Aquatic Vegetation Laboratory Study:

Phase 1: Observations of water quality changes and plant growth under various flows.

Phase 2: Effects of carbon dioxide level on aquatic plants found in the Comal and San Marcos Springs/River Ecosystems.

Final Report
Variable Flow Study: Project 802, Task 27
San Marcos National Fish Hatchery & Technology Center, San Marcos, Texas

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EXECUTIVE SUMMARY

A two-phase study was conducted during the summer and fall of 2003 to evaluate impacts of different flow levels on individual water quality parameters and the corresponding effects on aquatic vegetation found in the Comal and San Marcos Springs/River ecosystems. Aquatic vegetation in these two spring-fed systems plays a vital role as habitat for certain endangered species and suitability as habitat varies by plant type (Linam et al. 1993, USFWS 1996, BIO-WEST 2002a, 2002b, 2003a, 2003b). Abundant plant species and those that harbor high densities of threatened/endangered species were chosen for the study. The study was conducted at the San Marcos National Fish Hatchery and Technology Center (NFHTC) in San Marcos, Texas. The NFHTC supports a refugium for most of the endangered species from the Comal and San Marcos Springs, and has an aquatic nursery/greenhouse for experimentation. A series of "living streams" in the nursery that receive a continuous flow of Edwards Aquifer groundwater were used in the study. BIO-WEST manipulated conditions in the living streams to allow for different flows (Phase 1) and different water chemistry (Phase 2) among tanks.

Phase 1 of the study was observational and designed to evaluate changes in water chemistry that occur under various flow levels and also examine growth and overall health of *Vallisneria* sp. and *Ludwigia repens* under the same range of conditions. The experiment was not designed to allow for statistical analysis, but observations of plant response to the range of flow conditions indicate that the quantity of flow affects plant growth and health. The plants exposed to the lowest flow conditions were faded-green in color with leaves that exhibited weak structural support. Although all plants increased in size during the experiment, these plants grew less than those in tanks with greater flow. Among the observed water chemistry changes, variation in carbon dioxide (CO_2) was greatest and most likely to have an impact on the plants.

Phase 2 examined how different levels of CO_2 affect aquatic vegetation growth for six plant species found in the Comal and San Marcos Springs/River ecosystems including four angiosperms and two bryophytes. Three treatments provided CO_2 concentrations ranging from the high concentration found in spring openings to very low concentrations that may be found, at times, further downstream in either spring ecosystem. All six plant species increased in biomass in all treatments over the six-week study period, thus even when CO_2 was low, growth still occurred. Three of the four angiosperms had higher above ground, below ground and/or total biomass in higher CO_2 treatments at the end of six weeks (the fourth had significant differences when a far outlier was removed from the dataset). Both bryophyte species had a similar total biomass in all three treatments at the end of the study. The lack of differences among treatments for the bryophytes may be an artifact of the enclosure used to house the plants since plant health appeared greater in the higher CO_2 treatments. The results support the hypothesis that lower CO_2 concentrations, under similar water temperatures, results in less growth and/or less healthy appearance than higher CO_2 concentrations in all plants evaluated in the study. As the amount of CO_2 decreases in the natural habitat, the growth of many plants that provide important habitat for threatened/endangered species may be reduced. Therefore, dissolved CO_2 concentration appears to be an important parameter in shaping the aquatic vegetation community of the Comal and San Marcos Springs/River ecosystems. This CO_2-aquatic vegetation relationship has not been previously studied in the Comal and San Marcos Springs/River ecosystems and needs to be more closely monitored in future efforts.

Water quality monitoring that includes measuring CO_2 concentration is currently in place for the Critical Period monitoring component of the Authority's Variable Flow study. This information will be crucial in assessing the affects of water quality/flow on aquatic vegetation during low-flow conditions in the Comal and San Marcos Springs/River ecosystems. In the interim, low-flow experiments in a natural setting could provide a wealth of data concerning this and many other topics vitally important to the survival of endangered species in the Comal and San Marcos Springs/River ecosystems.

PHASE 1

1.0 INTRODUCTION

The purpose of Phase 1 was to explore how changing flow conditions in an artificial environment would alter water quality parameters and ultimately impact two types of aquatic vegetation found in both the Comal and San Marcos Springs/River ecosystems. This observational study was conducted at the NFHTC over a short time period (28-days) using Edwards Aquifer groundwater.

2.0 METHODS

Plant Collection

Plant species used in this study were obtained from the NFHTC. Thirty potted and established *Vallisneria* sp. and *Ludwigia repens* plants that appeared healthy and had similar stem lengths were selected from nursery stock. Potted plants were individually measured from the sediment surface to the tip of the longest leaf (*Vallisneria* sp.) or the tip of the apical leaves (*Ludwigia repens*). The majority of *Vallisneria* sp. ranged between 28cm to 35cm in leaf length, so only plants within that range with green, crisp, unbroken leaves were used. Most *Ludwigia repens* plants were 17cm to 22.5cm in stem length, so only plants within that range in good health were used. Plants with observed reproductive structures were not selected. The length of individual plants was recorded at the start and end of Phase 1.

Study Design

Six 950-L fiberglass tanks (Living Stream Model MT-1024, Frigid Units Incorporated, Toledo, Ohio) received Edwards Aquifer water directly from a water hose located at the north-end of the tank. Flow was restricted to a quantity of 5.0 gallons per minute (gpm), 2.0 gpm or 0.5 gpm. A portion of the water in each tank was re-circulated using a 0.5 hp pump (Hayward Power Flo II Pump Model SP 125J, Hayward Pool Products, Inc., Elizabeth, New Jersey). Water was pumped through a system of PVC pipes and entered back into the tank through adjustable nozzles lowered into the water that were angled to spray water just below the water surface. Since the lower flow tanks resulted in a higher residence time for the water in the tank, the same water was re-circulated more than in the high flow tanks where new water moved through the tanks more quickly. Thirty one-liter pots, 15 containing *Vallisneria* sp. and 15 containing *Ludwigia repens* were divided equally among the three treatment tanks (5.0, 2.0, and 0.5 gpm). Three more tanks had the same treatment conditions (5.0, 2.0, and 0.5 gpm) but contained no plants.

Data Collection

Water quality parameters (dissolved oxygen, temperature, pH, conductivity) were monitored every other day over the 28-day experiment with a Quanta Hydrolab data sonde (Hydrolab Corporation, Austin, Texas). Carbon dioxide (CO_2) levels were monitored with a LaMotte CO_2 titration kit.

Continuous temperature readings were recorded in ten-minute intervals in all six tanks with Onset Stow Away Tidbit Temperature Loggers.

Water quality parameters were collected at each end of the tank and in the middle, measurements were taken at mid-depth. Readings were taken in three places in order to record variations throughout the tank due to the distribution of water by the nozzles. Mid-depth readings prevented errors associated with air-water mixing at the surface. Temperature loggers were placed in the bottom of each tank for the duration of the study.

Water quality parameters were collected in tanks both with and without plants to determine if water chemistry changes due to flow variations were influenced by the presence/absence of aquatic vegetation. At the end of the study, *Vallisneria* sp. plant length was measured from the sediment surface to the tip of the longest leaf, and *Ludwigia repens* plant growth was measured from the sediment surface to the tip of the apical leaves.

3.0 RESULTS

Water Quality

A summary table of water quality data from the Phase 1 study is provided below.

Table 1. Phase 1 - Summary of water quality parameters

TREATMENT	TEMPERATURE (^{o}C)			pH			DO (mg/L)			CONDUCTIVITY (umhos/cm)			CO_2 (mg/L)		
	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min
5.0 gpm	24.27	24.85	23.77	6.92	7.00	6.82	6.37	7.16	5.82	646	663	616	16	17	15
5.0 gpm, Plants	24.16	24.66	23.69	6.94	7.04	6.84	6.72	7.42	6.02	645	663	613	16	18	15
2.0 gpm	24.58	25.31	24.00	7.00	7.08	6.91	7.04	7.91	6.51	645	662	615	16	18	12
2.0 gpm, Plants	24.36	25.31	23.69	6.95	7.08	6.82	6.77	7.91	5.82	640	663	613	14	17	11
0.5 gpm	26.73	28.32	25.00	7.54	7.64	7.42	8.33	9.20	7.20	635	654	600	3.5	5	2
0.5 gpm, Plants	26.27	27.92	24.68	7.53	7.68	7.40	8.84	9.80	8.14	616	637	584	3.25	5	2

Tanks receiving the least amount of flow (0.5 gpm) had the warmest temperatures, highest pH, lowest CO_2, highest dissolved oxygen (DO), and least amount of plant growth. Although temperatures in the 0.5 gpm tank initially rose from 23^{o}C to around 28^{o}C, they exhibited a linear decline over the 28-day experiment. This steady decline upon stabilization was evident for all treatments. Increases during warm periods and decreases during rain events were noted, but the overall decline in temperature would imply that external weather plays a major role in temperature regulation once flow is stabilized. In the natural environment, water volume and surface area would obviously play a key role on how quickly temperatures would stabilize and the level of fluctuation. Within flow treatments, water temperature was slightly higher in tanks without plants where leaves were not present to provide some shading.

Typically pH and CO_2 readings are negatively correlated, as pH increases CO_2 decreases and vice versa, therefore differences that were observed in CO_2 level among treatments also correspond to differences in pH level. Among flow treatments, the higher flow rates allowed shorter residence times and higher CO_2 concentrations were maintained in these tanks. Within flow treatments, the original thought was that the plants would reduce the CO_2 concentration and a difference would be observed between the tanks with and those without plants. This should have been most pronounced in the low-flow tank where long residence times would presumably allow the plants to take up measurable quantities of CO_2. However, the pH and CO_2 concentrations were very similar for the tanks with and without plants even in the low-flow treatment. This probably occurred because of the re-circulation process. The agitation that occurred with this process reduced the high CO_2 concentration directly from the aquifer. Since the high residence time of low-flow treatments resulted in more re-circulation of the same water, the CO_2 concentration was significantly reduced in the low-flow tanks. It appears that the mechanical reduction of the CO_2 concentration masked any reduction in CO_2 that may have occurred from plants. It is likely that the CO_2 concentration for each flow rate would have been higher without re-circulation and possible that CO_2 removal by plant biomass would have resulted in measurable differences between tanks. Nonetheless, the plants were exposed to a wide range of pH and CO_2 conditions during the experiment.

The higher DO concentrations in the 0.5 gpm treatments were also likely related to the re-circulation process where the longer residence time increased the amount of aeration. The warmer temperatures and longer residence times also resulted in abundant algal growth in the 0.5 gpm treatment (both with and without plants) and respiration from the algae may have contributed to increased DO concentrations (all water quality measurements were taken during the daylight hours, most were taken in the afternoon). Within treatments, tanks with plants generally maintained higher DO concentrations than the open tanks, which was also likely due to plant respiration.

Conductivity was slightly variable among all six tanks, but lower in the tank with plants receiving 0.5 gpm flow. It has been shown that $CaCO_3$ will precipitate out of the water when CO_2 concentrations in water are low (Wetzel, 1983). This precipitate was clearly evident on the plant leaves in the 0.5 gpm treatment.

Plant Growth

Plants receiving 5.0 gpm flow grew the most and appeared most healthy (greener, crisper leaves) (Table 2). Plants receiving 0.5 gpm flow grew the least, and at the end of Phase 1 the plants in this treatment were a dull green color, their leaf structure was weak and soft, and their leaves were coated with calcium carbonate residue.

Table 2: Phase 1 - Summary of plant growth

	Vallisnaria sp. (leaf length)			*Ludwigia repens* (length to apical tip)		
Treatment	0.5 gpm	2.0 gpm	5.0 gpm	0.5 gpm	2.0 gpm	5.0 gpm
Mean Growth Difference (cm)	23.95	28.55	43.75	16.4	21.75	25.75
Max Growth Difference (cm)	44	49.5	52	20	30	34
Min Growth Difference (cm)	10	22	36	12.5	16	17.5

4.0 DISCUSSION

Phase 1 results were consistent with what might be expected during the August/September time period and varying flow conditions (Table 3).

Table 3: Phase 1 – Summary of Observational Results per flow treatment

28-day Study Period (August/September 2003)

0.5 gpm	2.0 gpm	5.0 gpm
Water temperature rose to approx. 28°C , then gradually declined to <26 °C	Water temperature rose to just above 25°C, then gradually declined to <24 °C	Water temperature rose to just below 25 °C , then gradually declined to <24 °C
Highest pH, Lowest CO_2	Lower pH, Higher CO_2	Lowest pH, Highest CO_2
Highest dissolved oxygen	Lower dissolved oxygen	Lowest dissolved oxygen (yet still above 5.8 mg/L)
Lower conductivity for treatment with plants than without.	Stable conductivity (plants & no plants)	Stable conductivity (plants & no plants)
Abundant algal growth	Limited algal growth	Limited algal growth
Plants grew, but poor health	Plants grew well, crisp leaves	Plants grew best, crisp green leaves

Clearly the differences in water quality parameters among tanks were a result of the different flow treatments and these differences caused the differences in plant growth and health. However, because flow quantity affects a number of water quality parameters it is not possible to determine which one, or which combination, had the effect of higher plant growth at higher flow level. Two parameters that are considered important to plant growth are water temperature and CO_2 concentration and the Phase 1 study showed that under lower flows, water temperature was higher and CO_2 concentration lower than in the higher flow treatments. In addition, low-flow conditions are presumed to increase the boundary layer along the surface of aquatic plants and reduce the ability of the plant to uptake CO_2 and/or nutrients. Water flow often increases nutrient and dissolved inorganic carbon uptake by plants (Smith and Walker 1980, Stevens and Hurd 1997) by reducing this unstirred boundary layer surrounding plant foliage (Chambers et al. 1991). The boundary layer creates resistance to nutrient and dissolved inorganic carbon uptake (Smith and Walker 1980). When water flow is reduced, this boundary layer thickens, and the rate of diffusion is reduced, potentially reducing plant growth and development (Crossley et al. 2002).

Among the three variables mentioned above, variation in the thickness of the boundary layer at a plant's surface, water temperature, and CO_2 concentration, the latter was the most likely to produce the observed differences in plant growth in this study. Aquatic vegetation growth and development is usually enhanced with increasing temperature to a plateau that often occurs near the lethal level of the plant. Furness and Grime (1982) found that many bryophytes grew optimally at temperatures between 15-25 °C and that growth decreased at some level between 25-30 °C. The results of that study show that many of the plants maintained relatively high growth rates up to 30 °C, but died rapidly in water maintained at 35 °C. Olesen and Madsen (2000) grew two macrophytes, *Cllitriche cophocarpa* and *Elodea canadensis* in temperatures ranging from 7-25 °C and found the greatest growth at the highest temperatures (when CO_2 was maintained at high levels). Water temperatures in the Phase 1 study were generally close to 25 °C in all treatments. Although the 0.5 gpm treatments initially rose to 28 °C, the temperature declined gradually to less than 26 °C over the 6-week period. Thus it did not appear that temperature was the primary factor in observed differences in growth during the Phase 1 Study. It is possible that temperature may become an issue in the wild when residence times are significantly increased with lower flows, however, and this may be a valuable parameter to evaluate further.

The poor growth and development of plants under 0.5 gpm flow conditions could have been due to a thickened boundary layer. However, the agitation from re-circulated water was similar among treatments and produced a noticeable movement of water that should have reduced any boundary layer on the plants' surface. It would be possible to test this potential effect by altering the water velocity among several treatments and it may be beneficial to do so. However, relating laboratory results back to the natural environment would be extremely difficult. Only with intensive field measurements and complex modeling could a prediction of a possible discharge-to-velocity relationship be developed for each plant type. In addition, the fit of such a model would undoubtedly be low since the velocity of water at the plant surface depends on a great number of localized variables and each plant type is found in a wide range of conditions. For example, point velocities measured at 15 cm during the Variable Flow study conducted throughout the Comal and San Marcos Springs/River ecosystems ranged from 0.0 to very fast under a range of discharges. Due to the complexity of evaluating this parameter and potentially limited ability to extrapolate results from laboratory conditions into the wild, it was not explored further in Phase 2.

Finally, the CO_2 concentrations in the 0.5 gpm treatments (both with and without plants) were 2-5 mg/L, well below pumped NFHTC Edwards Aquifer water concentrations, which typically ranged between 20-25 mg/L (BIO-WEST, field observation). Although the mechanical reduction of the CO_2 concentration was an unexpected result of the design, the artificially reduced values may have been the factor that impacted plant growth the most. Aquarium literature suggests that optimal CO_2

concentrations for aquatic plant growth are 15-25 mg/L. Inorganic carbon in the aquatic environment is available in different forms, but is used by most plants as CO_2. If CO_2 is in short supply, plants must utilize bicarbonate. Some aquatic plants, such as *Elodea canadensis* (Olesen and Madsen 2000) and Chara (Raven 1970) are able to utilize bicarbonate, while others such as *Riccia fluitans* (Ballesteros et al 1998) and most moss species (Bain and Proctor 1980) cannot. A literature search could not determine whether *Vallisneria* sp. or *Ludwigia repens* are CO_2 obligates. However, the results of Phase 1 preliminarily indicate that the potential exists for both species. Texas wild-rice (*Zizania texana*) has been shown to be a CO_2 obligate (Powers, USFWS, personal communication). Therefore CO_2 concentration was chosen as the variable to isolate in Phase 2 to evaluate the growth of multiple plant species (found in the Comal and San Marcos Springs/River ecosystem) exposed to a range of CO_2 levels, with constant flow and temperature conditions.

PHASE 2

5.0 INTRODUCTION

On-going research and monitoring continues to confirm the importance of aquatic vegetation to several of the threatened and endangered species in the Comal and San Marcos Springs/River ecosystems. Aquatic vegetation not only provides essential cover/refuge but also harbors an abundant food supply for these species. The type and quality of the aquatic vegetation can greatly affect the density of fountain darters (*Etheostoma fonticola*) and San Marcos salamanders (*Eurycea nana*) in a specific area and in aggregate throughout the entire system. In addition, one of the federally endangered species is an aquatic plant, Texas wild-rice (*Zizania texana*), which is directly affected by changes in water quality and quantity conditions. Changes in springflow can alter water quality conditions as evidenced in Phase 1 of this study. Such alterations have the potential to affect both the quantity and quality of aquatic vegetation types in the Comal and San Marcos Springs/River ecosystems.

Fluctuations in carbon dioxide (CO_2) availability (Vadstrup and Madsen 1995, Hannan and Dorris 1970), water levels and flow (Rejmankova 1992) and changes in water temperature (Sanford 1979) have been found to affect aquatic vegetation growth. During the Phase 1 observational study it appeared that CO_2 concentrations had the most noticeable affect on the aquatic plants used. Therefore the purpose of the Phase 2 study was to explore how different levels of CO_2 affect aquatic vegetation growth for plants common in both the Comal and San Marcos Springs/River ecosystems.

6.0 METHODS

Plant Collection and Acclimation

Four angiosperm plant species and two bryophyte species found in either the Comal or San Marcos Springs/River ecosystems were used in this study. *Ludwigia repens*, *Vallisneria* sp., *Zizania texana*, *Hydrocotyle umbellata*, and *Amblystegium* sp. were obtained from the San Marcos National Fish Hatchery and Technology Center (NFHTC) greenhouse and/or raceways. *Zizania texana* was planted from seed obtained from the NFHTC seed bank. *Riccia* sp. was collected from Landa Lake, Comal River, treated and held as per NFHTC protocol to ensure all snails and other organisms were removed prior to placement in experimental tanks. All species were placed in the containers to be used during experimentation and moved into tanks prior to initiation of the experiment for acclimation and observation. *Ludwigia repens*, *Vallisneria* sp., and *Hydrocotyle umbellata* plants were placed in one-liter pots and moved to the acclimation tanks for 10 days. Only plants of similar length with green, crisp, unbroken leaves were used. In addition, plants with observed reproductive structures were not selected. *Ludwigia repens* sprigs were chosen with four root nodes and were approximately equivalent in length; *Vallisneria* sp. plants were chosen with one root ball and approximately the same number of leaves and biomass; and *Hydrocotyle umbellata* plants were chosen with eight root nodes and eight leaves of approximately the same length. Five *Zizania texana* seeds were planted per 1-liter pot and allowed to germinate and grow in the acclimation tanks at the NFHTC for two weeks prior to initiation of the study. Immediately prior to the acclimation period, *Vallisneria* sp. plants were trimmed to three inches in leaf length measured from the sediment surface to the tip of each leaf. After collection and treatment, the bryophytes, *Riccia* sp. and *Amblystegium* sp., were weighed and placed in a piece of PVC pipe, approximately 10 cm long, that was cut in half lengthwise. To contain the sample, the bryophytes and pipe were then covered with thin nylon netting (pantyhose). Each bryophyte sample container was then placed in the acclimation tanks for 10 days to observe the suitability of the containment design.

Study Design

Eleven 950-L fiberglass tanks (Living Stream Model MT-1024, Frigid Units Incorporated, Toledo, Ohio) were used during this experiment. The design included high, moderate, and low CO_2 treatments that were each replicated three times for a total of nine treatment tanks. The two additional tanks served as preparation tanks (prep tanks). As the Edwards Aquifer well water supplied to the NFHTC is high in CO_2, well water was moved through hoses directly into the three high CO_2 treatment tanks. The moderate and low CO_2 tanks required the use of prep tanks that received Edwards Aquifer water directly from a water hose located at the north-end of each tank. Water was re-circulated in the prep tanks through a system of PVC pipes connected to six adjustable nozzles that were angled to spray water just below or above the water surface to adjust the CO_2 concentration in the tank. Allowing water to spray into the air before entering the tank provided greater surface agitation and allowed a considerably greater reduction in CO_2. In addition, an aerator was placed in the low CO_2 prep tank to further agitate the water surface and maintain low CO_2 concentrations. Once water was treated in this way for the moderate and low CO_2 prep tanks it was moved via a system of PVC pipes out of the respective prep tank and into the appropriate treatment tanks (three for each treatment).

As CO_2 was the parameter of interest, efforts were made to equalize all other parameters that could affect aquatic vegetation growth. Flow was restricted to a quantity of approximately 1.5 to 2.0 gpm in each of the treatment tanks. A portion of the water moving through each tank was through a 0.5 hp pump (Hayward Power Flo II Pump Model SP 125J, Hayward Pool Products, Inc., Elizabeth, New Jersey). Additionally, each of the nine treatment tanks and two prep tanks were connected to individual heater/chiller units, each set at 24ºC.

Each of the nine treatment tanks were randomly assigned a low, moderate, or high treatment. The tanks were cleaned and allowed to operate at their respective treatment conditions for 10 days to allow fine tuning of the CO_2 concentration in prep tanks where necessary. Each plant/bryophyte was randomly assigned to one of the three treatments, then to one of the three tanks within a treatment, and ultimately to a specific location within each tank. Each of the nine tanks contained three one-liter pots of each of *Ludwigia repens*, *Vallisneria* sp., and *Zizania texana*, two one-liter pots of *Hydrocotyle umbellata* and three PVC pipe sections of each of the two bryophyte species during the experiment. Therefore, each tank contained 17 aquatic plants, 11 angiosperms and 6 bryophytes.

At the beginning of the experiment five plants/bryophytes from each species were dried and weighed to get a "before-treatment" biomass measurement. Since the biomass of experimental plants could not be measured before the experiment, similar sized plants were used to estimate the mean before-treatment biomass. These plants were chosen along with experimental plants based upon the same characteristics and were placed in the same conditions in the acclimation tank prior to drying and weighing. When collected for weighing, the roots were carefully rinsed to remove soil without losing any plant biomass. The bryophytes were gently washed to remove any snails or other foreign material. Plants and bryophytes were dried in a drying oven at 70 ºF. All plants and bryophytes were weighed (grams) within two hours of removal from the drying oven. In addition, the bryophytes were heated in a muffle furnace to a temperature of 500 ºC for four hours. All bryophyte ash was weighed within 30 minutes of removal from the muffle furnace. Growth of each species was measured at the end of the experiment as changes in biomass (dry weight in grams for all plants and bryophytes and ash-free dry weight in grams for bryophytes only).

Water Quality Data Collection

Water quality variables (dissolved oxygen, temperature, pH, conductivity) were measured two or three times per week for six weeks with a Hydrolab data sonde (Quanta, Hydrolab Corporation, Austin, Texas). CO_2 levels were measured with a LaMotte carbon dioxide titration kit two or three times per week for six weeks.

Water quality measurements were collected at each end of the tanks (the front third and the back third) at mid-depth. Readings were taken in two places in order to record variations throughout the tank and at mid-depth in order to avoid reading errors associated with air-water mixing at the surface. Upon completion of water quality measurements tanks and plants were cleaned as needed (plants and nylon netting were gently rubbed to remove periphyton growth). Additionally, tank sides were scrubbed of algae and excess material siphoned as needed to maintain similar conditions in each treatment tank.

Statistical analyses:

Data used in analyses included the total biomass of each plant or bryophyte at the end of the experiment as well as the above ground and below ground biomass of each angiosperm plant. A one-factor analysis of variance was used to assess whether differences in plant biomass occurred among treatments and Tukey's test was used to determine which treatments differed from one another. Each data set was examined for normality and data that were not normally distributed were transformed using the logarithm base 10 function or the exponential function. Box Plots were used to display distributions for each data set among the three treatments. All data sets with outliers were analyzed initially with outliers included and then with the outliers removed.

7.0 RESULTS

Water Quality

The water quality parameters measured over the six-week study period are presented in Table 4. CO_2 concentration ranged from 3 to 10 mg/l in the low treatment, 6 to 18 mg/l in the moderate treatment, and 13 to 27 in the high treatment. Average CO_2 concentrations were 6, 12, and 21.5 mg/l, respectively. While ranges did overlap between treatments, the overlapping values did not exceed the 25th percentile in the moderate or high categories and each treatment was significantly different from the other two (Tables 4 and 5, Figure 1). Generally, variation in CO_2 concentrations occurred concurrently between treatments. For instance, at least two cold fronts with stiff northern winds occurred during the study period, during which CO_2 concentrations declined in each of the tanks. This was presumably due to the surface agitation caused by the strong winds. The relative differences in CO_2 concentrations among treatment tanks were generally maintained and any overlap due to acute disturbances was short-lived.

TABLE 4. Water Quality Measurements per Treatment.

	CO_2 Treatment	Temp. °C	Cond. (μmhos/cm)	DO (mg/L)	pH	CO_2 (mg/L)
Minimum	Low	21.56	302	7.51	7.35	3
25th percentile	Low	22.60	597	8.51	7.44	5
50th percentile	Low	23.13	605	9.18	7.49	6
75th percentile	Low	23.66	625	9.53	7.53	7
Maximum	Low	26.90	634	10.57	7.65	10
	CO_2 Treatment	Temp. °C	Cond. (μmhos/cm)	DO (mg/L)	pH	CO_2 (mg/L)
Minimum	Moderate	21.35	603	6.74	7.00	6
25th percentile	Moderate	22.46	612	7.94	7.16	10
50th percentile	Moderate	22.98	618	8.82	7.21	12
75th percentile	Moderate	23.57	642	9.23	7.31	14
Maximum	Moderate	25.22	653	9.86	7.46	18
	CO_2 Treatment	Temp. °C	Cond. (μmhos/cm)	DO (mg/L)	pH	CO_2 (mg/L)
Minimum	High	21.83	607	5.84	6.76	13
25th percentile	High	22.61	614	7.01	6.91	20
50th percentile	High	23.18	620	7.68	6.96	21.5
75th percentile	High	23.56	644	8.31	7.04	23.25
Maximum	High	24.94	653	9.15	7.16	27

Table 5. Kruskall Wallis Test for comparison of Water Quality among CO_2 treatments.

Water Quality Variable	**Sample size**	**Df**	**KW Test Statistic**	**P-Value**	**Significance**
Temperature	279	2	1.027	0.598	NS
Conductivity	279	2	48.208	0.000	Significant
Dissolved Oxygen	279	2	102.280	0.000	Significant
pH	279	2	237.244	0.000	Significant
CO_2	252	2	217.583	0.000	Significant

Although water temperature was a controlled factor and attempts were made to equalize this parameter among treatment tanks, the heater/chiller units allowed some variability over the study period. Using the prep tanks for source water probably also contributed to this variability. As with CO_2, external weather conditions generally affected temperature in all treatment tanks equally. No statistical temperature difference was found among treatments (Table 5, Figure 2).

As demonstrated in the Phase 1 study, CO_2 concentrations are closely related to pH and conductivity. Average pH was 7.49, 7.21, and 6.96 for the low, moderate, and high CO_2 treatments, respectively (Table 4). The distribution pattern in the boxplots of pH and CO_2 reflect the negative correlation between the two parameters, which was expected considering the strong relationship between pH and CO_2 (See Figures 1 and 3).

Figure 1

Distributions of CO_2

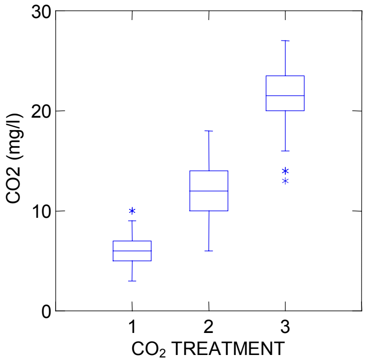


Figure 2

Water Temperature

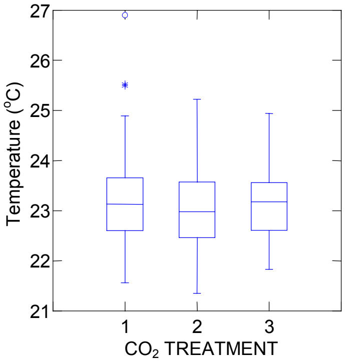


Figure 3

Distributions of pH

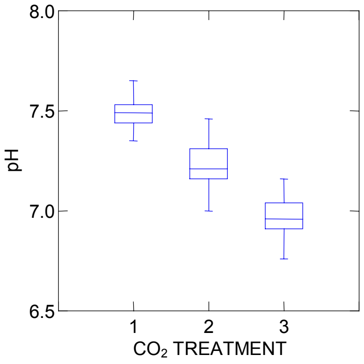


Figure 4

Conductivity

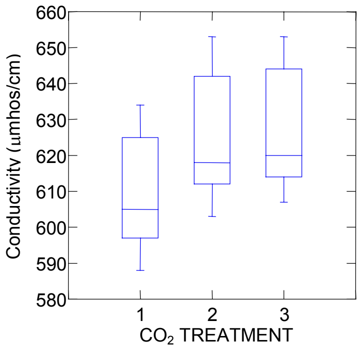
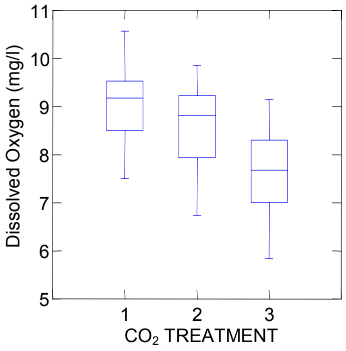


Figure 5

Dissolved Oxygen



CO_2 TREATMENT: 1 = LOW, 2 = MODERATE, 3 = HIGH

Conductivity values were significantly lower in the lowest CO_2 treatment (Table 4, Figure 4). These lower values occurred because calcium carbonate ($CaCO_3$) will precipitate out of the water when CO_2 concentrations are low (Wetzel, 1983). Partial pressure of CO_2 in spring-fed waters can be many times higher than that of atmospheric CO_2, but CO_2 is easily released from these highly saturated waters to the atmosphere which may not permit most of the CO_2 to be taken up during photosynthesis (Wetzel, 1983). Despite the statistically significant difference in conductivity among treatments, the range of conductivity that occurs in freshwaters suggests that the difference among treatments was actually minor and biologically insignificant.

Dissolved oxygen (DO) concentrations were lower in the high CO_2 treatments than in the low CO_2 treatments (Figure 5). This is likely a function of increasing aeration in the prep tanks for moderate and low CO_2 concentration treatments. As with pH and conductivity, no attempt was made to adjust DO concentrations directly, but the range of DO for all treatments were acceptable for aquatic life.

Comal and San Marcos Springs/River CO_2 Ranges

As part of the Authority's Variable Flow Study, BIO-WEST collected water quality data for a two-year period (August 2000 – August 2002) at numerous locations along the Comal and San Marcos Springs/River ecosystems. Although CO_2 was not measured directly, both pH and alkalinity measurements were taken at all stations and these data can be used to estimate CO_2 concentrations. Table 6 shows a subset of these data including CO_2 ranges for several sample locations in each ecosystem to provide a glimpse at actual conditions in the natural environment. The estimated CO_2 concentrations in the Comal and San Marcos Spring/River ecosystems span the range of concentrations used for the low, moderate, and high CO_2 treatment for the Phase 2 experiment.

Table 6: Calculated CO_2 concentrations at select locations for Comal and San Marcos Springs/Rivers.

San Marcos Springs/River		Comal Springs/River	
Station	CO_2 range (mg/L)	Station	CO_2 range (mg/L)
Slough	1-12	Blieders Creek	3-17
Spring Lake – Hotel Area	7-25	Landa Lake – Spring Island	5-20
Spring Lake – Landing	8-24	Spring Run One	6-19
Spring Lake – Above Dam	4-23	Spring Run Two	5-21
SMR – Below Dam	5-15	Spring Run Three	5-21
SMR – Lions Club	3-15	Old Channel - upstream	2-10
SMR – Rio Vista Dam	3-11	Old Channel – downstream	2-6
SMR – IH35	2-9	New Channel – upstream	2-16
SMR – Thompson's Island	2-8	New Channel – downstream	2-9
SMR – Animal Shelter	2-6	Downstream of Old/New confl.	2-8

Biomass

Table 7 presents the total biomass of each species prior to the study initiation and the total biomass upon completion of the study by CO_2 treatment (low, moderate and high). As evident in Table 7, all plant species exhibited considerable growth under all CO_2 treatments during the six-week study period. Only one individual plant (*Vallisneria* sp.) that apparently died during initial planting had a lower biomass at the end of the experiment than the mean of the five plants used to calculate biomass at the beginning of the experiment. The ability of each of these plants to continue growing even under the low CO_2 conditions is an important finding and will be covered in greater detail in the discussion section.

Table 7. Mean total biomass (grams) by species at the start and finish of the experiment.

Plant	**Method**	**TOTAL BIOMASS**			
		BEFORE	**AFTER**		
			Low	**Moderate**	**High**
Ludwigia repens	Dry weight	0.48	4.12	4.09	6.75
Vallisneria sp.	Dry weight	0.57	1.49	1.38	2.38
Zizania texana	Dry weight	0.30	1.02	0.95	1.38
Hydrocotyle umbellata	Dry weight	0.52	1.00	1.16	1.39
Riccia sp.	Dry weight	0.89	4.08	2.20	1.88
Riccia sp.	Ash Free Dry	---	0.58	0.54	0.62
Amblystegium sp.	Dry weight	1.66	5.01	2.99	2.30
Amblystegium sp.	Ash Free Dry	---	1.14	1.20	1.29

Angiosperms

Table 7 also shows that for all angiosperms, the high CO_2 treatment produced greater total mean biomass than either the low or moderate CO_2 treatments. Table 8 shows that there was a significant difference between treatments for at least one biomass component (total, above ground, or below ground) for each angiosperm species (*Hydrocotle umbellata* did require deletion of a far outlier before significant differences were found). *Ludwigia repens* total and above ground biomass results were significantly different among treatments when outliers remained in the data set as well as when outliers were removed from the data. *Vallisneria* sp. total and below ground biomass results were significantly different among treatments. Below ground biomass of *Zizania texana* also differed significantly among treatments. *Hydrocotyle umbellata* above ground biomass results changed from not significant to significantly different among treatments when the far outlier was removed from the data (Table 8).

Biomass ranges for the angiosperms can be found in Figures 6-17. Several outliers occurred in the data. An outlier is 1.5 to 3.0 deviations from the median and is marked by an asterisk in the box plots. A far outlier is greater than 3.0 deviations from the median and is depicted as an open circle in the box plots.

Table 8. ANOVA TABLE results for comparison of Plant Biomass among CO_2 treatments.

Angiosperm Plant	**Biomass** (grams dry wt.)	**Sample size**	**Df**	**Mean Square**	**F-ratio**	**P-value**	**Significance**
Ludwigia repens	**Total**	27	2	20.876	7.021	0.004	**Significant**
Ludwigia repens	**Above ground**	27	2	12.719	8.827	0.001	**Significant**
Ludwigia repens	Below ground	27	2	0.167	2.100	0.144	NS
Vallisneria sp.	**Total**	27	2	2.743	4.233	0.027	**Significant**
Vallisneria sp.	Above ground	27	2	1.006	3.143	0.061	NS
Vallisneria sp.	**Below ground**	27	2	0.428	5.891	0.008	**Significant**
Zizania texana	Total	27	2	0.470	2.860	0.077	NS
Zizania texana	Above ground	27	2	0.263	2.503	0.103	NS
Zizania texana	**Below ground**	27	2	0.051	4.373	0.024	**Significant**
H. umbellata	Total	18	2	0.234	3.464	0.058	NS
H. umbellata	Above ground	18	2	0.048	2.552	0.111	NS
H. umbellata	Below ground	18	2	0.072	2.248	0.140	NS
H. umbellata*	**_Total*_**	_17_	_2_	_0.256_	_4.459_	_0.032_	**_Significant_**
H. umbellata*	**_Above ground*_**	_17_	_2_	_0.059_	_4.165_	_0.038_	**_Significant_**

* A far outlier in the moderate treatment category was removed, which changed the results.

The test for differences in total biomass of *Zizania texana* among treatments had a p-value that was 0.077. Although not considered statistically significant for this study, it is a notable result (Table 7, Figure 12). The *Zizania texana* total and above ground biomass varied substantially in the low CO_2 treatment which influences the results (Figures 12 and 13). This large variation in the low treatment tanks may be a result of *Zizania texana* leaves growing to the water surface before the end of the experiment. Since the below ground biomass was significantly greater in the high CO_2 treatment than in the low CO_2 treatment, it appears that lower CO_2 conditions may cause *Zizania texana* plants to allocate more energy into above ground growth and less to below ground biomass to expedite reaching the water surface. Once plants reach the water surface, the leaves can draw CO_2 from the air, which in turn, would allow for increased plant growth. The *Zizania texana,* plants used this study were in the seedling/early growth stage where plants typically shift resources to below ground biomass, presumably to exploit minerals needed for continued growth and to anchor the plant before leaves encounter flowing water. Root biomass of plants in the high CO_2 treatment was significantly greater than low and moderate CO_2 treatments. This suggests that higher CO_2 tanks allowed higher photosynthetic rates to occur in submersed leaves and plants were able to shift the products of that photosynthesis to below ground biomass.

Ludwigia repens below ground biomass was transformed using the logarithm base 10 function to produce a normal distribution. No other angiosperm data sets required transformation.

Figure 6

LUDWIGIA TOTAL BIOMASS

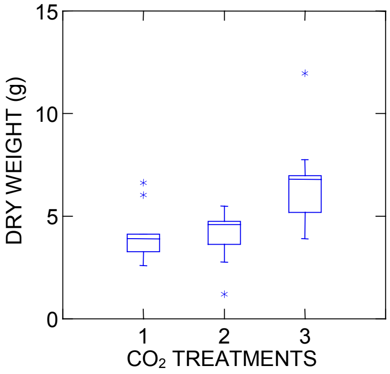


Figure 7

Ludwigia Above Ground Biomas

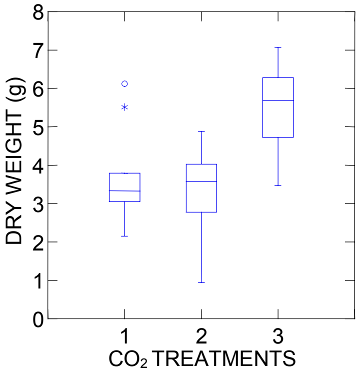


Figure 8

Ludwigia Below Ground Biomas

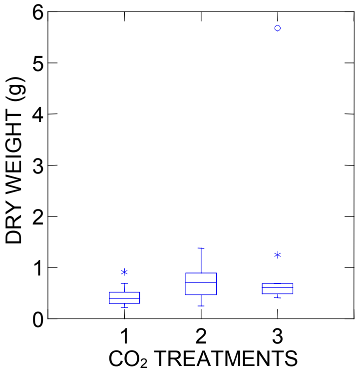


Figure 9

VALLISNERIA TOTAL BIOMASS

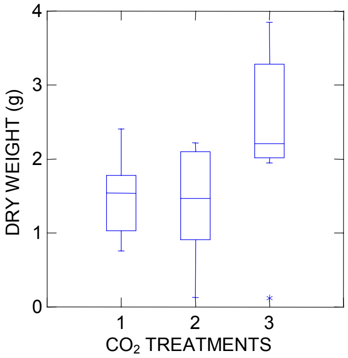


Figure 10

Vallisneria Above Ground Biomass

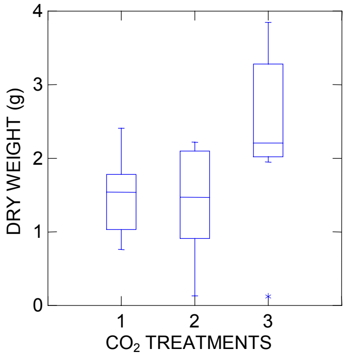


Figure 11

Vallisneria Below Ground Biomass

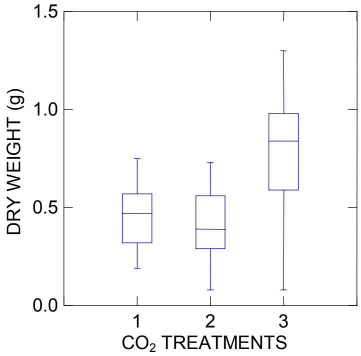


Figure 12

ZIZANIA TOTAL BIOMASS

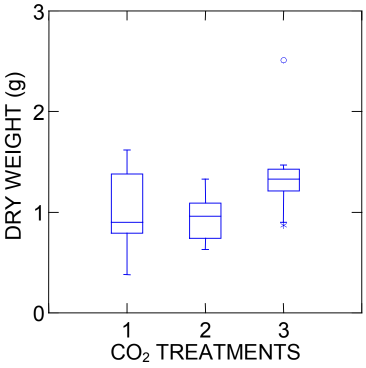


Figure 13

Zizania Above Ground Biomass

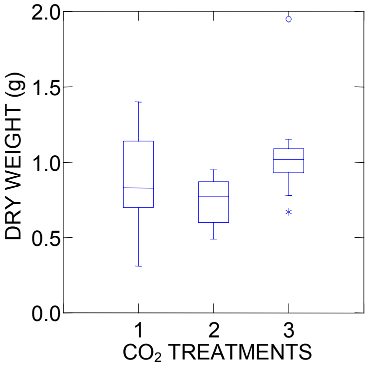
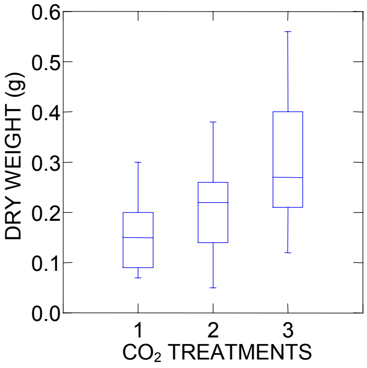


Figure 14

Zizania Below Ground Biomass



CO$_2$ TREATMENT: 1 = LOW, 2 = MODERATE, 3 = HIGH

Figure 15

HYDROCOTYLE TOTAL BIOMASS

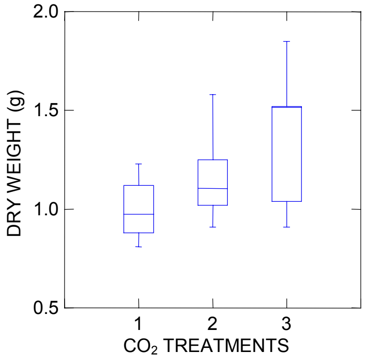


Figure 16

Hydrocotyle Above Ground Biomass

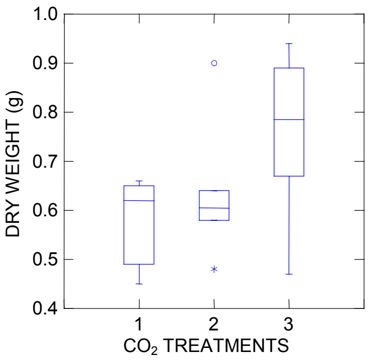
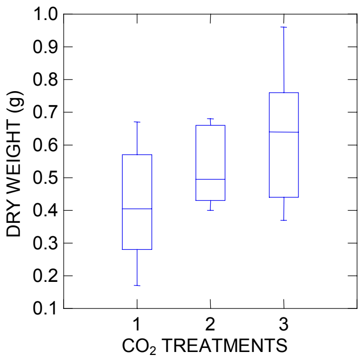


Figure 17

Hydrocotyle Below Ground Biomass



CO_2 TREATMENT: 1 = LOW, 2 = MODERATE, 3 = HIGH

Bryophytes

Amblystegium sp. and *Riccia* sp. dry-weight biomass were significantly different among treatments; bryophytes in the low CO_2 treatments had higher dry weights than bryophytes in the moderate and high CO_2 treatments (Table 7). These results were counter-intuitive since bryophytes in general (Bain and Proctor, 1980), and *Riccia* sp. in particular (Ballesteros, et al., 1998), are known to be CO_2 obligates. This means that they require CO_2 for photosynthesis and cannot use bicarbonate in low CO_2 conditions as some other plants can. However, the ash-free dry-weight biomass results showed that *Amblystegium* sp. and *Riccia* sp. organic biomass totals did not differ among the three CO_2 treatments (Tables 7 and 9). Since $CaCO_3$ will precipitate out of the water when CO_2 concentrations are low (Wetzel, 1983) the differences in dry weight were clearly attributable to the accumulation of this inorganic material on the plants in the low CO_2 concentration treatment..

Table 9. ANOVA TABLE results for comparison of bryophyte biomass among CO_2 treatments.

Bryophyte	Total Biomass (grams)	Sample size	Df	Mean Square	F-ratio	P-value	Significance
Amblystegium sp.	**Dry Weight**	27	2	0.275	37.434	0.000	**Significant**
Riccia sp.	**Dry Weight**	27	2	0.287	38.101	0.000	**Significant**
Amblystegium sp.	Ash-Free Dry Wt.	26	2	0.569	1.215	0.314	NS
Riccia sp.	Ash-Free Dry Wt.	27	2	0.014	2.928	0.073	NS

Amblystegium sp. and *Riccia* sp. were contained within nylon netting during the experiment. While the netting did keep the entire sample contained it also presented some problems. Algae grew on the netting, which shaded the bryophytes. In addition, $CaCO_3$ accumulated on the netting and within the sample, which affected the dry weight total biomass reading. The leaves of the angiosperms were lightly cleaned once or twice a week; however, the bryophytes could not be cleaned directly.

Instead, the netting was lightly rubbed to remove the algae and $CaCO_3$ build-up. Cleaning the netting probably allowed the $CaCO_3$ on the netting to settle within the netting.

To determine the organic biomass of the bryophytes, samples were burned in a muffle furnace to provide an ash-free dry weight for each bryophyte. *Amblystegium* sp. ash-free dry weight data was transformed using the exponential function (e^a) to produce a normal distribution. *Amblystegium* sp. and *Riccia* sp. dry weight data were transformed using the logarithm base 10 function to produce a normal distribution. The discrepancies between the two methods are clearly evident in Figures 18-21. When outliers were removed from the *Amblystegium* sp. ash-free dry-weight data, the results remained not significant. When outliers were removed from the *Riccia* sp. ash-free dry-weight data, the results became statistically significant (MS=0.009, F-ratio=5.667, p=0.012). However, the statistically significant difference was between the high and moderate treatments not the high and low treatments. This result may have occurred due to factors other than the different CO_2 treatments (such as the fouling of the netting that shaded the *Riccia* sp.).

Figure 18

Amblystegium Total Biomass

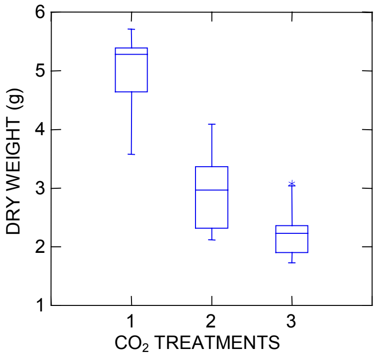


Figure 19

Amblystegium Ash-Free Total Biomass

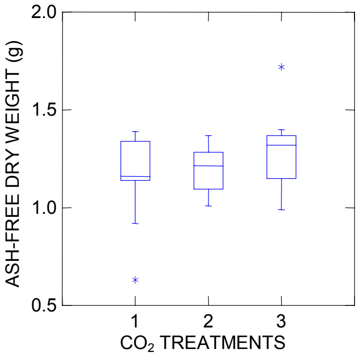


Figure 20

Riccia Total Biomass

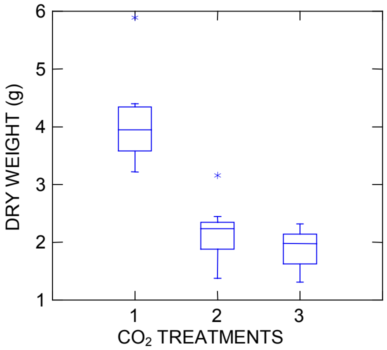
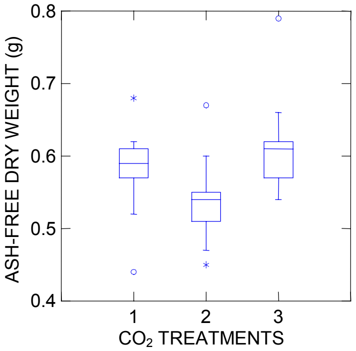


Figure 21

Riccia Ash-Free Total Biomass



CO_2 TREATMENT: 1 = LOW, 2 = MODERATE, 3 = HIGH

Even when using the ash-free dry weight comparison among treatments, clearly the more representative method, the bryophyte data remains intriguing. There was clear visual evidence that the bryophytes in the high CO_2 treatment were much healthier (bright green coloration and crisp leaf structure) than bryophytes in the low and moderate CO_2 treatments (much duller coloration and leaf structure crispness. This observation suggests that high CO_2 concentrations would promote greater increases in the biomass of bryophytes in an unrestricted growing environment.

8.0 DISCUSSION

In summary, all plant species in all treatments increased in biomass over the six-week study period. Each of the four angiosperms had a significantly lower biomass (either total, above ground, or below ground) in the low CO_2 treatments compared to the other treatments (one angiosperm required deletion of a far outlier for significant differences). The bryophytes responded to the different CO_2 treatments by having similar biomass totals among the CO_2 treatments, however as previously mentioned this may be an artifact of the enclosure. The health of bryophytes was clearly greater in the high CO_2 concentration treatments compared to the low CO_2 concentration treatments. From this study it seems clear that the ranges of CO_2 in the high concentration treatment (when water temperature is held constant) provides for greater plant growth and health. A reduction in growth or a change in growth strategy occurs when CO_2 concentrations are within the ranges of the low concentration treatment (under constant water temperatures). It is clear from the Phase 1 study that water quality parameters change in response to changes in flows. The same holds true in the natural environment as increases or decreases in water quantity will affect the water quality by collectively changing water temperature, CO_2, pH, conductivity, and DO concentrations. Therefore, further experimentation in the laboratory and in the natural environment would be beneficial to add to these preliminary findings.

In the natural setting, CO_2 concentrations measured in the Comal and San Marcos Springs/River ecosystems between summer 2000 and fall 2002 tended to be similar, at most stations, to the concentrations represented by the low and moderate CO_2 treatments in this experiment (BIO-WEST 2003a, 2003b). The higher CO_2 concentrations in both systems for all sampling events during that time period were found closer to the spring outlets (i.e. spring runs and Landa Lake in the Comal Springs/River ecosystem and Spring Lake in San Marcos Springs/River ecosystem). The diversity and biomass of aquatic vegetation is also high in these areas. Downstream from the spring openings, concentrations of CO_2 decrease along with diversity and biomass of aquatic vegetation.

In the spring of 2002 and following the large recharge event in the summer of 2002, CO_2 concentrations near the spring openings at both Comal and San Marcos were more similar to the high CO_2 treatment used in this experiment. During these time periods there was a rapid growth of aquatic vegetation in both systems including a substantial increase in the amount of bryophytes in

Landa Lake. Although other water quality parameters (temperature, nutrients, etc.), physical properties (substrate, channel depth, light, etc.), and physical processes (scouring of old vegetation and sediment accumulation) are important to the dynamics of the aquatic vegetation community, CO_2 appears to be an important factor that may have strongly influenced these observed changes. The relationship between CO_2 and aquatic vegetation has previously been poorly understood or defined for the Comal and San Marcos Springs/River ecosystems.

In addition, the CO_2 concentrations were in the upper range of the low CO_2 treatment or lower range of the moderate CO_2 treatment in key habitat areas during the summer and fall of 2000 when flows at Comal Springs were below 150 cfs and flows at San Marcos Springs were below 110 cfs. Nonetheless, the interesting observation is that at these CO_2 levels all aquatic vegetation in the study continued to grow over the experimental period, although growth was lowest in the low CO_2 treatment. From the results of this initial experiment, one might jump to conclusions regarding how aquatic vegetation will respond during low flows in the Comal and San Marcos Springs/River ecosystems, but with the small amount of laboratory data and limited field data collected during low-flow periods, it is truly speculation at this point. Additional monitoring that is currently in place for the Critical Period monitoring component of the Authority's Variable Flow study will provide crucial data for assessing the affects of water quality on aquatic vegetation during low-flow conditions in the Comal and San Marcos Springs/River ecosystems. In the interim, low-flow experiments conducted in a natural setting could provide a wealth of data concerning this and many other topics vitally important to the survival of endangered species in the Comal and San Marcos Springs/River ecosystem.

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