sourced from a mother colony grown at the ARC greenhouse for experimental purposes. As previously described in the Lab vs. Field Study Methods (Section 2.4), plant material collected from the river was treated and propagated in quart-size pots filled with 900 mL of sandy clay loam soil. Parent plant material was cut into 8-cm-long apical fragments. Two apical fragments (*Cabomba* and *Ludwigia*) or a single rosette (*Vallisneria*) were planted in each pot for a total of 100 pots per species. The potted plants were placed into quarantine troughs in the ARC greenhouse under 30 percent shade for establishment and monitoring for nuisance aquatic species. Due to slow growth rate *Cabomba* and *Vallisneria* were allowed to establish for a period of 1 month, while *Ludwigia* plants were allowed to establish for a period of 2 weeks.

For the study, four 950-L fiberglass tanks (Living Stream Model MT-1024, Frigid Units Incorporated, Toledo, Ohio) were divided into eight independent tanks. Seven of them were subsequently used as treatments. Six of these tanks were plumbed to receive low CO2 water from a 750-L reservoir tank. One tank was plumbed to receive Edwards Aquifer well water directly. To produce the required temperatures for this study, a single, 120-volt, industrial, L-shaped submersible heater (Process Technology, Mentor, Ohio) was placed into each of the six treatment tanks.

To initiate the study, six tanks were randomly assigned a water temperature treatment of either 28 °C (tanks 3, 5, and 6) or 34 °C (tanks 1, 2, and 4) with one tank (7) assigned to act as the control tank. Heaters were placed into treatment tanks 1–6. Next, 84 plants of each vascular plant species were randomly selected, measured for initial total stem length, and placed into the tanks. Plants were also placed into randomly selected positions within each tank. Prior to study initiation, 10 randomly selected vascular plants of each species were harvested to gain initial above and belowground biomass measurements. *Riccia* was removed from the parent colony and placed into 84 clumps of a standard 8 g wet weight roughly 5 cm in diameter. These clumps were then placed into clear plastic parfait cups 400 mL in volume with domed tops and filled three quarters full with pea gravel for ballast. Mesh covering was placed over the dome top opening and holes were drilled into the side of the cup to allow water exchange. The experimental cups were placed on upturned ceramic bricks to make them equal in height with vascular plants in the treatment. Ten additional clumps of *Riccia* were also harvested for initial biomass data. Initial harvests were conducted on June 19, 2013.

Plants were then placed into tanks that were initially filled with well water (Figure 20). Reservoir water was slowly introduced to the tanks and water temperatures were increased over a period of 72 hours toward their respective treatment conditions to allow for acclimatization. Incoming water flow was set at approximately 1.9 L/m in all tanks. The 28 °C/34 °C study was run for a total of 47 days (June 19 to August 5, including the acclimatization period). Over the course of the study period, four plants of each species from each treatment were harvested every 2 weeks (July 8, July 22, August 5). At the end of the study period, final total stem length measurements were recorded and all remaining plants harvested to assess biomass. Standard brown paper bags were dried for 24 hours at 60 °C then weighed on an electronic balance to obtain a tare weight to the nearest 0.001 g. Aboveground growth (shoots) and belowground growth (roots) were divided to be analyzed

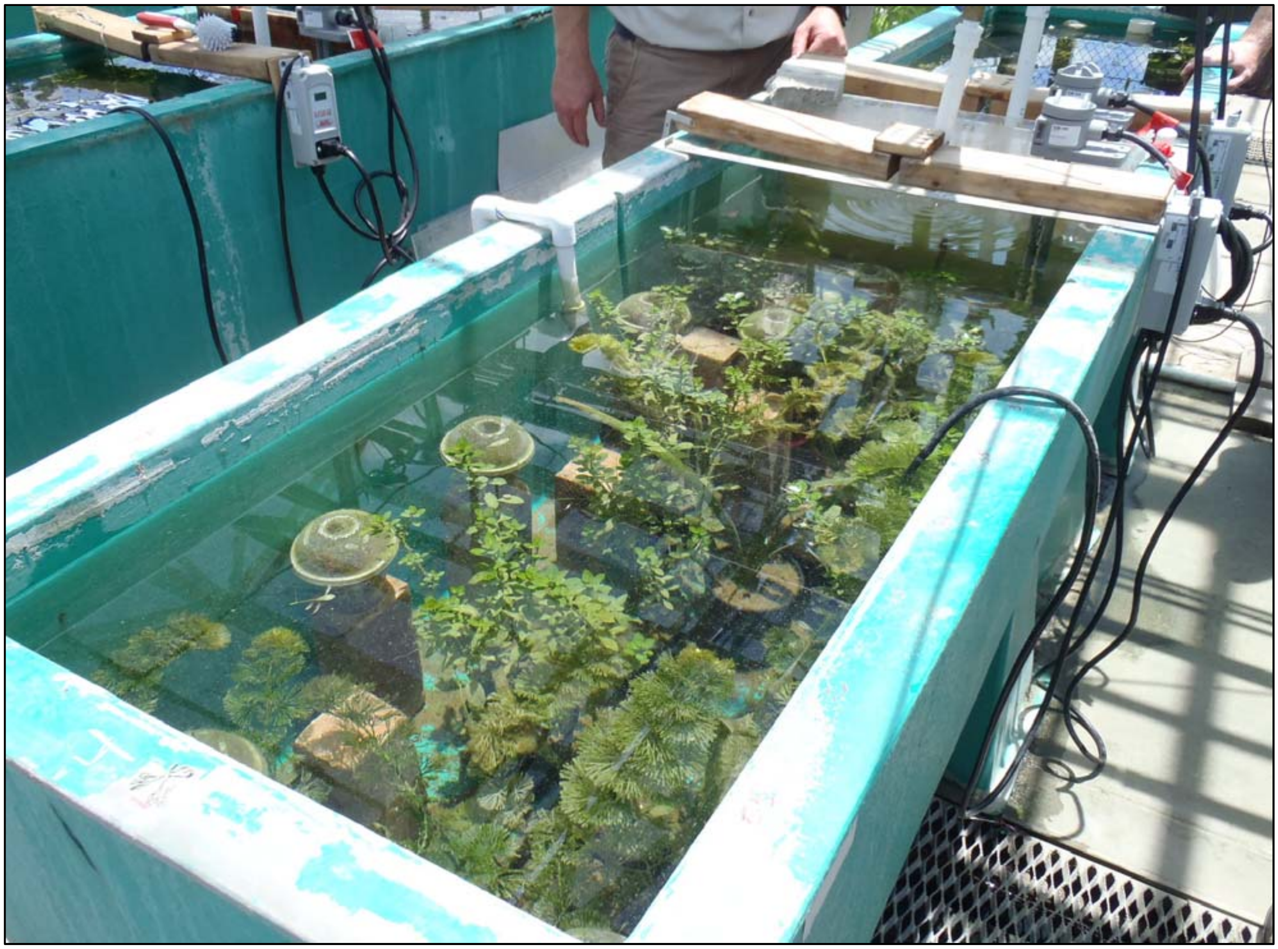


Figure 20. Formal laboratory experiment treatment tank with four aquatic plant species.

separately. Shoots were clipped at the soil surface, and divided plants were rinsed and placed into separately tared, labeled paper bags. *Riccia* were processed as whole plants because bryophytes lack the root-shoot system. Bagged plants were transported to Baylor University where they were dried for approximately 96 hours at 60 °C then weighed to the nearest 0.001 g.

During the experiment, water quality parameters were measured twice a day, morning and evening, 5 days per week. Dissolved oxygen, temperature, and pH were measured at the opposite end of incoming water using a YSI multi-parameter sonde with a pro series handheld data unit (YSI Inc., Yellow Springs, Ohio). Dissolved CO2 was measured using the Oxyguard portable CO2 Analyzer (Oxyguard International AS, Berkerød, Denmark). Additionally, continuous temperature readings were measured at 15-minute intervals with Tidbit Temperature data loggers (Onset Computer Corporation, Cape Cod, Massachusetts). Measurements of PAR were taken from three locations along the middle of each tank, approximately 2 cm below the water surface and directly over the plants. The PAR was measured with the MQ-200 Quantum meter (Apogee Inc., Logan, Utah).

After the 28 °C and 34 °C treatments were completed and analyzed, the project team decided to continue with an additional follow up treatment of 37 °C. This experiment was conducted following a similar design and methods as described above. All vascular plants were collected, quarantined, propagated, measured, and harvested using the previous methods and were allowed 2 weeks for establishment. With only one temperature treatment, this experiment used only four fiberglass troughs, three of which were randomly designated as replicates for the 37 °C treatment and one trough again designated as a control. A total of 20 plants (5 of each species) were placed into each tank. Prior to study implementation, an additional five plants per species were harvested for initial biomass. Plants were allowed to remain in their treatment for a period of 21 days, at which time they were measured and harvested for final biomass. The main difference in the 37 °C follow-up study is that the first study extended for 47 days with partial harvest occurring at the second- and fourth-week intervals. The 37 °C study did not conduct partial harvest during the study period. Plants that were living at the end of this trial were measured for length, harvested and processed for biomass and the number of dead plants was recorded.

Response Variables

Survival

The total survival for each species for each tank was computed simply as the total number of surviving plants of each species.

Relative Growth Rate (RGR)

Plant RGR is a measure of growth over a specified time period relative to the current plant size and has units of [mass / (mass x t)]. In this study RGR was estimated on a per-day basis and mass (in g) on a dry mass basis (RGR units are g g-1d-1 or more simply, d-1). Conceptually, this is a measure of proportional change in mass per day. Since there were data from multiple harvests (initials, 2 week, 4 week, 6 week), an average RGR was calculated for each tank by computing the slope of the line of Logarithm (natural) (ln) Total Biomass (g) vs. days of growth. Figure 21 shows an example of data computed for *Ludwigia* growing in the 22 °C control tank.

Change in Number of Leaves/Stems

The change (increase or decrease) in total number of leaves (*Vallisneria*) or stems (*Ludwigia*, *Cabomba*) was computed for each surviving plant by subtracting the average number of leaves/stems of the initial plants from the number in each pot at the end of the experimental growth period. The per-tank average was computed as the mean for all surviving pots in each laboratory tank. A change in the total number of leaves or stems reflects the plants basal area growth.

Change in Total Leaf/Stem Length

The change (increase or decrease) in total combined length of all leaves (*Vallisneria*) or stems (*Ludwigia*, *Cabomba*) was computed for each surviving plant by subtracting the average of the initial plants from the number in each pot at the end of the experimental growth period. The per-tank average was computed as the mean for all surviving pots in each laboratory tank. A change in the total combined leaf or stem length is a measure of total energy investment in surface area for capturing light.

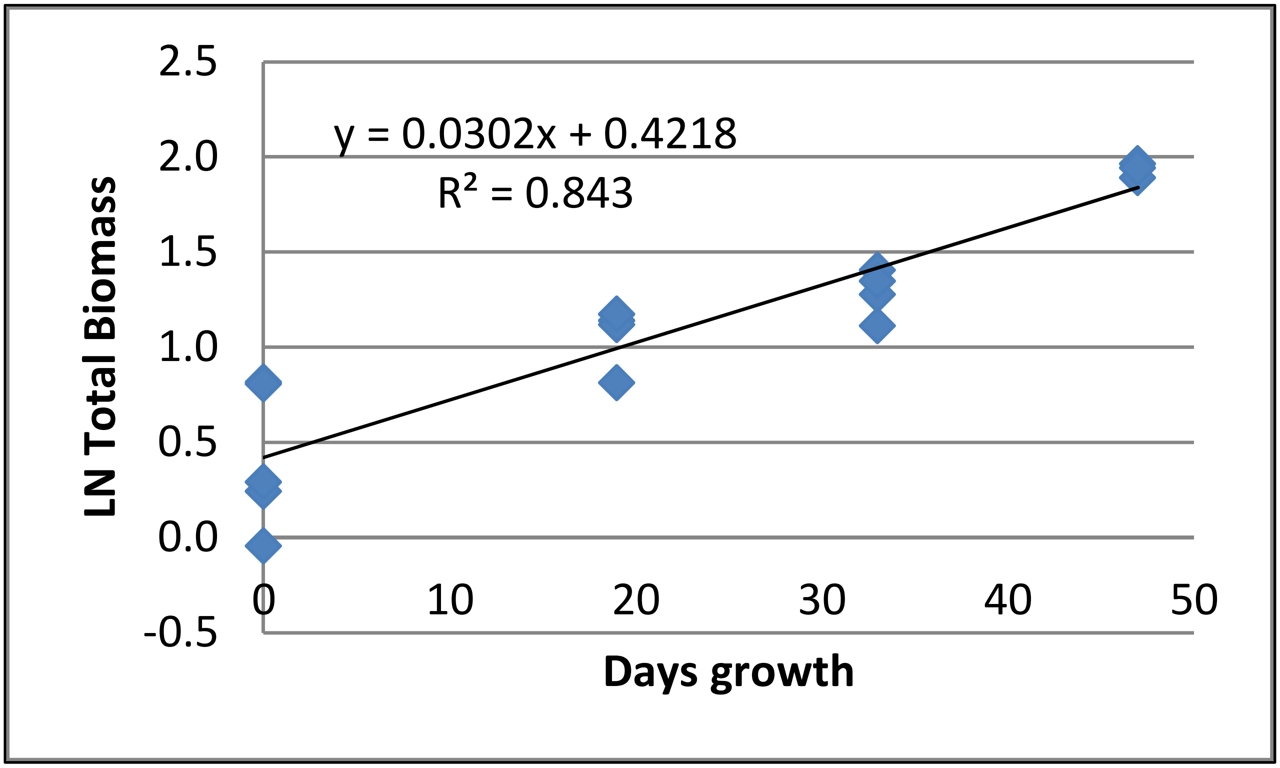


Figure 21. **Logarithm (natural) Total Biomass of *Ludwigia repens* grown in the control tank (22 degrees Celsius). The slope of the line reflects the rate of biomass increase per day.**

Aboveground:belowground Ratio (AGBG)

The AGBG of surviving plants was computed as the ratio of dry biomass aboveground (leaves/stems) to belowground (roots, rhizomes). The per-tank average was computed as the mean for all surviving pots in each laboratory tank. Shifts in AGBG arise from differences in allocation of biomass to roots (=nutrient absorption, anchorage) and shoot (light harvesting and photosynthetic potential). Historically, shifts in allocation patterns are regarded as plant optimization for current growth conditions (e.g., increase in roots when nutrients are in short supply, or an increase in shoots when light is limited), although recent analyses caution against simplistic interpretations of these ratios (Reich 2002).

Statistical Analysis

Statistical analyses of plant biotic responses were made using a *t*-test to determine if significant differences existed between plants grown in the 28 °C tanks vs. the 34 °C tanks. Standard Skewness values were examined to confirm that normality assumptions of this test were not violated. Values of the 22 °C control tank were visually compared to those of the 28 °C and 34 °C tanks, but the lack of replication in control tanks prevents statistical comparisons.

3.3.3 Results

Table 8 summarizes the water quality and light data measured in each treatment during the study. For the 28 °C / 34 °C study, water temperatures were maintained at their respective goals, although diurnal differences were significant in some tanks due to cool or extremely hot periods occurring

Table 8. Water quality and light parameters measured over the course of both experiments.

TREATMENT		DO [a] (mg/L) [b]	pH	PAR [c] (µmol m-2 s-1)	TEMP (°C) [d]	CO2 [e] (mg/L)
ARC Well water (28 °C vs. 34 °C experiment)	mean	6.74	7.34	122.31	21.23	31.25
	standard deviation	1.79	0.16	69.00	1.00	3.94
	range	7.22	0.71	269.33	4.00	19.00
	minimum	4.17	6.96	30.67	19.50	22.00
	maximum	11.39	7.67	300.00	23.50	40.00
28 °C	mean	6.75	8.17	152.02	28.43	2.52
	standard deviation	1.24	0.22	161.00	1.01	0.91
	range	5.89	1.44	1682.00	10.20	4.00
	minimum	3.85	7.10	24.00	27.10	1.00
	maximum	9.74	8.54	1706.33	37.30	5.00
34 °C	mean	6.25	8.27	112.30	34.00	2.60
	standard deviation	1.50	0.26	87.66	1.41	1.24
	range	6.85	1.60	491.67	15.10	6.00
	minimum	2.88	7.01	16.00	21.30	1.00
	maximum	9.73	8.61	507.67	36.40	7.00
37 °C	mean	4.87	8.49	106.43	36.87	2.29
	standard deviation	0.59	0.29	84.09	0.18	1.05
	range	2.91	1.33	350.00	15.70	7.00
	minimum	3.09	7.48	22.00	23.90	1.00
	maximum	6.00	8.81	372.00	39.60	8.00
ARC Well water (37 °C experiment)	mean	5.92	7.29	115.45	20.36	30.10
	standard deviation	1.16	0.22	80.70	1.22	3.85
	range	4.30	0.99	287.50	5.20	18.00
	minimum	4.47	6.80	21.50	19.00	18.00
	maximum	8.77	7.79	309.00	24.20	36.00

[a] DO=dissolved oxygen.
[b] mg/L=milligrams per liter.
[c] PAR= photosynthetically active radiation.
[d] C=Celsius.
[e] CO2=carbon dioxide.

during the study period. Not surprisingly, temperatures were significantly cooler and CO2 concentrations significantly higher in the control tank receiving Edwards Aquifer water. The CO2 readings were maintained at the goal <5 mg/L for the majority of the study period. Carbon dioxide was noted to increase toward the end of the experiment as the degassing tower and spray apparatus in the reservoir became coated with calcium precipitate and therefore less efficient. As CO2 and pH are negatively correlated, pH readings were higher across all treatment tanks receiving low CO2 water compared to the control tank.

The PAR is a highly variable measurement ranging from 0 µmol m^{-2} s^{-1}, indicating absolute darkness, to approximately 2,200 µmol m^{-2} s^{-1}, indicating direct sunlight. PAR within the treatment tanks was wide ranging (24 to 1,706 µmol m^{-2} s^{-1}) and influenced by shadows produced from overhanging structures in addition to the 30-percent shade cloth present on the ARC greenhouse.

Plants placed into treatments were well established with fully submersed growth. Three types of algae were observed in the treatment tanks. *Spirogyra* sp. was observed to quickly invade the control tank (Figure 22). By day 4 it was quite prolific, already growing on plants and water surface, but was entirely absent in the control tank by day 34. On day 7, *Oscillatoria* sp., a blue-green algae, was noted growing in the *Riccia* cups in all tanks except the control. This algae type persisted on *Riccia* and became quite dense later in the study, entirely covering some *Riccia* clumps. *Pithophora* sp. did not become prevalent until day 22, when it was observed in tanks 1, 2, and 4. It did not colonize the other treatment tanks. At the final harvest, tanks 1, 2, and 4 (34 °C tanks) contained extensive floating algae clumps, while tanks 3, 5, 6, and 7 (28 °C and control) remained clear. At the conclusion of the experiment, algae were still present in *Riccia* clumps across all treatments except the control tank. In some instances this algae completely covered or replaced most of the *Riccia* biomass. Growth of algae species is generally highly temperature dependent with community structure linked to temperature gradients (Roberts and Zohary 2010). In general, as temperatures increase, highest growth rates for algal groups change from diatoms, toward green algae to cyanobacteria (Canale and Vogel 1974) although species-specific responses are highly variable (Reynolds1984). O'Neal and Lembi (1995) found that growth of *Pithophora* sp. is inhibited in water temperatures of 15 °C with maximum growth rates occurring at 35 °C. *Spirogyra* sp. growth rates were only moderately inhibited at 15 °C and 35 °C with maximum growth rates at 25 °C.

On day 7, $CaCO_3$ precipitate was observed covering plants in Tank 4 (a 34 °C tank). This tank seemed to suffer from the most $CaCO_3$ precipitation although slight buildup was noticed on plants in the other treatments as well.

28/34 °C Experiment: Biotic Response Results

All species tested showed excellent survival at 22 °C, 28 °C, and 34 °C (Table 9, Figure 23). *Vallisneria* and *Riccia* showed 100 percent survival at all temperatures while *Ludwigia* and *Cabomba* showed one dead plant in each of several experimental troughs. While both *Ludwigia* and *Vallisneria* appeared to be healthy at all temperatures, the *Cabomba* plants were noted as being noticeably stressed and coated with epiphytic algae. Similarly, the *Riccia* clumps were all heavily overgrown by algae that could not be completely separated from the *Riccia*. Therefore, the biomass values for this species may overestimate the actual target species biomass.

The temporal pattern of total biomass is presented for all four target species (Figure 24). While AGBG ratio is small (Figure 25), total number of stems or leaves (Figure 26), and total stem or leaf length (Figure 27) is shown for the vascular aquatic macrophytes (*Cabomba*, *Ludwigia*, and *Vallisneria*). These graphs show that, in general, the pattern of biomass accumulation and shoot/root development (for the three vascular plants) did not differ greatly among the temperature treatments. Although some minor differences may be evident in the 2-week and 4-week harvests, by the 6-week harvest there was no evidence of a difference among any of the temperature treatments. This visual observation is confirmed by statistical comparison of the average values of the three tanks at 28 °C vs. the three tanks at 34 °C (*t*-test between 28 °C and 34 °C treatments) (Figure 28). *Cabomba* and *Ludwigia* both show clear evidence of growth at both 28 °C and 34 °C, with average RGRs clearly above the zero line (Figure 28). In contrast, the RGRs of *Vallisneria* are slightly positive for 28 °C and slightly negative for 34 °C, but neither differ significantly from zero, indicating that this plant species is surviving, but not growing, under these environmental conditions.

Figure 22. Control tank with well water and excessive algae.

Table 9. Number of plants in each treatment which did not survive until the final harvest (#dead/total# at start). Comments reflect views of personnel during the harvest period.

SPECIES	22 °C [a]	28 °C	34 °C	COMMENTS
Cabomba	0/12	1/36	2/36	Plants in 34 °C were noticeably stressed with heavy algal cover. Many plants in both 28 °C and 34 °C produced flowers during the trial.
Ludwigia	1/12	0/36	0/36	Generally healthy, plants in 34 °C tanks had numerous emergent tips while those in 28 °C had a few. The 34 °C groups were experiencing algal growth and $CaCO_3$ deposition.
Riccia	0/12	0/36	0/36	Clumps in 28 °C and 34 °C were heavily suffused with algae and bladderwort, and separation of the amalgam was not possible.
Vallisneria	0/12	0/36	0/36	Plants appeared healthy in all groups with some showing rhizomatous growth producing new rosettes.

[a] °C=degrees Celsius.

Figure 23. **Examples of growth over study period in the 28 degrees Celsius (°C) and 34 °C treatments. *Cabomba caroliniana* (left), *Vallisneria* sp. (center), and *Ludwigia repens* (right).**

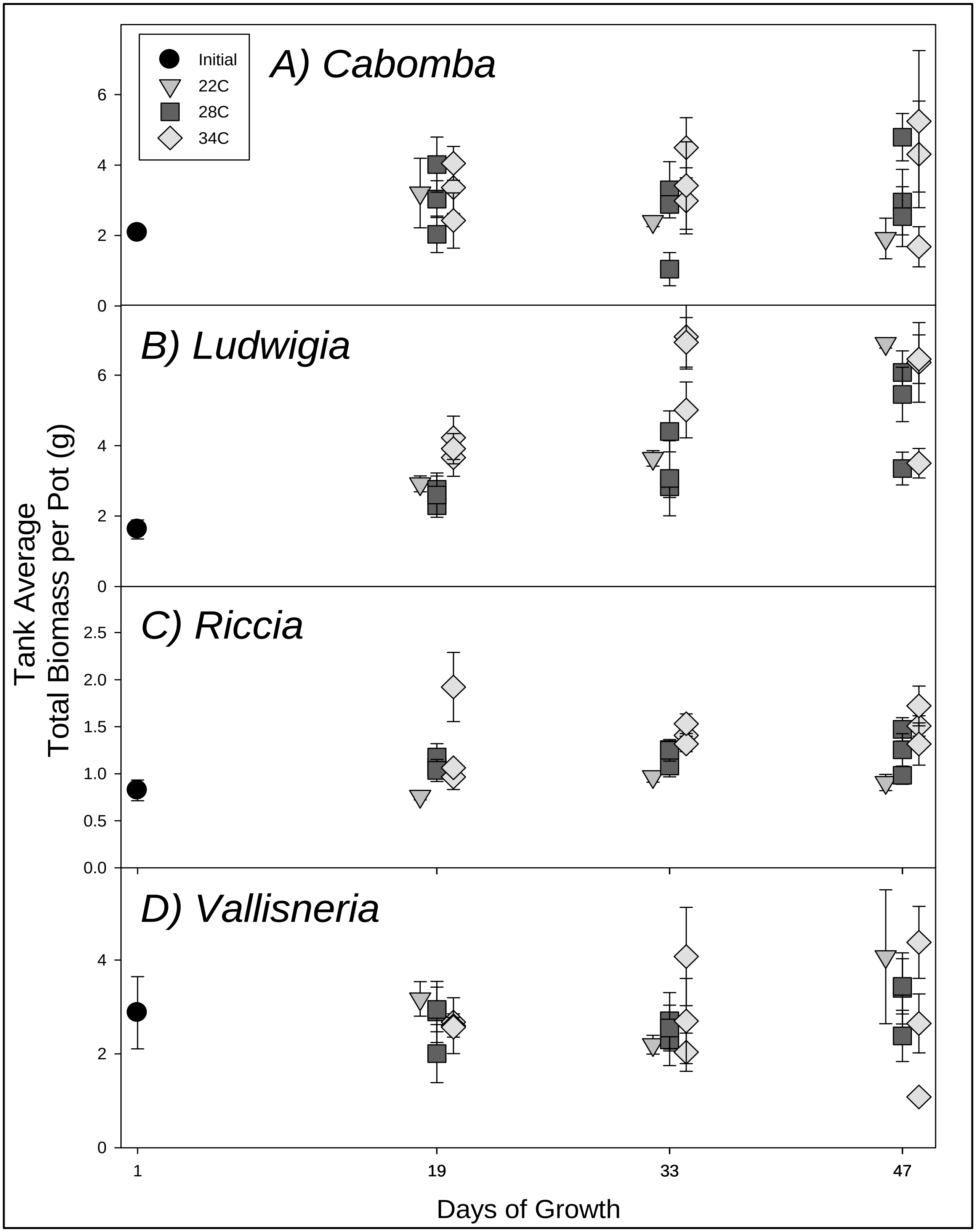


Figure 24. **Time course of total biomass through the experimental growth period. Data shown are the means ± SE (n=3 tanks) at the beginning of the growth period and at each sequential harvest.**

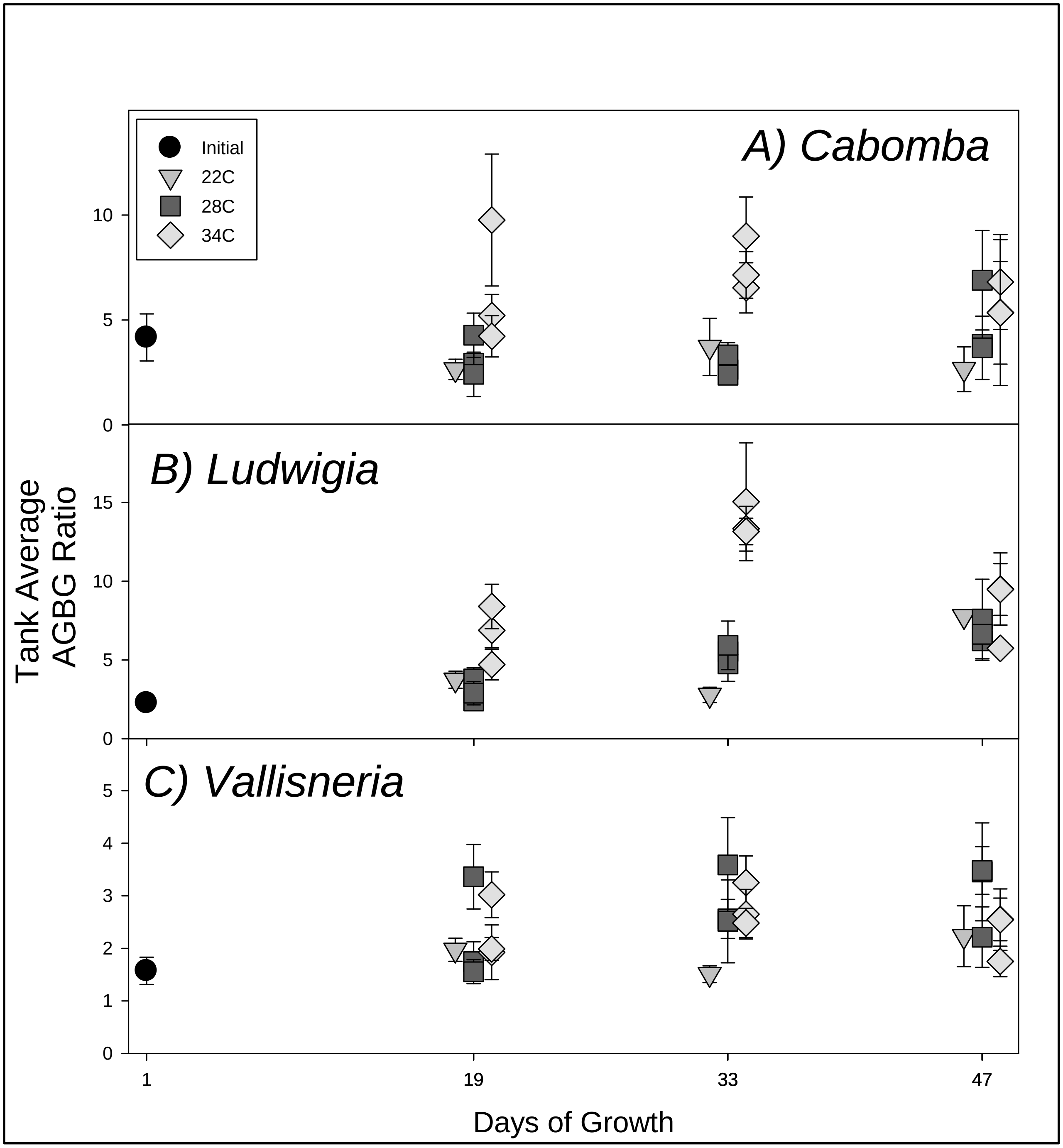


Figure 25. **Time course of aboveground:belowground ratio (AGBG) through the experimental growth period. Data shown are the means ± SE (n=3 tanks) at the beginning of the growth period and at each sequential harvest.**

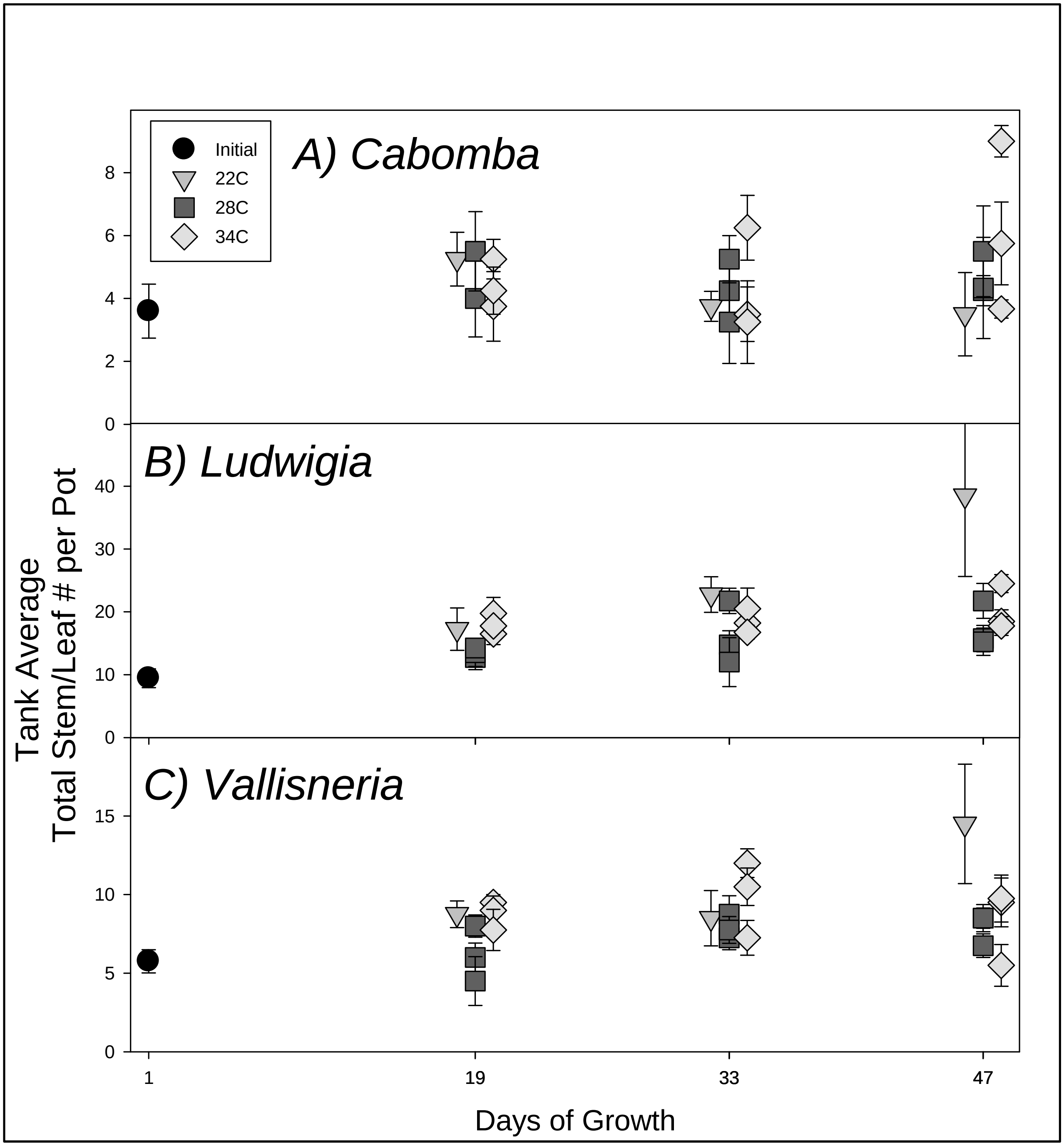


Figure 26. **Time course of total number of stems (*Cabomba caroliniana* and *Ludwigia repens*) or leaves (*Vallisneria* sp.) through the experimental growth period. Data shown are the means ± SE (n=3 tanks) at the beginning of the growth period and at each sequential harvest.**

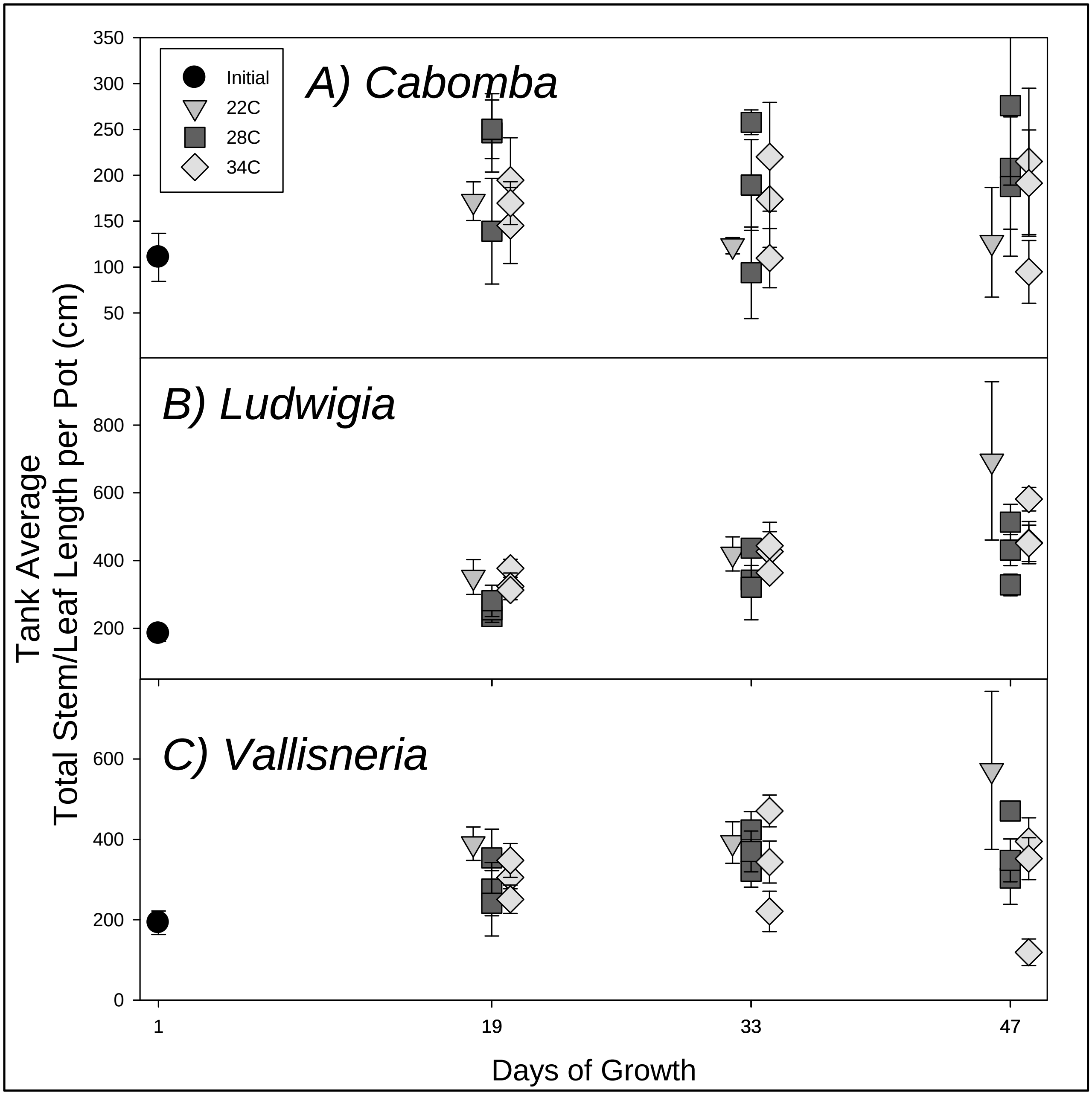


Figure 27. **Time course of total length of all stems (*Cabomba caroliniana* and *Ludwigia repens*) or leaves (*Vallisneria* sp.) through the experimental growth period. Data shown are the means ± SE (n=3 tanks) at the beginning of the growth period and at each sequential harvest.**

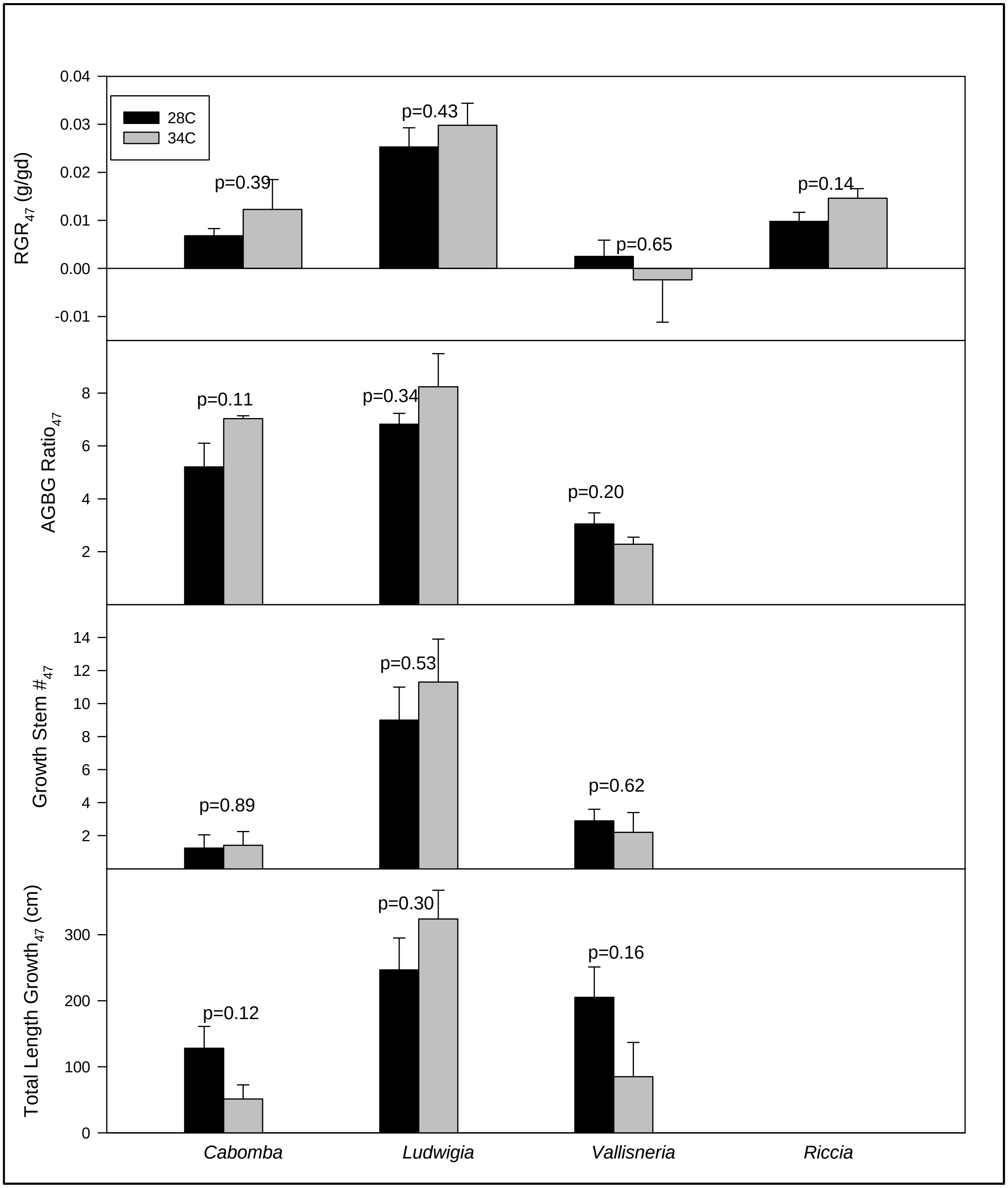


Figure 28. **Results at 6-week harvest (day 47). Shown are mean ± SE (n=3 tanks) for each parameter. The p values shown are the results of a *t*-test comparing 28 °C vs. 34 °C.**

The results of the 37° C treatment indicate that this high temperature was detrimental to the aquatic plants (Figure 29), leading to rapid and complete death of *Riccia* and *Cabomba* and near-death of *Vallisneria*. *Riccia* was the first species with a negative response towards the treatment, with noticeable death by day 7. *Cabomba* suffered 100 percent mortality by day 12. *Vallisneria* suffered almost 100 percent mortality by day 21. No mortality was observed in *Ludwigia*. However, all plants showed little development with no lateral branching and little apical growth. During the 37 °C trial, only *Spirogyra* sp. was observed growing in the control tank as it did in the previous study. All of the treatment tanks remained algae free.

37 °C Experiment: Biotic Response Results

The results of the follow-up 37 °C experiment show that this higher temperature is much more problematic for the species tested (Table 10, Figure 30). While all plants of all species survived and at least maintained stable biomass in the 22 °C control tank, all *Cabomba* and *Riccia* plants died prior to the end of the 23-day experimental growth period. While a few very small *Vallisneria* plants were still alive in some of the 37 °C tanks at the end of the experiment, it was clear that these plants were dying, and the investigators doubt that the plants could have survived, although a recovery growth period was not attempted. The average biomass of the few surviving plants at 37 °C was <15 percent of the average biomass in the 22 °C control tank (Figure 30) and the plants were fragile and broke apart easily when handled. Surprisingly, all *Ludwigia* plants survived the 23 days at 37 °C. Furthermore, the total biomass of the plants grown at 37 °C was similar to that in the control 22 °C tank. However, the investigators who conducted the final harvest noted that the *Ludwigia* plants appeared stressed.

3.3.4 Discussion

Examination of the data generated in the 28 °C vs. 34 °C experiment (Table 9, Figures 24–28) leads to four key generalizations:

1. Although some species showed qualitative evidence of stress, the four tested species were all able to survive the 6 weeks growth period under the test conditions of low CO2 and temperatures of 28 °C and 34 °C.

2. All species maintained (*Vallisneria*) or increased (*Cabomba, Ludwigia,* and perhaps *Riccia*) biomass when grown at 28 °C or 34 °C relative to the initial plant biomass.

3. The accumulation of total biomass was not impacted by the temperature treatment (28 °C vs. 34 °C) and the response of plants in the 22 °C control tank was not outside the range of those observed in the warmer tanks.

4. At the final harvest (6 weeks growth), there were no significant differences between the 28 °C and 34 °C tanks in the plant architecture of the vascular plant species (AGBG ratio, # stems/leaves, total length stems/leaves). The number of leaves/stems and the total leaf/stem length were numerically higher for the 22 °C tanks for *Ludwigia* and *Vallisneria*, although the lack of replication on the control tank makes statistical comparisons impossible.

Figure 29. Examples of plant condition in 37 degrees Celsius treatment: *Cabomba caroliniana* (left) and *Vallisneria* sp.

Table 10. Number of plants in each treatment which did not survive the experimental growth period (#dead/total# at start). Comments reflect views of personnel during the harvest period.

SPECIES	22 °C [a]	37 °C	COMMENTS
Cabomba caroliniana	0/5	15/15	All plants in the 37 °C treatment died and disintegrated (Figure 29).
Ludwigia repens	0/5	0/15	All 37 °C plants survived but appeared stressed with heavy $CaCO_3$ deposition.
Riccia fluitans	0/5	15/15	Clumps in the 22 °C treatment were green and healthy with some bladderwort growth noted. However, all 37 °C clumps were dead and brown in color with some algal and bladderwort growth noted.
Vallisneria sp.	0/5	11/15	Most 37 °C plants died, with the few surviving plants clearly deteriorating (Figure 29).

[a] °C=degrees Celsius.

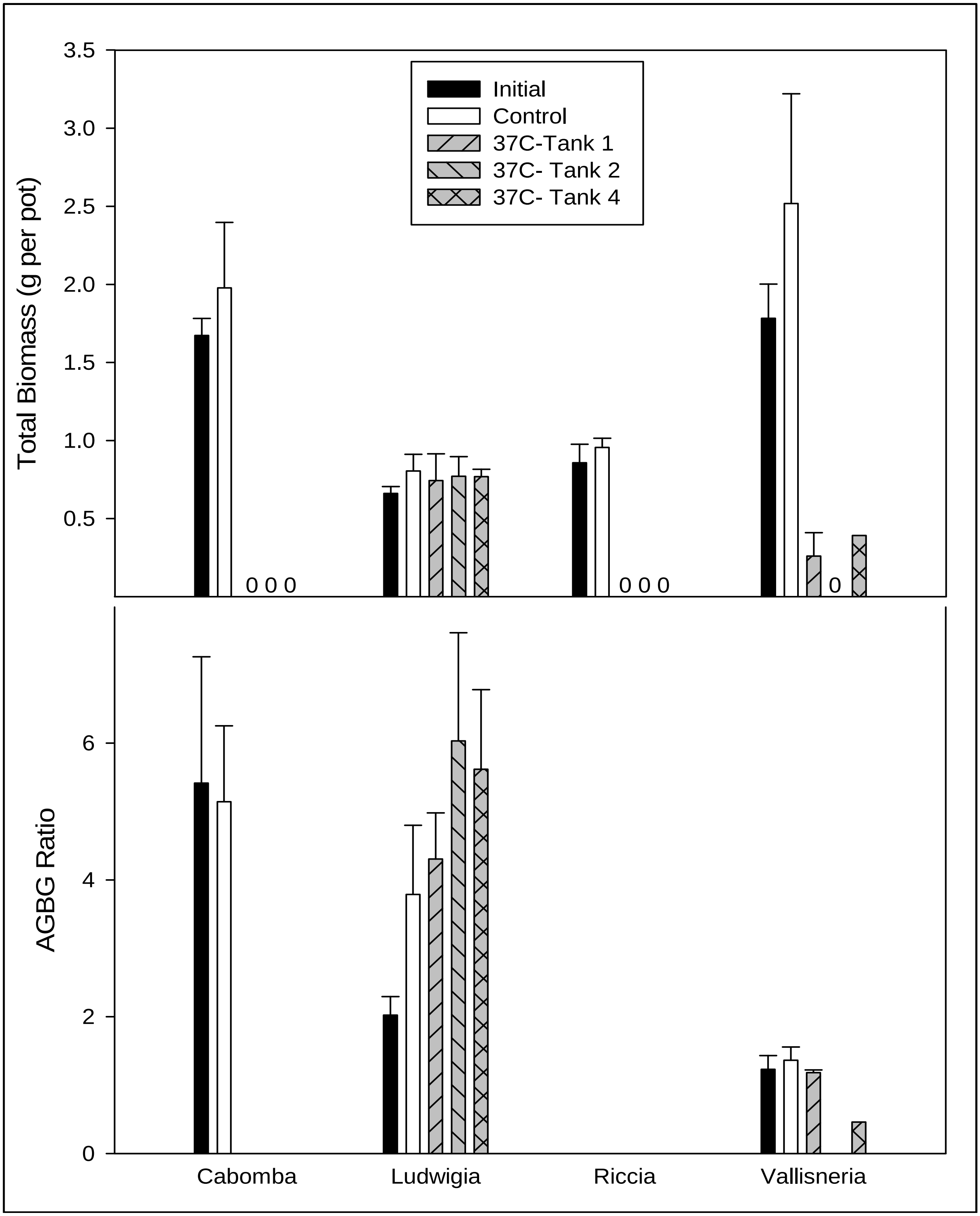


Figure 30. **Results of 23-day growth period at 37 degrees Celsius (°C) or 22 °C. Shown are mean ± SE for each individual tank at each temperature (n=5 pots per tank) for total plant biomass and aboveground:belowground ratio (AGBG).**

The follow-up 37 °C experiment (data shown in Table 10, Figure 30) permit one additional generalization:

5. Of the four species tested, only *Ludwigia* survived the 23-day growth period at 37 °C. Although this plant was likely stressed, it appeared to the investigators that it retained the ability to survive if the growth conditions had been returned to normal range.

The key generalizations described above lead to the conclusion that the four tested aquatic plants would likely survive short-periods (≈month) of water temperatures up to around 34 °C with relatively low free CO2. They also suggest that the thresholds for these species are not much higher as drastically different results were experienced at 37 °C. It was extremely encouraging that *Ludwigia* (a plant of known importance to the fountain darter and a target for extensive habitat restoration) would likely survive short durations of elevated water temperatures and low CO2. It must also be noted that, although qualitative in nature, the build-up of different types and levels of algae over the course of these experiments raises questions and concerns to the potential affect algae will have on aquatic plants during low-flow conditions in the wild.

Please refer to Section 6 for a discussion regarding lessons learned and cautions for interpretation, HCP ecological model application, and recommended future applied research relative to the 2013 HCP vegetation tolerance laboratory studies described in this section.

3.4 Pond Experiment

The Pond Experiment focused on the following two aspects of fountain darter habitat: (1) native aquatic vegetation and (2) water quality. The Pond Experiment was designed based on the available literature and information learned during the vegetation tolerance laboratory studies (see Section 3.3) and pond pre-study trial described below. The Pond Experiment is an outdoor evaluation of extremely low-flow or no-flow conditions that may be experienced in portions of the Comal and San Marcos systems within the context of the HCP flow regime.

3.4.1 Materials and Methods

The Pond Experiment encompassed both a pre-study trial and formal experiment both described in each of the methods, results, and discussion sections below. Although the pre-study trial was more limited in scope, it did provide several noteworthy observations as well as help guide the design and implementation of the formal experiment. The formal Pond Experiment study design and methodology was presented to the HCP Science Committee in August with subsequent approval to proceed.

Pre-study Pond Trial

For the pre-study trial, existing *Ludwigia* and *Cabomba* plants reared in ARC greenhouse troughs and adjacent outdoor pond were used. This resulted in three experimental groups based on plant source and species (Table 11). Experimental plants from each group were randomly placed into groups of 10 to provide experimental units. Experimental units were placed in the pond at randomly generated GPS locations with a Trimble GeoXT 6000 (Trimble, Sunnyvale, California) submeter GPS unit (Figure 31) and protective wire cages were placed around each unit to prevent interference and damage from turtles that periodically migrate into the pond (Figure 32). Experimental plants

Table 11. **Distribution of plant groups into experimental units in the pond experiment pre-study. Experimental plants were grouped by source from which plants were obtained and species. The total number of plants exposed to no-flow conditions (*n*) and the number of experimental units composed of plants from each group (N) are denoted.**

SOURCE	SPECIES	*n*/N
Greenhouse	*Cabomba caroliniana*	10/1
Greenhouse	*Ludwigia repens*	30/3
Pond	*Cabomba caroliniana*	50/5

Figure 31. **Randomly generated locations in the San Marcos Aquatic Resource Center (ARC) pond to which experimental units (aggregations of 10 plants) were randomly assigned.**

were allowed to acclimate to pond conditions with flow (≈ 0.023 cubic feet per second [cfs]) for 1 week. Following acclimation, 10 individuals from each group were randomly selected to provide an initial biomass sample and total initial stem length was measured for all plants. After all initial data were collected; flow to the pond was terminated (30 July 2013). These no-flow conditions were allowed to persist for 2 weeks, after which total stem length of all individuals was recorded. Ten individuals from each of the three groups were randomly selected to provide a post-treatment biomass sample.

Figure 32. Protective cages in place around experimental plants to protect experimental subjects from mechanical damage by turtles and other vertebrates.

Water quality parameters (DO, temperature, pH, PAR, CO_2) were monitored daily throughout the study period. All water quality parameters were collected at four points in the study area three times daily (morning, mid-afternoon, and night) to capture maximal variation in parameters. Temperature, DO, and pH data were measured using a YSI multi parameter sonde with Pro Series handheld data unit (YSI Inc., Yellow Springs, Ohio). Carbon dioxide was measured along with other water quality parameters using an Oxyguard portable CO_2 analyzer (Oxyguard International AS, Berkerød, Denmark). All PAR data was collected using a MQ-200 Quantum meter (Apogee Inc., Logan, Utah).

Statistical Analysis

Differences in water quality parameters between the flowing and non-flowing portions of the study were investigated using two-sample *t*-tests, or Wilcoxon rank-sum tests if the assumptions of the *t*-test were not satisfied by the data. The statistical significance of mean growth for each species was assessed by analyzing the difference between final and initial total stem length of each plant using one sample *t*-tests. Prior to conducting *t*-tests, the distribution of the data was assessed for normality via visual inspection of quantile/quantile plots and Shapiro-Wilk normality tests. The difference between initial and final biomass samples for each species was analyzed by two-sample *t*-tests using methods analogous to those outlined above.

Formal Pond Experiment

The formal pond experiment was designed to incorporate insights from the pre-study trial to provide a more formal test of the hypothesis that loss of flow will result in less-favorable environmental conditions and consequently adverse effects on plant growth. The pond was divided lengthwise with approximately 400 sandbags, which produced a flowing treatment area where water exchange was present for the duration of the experiment, as well as a non-flowing treatment area lacking water exchange (Figure 33). The native species *Ludwigia* and *Vallisneria* were used for this experiment because they are inhabited by fountain darters and likely to be present in Landa Lake during extremely low-flow conditions. Plants to be used in the experiment were grown using wild stock clippings from the Comal system, which were allowed to grow in well water at the ARC greenhouse for 2 weeks prior to the experiment. Twenty individuals of each species were randomly selected at the end of the greenhouse growth period to provide an initial biomass sample, with this process repeated at the conclusion of the experiment with plants of each species from each treatment.

Figure 33. **Division of the experimental pond into two treatment areas. The flowing treatment is on the right, the inlet pipe providing fresh water can be seen in the lower right.**

For each species, five units of ten plants were randomly placed in each treatment area using randomly generated GPS coordinates and a Trimble GeoXT 6000 (Trimble, Sunnyvale, California) submeter GPS unit (Figure 33). Total stem length was measured for all plants at the initiation (19 August 2013) and conclusion (10 September 2013) of the experiment. Water quality parameters were collected at four points in the study area twice daily (morning: ~08:00, mid-afternoon: ~16:30) to capture maximal diel variation in parameters. Temperature, DO, and pH data were measured using a YSI multi parameter sonde with Pro Series handheld data unit (YSI Inc., Yellow Springs, Ohio). Carbon dioxide was measured along with other water quality parameters using an Oxyguard portable CO2 analyzer (Oxyguard International AS, Berkerød, Denmark). PAR data was collected using a MQ-200 Quantum meter (Apogee Inc., Logan, Utah). Velocity of the flowing treatment was monitored using a Flo-mate 2000 portable flowmeter (Marsh-Mcbirney Hach Inc., Loveland, Colorado).

Statistical Analysis

To assess differences in water quality parameters between the treatments with (flowing) and without (non-flowing) water exchange in the formal pond experiment, two-sample *t*-tests (or Wilcoxon rank-sum tests if the assumptions of the *t*-test were not satisfied by the data) were used. Difference in growth (assessed as initial total stem length subtracted from final total stem length) of plants between the two treatments were analyzed using two-sample *t*-tests. Prior to conducting *t*-tests, the data was assessed to ensure analysis assumptions were met via visual inspection of quantile/quantile plots, Shapiro-Wilk normality tests, and F-tests of two variances. In the event that assumptions of parametric tests (*t*-test) were not met, non-parametric tests (Wilcoxon rank-sum) were applied instead. Difference in final biomass samples between treatments from plants of each species were analyzed by two-sample *t*-tests using methods analogous to those outlined above. Correlation of species growth and depth were investigated using Spearman's rank correlation test conducted for each species by treatment.

3.4.2 Results

Pre-study Pond Trial

Temperature (Wilcoxon rank sum test, W=2360.5, p<0.001), DO (Welch two-sample *t*-test, t=4.88, df=174.8, p<0.001), pH (Wilcoxon rank sum test, W=1092.5, p<0.001), and CO2 (Wilcoxon rank sum test, W=7615, p<0.001) were found to differ significantly between the non-flowing and flowing portions of the experimental pond (Table 12), while PAR (Wilcoxon rank sum test, W=1709.5, p=0.5587) did not. Values of water quality data collected at night were intermediate between morning and afternoon values, suggesting that no additional variation was accounted for by night measurements, and these were dropped from the formal pond experiment (Figure 34). Figure 35 depicts the changes in water temperature, DO and CO2 over the course of the pre-study trial.

Growth in total stem length (cm) was found to be significantly different from zero by one-sample *t*-tests for laboratory grown *Ludwigia* (t=5.1048, df=28, p<0.001, mean=55.22) and pond-grown *Cabomba* (t=4.67, df=49, p<0.001, mean=22.63), while lab-reared *Cabomba* growth was not significant (t=1.80, df=9, p=0.105, mean=20.1). No significant difference was found in biomass for lab-grown *Ludwigia* (t=0.0315, df=12.107, p=0.9754), pond-grown *Cabomba* (t=-0.3548, df=13.586, p=0.7282) or lab-grown *Cabomba* (t=-0.5627, df=16.974, p=0.581) by Welch's two-sample *t*-tests.

Table 12. Summary of water quality parameter values for each treatment condition in the pond experiment pre-study.

VALUE	FLOW				NO FLOW			
	maximum	minimum	mean	standard deviation	maximum	minimum	mean	standard deviation
Temperature[a]	33.6	24.6	28.5	3.2	36.3	25.7	30.6	3.6
Dissolved oxygen[b]	10.74	4.56	6.90	1.63	8.80	2.29	5.64	1.82
pH	8.38	7.86	8.11	0.14	8.98	8.03	8.42	0.24
Carbon dioxide[b]	6.0	2.0	3.7	0.9	4.0	0.0	1.7	1.0
PAR[c]	1,774	85	767	614	1,680	2.5	771	629

[a] Measured in degrees Celsius.
[b] Measured in milligrams per liter.
[c] PAR= photosynthetically active radiation, measured in (µmol m-2 s-1).

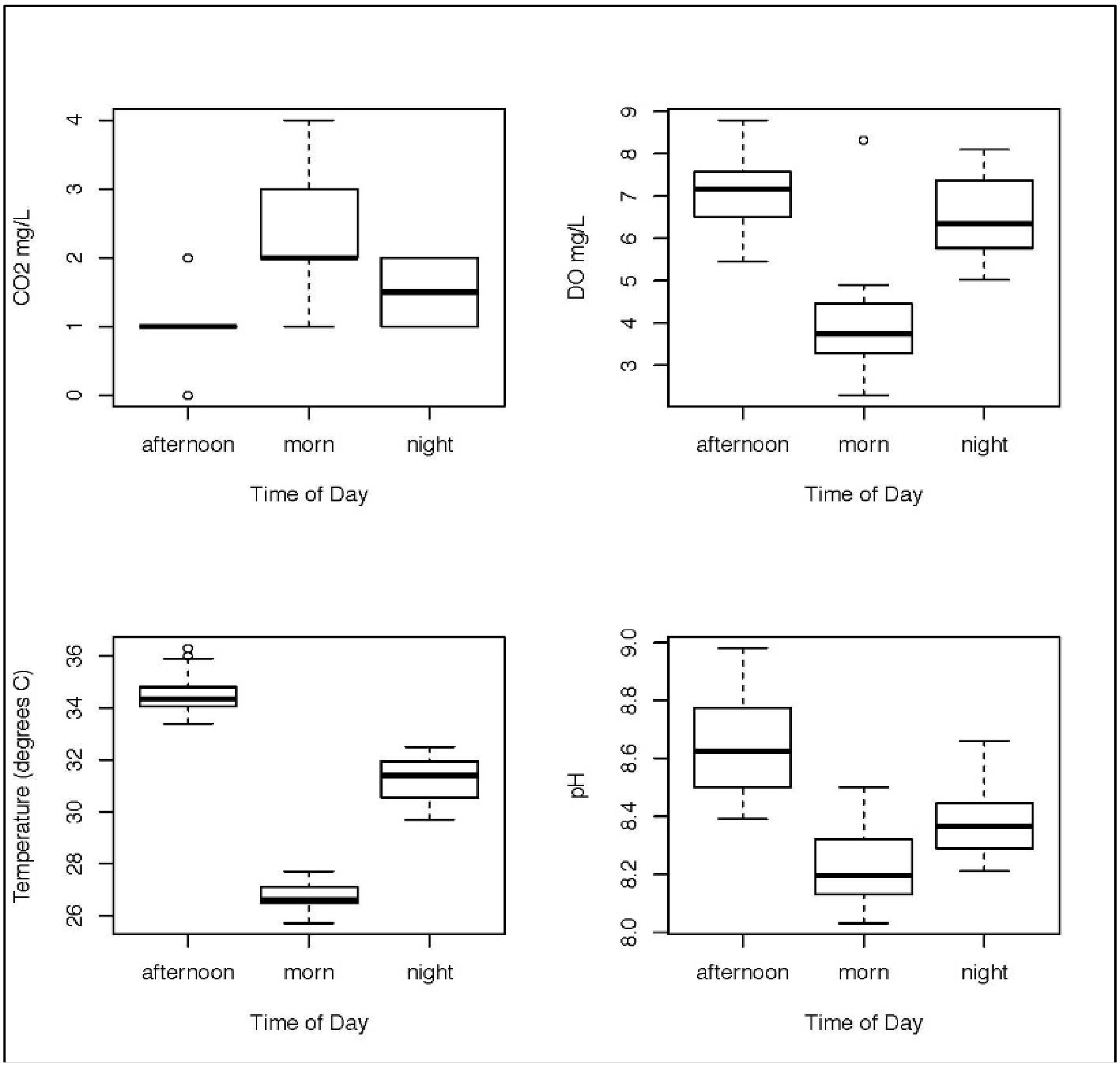


Figure 34. Box-whisker plots of water quality parameter data taken at different periods.

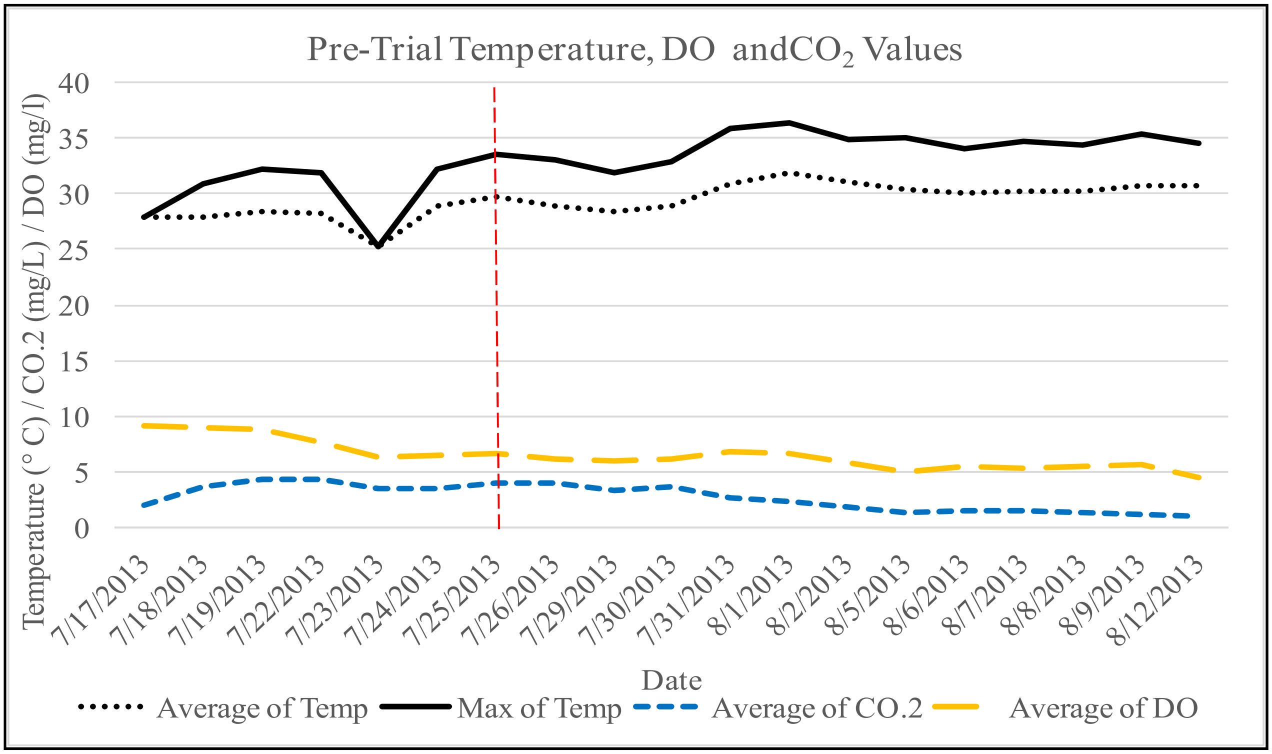


Figure 35. **Daily maximum and average temperature values (°C) and average CO2 (in milligrams/liter) values over the course of the pond study pre-trial. Vertical dashed line represents the cessation of inflow to the experimental pond.**

Formal Pond Experiment

Values for all water quality parameters (temperature, DO, pH, and CO2) except PAR were significantly different between the two treatments (Tables 13 and14). Figure 36 depicts the changes in water temperature, DO and CO2 over the course of the study for both the flow and no-flow treatments.

Growth (initial total stem length subtracted from final total stem length) in stem length was significantly different between flowing and non-flowing treatments for *Vallisneria* (Welch two-sample *t*-test, t=-3.15, df=97.98, p=0.002) as well as *Ludwigia* (Welch two-sample *t*-test, t=4.26, df=92.7, p<0.001). Mean growth for Ludwigia was 119.85 cm in the flowing treatment and 83.01 cm in the non-flowing treatment. Mean growth of *Vallisneria* was -13.7 cm in the flowing treatment and 56.36 cm in the non-flowing treatment. Depth was significantly correlated with growth for *Vallisneria* in both treatments (Spearman's rank correlation, flow: s=11324.24, p=0.00087, rho=0.456; non-flow: s=15008.63, p=0.0495, rho=0.279) though the correlation was less strong for the non-flow treatment. In *Ludwigia*, depth was only correlated with growth in the non-flowing treatment (Spearman's rank correlation, flow: s=17568.75, p=0.2782, rho=0.156; non-flow: s=33418.15, p<0.001, rho=-0.605). Final biomass of *Ludwigia* was significantly different between treatments (Welch two-sample *t*-test, t=10.8655, df=45.578, p<0.001), with mean biomass being greater in the flowing treatment (2.014 g) than the non-flowing treatment (0.931 g). Final biomass of *Vallisneria* was not significantly different between treatments (Welch two-sample *t*-test, t=-0.8556, df=45.095, p=0.3967) (Figure 37).

Table 13. Mean water quality values for parameters monitored over the course of the pond study experiment.

VALUE	FLOW				NO FLOW			
	maximum	*minimum*	*mean*	*standard deviation*	*maximum*	*minimum*	*mean*	*standard deviation*
Temperature[a]	32.4	21.5	26.2	3.2	35.4	24.3	29.5	3.5
Dissolved oxygen[b]	17.49	4.48	7.87	2.43	9.67	3.77	6.66	2.06
pH	8.75	7.55	8.05	0.24	9.03	6.30	8.44	0.31
Carbon dioxide[b]	15.0	2.0	6.8	3.1	5.0	1.0	2.3	1.2
PAR[c]	1,646	53	611	452	1,552	42	700	444

[a] Measured in degrees Celsius.
[b] Measured in milligrams per liter.
[c] PAR= photosynthetically active radiation, measured in (µmol m-2 s-1).

Table 14. Significance values for comparisons of water quality parameters between pond study experiment treatments.

VALUE	*T*-TEST			WILCOXON RANK SUM	
	t	df	p	W	p
Temperature	7.526	235.67	<0.001	-	-
Dissolved oxygen	-4.1426	232.08	<0.001	*	*
pH	*	*	*	12577	<0.001
Carbon dioxide	*	*	*	236	<0.001
PAR[a]	-0.2025	237.9	0.84	*	*

[a] PAR= photosynthetically active radiation.

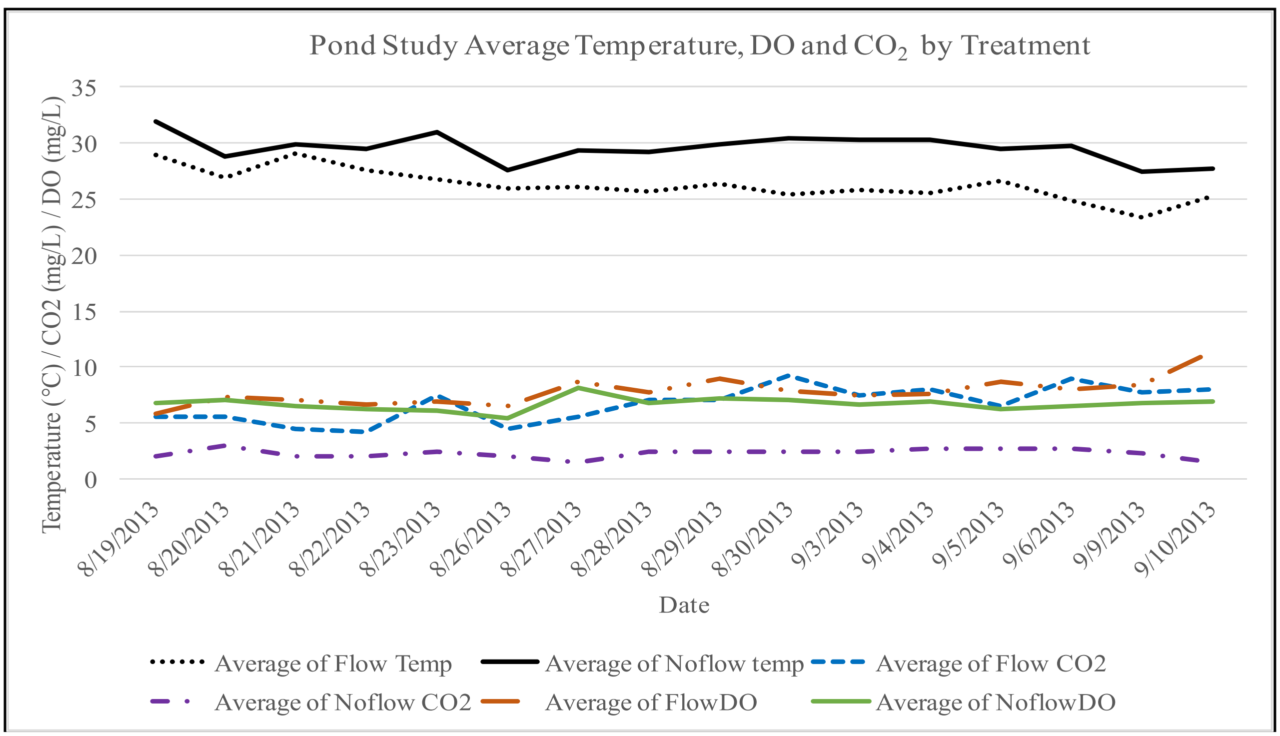


Figure 36. Daily average temperature and carbon dioxide (CO2) over the course of the vegetation tolerance formal pond study.

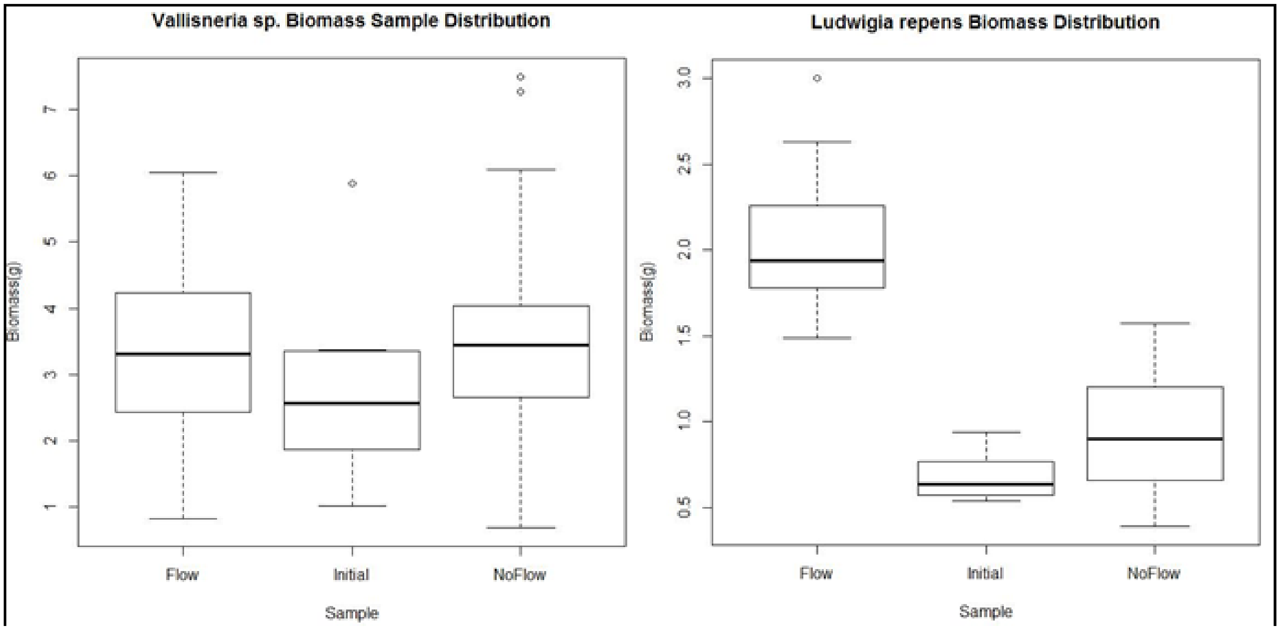


Figure 37. Box-whisker plots illustrating the distribution of initial biomass samples and final biomass for each treatment.

3.4.3 Discussion

Pre-study Pond Trial

It is quite obvious by viewing the measured water quality parameters (Figure 35) at what point in time the flow to the pond was shut off. Not surprisingly, almost immediately following cessation of flow, an increase in water temperature and decrease in DO and CO2 began. Although DO and CO2 appeared to still be declining at the conclusion of the trial, water temperature seemed to mostly stabilize after approximately 1 week. The effect of these changes on plant growth is discussed below. However, plant growth is not the only factor that will be impacted by water quality changes during extended periods of low flow. After flows were shut off, average water temperatures climbed to over 30 ˚C during the course of the first week and stayed above that mark for the remainder of the experiment (Figure 35). In addition, upon cessation of flow, maximum temperatures initially increased to 33 ˚C, and then climbed again and stabilized near 35 ˚C after approximately 1 week. These elevated temperatures can have impacts on fountain darters and other fishes. Fountain darter egg production is shown to be reduced at temperatures greater than 26 ˚C, with larval production impacted at temperatures of 25 ˚C or higher (BIO-WEST 2002, Bonner et al. 1998, McDonald et al. 2007). Therefore, temperatures observed in the pond would likely result in the cessation of all fountain darter reproduction, as well as egg and larval development.

Additionally, temperatures of 33–35 ˚C approach or exceed the listed critical thermal maximum (CTM) for many fish species (Beitinger et al. 2000). A CTM is defined as "… the arithmetic mean of the collective thermal points at which locomotory activity becomes disorganized and the animal loses its ability to escape from conditions that will promptly lead to its death when heated from a previous acclimation temperature at a constant rate" (Cox 1974). For fountain darters, this value is approximately 34.8 ˚C (Brandt et al. 1993); therefore, they are unlikely to live for an extended period of time in an environment (no flow, elevated summer air temperatures) like the one in this study. These high temperatures and no-flow conditions also resulted in low DO readings (2 mg/L–3 mg/L) in the mornings near the end of the experiment. Dissolved oxygen below 4 mg/L is stressful to most fishes, and DO of approximately 2 mg/L can be lethal (Ostrand and Wilde 2001, Rutledge and Beitinger 1989). Dissolved oxygen readings in the mornings regularly fell within this range. Therefore, DO concentrations may have detrimental effects to fountain darter survival when flows are severely reduced and water temperatures greater than 30 ˚C are observed.

Although little growth was apparent based on comparison of initial and final biomass across the plant groups in the pre-study, it was clear from examination of stem length data that significant growth occurred in all groups with the exception of laboratory grown *Cabomba* (most likely attributable to the very small sample size of this group). The experimental plants did not succumb to the changing water quality conditions caused by lack of flow in this experiment, nor did they experience decreases in biomass or loss of stem length during the study period. No plants were noted as dead at the end of the study. It was noted that by the end of the trial period most *Ludwigia* plants had grown into a prostrate mat at the surface and produced emergent apical tips. In this form, most submerged leaves are senesced and stems are greatly elongated to reach the surface, resulting in a majority of the biomass being apportioned to the top few centimeters of the plant where CO2 is readily available.

Unlike *Ludwigia*, *Cabomba* is not an amphibious species. While it commonly produces small floating leaves at the surface, these structures are associated with flowering rather than utilizing atmospheric CO2 (Ogaard 1991, Williamson and Schneiber 1993). James (2011) suggested *Cabomba* as a species that can only utilize free CO2 for photosynthesis. Data from the pH drift chapter support this conclusion. The low ambient CO2 values exhibited in this trial (Table 12) likely suppressed the growth of *Cabomba*. Although CO2 levels may decrease significantly in pooled waters, many submersed plants have the ability to become more efficient at utilizing available CO2 or can utilize carbon from other sources, such as sediment (Winkel and Borum 2009) or HCO3- in order to maintain biomass (see Section 4).

Formal Pond Experiment

It is interesting to note that a few days after starting the experiment (20–22 August), and again on 26 August, several cloudy days occurred, which closed the water temperature gap temporarily between treatments. A cooler period was also experienced at the tail end of the experiment and evident in the temperature response. It is also interesting to note that with only 0.023 cfs flowing in one treatment and essentially no flow in the other, water temperatures seemed to stabilize fairly quickly to ambient air temperatures and ever so slowly declined as slightly cooler air temperatures were experienced as the study progressed. Although not shown in the daily average figure, diurnal temperature fluctuations were evident in both treatments. As evident in Figure 36, temperature, DO and CO2 were all higher in the flow treatment as expected. The effects on plant growth are discussed below, but as mentioned in the pre-trial discussion, direct impacts to the fountain darter and other aquatic animals will likely occur at these levels (if experienced in the wild).

Water temperature and dissolved CO2, are two important yet often overlooked factors influencing growth of aquatic plants. Research has shown that the Comal River is a stable system with little fluctuations in water temperature and other environmental parameters. However, these qualities will undoubtedly change during times of severe drought and lower spring discharges. Field monitoring data is needed to define the boundaries of these changes in the system, and make predictions of changes likely to occur under extreme conditions. This study illustrates that *Ludwigia* and *Vallisneria* can survive and continue to grow when water temperature increases to a daily mean of 30°C with fluctuation as high as 35°C, and mean dissolved CO2 decreases below 10 mg/L.

In addition to temperature and CO2, ecological factors play a role in the growth of macrophytes. At week 3 of the formal pond experiment, an extensive algae bloom of *Spirogyra* sp. occurred in the flowing treatment, while no algae growth occurred in the no-flow treatment (Figure 38). The experiment was subsequently ended to prevent confounding effects of algae growth on the plant response in the study. Algae can play an important role in the aquatic plant community and has been known to suppress growth of some macrophytes by increasing pH and decreasing CO2 levels (Simpson and Eaton 1986, Ozimek et al. 1991). Availability of CO2 may play a direct role in biomass production of filamentous algae species which thrive in high CO2 waters while their growth is inhibited in low CO2 waters (Andersen and Andersen 2006).

Figure 38. Algae growth in the flow treatment (right) vs. the no-flow treatment (left).

It was noted that *Vallisneria* plants in the flowing treatment retained their leaves while those in the no-flow treatment grew new leaves and, in most cases, additional daughter plants. *Vallisneria* leaves in the flowing treatment also retained epiphytic algae, which colonizes most of the *Vallisneria* population in Landa Lake. Presence of epiphytic algae plays an integral role in aquatic communities and aquatic plant growth. Leaves of *Vallisneria* have shown to be an excellent substrate for epiphytic communities (Hutorowicz and Hutorowicz 2008), which can decrease photosynthetic rates in *Vallisneria* species (Sand-Jensen 1977). *Vallisneria* in Landa Lake is noticeably encrusted with marl, a mix of calcium carbonate and epiphytes, which was present on all *Vallisneria* collected. In this study the warm water of the no-flow treatment may have stimulated new growth of *Vallisneria* while limiting the colonization and growth of epiphytic algae on new leaves.

Please refer to Section 6 for a discussion regarding lessons learned, HCP ecological model application, and recommended future applied research relative to the 2013 HCP vegetation tolerance studies described in this section.

4.0 pH DRIFT STUDY

4.1 Introduction

The objective of this study was to determine which of the major submersed aquatic plant species of the Comal River are capable of utilizing HCO3- as a carbon source for photosynthesis. It is projected that under reduced flows and warmer water temperatures, the pH of the rivers may rise resulting in significantly lower CO2 availability than currently experienced. Hence, knowing which species would be capable of HCO3- utilization will be a key aspect of planning for reduced flows.

4.2 Data Review and Available Literature

The Comal and San Marcos rivers are dominated by submersed aquatic plants that must obtain inorganic carbon for photosynthesis from the surrounding water. Fortunately for these plants, the waters in these spring-fed systems have high total dissolved inorganic carbon (CT) levels (ALK > 200 mg l^{-1} $CaCO_3$ or >4 meq l^{-1}) and relatively low pH (about 7.2), such that abundant levels of CO2 are available in the water. Therefore, in the Comal and San Marcos rivers, even the species capable of producing emergent leaves usually don't since sufficient available carbon is present in the water column. However, projections of lower future flow conditions may result in lower levels of available CO2 and introduce a stress factor that has historically not been present to these aquatic plant communities.

The availability of the various forms of CT in water is governed by well-known pH dependencies. Under low pH conditions, most CT is found as CO2 but as pH rises the equilibrium shifts to HCO3- and finally to CO3 (Figure 39). All submersed aquatic plants (like their terrestrial counterparts) can utilize CO2 as a carbon source for photosynthesis. Furthermore, no aquatic plant can directly utilize CO3 for photosynthesis. However, the ability of different species to utilize HCO3- varies widely. Some species are unable to utilize this form of carbon (obligate CO2 users), while others show varying efficiencies in extracting HCO3- (Allen and Spence 1981, Maberly and Spence 1983, Sand-Jensen 1983, Spence and Maberly 1985, Power and Doyle 2004).

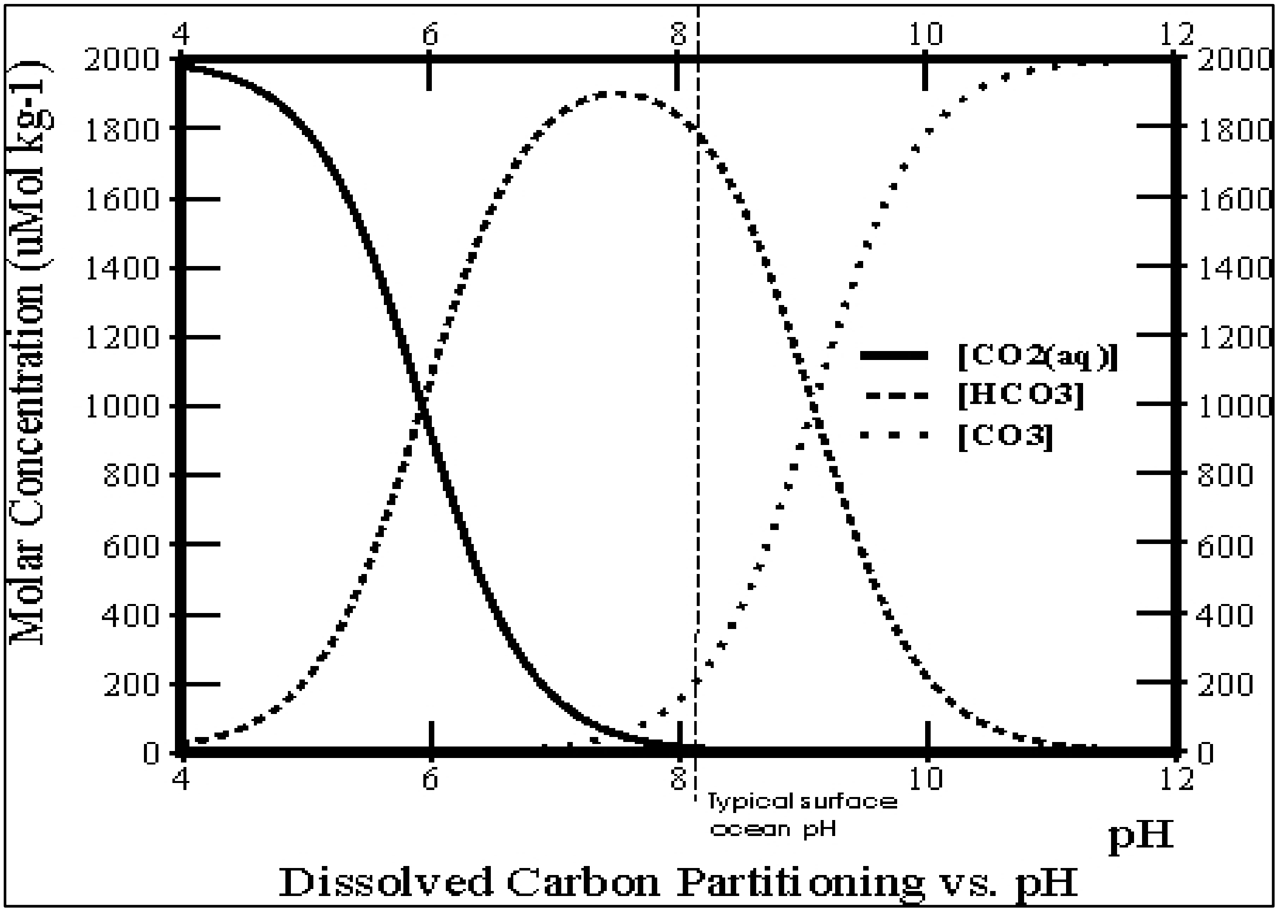


Figure 39. Dissolved inorganic carbon (CT) speciation vs. pH.

To date, the only plant collected directly from the Comal and San Marcos rivers to have been assayed for HCO3- utilization potential is *Zizania texana* (Texas wild rice). Power and Doyle (2004) demonstrated that freshly collected plants and seedlings grown at the ARC were CO2-obligate plants. From this review, a study plan to examine the HCO3- utilization potential of other common aquatic plant species in these two spring/river systems was prepared and presented to the HCP Science Committee in February 2013 with subsequent approval.

Several of the species (or at least genera) of aquatic plants present in the Comal or San Marcos system that were assayed in this study have some amount of published data in the literature related to inorganic carbon sources (Table 15). In summary, the literature indicates that *Riccia* and *Cabomba* are CO2-obligates, while *Hygrophila polysperma*, *Ludwigia*, *Sagittaria,* and *Vallisneria americana* may exhibit HCO3- utilization.

Table 15. Available data on inorganic carbon usage potential for the species (or related species within the same plant group) assayed in this study.

PLANT GROUP	INORGANIC CARBON USAGE INFORMATION	REFERENCES
Aquatic bryophytes (*Riccia* sp.)	Two studies conclude that aquatic bryophytes in general (including a *Riccia* sp.) are carbon dioxide (CO2) obligates.	Bain and Proctor 1980, Ballestros et al. 1998
Cabomba caroliniana	One report concludes that *C. caroliniana* is a CO2-obligate.	Hiscock 2003
Hygrophila polysperma	Two studies report low CO2 compensation points and conclude that *H. polysperma* is a potential bicarbonate (HCO3-) user.	Maberly and Madsen 2002, Spencer and Bowes 1985
Ludwigia spp.	*L. repens* from Florida springs did not show evidence of bicarbonate utilization; *L. natans* listed as CO2 obligate.	Lytle 2003 (*L. repens* data), Prins et al. 1980 (*L. natans* data)
Sagittaria spp.	Some *Sagittaria* species appear to show CAM-like photosynthesis, which would indicate bicarbonate utilization.	Keeley 1998
Vallisneria americana	Numerous studies support *Vallisneria americana* as a strong bicarbonate user.	Keeley 1998, Maberly and Madsen 2002, Nishihara and Ackerman 2006, Titus and Stone 1982, Holaday and Bowes 1980

4.3 Materials and Methods

4.3.1 Plant Field Collection and Laboratory/Greenhouse Cultures

Bicarbonate utilization potential was examined in both freshly collected samples from the field and plants cultured under CO2-controlled conditions (no CO2 or with CO2). Apical shoots of *Ludwigia*, *Hygrophila polysperma*, *Cabomba*, *Sagittaria*, and *Vallisneria*, as well as portions of bryophyte clumps (*Riccia*) were collected at various times during the spring and summer 2013 from established plant communities throughout the Comal River system (Figure 40). All harvested material was placed in an insulated container of river water and transported to Baylor University. Assays from freshly collected field samples were initiated within 36 hours of collection.

The ability to use HCO3- has been shown to be an inducible trait in some aquatic plants under CO2-limited conditions (Sand-Jensen and Gordon 1986, Magnin et al, 1997). To assess this potential for Comal species, plants were cultured in aquaria on the Baylor University campus under CO2-controlled conditions. Mature plants were collected from the Old Channel and planted in individual pots with a three-way soil mixture of clay loam topsoil, compost and green sand submersed in Edwards Aquifer well water collected from the ARC. Aquaria at Baylor were maintained in either CO2-stressed (aeration with air only) or CO2-sufficient (air stream amended with CO2) conditions. Aquaria were aerated with a standard aquarium air pump and air stones with CO2 being supplied to one aquarium from a compressed gas cylinder at a rate sufficient to maintain pH below 8.0. Plants were allowed to acclimate to aquaria conditions before being subjected to pH drift trials (described below). Plants were also selected during the final harvest of the vegetation tolerance laboratory experiment (section 3.3) conducted at the ARC to assess their ability to utilize HCO_3^-. For that experiment plants were cultured at both 28 °C and 34 °C temperature under CO2-stressed conditions (CO2 3 mg l^{-1}) in the troughs at the ARC. Samples were collected from both the 28 °C and 34 °C experimental groups for bicarbonate utilization assays.

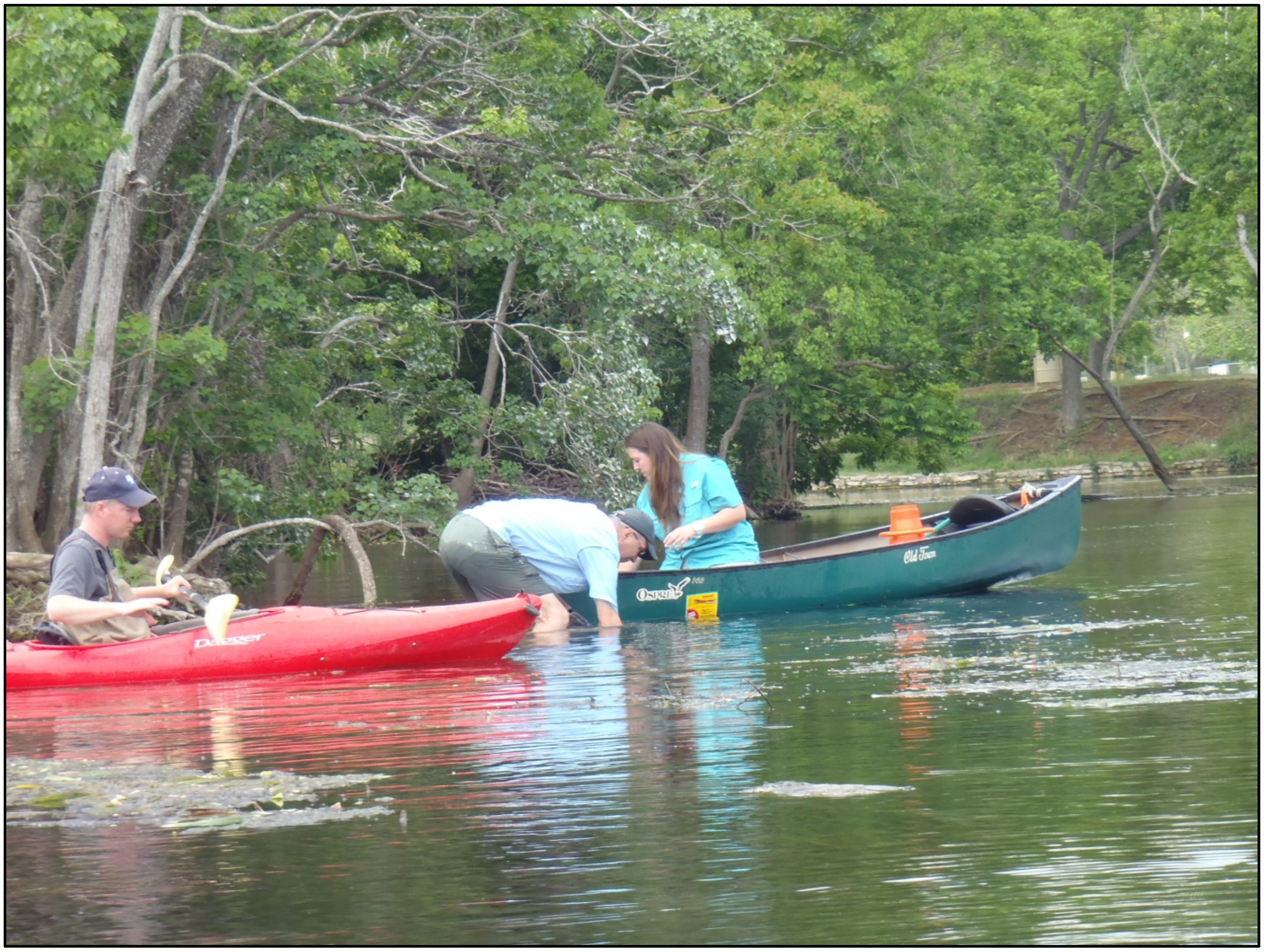


Figure 40. Field collection of plants from Landa Lake, Comal River.

pH Drift Experiment: Background and Rationale

To determine the HCO3- utilization potential of the six plant species, a pH drift method similar to that described by Allen and Spence (1981) and utilized by Power and Doyle (2004) to examine the bicarbonate uptake potential of Texas wild rice was used. The maximum pH endpoint for the pH drift experiments is determined by the pool of total CT, the plant's ability to utilize HCO3-, and the buffering capacity (i.e., ALK) of the culture solution. The proportion of CO2 or HCO3- in solution at the pH associated with photosynthetic cessation is referred to as the CO2 or HCO3- compensation points, respectively. Plants that are CO2 obligate cannot push the pH beyond the threshold at which H_2CO_3 disappears and equilibrium reactions favor HCO3- and CO_3^{2-} (ca. 9.2), but plants with the ability use HCO3- will continue to exhibit positive net photosynthesis at a pH well beyond this point.

Carbon dioxide consumption is associated with an increase in pH, but it has no effect on alkalinity (ALK) and only a slight impact on CT. Bicarbonate consumption has a more pronounced effect on CT and involves the evolution of OH^- ions which further increase pH and balance the buffer capacity (i.e., ALK) lost through bicarbonate removal. The CT:ALK ratio at the final pH is an indicator of a plant's ability to consume HCO3-, and a ratio close to 1.0 is indicative of CO2 obligates while HCO3- users yield a much lower ratio (Maberly and Spence 1983, Prins et al. 1980).

Experimental Assay Procedure

The assays were conducted in an artificial aquatic plant culture media of known composition and moderate ALK as described by Smart and Barko (1984, 1985). This solution has an ALK of 0.85 meq l^{-1} (42.5 mg $CaCO_3$ l^{-1}) and a pH of 8.0 when in equilibrium with air. The expected ALK was verified by titration with dilute hydrochloric acid according to standard methods. The solution contains:

91.7 mg/L of $CaCl_2$*$2H_2O$
69.0 mg/L of $MgSO_4$*$7H_2O$
58.4 mg/L of $NaHCO_3$
15.4 mg/L of $KHCO_3$

Apical shoot portions were cleaned with tap water to remove epiphytic material and then placed in a 300mL glass BOD bottle containing an oxygen-depleted general purpose culture solution. The culture solution was initially bubbled with a nitrogen (N2) and CO2 gas mixture (350 ppm CO2 balance N_2) to reduce oxygen (O2) while maintaining normal pH and CO2 concentrations. This is necessary to avoid O2 supersaturation during the assay. Supersaturation induces photorespiration—a process not connected to normal metabolic respiration in which plants convert O2 to CO2. At the start of the assays the O2 concentrations were typically about 20 percent of air saturation (1.5-2.0 mg O_2 l^{-1}) and pH was typically between 8.0 and 8.1.

Bottles were stoppered with ground-glass stoppers and capped to create air-tight conditions. Bottled plants were then placed in a continuously circulating, temperature-controlled water bath (22 °C) under high-output fluorescent aquaria lights with saturating light intensities (ca. 500 µmol m^{-2} s^{-1} PAR) (Figure 41). Control bottles were set up with aerated culture solution or DI water, and plants were allowed a 30-minute acclimation period before the first measurement was taken.

Individual bottles were removed from the experimental treatment just long enough to measure DO and pH. Photosynthetic activity was calculated as the change in O2 concentration (mg O_2 l^{-1}) over time using discrete measurements obtained with a YSI 5010 BOD probe connected to a YSI 5000 Dissolved Oxygen Meter. pH was measured with an epoxy pH electrode (sensitivity of 0.01 units) connected to a pH/conductivity meter (VWR symphony SB80PC). Experimental trials lasted from 24 to 100 hours, and were allowed to proceed until they reached a point at which the O2 concentration and pH stabilized. Measurements were initially taken in 1- to 2-hour intervals with decreasing frequency as the rates of change slowed. In some cases, DO levels rose to near-saturating conditions before photosynthesis stopped. In such cases, pure N2 gas was bubbled into BOD bottles to reduce O2 without adding CO2 or changing pH. Dissolved oxygen and pH were re-measured post-N2 exposure to verify that this activity did not impact the pattern of change in net photosynthesis or change the pH.

At the end of each trial, plants were removed from their respective BOD bottles, cut into pieces and placed into tarred and labeled aluminum weighing boats. Plants were dried at 60 °C for approximately 72 hours and weighed on an electronic balance to the nearest 0.001 g.

Figure 41. **Subsamples of plant tissue were incubated in glass BOD bottles under light-saturating conditions at controlled temperatures (left). Periodically, the bottles were removed and pH and dissolved oxygen (DO) levels measured (right).**

Data Analysis and Statistics

Biomass-specific rates of net photosynthesis (mg O_2 mg dry mass-1 h^{-1}) were estimated for each sample interval. The average pH of the interval was computed as the pH equivalent of the average hydrogen ion concentration at the beginning and end of the interval. Typically, the rates of net photosynthesis will decline to zero as the pH approaches the CO2 or HCO3- compensation point of the plant.

The CT:ALK ratio was computed for the maximum pH observed during the assay or the pH at which net photosynthesis dropped to zero. The CT and ALK of this endpoint were computed using the equilibrium equations provided by Stumm and Morgan (1981).

Comparison of the CT:ALK data among the six species grown under two different growth conditions (fresh samples and CO2-stressed conditions) was made by One Way ANOVA. This approach yielded 12 species x growth condition combinations.

4.4 Results

Example results of net photosynthesis vs. pH for *Cabomba* (a species which appears to not utilize HCO3-, see below) and *Vallisneria* (a well-known HCO3- user, according to Prins et al. [1980]) are shown in Figure 42. Net photosynthesis of *Cabomba* drops to zero as the pH approaches 9 (maximum observed pH in this assay was 8.93). In sharp contrast, *Vallisneria* maintains positive net photosynthesis until a pH above 10.5 (maximum pH in this assay was 10.53).

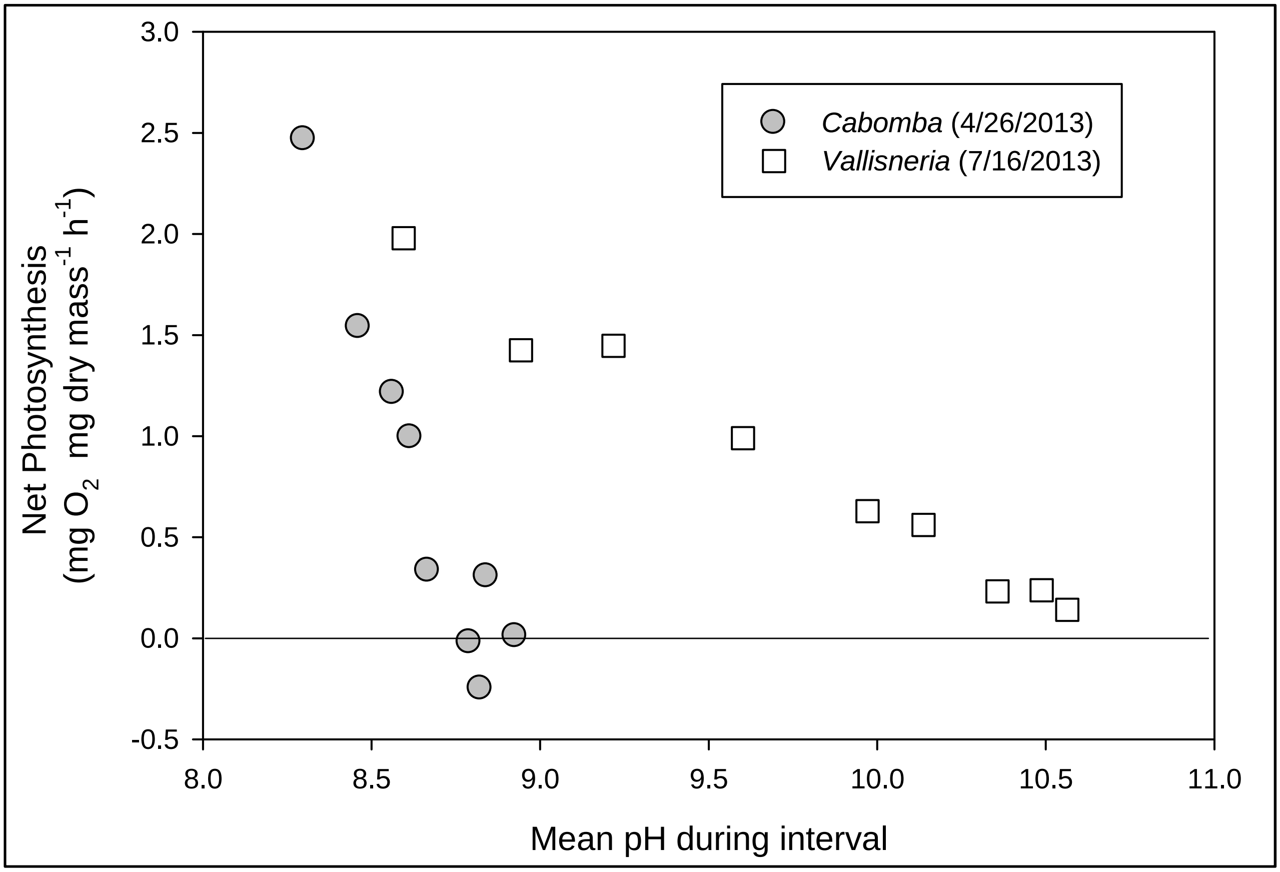


Figure 42. **Net photosynthesis vs. pH for each time interval for a pH drift assay for *Cabomba caroliniana* and *Vallisneria* sp. Net photosynthesis for *Cabomba* drops to zero at a pH near 9.0 (a pH near the 9.2 threshold where CO2 disappears from solution). In contrast, *Vallisneria* maintains positive net photosynthesis at much higher pHs, indicating robust bicarbonate (HCO3-) utilization.**

Figure 43 displays the results of the HCO3- utilization experiments for the six species assayed during spring 2013. Three species (*Riccia, Cabomba, Sagittaria*) appear to be CO2 obligates (unable to utilize HCO3-); two species (*Ludwigia* and *Hygrophila*) do not usually utilize HCO3- under normal field conditions, but can induce HCO3- utilization when growing under CO2-stressed conditions; and one species (*Vallisneria*) shows evidence of strong HCO3- utilization under both field and laboratory conditions.

The CT:ALK ratio of the six field collected samples of *Riccia* averaged 0.96 (range 0.89–0.99) strongly indicating no HCO3- utilization under field conditions. This species also showed no evidence of HCO3- utilization when grown in the laboratory under CO2-stressed condition in the laboratory (Figure 43).

Thirty-three samples of freshly collected *Cabomba* were assayed for this study and almost all of them indicate no HCO3- utilization under field conditions (Figure 43). A single outlier exists from the four samples collected in August 2013 from near Thompson's Island (San Marcos River) at a location noted to be "turbid, warm and not flowing." Although contamination by epiphytes was not noted, these are the conditions when such contamination would be most likely. Without this outlier

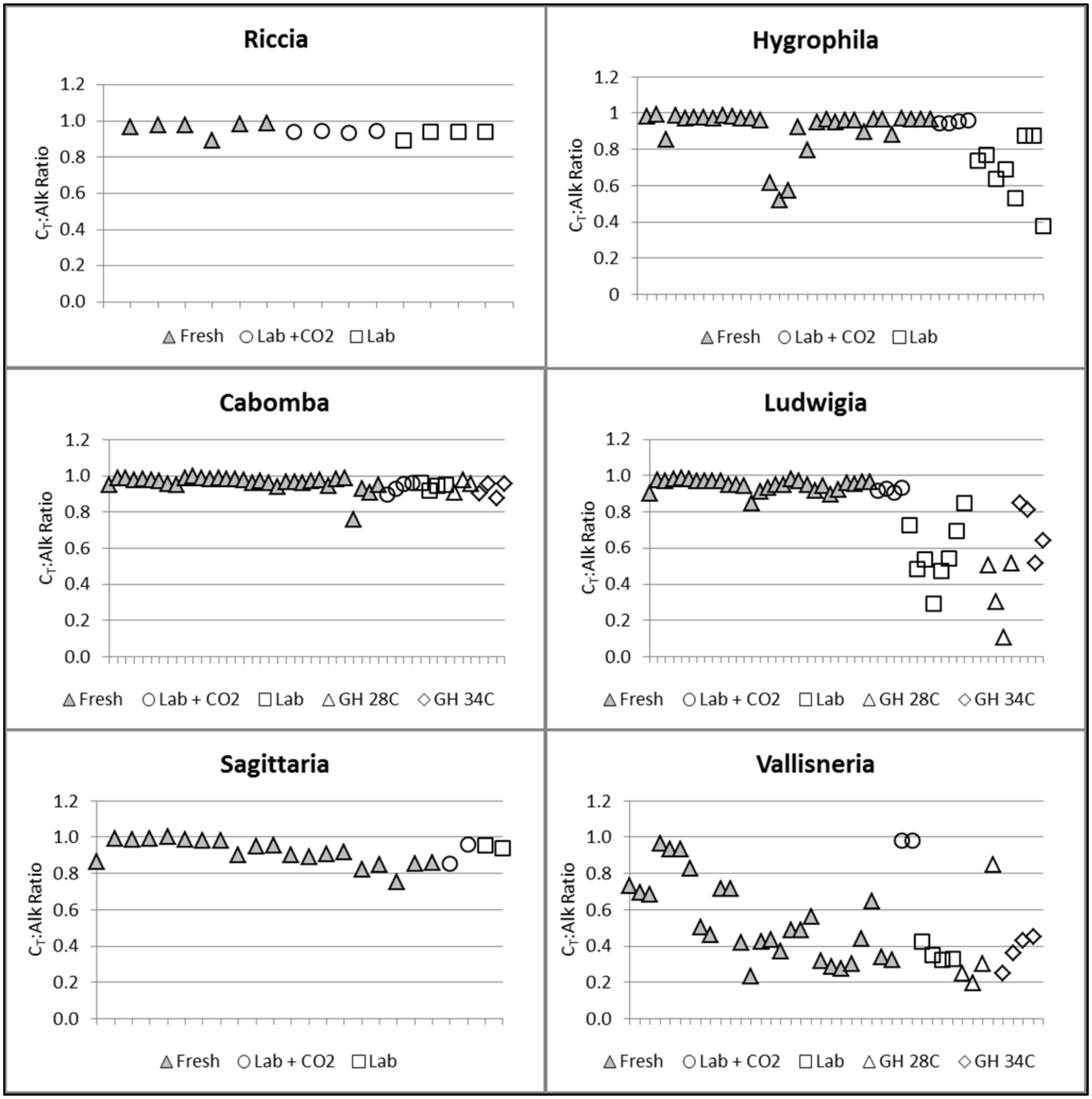


Figure 43. Ratio of total dissolved inorganic carbon (CT) to alkalinity (ALK), or CT:ALK, of freshly collected plants (shaded triangle indicates fresh), lab cultures amended with carbon dioxide (CO2) (indicated by open circle), lab cultures with no CO2 (open square), and plants grown as part of the temperature threshold study (open triangle=28 °C, open diamond=34 °C) at the San Marcos Aquatic Resource Center (ARC).

sample, the CT:ALK ratio averaged 0.97 (range 0.91–1.00). Supporting the view that *Cabomba* does not utilize HCO3- are the data from laboratory cultures, laboratory cultures plus CO2, and the plants growing in the 28 °C and 34 °C conditions in the ARC greenhouse (temperature threshold experiment). The average C_T:ALK ratio of the plants growing under these CO2-stressed conditions was 0.94.

Twenty samples of freshly collected *Sagittaria* were assayed for this study and indicate no or very weak HCO3- utilization under field conditions (Figure 43). The CT:ALK ratio of field-collected *Sagittaria* averages 0.92, although a few samples collected in the summer showed ratios of around 0.80–0.85. The slightly lower ratios could have been caused by attached epiphytes, which researchers were unable to scrape from the leaves. Supporting the view that *Sagittaria* is a CO2 obligate is the absence of HCO3- utilization by the plants cultured in the laboratory. Since this species was not used for the vegetation tolerance threshold study, there are no samples from growth at 28 °C and 34 °C.

Ludwigia and *Hygrophila* both appear to be able to induce HCO3- utilization when grown under CO2-stressed conditions (Figure 43). However, these species also appear to not usually utilize this pathway under field conditions.

The CT:ALK ratio of 29 freshly collected *Ludwigia* samples averaged 0.95 indicating little or no HCO3- utilization. Two samples with slightly lower CT:ALK ratios (0.85–0.89) may indicate weak utilization under field conditions or epiphytic growth on the samples. When grown in the laboratory with CO2 amendments, *Ludwigia* shows no sign of HCO3- utilization (ratio 0.91–0.93). However, when grown in the laboratory or the ARC greenhouse under CO2 deficient conditions, the CT:ALK ratio drops to an average of 0.55 indicating robust HCO3- utilization. Two samples collected from the ponds at the ARC (data not shown) had an average CT:ALK ratio of 0.77 indicating moderate HCO3- utilization.

The CT:ALK ratio of 31 freshly collected *Hygrophila* samples averaged 0.91. This average is lowered by four samples from April 2013, which had valued in the 0.52–0.79 range. While the data suggest occasional HCO3- usage, it is also clear that the vast majority of the time the plants are not utilizing HCO3- under field conditions. However, the laboratory cultures indicate that this species is capable of inducing at least moderate HCO3- use. The average CT:ALK ratio of the samples cultured without CO2 averaged 0.69. The four samples grown under CO2 amended conditions never developed the ability to utilize HCO3- (ratio=0.95). Because this exotic species was not utilized in the temperature threshold study, there are no data from plants grown at 28 °C and 34 °C.

Finally, *Vallisneria* in the Comal River appears to be using HCO3- under field conditions. The CT:ALK ratio of field-collected samples varied from 0.23 to 0.96 and averaged 0.55, indicating HCO3- utilization much of the time. The samples from laboratory culture without CO2 and from the temperature threshold study at the ARC averaged 0.38 and varied from 0.20 to 0.85, indicating very robust HCO3- utilization. *Vallisneria* is a species that is well known to have a strong affinity for HCO3- (Prins et al. 1980).

Figure 44 shows the results of a One Way ANOVA comparing the averages of both fresh plants and plants grown under CO2-stressed conditions. The average CT:ALK ratio of fresh samples of *Riccia, Cabomba, Sagittaria, Hygrophila*, and *Ludwigia* are all very high (0.91–0.96) and not statistically different from each other, supporting the conclusion that these species were not utilizing HCO3- under the field conditions of spring and summer 2013. In contrast, the average CT:ALK ratio of fresh *Vallisneria* was significantly lower (0.55), supporting the conclusion that it does utilize HCO3- under the field conditions.

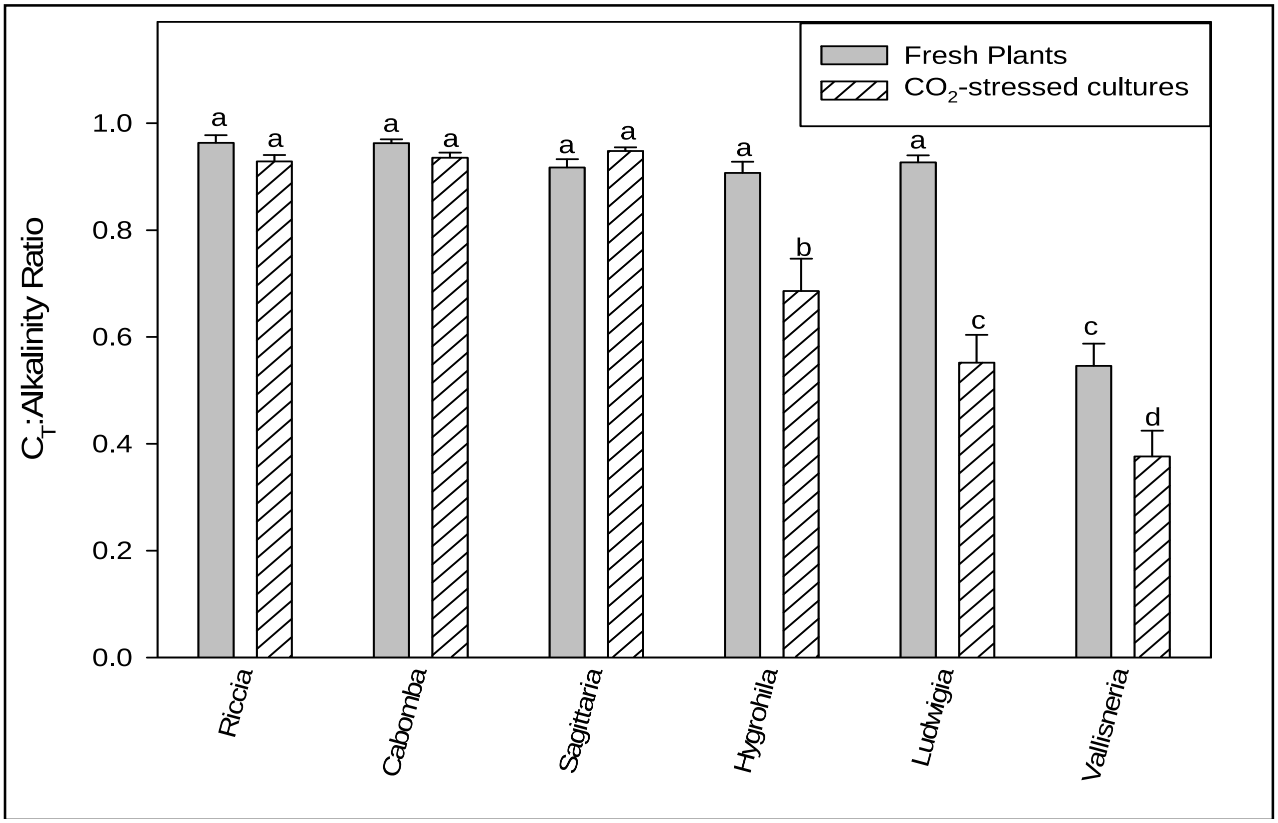


Figure 44. Ratio of total dissolved inorganic carbon (CT) to alkalinity (ALK), or CT:ALK (± SE), for each species. Grey bars show data for freshly collected samples, while hatched bars are for plants cultured for less than 2 weeks under carbon dioxide (CO2)-stressed culture conditions in the lab. Letters show statistically significant differences (One Way ANOVA, LSD multiple range test p<0.05).

When *Riccia, Cabomba,* and *Sagittaria* were cultured under CO2-stressed conditions, they did not induce the ability to utilize HCO3-. The average CT:ALK ratio of plants cultured under CO2-stressed conditions remained high (0.93–0.95) and were not statistically significantly different from freshly collected samples.

Hygrophila and *Ludwigia* did not usually utilize HCO3- under current field conditions, but developed the ability to utilize this carbon source when cultured under CO2-stressed conditions. The average CT:ALK ratio of *Hygrophila* plants grown under carbon stress dropped from 0.91 to 0.69, while that of *Ludwigia* dropped even more sharply, from 0.93 to 0.55 (Figure 44). Changes in both averages were statistically significant. Finally, the CT:ALK ratio of *Vallisneria* grown under carbon-stress dropped significantly from 0.55 to 0.38.

4.5 Discussion

These data directly establish the HCO3- utilization potential of six species of aquatic plants within the Comal/San Marcos systems.

Riccia and *Cabomba* are shown to not utilize HCO3- under field conditions present during the March–July 2013 period. Furthermore, neither species shows evidence of developing the capacity to utilize HCO3- under CO2-stress conditions. These data support the previous conclusions related to HCO3- utilization potential for these two species as summarized in Table 15 (Bain and Proctor 1980, Ballestros et al. 1998, Hiscock 2003). *Sagittaria* also was not able to utilize HCO3- during the time period tested. However, unlike *Cabomba* and *Riccia*, *Sagittaria* is capable of forming emergent leaves and avoiding CO2 limitation by obtaining the gas from the atmosphere instead of water. These data differ from that of Keeley (1998) who reports that some *Sagittaria* species exhibit CAM-like photosynthesis. However, data from this study include relatively few samples from plants grown under CO2 stress, so further investigation on this species may be in order.

Both *Hygrophila* and *Ludwigia* develop the ability to utilize HCO3- when growing under CO2-stressed conditions. Additionally, since both species are heterophylous and capable of forming emergent leaves, they can also alleviate CO2 stress by sending out emergent stems and leaves. The observed results for *Hygrophila* support the previous information on this species presented by Maberly and Madsen (2002) and Spencer and Bowes (1985). However, data from this study differs from previous reports by Lytle (2003) for *Ludwigia repens* and data from Prins et al. (1980) for *Ludwigia natans*. However, had only freshly collected samples been analyzed (a common practice in many of the studies to date), results would have concluded that these species were not capable of HCO3- utilization.

Finally, the *Vallisneria* species growing in the Comal River is a strong HCO3- user. Samples collected from throughout the Comal system (Landa Lake, Old Channel, New Channel) all showed strong ability to utilize HCO_3. These observations make sense in light of the widespread distribution of this species in lakes and reservoirs where CO2 deficient conditions are likely. These data are in line with a strong literature supporting the efficient use of HCO3- by members of this genus (Ackerman 2006, Holaday and Bowes 1980, Keeley 1998, Maberly and Madsen 2002, Nishihara and Titus and Stone 1982).

Since CO2 availability to the submersed leaves of aquatic plants is a function of the affinity of the species for HCO3-, as well as water current velocity (which controls boundary layer thickness), lower CO2 concentrations in the spring water may be partially offset by increased flow. Hence, survival of some aquatic plant species under low-flow conditions may be facilitated in reaches with relatively higher flow. So the current situation in which both HCO3- users as well as CO2-obligate plant species are widely distributed within the rivers may shift so that only strong HCO3- users survive in the more stagnant, lake-like conditions, and the other species are much more limited in distribution.

Please refer to Section 6 for a discussion regarding lessons learned, HCP ecological model application, and recommended future applied research relative to the 2013 HCP pH drift study described in this section.

5.0 FOOD SOURCE STUDY

5.1 Introduction

A major data gap identified in the development of the HCP is whether the macroinvertebrate food base that presently supports the diet of the endangered fountain darter will continue to persist during extreme drought conditions. To test the food base response to extreme conditions, a low-flow, food-source study was designed based on the available literature, information acquired during 2013 HCP aquatic vegetation studies, and the pre-study food source investigations.

The focus of the fountain darter food source study is to determine if conditions anticipated to result from low- or no-flow conditions can be expected to adversely impact availability of a significant prey source of the fountain darter. It is expected that aquatic macroinvertebrates, specifically the amphipod *Hyalella azteca* (used as a surrogate), will be unable to tolerate increased water temperatures that may result from low flow at some threshold. Fountain darters are a federally listed endangered species only found in the Comal and San Marcos rivers, Texas. Although the extreme conditions noted in 1956 (cessation of spring flow) for the Comal River have not been repeated, summer flows in the Comal River have dropped below 100 cfs in 1989, 1990, and 1996 (USGS gage 0816900). The present HCP flow regime incorporates total discharge in the Comal system well below 100 cfs. As such, it becomes important to understand how elevated water temperatures due to decreased spring flow will affect the fountain darter beyond just reproductive success and organism survival.

5.2 Data Review and Available Literature

The fountain darter typically inhabits the bottom third of the water column, preferring aquatic vegetation for cover. This vegetation also provides cover for aquatic invertebrates, the preferred food of fountain darters (Bergin et al. 1995, Schenck and Whiteside 1977). Fountain darter diets are made up of many types of invertebrates, but copepods, dipterans, mayflies, and amphipods are most frequently found in their stomachs (Schenck and Whiteside 1977). The most common amphipod in the Comal Springs/River ecosystem is *Hyalella azteca*, which is widely distributed across North America (Strong 1972). These invertebrates likely reproduce most of the year due to constant water temperatures (Strong 1972). *Hyalella azteca* are shown to have inter-population variation in body size (Strong 1972, Wellborn 1994) across regions and water systems (e.g., springs [Wellborn et al. 2005]). In addition, this amphipod is known to be different morphologically in the presence of predators (e.g., fish [Cooper 1965, Strong 1972, Wellborn 1994]). As such, using experimental stock taken from the population in Comal Springs was deemed necessary rather than solely relying on information from other watersheds.

Hyalella azteca is common in fountain darters' preferred vegetation types (bryophytes, filamentous algae, *Hygrophila*, *Ludwigia* [BIO-WEST 2013a]) and, therefore, a widely available prey source. *Hyalella* spp. are widely distributed in North America and can tolerate a wide range of water temperatures. While researchers have observed adverse effects of high temperatures on *Hyalella* spp. in some regions (Pickard and Benke 1996 [>26° C], Wellborn and Robinson 1996 [30° C]), these limits are not necessarily indicative of all inhabited locations. A common method for determining an upper thermal limit is to determine the CTM. Studies using various acclimation methods have shown LT_{50} values of *Hyalella azteca* at 33.2 °C (Sprague 1963), as well as CTM and LT_{50} values of closely related crustaceans to range from 29.6 °C to as high as 38.6 °C (Buchanan et. al. 1988, Dallas and Rivers-Moore 2012, Gaston and Spicer 1998, Morritt and Ingólfsson 2000, Sprague 1963). However, these studies tested amphipods collected from sources that varied in location and habitats that are not representative of what is found in the Edwards Aquifer region.

As such, a CTM was determined for *Hyalella azteca* from the Comal system, which could assist in parameterization of the fountain darter food source input to the HCP ecological model(s). The CTM uses temperature as a one-variable test and will serve as a surrogate for when amphipods cease to be available as a food source to the fountain darter. This cessation of feeding is based on the literature that describes fountain darters (in laboratory studies) as only feeding on moving prey (Schenck and Whiteside 1977). In collaboration with results found in CTM trials, additional fountain darter food source studies involving laboratory and pond experiments tested the effects of prolonged exposure to temperatures similar and in excess of what is conventionally found at Comal spring systems. These studies will help demonstrate how temperature will affect a main food source of the fountain darter and, further, our understanding of the ecological impact of rising water temperatures associated with low-flow conditions.

5.3 Materials and Methods

The amphipod, *Hyalella azteca*, was collected from Landa Lake and Old Channel reaches of Comal River in New Braunfels, Comal County, Texas. Amphipods were netted using 1,000-micrometer (µm) mesh and held in 5-gallon containers along with their natural substrate and vegetation that were incidentally collected while sampling. All holding, acclimation, and experimental trials used fresh, untreated Edwards Aquifer well water, with particular treatments heated accordingly. Experimental trials were undertaken at facilities provided by the ARC. The formal food source study design and methodology was presented to the HCP Science Committee in August with subsequent approval to proceed.

5.3.1 Critical Thermal Maximum (CTM) Experiments

Prior to acclimation, there was an initial holding period of ca. 20.3 °C for 2 days using Edwards Aquifer water. Experiments tested critical thermal maxima of amphipods acclimated to treatments of 20 °C, 24 °C, and 28 °C. Amphipods were separated into their respective treatment containers with an increase of 1 °C per day until reaching acclimation temperature. Consumer-grade aquarium heaters were used to increase and maintain desired acclimation conditions. After 10–12 days of acclimation, 50 amphipods from each acclimation treatment were tested by placing ten individuals in a water bath increasing in temperature at 0.3 °C per minute. Setup and equipment (Figure 45) was the same as that which was used by Fries and Gibson (2010). The temperature sensor of a BASIC pH Meter (Denver Instrument Co., Arvada, Colorado) was used to determine CTM and calibrated using a National Institute of Standards and Technology (NIST)-certified mercury thermometer (Thermo Probe Inc., Pearl, Mississippi). Along with the temperature sensor, a Hobo TidbiT v2 Temperature data logger (Onset Computer Corporation, Cape Cod, Massachusetts) was added to the

Figure 45. Setup for critical thermal maximum (CTM) experiment.

experimental chamber to ensure recorded temperatures were accordant. Amphipods were observed constantly while temperatures increased, and ecological death, or critical thermal endpoint, was established when individuals were perceived to have lost equilibrium and were unresponsive to gentle probing or pipetting. When an individual reached thermal endpoint, the temperature and time of the event was recorded and the individual was placed into room temperature water (ca. 21 °C) and returned to its original acclimation temperature for 18–24 hours, after which living amphipods were counted and recovery rate established.

Statistical Analysis

A paired *t*-test was used to determine if any differences existed between temperature sensors. Comparison of CTM used single factor ANOVA.

5.3.2 Laboratory and Pond Studies

Experimental Unit and Enclosure Design

In both experiments, amphipods and bryophytes were enclosed in specifically designed enclosures (Figure 46) to provide a controlled study area. The enclosure base was weighted down using gravel and had 5-mm holes drilled into the unit base, allowing water movement between the enclosures and the aquarium they were housed in. The base and lid opening were covered with 790-µm mesh using a lid to secure the mesh in place, completely enclosing the study area while allowing water exchange throughout the enclosure and the surrounding aquatic environment. An experimental unit

Figure 46. Experimental enclosures for amphipod testing.

included enclosures with only amphipods, whereas the other half included an aquatic bryophyte, *Riccia*, along with amphipods. It was hypothesized that aquatic vegetation might serve as a food source for the amphipods, thus potentially eliminating starvation as a cause of death in the *Riccia* units. To test the applicability of the enclosures, 10 amphipods were added to each one with bryophytes added only to designated units. Enclosures and individual amphipods were checked regularly over a 2-week period via gentle probing to determine if individuals were living or deceased. From these preliminary tests, it was determined that the specially designed experimental enclosure would support survival of amphipods for a minimum of 2 weeks, and thus experimentation using these enclosures was carried forward.

Pre-Trial Study

Preliminary laboratory food-source trials tested amphipod survival rates at two temperature treatments. After 48 hours of acclimation to treatment temperatures, amphipods were separated into two treatments of 28 °C and 34 °C and survival was assessed incrementally. These temperatures were determined by established biological limits of the fountain darter, as 28 °C has been shown to reduce offspring viability and larval survival while 34 °C is approximately the CTM for fountain darters (Bonner et al. 1998, Brandt et al. 1993). Death rates of *Hyalella azteca* were higher in the higher-temperature treatment (Table 16). Using the results from preliminary trials, and the results from CTM trials, laboratory and pond food-source study protocols were established, and results described herein.

Table 16. Preliminary laboratory food-source trials using *Hyalella azteca*, showing death rates (percent) at treatment temperatures throughout the 16-day study. No surviving individuals were found at 34 °C treatments 8 days after acclimation.

STUDY DAY	TEMPERATURE TREATMENT	
	28 °C [a]	34 °C
Day 4 - % Death rate	46.2	92.2
Day 8 - % Death rate	70.8	100
Day 16 - % Death rate	86.2	100

[a] **°C**=degrees Celsius.

5.3.3 Laboratory Study

Laboratory food-source trials included two replicates of three water temperature treatments (Figure 47): 20 °C, 28 °C, and 34 °C. Amphipods were acclimated to their respective temperature treatments 48-h prior to testing. Treatments were conducted in 950-L fiberglass tanks (Living Stream Model MT-1024, Frigid Units Incorporated, Toledo, Ohio) using two smaller 110-L fiberglass tanks (Red Ewald Inc., Karnes City, Texas) within each treatment tank as replicates (Figure 48). Each replicate was randomly assigned six total experimental units: three units with amphipods and bryophytes, and three units with amphipods but no bryophytes, for an experiment total of 36 units. Edwards Aquifer water was used to sustain environmental conditions, in conjunction with a single 120-volt industrial L-shaped submersible heater (Process Technology, Mentor, Ohio)**,** which was used to keep treatment temperatures constant throughout the duration of the 2-week study. Daily water quality measurements (temperature in °C, DO in mg/L, and pH) were made using an YSI multiparameter sonde with a Professional Series handheld data unit (YSI Inc., Yellow Springs, Ohio).

Statistical Analysis

Water quality differences among treatments were assessed using single factor ANOVA and Kruskal-Wallis tests. Water quality data among replicates within treatments were assessed for normality using Shapiro-Wilk tests and homogeneity of variance using F-tests. If data met test assumptions, a two-sample *t*-test was used for analysis (Wilcoxon rank-sum tests were used if the assumptions of the *t*-test were not satisfied by the data). Amphipod survival data from laboratory food source experiments were analyzed using the approach described by Crawley (2013) for dealing with proportion data and categorical explanatory variables. Responses of death to treatment conditions were treated as "successes" and survival responses as "failures" (i.e., viewed as proportion of deaths rather than proportion surviving). The data were fit with a generalized linear model (glm) with binomial errors using a logit link to linearize the data incorporating presence or absence of *Riccia* as a food source. The data were found to be over-dispersed (residual deviance/residual df=1.45). To account for this, a new glm model was fit using the quasi-binomial error family. Simplification of the maximal model (y~treatment*riccia) was achieved by removing non-significant interaction terms and main effects to produce the minimal adequate model.

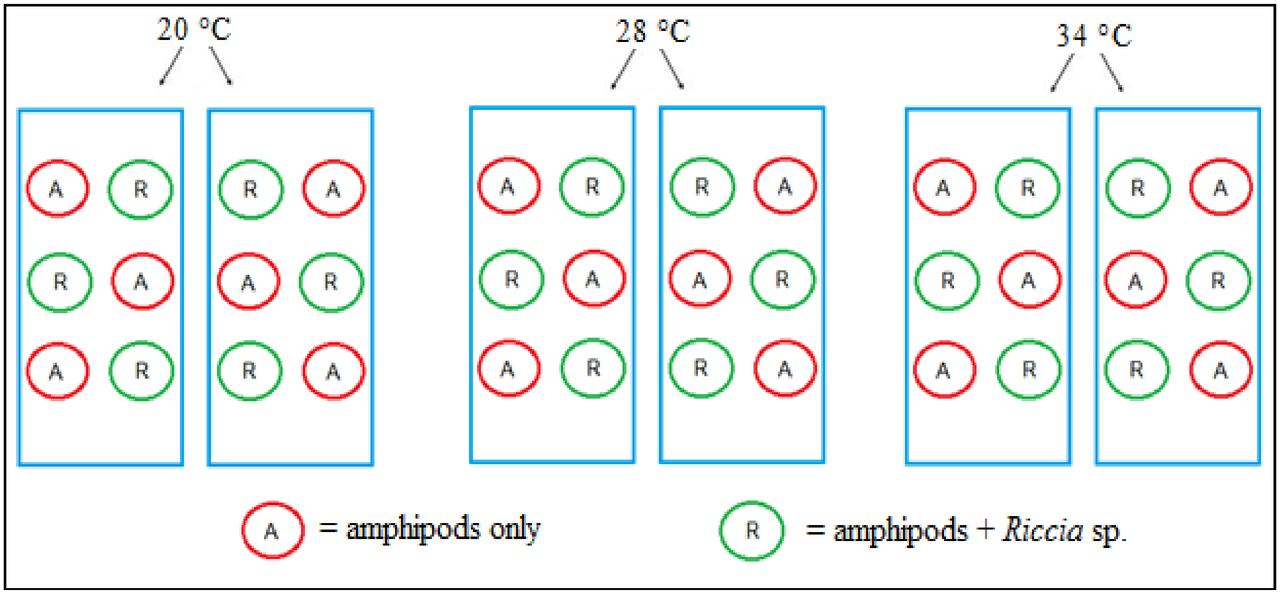


Figure 47. **Experimental design of the laboratory food source experiment; shows three treatment temperatures each with two replicates.**

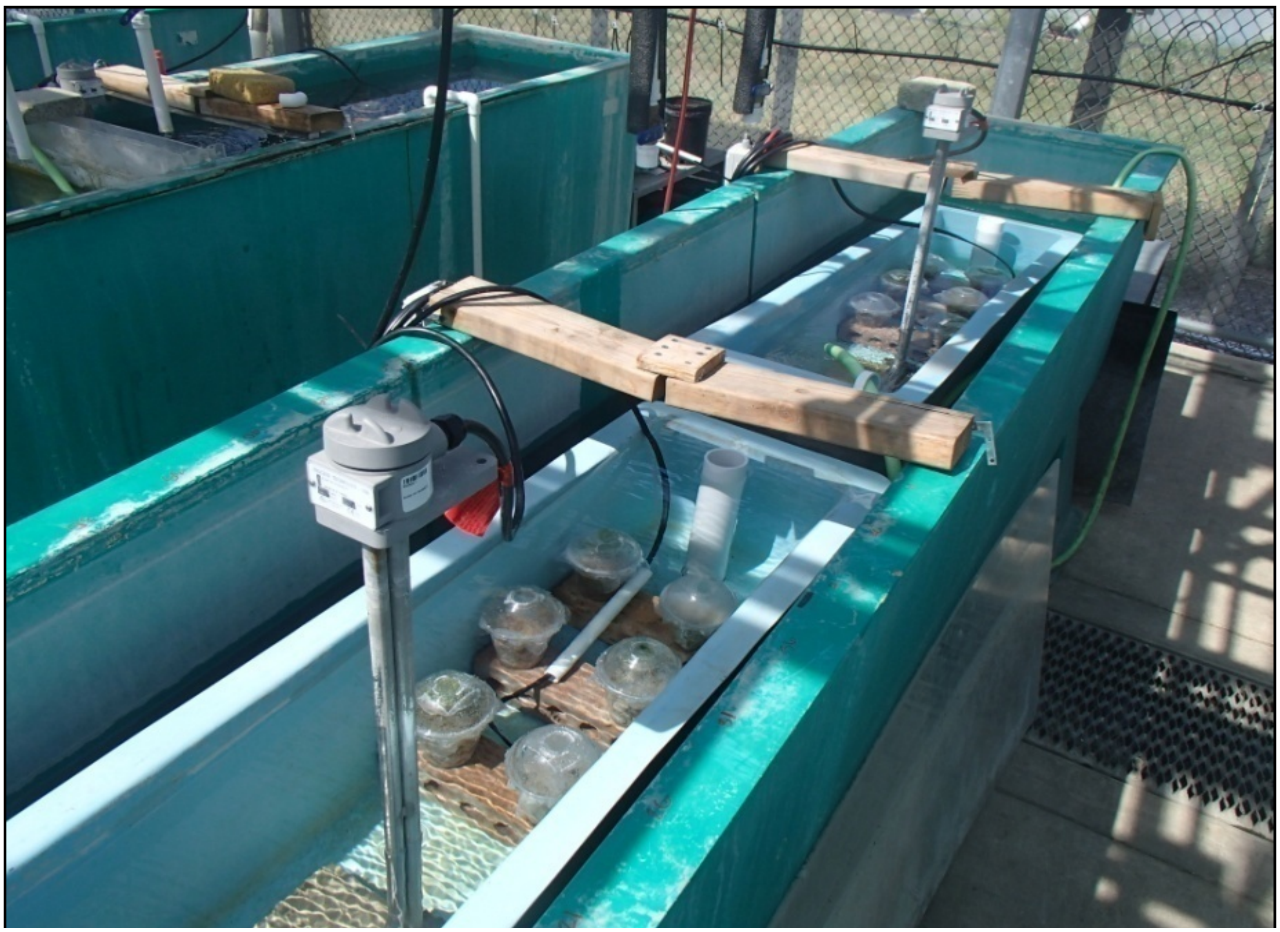


Figure 48. **Amphipods in experimental cups with and without *Riccia* in two replicates of 28 degrees Celsius.**

5.3.4 Pond Study

In conjunction with the pond vegetation study, the pond food-source study included five replicates in two treatments of differing temperature regimes due to flow or lack thereof (Figure 49). The experimental pond was divided by a sandbag barrier, leaving approximately one half of the pond with flow and the remaining area ponded. Amphipods were acclimated to pond conditions 48-h prior to testing. Each treatment contained 10 experimental units, with each replicate containing one unit with amphipods and bryophytes, as well as one unit with amphipods but no bryophytes, for a total of 20 experimental units. Amphipod units were randomly assigned to and placed in one of 20 experimental plant units composed of 10 individuals of either *Ludwigia* or *Vallisneria*. Placement within vegetation is more representative of the habitat where they would be found in the wild as well as habitat where they would likely be encountered by fountain darters.

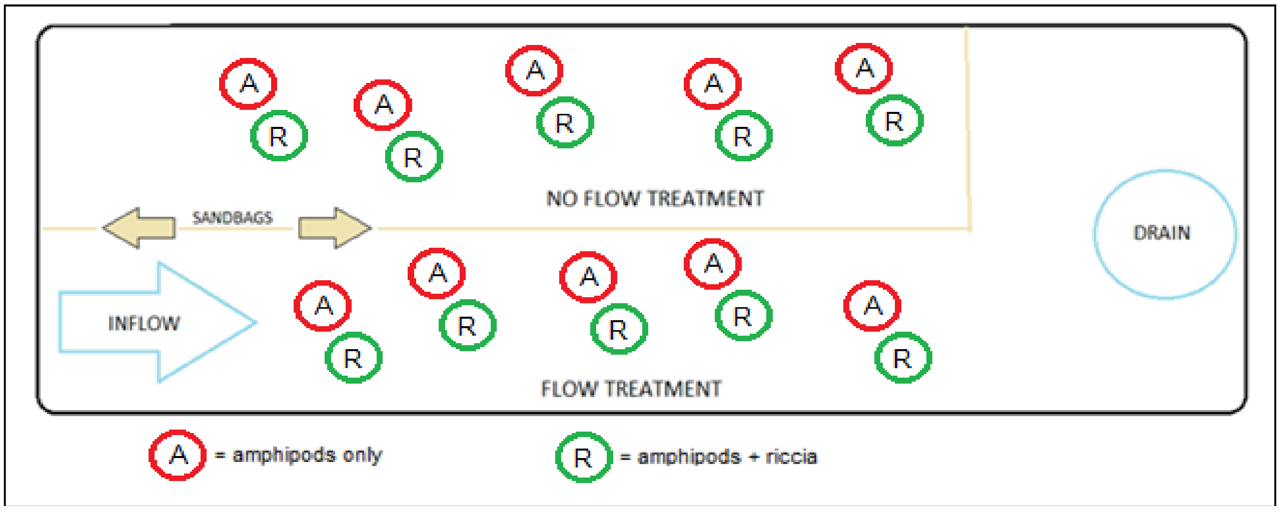


Figure 49. Representation of the experimental scheme for the low-flow, food-source study pond experiment. Red and green circles represent experimental units containing *Hyalella azteca* only or *Hyalella azteca* with *Riccia*, respectively.

Daily water quality measurements were made using an YSI multiparameter sonde with a Professional Series handheld data unit (YSI Inc., Yellow Springs, Ohio) and dissolved CO2 was measured using the Oxyguard portable CO2 Analyzer (Oxyguard International AS, Berkerød, Denmark). In addition to water quality measurements, PAR was measured using the MQ-200 Quantum meter (Apogee Inc., Logan, Utah). A Flo-mate 2000 portable flowmeter (Marsh-McBirney Hach Inc., Loveland, Colorado) measured flows within the study area. Environmental variables (DO in mg/L, PAR in $\mu E\ m^{-2}\ sec^{-1}$, CO2 in mg/L, temperature in °C, pH, flow in cfs, and depth in m) were measured twice daily (once in the early morning and again mid-afternoon) to capture diurnal fluctuation of these variables.

Statistical Analysis

To assess differences in water quality parameters between the treatments with (flowing) and without (non-flowing) water exchange in the formal pond experiment, two-sample *t*-tests (or Wilcoxon rank-sum tests if the assumptions of the *t*-test were not satisfied by the data) were used. Death rates were analyzed using similar methodology as described in laboratory food source studies.

Generalized linear model data incorporated both flow and no-flow treatments, accounting for temperature differences, where factors were found to be over-dispersed (residual deviance/residual df=2.24). To account for this, a new glm model was fit using the quasi-binomial error family. Simplification of the maximal model (y~treatment*riccia) was achieved by removing non-significant interaction terms and main effects to produce the minimal adequate model.

5.4 Results

5.4.1 Critical Thermal Maximum (CTM) Experiments

Analysis showed differences to be significant between the two temperature sensors (=10.573, df=150, p<0.001) with a 95-percent CI [0.174 °C, 0.254 °C]. However, the average difference between sensors was found to be 0.214 °C, which is similar to the accuracy interval provided by the Hobo temperature logger manufacturer (±0.21 °C) and within the accuracy of the BASIC pH meter temperature sensor (±0.4 °C). This led to the interpretation of CTM values to be determined using the BASIC meter readings. Data passed assumptions of a Shapiro-Wilk normality test; therefore, CTM values were analyzed using single factor ANOVA, which suggested no difference among acclimation treatments ($F_{2, 12}$=0.182, p>0.05) (Table 17). Given that there did not appear to be any effect of acclimation temperature on CTM, all response values were used to calculate the mean CTM of 37.89 °C with 95-percent CI (37.78 °C, 38.01 °C). Immediately following trials, amphipods were returned to original acclimation temperature for 18–24 hours, with 77.6 percent surviving after the recovery period. While the CTM of *Hyalella azteca* only tests one factor (temperature) of survival, it does provide a starting point to examine how this species might be affected by low-flow conditions.

Table 17. Preliminary summary of critical thermal maximum (in degrees Celsius) for *Hyalella azteca* showing number of individuals tested (N), minimum, maximum and mean values, and standard deviation. No differences were found among acclimation treatments (p>0.05).

TEMPERATURE	N	MAXIMUM	MINIMUM	MEAN	STANDARD DEVIATION
20 °C	50	39.2	36.1	37.95	0.82
24 °C	51	39.3	36.0	37.94	0.72
28 °C	50	39.1	35.8	37.79	0.62

5.4.2 Laboratory Study

To further the understanding of *Hyalella azteca* as a food source for the fountain darter, laboratory experiments tested the effect of the interaction with *Riccia* at varying temperatures. Significant differences among temperature treatments were found by single factor ANOVA for temperature ($F_{2, 97}$=4975, p<0.001) and pH ($F_{2, 97}$=35.93, p<0.001). Dissolved oxygen was also found to differ significantly among temperature treatments by Kruskal-Wallace test (H=18.66, df=2, p<0.001) (Table 18). No significant differences were found in water quality parameter means between replicates within temperature treatment groups (Table 19) with the exception of temperature between replicates in the 34 °C treatment (t=-2.4813, p=0.02385), though this difference was <1 °C (95% CI [0.81415, 0.06585]).

Table 18. Water quality values for parameters monitored over the course of the laboratory experiment.

PARAMETER	TREATMENT											
	34 °C				28 °C				20 °C			
	max	min	mean	SD[a]	max	min	mean	SD	max	min	mean	SD
Temperature[b]	34.7	33.1	33.9	0.45	29.4	26.5	28.0	0.63	21.4	19.0	19.7	0.51
pH	8.06	7.41	7.79	0.18	7.83	5.57	7.44	0.34	7.83	6.42	7.15	0.25
Dissolved oxygen[c]	5.09	4.09	4.66	0.25	8.25	4.24	5.20	0.91	5.75	4.32	5.07	0.31

[a] SD=standard deviation.
[b] Measured in degrees Celsius.
[c] Measured in milligrams per liter.

Table 19. Summary of mean (SD) values of water quality parameters (temperature in degrees Celsius, dissolved oxygen in milligrams per liter, and pH) of replicates within treatments. These data were analyzed by parameter between replicates within each treatment using *t*-tests (or Wilcoxon rank-sum tests as required by data distribution).

TREATMENT	PARAMETER					
	temperature		dissolved oxygen		pH	
	replicate 1	replicate 2	replicate 1	replicate 2	replicate 1	replicate 2
34°C	33.67 (0.44)	34.11 (0.34)	4.59 (0.26)	4.73(0.22)	7.81 (0.14)	7.77 (0.21)
28°C	28.11 (0.20)	27.96 (0.87)	5.39 (0.19)	5.01 (0.46)	7.52 (0.16)	7.37 (0.45)
20°C	19.65 (0.45)	19.76 (0.57)	5.10 (0.30)	5.04 (0.33)	7.23 (0.26)	7.08 (0.22)

The minimally adequate glm model was determined to be “y~treatment+riccia”, as the interaction between temperature treatments and *Riccia* presence was not significant (p=0.4375) and was removed from the model. This allows for the conclusion that the effect of *Riccia* presence/absence was consistent throughout different temperature treatments. Treatment temperature (F=61.67, p<0.001) and *Riccia* presence/absence (F=45.05, p<0.001) were both found to have significant effects on amphipod survival. Death rates were higher for units without *Riccia* across all temperature treatments, and highest in 34 °C treatments (Table 20). As expected, providing a food source (*Riccia*) for *Hyalella azteca* was important in its survival and may be a key reason it is associated with *Riccia* in Landa Lake of the Comal system.

Table 20. Laboratory food source experiment death rates (percent) in the presence or absence of *Riccia* throughout the 2-week study (temperature in degrees Celsius).

CONDITION	TREATMENT		
	34 °C	28 °C	20 °C
With *Riccia* - % Death rate	100[a]	38.3	11.7
Without *Riccia* - % Death rate	100[a]	85.1	54.9

[a] No surviving amphipods were found in 34 °C treatments one week into study.

5.4.3 Pond Study

Using two-sample *t*-tests and Wilcoxon rank sum tests, values for all water quality parameters except PAR (temperature in °C, DO in mg/L, pH, and CO2 in mg/L) were significantly different between the two treatments (Tables 21 and 22).

Table 21. Water quality values for parameters monitored over the course of the pond experiment.

PARAMETER	FLOW				NO FLOW			
	maximum	minimum	mean	standard deviation	maximum	minimum	mean	standard deviation
Temperature[a]	32.4	21.5	26.2	3.2	35.4	24.3	29.5	3.5
Dissolved oxygen[b]	17.49	4.48	7.87	2.43	9.67	3.77	6.66	2.06
pH	8.75	7.55	8.05	0.24	9.03	6.30	8.44	0.31
Carbon dioxide[b]	15.0	2.0	6.8	3.1	5.0	1.0	2.3	1.2
PAR[c]	1,646	53	611	452	1,552	42	700	445

[a] Measured in degrees Celsius.
[b] Measured in milligrams per liter.
[c] PAR= photosynthetically active radiation, measured in (µmol m-2 s-1).

Table 22. Significance values for comparisons of water quality parameters between pond study experiment treatments.

PARAMETER	TEST				
	t-test			Wilcoxon rank sum	
	t	df	p	W	p
Temperature	7.526	235.67	<0.001		
Dissolved oxygen	-4.1426	232.08	<0.001	*	*
pH	*	*	*	12577	<0.001
Carbon dioxide	*	*	*	236	<0.001
PAR[a]	-0.2025	237.90	0.84	*	*

[a] PAR=photosynthetically active radiation.

The minimally adequate glm model was determined to be "y~treatment+riccia", as the interaction between temperature treatments and *Riccia* presence was not significant (p=0.91) and was removed from the model. This allows for the conclusion that the effect of *Riccia* presence/absence was consistent throughout different temperature treatments. Treatment temperature (F=5.23, p=0.03526) and *Riccia* presence/absence (F=8.32, p=0.01) were both found to have significant effects on amphipod survival. Death rates were higher for units without *Riccia* at both treatments, and highest in no-flow conditions (Table 23). Again, the presence of *Riccia* sp. resulted in an increase in survival of *Hyalella azteca* both with and without flow. This is important because areas like Landa Lake in the Comal system contain habitats with varying flows that will shift as discharge decreases under an extreme drought scenario.

Table 23. Pond food source experiment death rates (percent) in the presence or absence of *Riccia* throughout the 2-week study.

CONDITION	FLOW	NO FLOW
With *Riccia* - % Death rate	54.4	79.6
Without *Riccia* - % Death rate	83.6	94.4

5.5 Discussion

5.5.1 Critical Thermal Maximum (CTM) Experiments

Experiments tested if acclimation temperatures had any effect on amphipod thermal resistance when rapidly increasing water temperatures (0.3 °C per minute). Bovee (1949) subjected *Hyalella azteca* to various (0.375 °C/min, 0.261 °C/min, 0.150 °C/min, 0.036 °C /min) rising temperature baths and found survival to occur at temperatures less than 39 °C, never exceeding 41°C. Similar to Bovee (1949), average CTM between treatments in this experiment was found to be 37.89 °C with 77.6 percent recovering from the thermal stress. No differences were found among acclimation treatments, comparable to that reported by Sprague (1963), who found that acclimation of *Hyalella azteca* had no significant effect on heat resistance in LT_{50} trials. These CTM experiments are not intended to model natural occurrences, but rather used to test the upper thermal tolerances of subject organisms. It should be noted that mean *Hyalella azteca* CTM (37.89 °C) exceed fountain darter CTM (34.8 °C) reported by Brandt et al (1993), suggesting that if environmental conditions caused water temperatures to increase, CTMs of the fountain darter would occur before the limits found for their food source. Unfortunately, this is only a single-factor experiment, and thus only provides baseline data on how *Hyalella azteca* may respond to decreasing discharge under extreme drought. Interpretation of the laboratory and pond food-source experiments, in conjunction with vegetation availability, suggests *Hyalella azteca* might actually cease to be a food source before CTM temperatures occur in the wild.

5.5.2 Laboratory study

The addition of a food source (*Riccia*) was found to significantly increase survival of *Hyalella azteca* in all treatments. This is similar to habitats where these amphipods are found in the Comal River. Wellborn and Robinson (1996) studied the effects of thermal pollution on macroinvertebrate communities, and found that, during summer months when water temperatures ranged from 38 °C–42 °C, all macroinvertebrates, including *Hyalella azteca*, attained their lowest abundance. As such (similar to what was found in the preliminary studies) laboratory settings show amphipods were unable to tolerate water temperatures of 34 °C for more than 7 days (Table 18 and Table 20). If springflows under drought scenarios result in elevated water temperatures for longer than 7 days, one could speculate that *Hyalella azteca* might cease to be a food source for fountain darters. Therefore, if fountain darters are still feeding at these high temperatures (remember the fountain darter CTM is 34.8 °C), they will be forced to find another food source or starve. Dissolved oxygen was found to be significantly less at 34 °C (4.59–4.73 mg/L), but based on DO levels in Sprague (1963), where only extremely low DO (<1.0 mg/L) resulted in *Hyalella azteca* death, it is unlikely to have affected survival of *Hyalella azteca* independent of temperature. While these laboratory studies stress the significance of the interaction between amphipods and their food source (e.g., *Riccia*), they do not consider other important factors like flow.

5.5.3 Pond Study

Pond experiments altered flow regimes in an attempt to simulate the Comal system during low-flow conditions. This alteration had an important role in the differences among treatments for all water-quality parameters. During the 2-week study, flow treatments created a maximum temperature of 32.4 °C of with an average of 26.2 °C and no-flow treatment assumed a maximum of 35.4 °C and an average of 29.5 °C. Dissolved oxygen and CO2 were significantly lower in the absence of flow. As stated before, the mean water temperatures observed (to say nothing of the maximum temperatures or low DO) are higher than temperatures that are known to reduce fountain darter reproduction, and egg and larval development (BIO-WEST 2002, Bonner et al. 1998, McDonald et al. 2007).

Amphipod death rates were more common in the no-flow treatment, and more importantly there was less than 50 percent survival throughout all experiment conditions (Table 23). This may indicate that *Hyalella azteca* have a minimum flow rate at which survival rates are higher over extended periods of time, but further study is required to answer this question. Similar to the aforementioned laboratory experiments, *Hyalella azteca* does not only have better survival at cooler temperatures, but also in the presence of *Riccia*, which had a significant effect on the death rate of amphipods in both treatments (Table 23). Therefore, when considering protection of fountain darter populations, it is important to realize that protection of food source habitat (i.e., *Riccia*) is important.

While this study addressed several parameters associated with a prey source's survival (and defined a narrower range of values where death occurs), the larger question of how prey interacts with fountain darters in extremely low-flow conditions remains unknown. That being said, this food-source study furthers our knowledge of when to expect harmful impacts on fountain darter prey to occur in the environment, and provides baseline data to populate the HCP ecological model to evaluate survival of the covered species.

Please refer to Section 6 for a discussion regarding lessons learned, HCP ecological model application, and recommended future applied research relative to the 2013 HCP food source studies described in this section.

6.0 EDWARDS AQUIFER HABITAT CONSERVATION PLAN (HCP) APPLICATION AND FUTURE APPLIED RESEARCH

As described in detail in Sections 2 through 5, a great deal of work was conducted and analyzed relative to 2013 HCP applied research. This section condenses that information to focus on (1) lessons learned, (2) HCP ecological model application, and (3) recommendations for future HCP applied research. These topics will be presented by study (Section) followed by an overall conclusion.

6.1 Field vs. Laboratory Study

6.1.1 Lessons Learned

Several key points were gleaned from this study. Although there were statistically significant differences in actual plant growth measures when comparing laboratory vs. field results, overall trends or patterns in results were similar between the laboratory and the field. Additionally, certain field parameters appeared to play a larger role than anticipated. Two parameters not anticipated by the project team were fine sediment deposition in slow moving areas of the Old Channel and herbivory. The fine sediment deposition triggered routine cleaning of field treatments in the Old Channel which caused additional stress on those plants relative to the controlled laboratory environment. Herbivory was qualitatively observed in the field throughout the study, but was not present in the laboratory. Although not quantifiable in this study, these factors undoubtedly attributed to differences in growth between the laboratory and field plants.

Regardless of explanations for laboratory vs. field differences, the fact remains that there are variables in the wild that often times are difficult, if not impossible, to simulate in the laboratory environment. Lessons learned during this study suggest that a similar study set up in open areas of Landa Lake would likely yield results closer to those of the laboratory. However, this would not accurately describe conditions in the Old Channel. This study clearly highlights the uncertainty surrounding using laboratory results solely to make management decisions or to populate ecological models. It can be done, but must be done with caution and understanding of the uncertainty involved.

Although magnitudes of responses were different between the laboratory and field, overall patterns were relatively similar. This allows the use of greenhouse data to project at least the direction of response of varying environmental conditions with some level of confidence. This is critical, and very much applicable to the HCP ecological model, since some factors simply cannot be manipulated nor tested in the field.

Finally, data from this study supports the intuitive conclusion that, where possible, field data should be used to project plant growth response. However, the overall strength of that recommendation varies by species and is likely less true for *Sagittaria* (many factors not different and overall magnitude of differences were relatively modest) than for *Cabomba* (all factors showed very strong lab vs. field responses, and in some cases showed consistent declines in the field and increases in the laboratory).

6.1.2 Habitat Conservation Plan (HCP) Ecological Model Application

The field vs. laboratory study was not designed to provide direct input into the HCP ecological model, but rather to test theories behind applied research to better inform decision making and model formulation as the HCP moves forward. From this exercise, it is clear that both the laboratory- and field-generated data are valuable, but should be used with caution in an ecological model attempting to simulate actual conditions in the wild. It is recommended that results from the 2013 applied research studies (Sections 3 through 5) be used to populate the HCP ecological model. However, there will always be more confidence surrounding the prediction of trends rather than the use of actual values (e.g., water temperature, growth rate). When the latter is necessary (which will likely frequently be the case), applied research results should first be entered and evaluated in the ecological model(s) as ranges around the results, rather than specific numbers. Subsequent model runs will identify the sensitivity of said parameters and ultimately model validation will help narrow down those ranges where applicable.

6.1.3 Future Applied Research Studies

No future applied-research studies are recommended for this topic at this time. Should certain plant species, environmental parameters, or spatial locations relative to aquatic vegetation and plant growth turn out to be highly sensitive in the ecological model(s), then future investigation may be warranted. It is recommended that any *in situ* work be conducted in MUPPTs (or similar device) where possible to allow the most direct application of the results.

6.2 Vegetation Tolerance studies (Laboratory and Pond)

6.2.1 Lessons Learned

The vegetation tolerance studies were multi-faceted with several lessons learned or implied from these investigations. The laboratory data indicates that the four species tested would likely survive short-term exposure to much warmer conditions with relatively low free CO2. This would suggest that if brief periods of low-flow conditions occur, these aquatic plant species would survive exposure up to 34 °C for a period of a few weeks under similar ecological conditions. While there are clearly limits to what the plants can survive (most died at 37 °C), these species appear resilient to short-term perturbations. The greenhouse conclusions are supported by the pond studies, which conclude that the aquatic plants tested can survive and continue to grow when water temperature increases to a daily mean of 30 °C with fluctuation as high as 35 °C, and mean dissolved CO2 decreases below 10 mg/L. Unlike the laboratory studies (which did not have a flow component), this also included an evaluation of very limited flow (0.023 cfs) and no flow at all.

Specific to plant survival during short periods of sub-optimal water temperature and CO2, the outcome of the greenhouse and pond experiments is excellent news for the long-term ecological integrity of the Comal and San Marcos river systems. The overall habitat which the aquatic plants of the Comal and San Marcos rivers provide to the listed species would be unlikely to completely disappear with limited temporal exposure to extreme low-flow conditions. With that said, two overarching cautions are in order: (1) data apply only to short-term exposures to the warm, low CO2 growth conditions and assumes the plants remain in the water (not left exposed by low water levels), and (2) data suggest the plants can survive these conditions when all other growth conditions are optimal. The plants were capable of surviving the *physiological challenge* of high temperature and low CO2 as established in the greenhouse and pond, but it may not mean that they would as easily survive the *ecological challenge* that would accompany such conditions in a field setting. In particular, replication of herbivory or other forms of disturbance (exposure and desiccation, excessive epiphytic growth), which occur simultaneously with these physiological challenges to the plants is difficult.

It was extremely encouraging that *Ludwigia* (a plant of known importance to the fountain darter and a target for extensive habitat restoration) would likely survive short durations of elevated water temperatures and low CO2. This was a major HCP unknown and directly affected the development of the long-term biological goals for the fountain darter. Not knowing the tolerance and HCO3- use potential of *Ludwigia* (a native plant), and with the assumption that *Hygrophila* (a nonnative plant) would be more tolerant, long-term biological goals were established for *Hygrophila* in both the Comal and San Marcos rivers. Although *Hygrophila* was not tested in the greenhouse study because of the restrictions of bringing nonnative plants on station, it was tested in conjunction with *Ludwigia* in the pH drift study with similar results. Based on the tolerance exhibited by *Ludwigia* in the laboratory and pond study, coupled with the understanding of HCO3- use potential and comparison to *Hygrophila* from the pH drift study, it is recommended that the long-term biological goals established for *Hygrophila* (at least in the Comal system) be reevaluated.

The build-up of different types and levels of algae over the course of both the laboratory and pond experiments raises questions and concerns as to the potential affect algae will have on aquatic plants in the wild during low-flow conditions. This is especially pertinent in that the areas that tend to exhibit springflow decline most quickly in the wild (i.e., Upper Spring run reach in the Comal River) experience high levels of algal build-up on aquatic plants each summer, with conditions intensified during low flow. The phenomenon of algal growth overtaking *Riccia* in the laboratory was also witnessed (summer/fall 2013) in the Upper Spring run reach of the Comal River.

Finally, another observation of the pond study goes beyond the effect of plant survival and/or growth in an outdoor limited or no-flow environment. The water-quality parameters experienced in both the pond pre-trial and formal experiment have the potential to directly impact fountain darters and other fishes. Fountain darter egg production is shown to be reduced at temperatures greater than 26 ˚C, with larval production impacted at temperatures of 25 ˚C or higher (BIO-WEST 2002, Bonner et al. 1998, McDonald et al. 2007). Therefore, temperatures observed in the pond would likely result in the cessation of all fountain darter reproduction, as well as egg and larval development. Additionally, temperatures of 33–35 ˚C approach or exceed the listed CTM for many fish species (Beitinger et al. 2000). For fountain darters, this value is approximately 34.8 ˚C (Brandt et al. 1993); therefore, they would be unlikely to live for an extended period of time in an environment (no flow, elevated summer air temperatures) like the ones tested in these studies. High temperatures and no-flow conditions also resulted in low DO readings (2 mg/L–3 mg/L) in the mornings near the end of the experiment. Dissolved oxygen below 4 mg/L is stressful to most fishes, and DO of approximately 2 mg/L can be lethal (Ostrand and Wilde 2001, Rutledge and Beitinger 1989). Therefore, DO concentrations similar to those observed may have detrimental effects to fountain darter survival when flows are severely reduced and water temperatures greater than 30 ˚C are observed in the wild.

As with all potential concerns, it is important to put the extremity of these studies in context with what might be anticipated to occur under the proposed HCP flow regime. For example, the flow that caused the conditions experienced in the pond experiment was either 0.023 cfs or none at all. The experiment was conducted under hot, summertime conditions in a black-lined pond with water temperatures maxing out above 35 °C with very low plant density. Even such, water temperatures did not continue to rise but seemed to stabilize fairly quickly to ambient air temperatures and ever so slowly declined as slightly cooler air temperatures were experienced as the study progressed. This stabilization is encouraging and highlights the effect of ambient air temperature and diurnal conditions. However, in pools with high aquatic plant density, water quality conditions could possibly be different. Dense plant stands are known to stratify the water column, limit water mixing, and decrease diel fluctuations, all of which can produce water quality conditions detrimental to aquatic organisms (Frodge et al. 1990). To put tested conditions in context, the HCP modeled water temperature in the upper portion of the Old Channel (above Elizabeth Street) during a month of 30 cfs total system discharge in the Comal system is below 28 °C (Hardy et al. 2010). On a similar note, it is unknown if CO_2 concentrations will even get to below 5 mg/L in the wild during the HCP projected flow conditions. The intent of the extremity of these applied research studies is to parameterize the HCP ecological model to the degree practical to allow for future evaluation of the HCP proposed flow regimes for both the Comal and San Marcos rivers.

6.2.2 Habitat Conservation Plan (HCP) Ecological Model Application

The vegetation tolerance studies provide several results for consideration in HCP model parameterization. These apply to the aquatic vegetation model and possibly to water quality model validation at low flows as follows:

Aquatic Vegetation Module

- Upper temperature threshold ranges (<35 °C) for plant survival for three species tested (*Cabomba*, *Vallisneria*, *Riccia*)
- Upper temperature threshold ranges (≈37 to 40 °C) for *Ludwigia* survival
- Lower CO_2 threshold ranges for plant survival and continued growth for all species tested (<5 mg/L)
- Relative growth rates for species tested relative to temperature and CO_2 changes
    - Water temperature - 22 °C, 28 °C, 34 °C, 37 °C
    - CO_2 - <5 mg/L, 9-12 mg/L, 30-40 mg/L
- Preliminary data on algal growth in temperature and CO_2 treatments

Water Quality Model

- Water temperature and DO data collected in pond studies may be applicable for water quality model refinement or validation under extremely low-flow or no-flow conditions in the summer.

The specific use of any of these parameters will be determined by the HCP ecological model team with guidance from the HCP Science Committee. This project team will collaborate with the ecological modeling team to provide all raw data and analysis that they determine applicable to their efforts. As previously stated, the use of these results for populating the ecological model should be done with care and an acknowledgement of the uncertainty surrounding the results.

6.2.3 Future Applied Research Studies

The apparent suitability of *Ludwigia* to substitute for *Hygrophila* with regard to providing fountain darter habitat is encouraging. However, additional information regarding these species is warranted. Thus, the following two studies concerning *Ludwigia* and *Hygrophila* are proposed. The first involves using MUPPTs to investigate *Ludwigia* growth under varying environmental conditions in the Comal River (Landa Lake, Old Channel, and New Channel) and in the San Marcos River (upstream and lower sections). While the 2013 greenhouse and pond studies strongly support the use of *Ludwigia*, the results of the laboratory vs. field experiment cautions against too much extrapolation.

The second proposed effort is *in situ* plant competition studies utilizing *Ludwigia* and nonnative species. Previous work conducted by Baylor University researchers has shown that, under relatively stagnant flow conditions, *Hygrophila* strongly outcompetes *Ludwigia.* However, it would be very useful to have data under more reasonable field conditions, and MUPPTs now provide the tool needed to make those measurements. In addition, studying the competitive ability of both of these species vs. *Hydrilla verticillata* is warranted for understanding the San Marcos River plant dynamics.

Finally, based on the laboratory and field experiments, in conjunction with the annual build-up of algae in portions of the San Marcos and Comal rivers, applied research studies directed at understanding the effect of water quality on algal growth, as well as the effect of algal growth on the survival of aquatic vegetation are both recommended.

6.3 pH Drift Study

6.3.1 Lessons Learned

The results from the pH drift study are the most easily interpreted of any of the 2013 applied research efforts conducted. Two native species with relatively high utilization as fountain darter habitat (*Riccia, Cabomba*) are shown to not utilize HCO3- under field conditions. Furthermore, neither species shows evidence of developing the capacity to utilize HCO3- under CO2-stress conditions. Therefore, these species seem to have elevated risk of being lost from these river systems if flow conditions change and result in lower flows, longer water residence time, lower flushing rates, higher pH, and significantly lower CO2 availability. Even if conditions don't drop to the point where the plants are physiologically unable to survive, they may be competitively disadvantaged during such conditions and displaced by other species.

Sagittaria also was not able to utilize HCO3- during the time period tested. However, unlike *Cabomba* and *Riccia*, *Sagittaria* is capable of forming emergent leaves and avoiding CO2 limitation by obtaining the gas from the atmosphere instead of water. Therefore, at least in shallow waters (<0.5m), *Sagittaria* may not be lost from this system, but is projected to survive as an emergent aquatic macrophyte. In fact, under stagnant greenhouse growth conditions, this species typically grows in its emergent form.

Both *Hygrophila* and *Ludwigia* develop the ability to utilize HCO3- when growing under CO2 stressed conditions. Additionally, since both species are heterophylous and capable of forming emergent leaves, they can also alleviate CO2 stress by sending out emergent stems and leaves. While neither species currently commonly grows as an emergent plant in the Comal or San Marcos rivers, more stressful conditions related to CO2 limitation could well stimulate such a growth strategy. As discussed in Section 6.2, this is an important finding with respect to *Ludwigia*. First, it supports the continued use of this species for ongoing restoration efforts in both systems. Secondly, it allows for a re-evaluation of the necessity of having the nonnative *Hygrophila* included in the long-term biological goals for the fountain darter.

Finally, the *Vallisneria* species growing in the Comal River is a strong HCO3- user. Samples collected from throughout the Comal system all showed strong ability to utilize HCO3-. These observations make sense in light of the widespread distribution of this species in lakes and reservoirs where CO_2 deficient conditions are likely. As such, it is likely that this species will survive in the San Marcos and Comal rivers under extreme low-flow conditions. As with *Sagittaria*, *Vallisneria* does not provide key fountain darter habitat unless bryophytes are present within (which won't likely be the case under extremely low flows), but it does provide stability to the system. Additionally, a key concern was that the expanses of *Vallisneria* in Landa Lake might crash under elevated water temperatures, which would cause massive vegetation decay and subsequent high levels of oxygen demand in the water column. Coupled with the vegetation tolerance studies, this study has alleviated this concern at least up to ≈35 °C.

6.3.2 Habitat Conservation Plan (HCP) Ecological Model Application

The pH drift data directly establishes the HCO_3^- utilization potential of six species of aquatic plants within the Comal/San Marcos river systems for consideration in HCP aquatic vegetation module parameterization as follows:

Aquatic Vegetation Module

- HCO_3^- use inputs for six species prevalent in the Comal and San Marcos systems (*Cabomba*, *Vallisneria*, *Riccia, Hygrophila, Ludwigia, Sagittaria*)

The specific use of a HCO_3^- utilization parameter in the aquatic vegetation module of the HCP ecological model will be determined by the HCP ecological model team with guidance from the HCP Science Committee.

6.3.3 Future Applied Research Studies

With information on six key aquatic plant species in the Comal and San Marcos rivers added to the known HCO_3^- use patterns of Texas wild rice and *Hydrilla verticillata*, no additional pH drift studies are recommended at this time.

6.4 Food Source Studies (Laboratory and Pond)

6.4.1 Lessons Learned

A CTM (37.89 °C) was established for *Hyalella azteca* taken directly from the Comal River. It is noteworthy that the mean *Hyalella azteca* CTM (37.89 °C) exceeds the fountain darter CTM (34.8 °C) reported by Brandt et al (1993), suggesting that if environmental conditions caused water temperatures to increase, the CTM of the fountain darter would occur before that of their food source. However, CTM is only a single factor experiment, and thus this data only provides a glimpse of how *Hyalella azteca* or the fountain darter may respond to increasing temperatures under extreme drought.

In both the laboratory and pond experiments, cooler temperatures and the presence of *Riccia* both significantly improved the survival of *Hyalella azteca*. The fact that all amphipods in the laboratory study died at 34 °C and nearly all in the no-flow pond treatment died, irrespective of *Riccia*, suggests the tolerance of this species when confronted with multiple stressors is considerably less than the CTM. It is encouraging that *Riccia* made a significant difference considering the aforementioned results regarding tolerance of aquatic vegetation. The interpretation of the laboratory and pond food source experiments, in conjunction with vegetation availability, suggests *Hyalella azteca* would likely cease to be a food source before CTM temperatures occur in the wild.

6.4.2 Habitat Conservation Plan (HCP) Ecological Model Application

The food-source study emphasizes the importance of this parameter in the development of the HCP ecological model.

Data available for parameterization of the fountain darter module includes:

Fountain Darter Module

- Food source input (using *Hyalella azteca*)
    - Water temperature thresholds
    - *Riccia* benefit

The specific use of a food source parameter in the fountain darter module of the HCP ecological model will be determined by the HCP ecological model team with guidance from the HCP Science Committee.

6.4.3 Future Applied Research Studies

Based on the laboratory and pond food source study results, two additional applied research topics are proposed. The first is to evaluate the temperature range between 28 °C and 34 °C to more accurately determine a threshold temperature for amphipods. This is important in that, at 28 °C, fountain darters can exist just fine and reproduce to a limited degree, but near 34 °C their reproduction shuts off and survival becomes tenuous. If food really should become limiting at 29 °C rather than 33.5 °C, there is the potential for this parameter to be extremely important. However, if the threshold is truly more near 34 °C, then direct temperature impacts to fountain darters would likely trump any food source response in the ecological model.

The second proposed investigation is to evaluate whether *Vallisneria* or *Ludwigia* provide similar benefits to what was experienced with *Riccia*. The reason for this investigation is that the bryophytes are likely the first plant species to be eliminated from the system during low flows while *Vallisneria* and *Ludwigia* should persist.

Additional studies could investigate longer test periods and other macroinvertebrates known to be darter prey items. However, it is recommended that the food source component of the ecological model first be developed and the sensitivity of this parameter tested using the results from this study and proposed efforts prior to conducting work with either of these additional topics.

6.5 Conclusion

The data and results presented in this report and summarized in Section 6 provide a wealth of information to assist the HCP process particularly with (1) HCP ecological model parameterization, (2) future HCP applied research, and (3) re-evaluation of HCP long-term biological goals. The complexity of the interactions are immense but will continue to be sorted out over time with the assistance of the HCP ecological model(s), more-refined applied research, and continued bio-monitoring. Applied research conducted in 2013 helped break down and clarify the picture in many instances. However, as with all research, other questions arise along the path, which then must be assessed for importance by future investigations.

In conclusion, the 2013 HCP applied research has provided valuable information on several of the HCP unknowns and emphasizes the importance of the HCP applied research efforts and ecological modeling moving forward.

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