Appendix 9.C. Salinity-Induced Enzymatic Stress Response In Vallisneria americana

Prepared by

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Introduction

Submerged aquatic vegetation (SAV) in the lower St. Johns River is regularly exposed to high salinity events due to salt water intrusion. Long or acute exposure removes SAV from large expanses of the Lower Basin and results in a net loss of SAV habitat. To protect and restore SAV we need to understand how salinity affects SAV on the biochemical level and predict a threshold at which the plant's integrity begins to be compromised.

Recent advancements in cellular diagnostic assays have shown promising results linking environmental stressors to the health of a variety of aquatic organisms including gastropods, cnidarians and crustaceans (Downs et al. 2000, 2001). By evaluating an organism's responses on the cellular level (i.e. anti-oxidant potential, protein metabolic condition) the damaging effects of both biotic and abiotic stressors may be evaluated much like medical diagnostics used in human pathology.

In a pilot study associated with this work (Spring 2008) it was determined that when *Vallisneria americana* encountered abiotic stressors, such as elevated salinity, it had the capability of producing or upregulating cellular markers of stress that could be measured and quantified. This was the basis of establishing a molecular biomarker system (MBS). This tool could serve as an accurate means of quantifying sublethal stress that could provide an early detection of cellular damage prior to the onset of organismal death or population decline. This technique represents an extremely powerful tool that can detect cellular damage well before any phenotypic response can be observed.

The goal of this current project was to expand on our initial pilot study and determine the precise threshold at which elevated salinity would negatively affect the physiology of *V. americana*. This would be the basis of a rapid assessment for measuring real time plant health.

Cellular parameters that were measured included total soluble protein content, reactive oxygen species (ROS) production, lipid peroxide levels, protein carbonylation levels, dark adapted respiration, catalase and superoxide dismutase activities. These selected parameters were chosen since they reflect specific cellular physiological functions or signs of damage (Table 1). Measuring these parameters indicate: (1) whether metabolic processes are compromised, (2) whether the structural integrity of the cell is challenged, (3) whether there is a response to oxidative stress as a function of increasing salinity.

Methods

Two different studies were conducted during the span of this project. The first study used a mesocosm (microcosm)-based approach to specifically account for salinity levels that any plant would be exposed to. In the second study SAV samples were collected on a weekly basis throughout part of the summer growing season. These samples of *V. americana* were obtained from an oligohaline (Buckman) and a freshwater site (Orangedale) in conjunction with the current intensive St. Johns River Water Management District SAV monitoring project. Samples were transported to UNF for laboratory analysis.

Table 1. Cellular biomarkers and their physiological impact

Cellular Biomarker	Impact
Reactive Oxygen Species (ROS)	When organisms (or cells) are exposed to any number of abiotic or biotic stressors (including salt stress) mitochondrial and photosynthetic electron transport chains are disrupted and produce a number of oxygenated free radical species termed ROS. Such examples include hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), and hydroxyl radical ($OH^{\cdot}$). ROS production induces oxidative stress.
Protein carbonylation	The production of ROS can oxidize and severely damage lipids, proteins, and DNA. The most general indicator and by far the most commonly used marker of protein oxidation is protein carbonyl content. Redox cycling cations such as Fe^{2+} or Cu^{2+} can bind to cation binding locations on proteins. Subsequent attack by H_2O_2 or $O_2^{\cdot-}$ can transform side-chain amine groups on several amino acids (i.e. lysine, arginine, proline, or histidine) into carbonyls. Carbonyl formation can serve as a reflection of oxidative damage.
Lipid Peroxidation	Unsaturated lipids of cell membranes or other membranous systems are common targets of oxidative attack by ROS. The end result is lipid peroxidation, a destructive process that compromises normal cellular function. Quantification of lipid peroxidation is one valuable biomarker to assess the role of this damage.
Catalase and Superoxide Dismutase activity	Catalase is a widely distributed enzyme that destroys H_2O_2 by dismutation to $O_2^{\cdot-}$ and H_2O. Superoxide dismutases are metalloenzymes that catalyze the dismutation of superoxide anion into the less damaging H_2O_2. Upregulation of these enzymes is indicative of an organism's attempt to counteract the presence of damaging ROS.

For mesocosm-based work, all specimens of *V. americana* were collected from the St. Johns River near Palatka, FL on several dates between March and July 2009(GPS: 29 32.259'N 81 41.590'W, Salinity: ~0.04 ‰, Temp: 23-27 °C) and transported to the University of North Florida. Plants were maintained in glass aquaria at 24 °C on a 12:12 h light:dark cycle (photosynthetic photon flux = 45 μmol * m^{-2} s^{-1}) until use.

Post-acclimation, individual plants were placed in microcosm containers and exposed to a single salinity treatment of either 0‰, 5‰, 10‰, 15‰, or 20‰ (n=5) for either 24 hours (acute) or 7 days (short term) at 24 °C on a 12:12 h light:dark cycle (photosynthetic photon flux = 115 μmol * m^{-2} s^{-1}). After treatment, a small amount of tissue (< 200 mg) was excised from adult blades and assayed for oxygen consumption using an Oxygraph system fitted with a DW3 liquid-

phase electrode chamber (Hansatech Instruments; Norfolk, UK). The remaining blades were flash frozen in liquid nitrogen and stored at -20 °C until further analysis. Five grams of ground tissue was homogenized with 25 mL of ice cold Tris-HCL buffer, pH 8.0, containing 1% PVPP (w/v) and 20 mM protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The homogenate was centrifuged for 15 min at 4,000g. The supernatant was collected and assayed for protein content using the Pierce ® BCA Protein Assay Kit (Rockford, IL, USA). Extracts were subsequently assayed for biochemical markers of oxidative stress using commercially available kits as per the manufacturers' instructions. Superoxide dismutase, protein carbonyl, and lipid hydroperoxide were assayed using kits from Cayman Chemical Company (Ann Arbor, MI, USA). Catalase was assayed using the Amplex Red kit (Eugene, OR, USA). All results were normalized for protein content.

To evaluate the production of ROS in *V. americana* tissue, the ROS specific probe 2',7'-dichlorofluorescein-diacetate (DCFH-DA) was employed. Briefly, DCFH-DA is a nonfluorescing nonpolar compound. When this compound reacts with cellular esterases, the diacetate group is cleaved off to yield the polar compound DCFH. Oxidation of DCFH by ROS yields the fluorescent product DCF. Fluorescence was monitored via epifluorescent microscopy. In essence, the higher the concentration of ROS the brighter the fluorescent signal. Stock vials of DCFH-DA were dissolved in DMSO in 10-mM aliquot stocks (stored at –80º C). Post-treatment, 2 cm leaf clippings were incubated for 15 min in 10 mL 0.22 µm filtered nanopure water with 5 µL of DCFH-DA. Prior to examination, samples were rinsed in fresh filtered distilled water to remove any unbound probe. Fluorescent imaging were taken using a Zeiss Universal microscope in conjunction with the Pixera 120es Application suite photo program.

In a second microcosm-based experiment (to narrow in on the specific zone of salt stress) plants were incubated at 0‰, 11‰, 13‰, or 15‰ (n=5) for either 24 hours (acute) or 7 days (short term) at 24 °C on a 12:12 h light:dark cycle (photosynthetic photon flux = 115 µmol * m^{-2} s^{-1}). Post-incubation samples were processed as mentioned above.

For the field-based component of this work *V. americana* samples were collected weekly over the course of a growing season from April to July, 2009. The two collection sites were along the St. Johns River; one near the Buckman Bridge (30°10'52.67" N, 81°38'34.07" W; Oligohaline site), and one in Orangedale (30°00'21.37" N, 81°36'50.37" W; Freshwater site). The plants were immediately frozen and subsequently transported to UNF where they were stored at -20 °C until analysis. Samples were processed as mentioned above.

Results and Discussion

The basis of this work was to examine the stress induced upon *V. americana* by elevated salinity. We were particularly focusing on oxidative stress. Any perturbation in mitochondrial or chloroplast function will cause electrons to accidentally flow away from their respective electron transport chains. Since these organelles exist in an aerobic environment, the surrounding O_2 has the capability of becoming reduced into a ROS. That is the problem! These ROS can cause cellular damage [oxidative stress] (carbonylate amino acids rendering them useless; oxidize lipids breaking down plasma membranes). Fortunately, cells have the ability to respond to oxidative stress and upregulate their antioxidant machinery (see Table 1 for CAT/SOD description).

In essence, we can measure 3 items associated with oxidative stress: (1) The initial production of ROS at specific time points and salinity treatments; (2) The production of damage induced by ROS (lipid peroxidation and protein carbonylation) and; (3) The cell's ability to recover (CAT/SOD activity)

Microcosm study

ROS Production

Figure 1 represents the detection of ROS as a function of time and salinity exposure. Recall, DCFH-DA is a fluorogenic probe that turns green in the presence of ROS. Using the same filter setup, the color red is due to the natural autofluorescence of chlorophyll. Briefly, even after a 24 hour exposure, 11 ppt has the capability of inducing ROS formation. After a 7 day exposure all treatments induced ROS production (even 0 ppt, which was probably due to the fact that the plant was out of its natural environment and was undergoing some stress). While the cells are still stressing at the 7 day period it is anticipated that they have the capability of upregulating their antioxidant response by this time (see CAT data below).

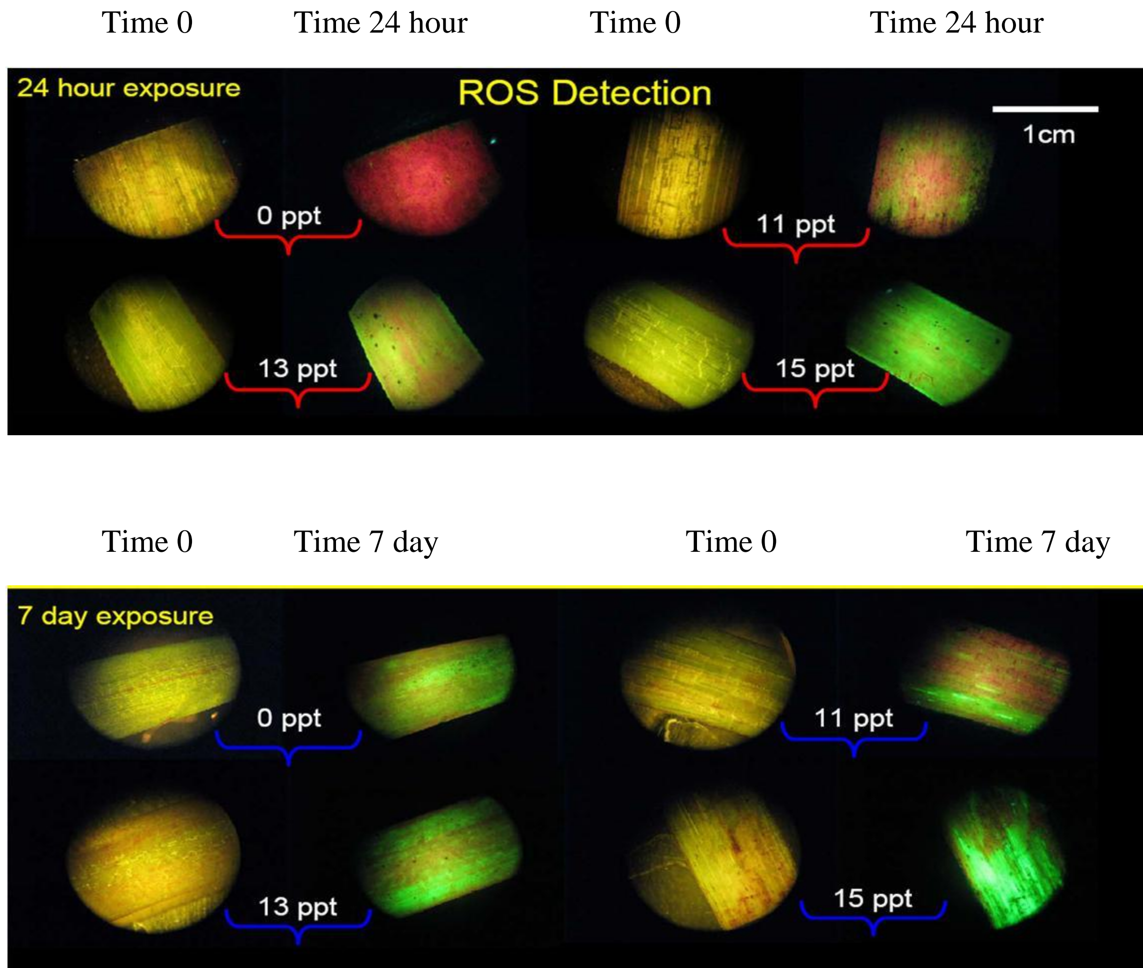

Figure 1. ROS detection in *V. americana* as a function of salinity exposure.

Respiration Data

Plants were incubated at selected salinities for 24 hours and 7 days. In experiment #1 we took a broad approach and exposures ranged from 0-20 ppt. In experiment #2 we narrowed in on 11-15ppt. As demonstrated in figures 2 and 4, within 24 hours plants exposed to 15ppt show a strong increase in oxygen consumption. This increase in respiration persisted through day 7 (figure 2). By 7 days we can see in increase in oxygen consumption in plants exposed to 13ppt (figure 5).

Catalase activity (anti-oxidant enzyme)

At the 24 hour time point there was no significant increase in CAT compared to control plants maintained at 0ppt. The same trend appears for plants incubated for 7 days. [Compare 13 ppt data point for 24 hour vs. 7 day incubation.-increase may be noted]

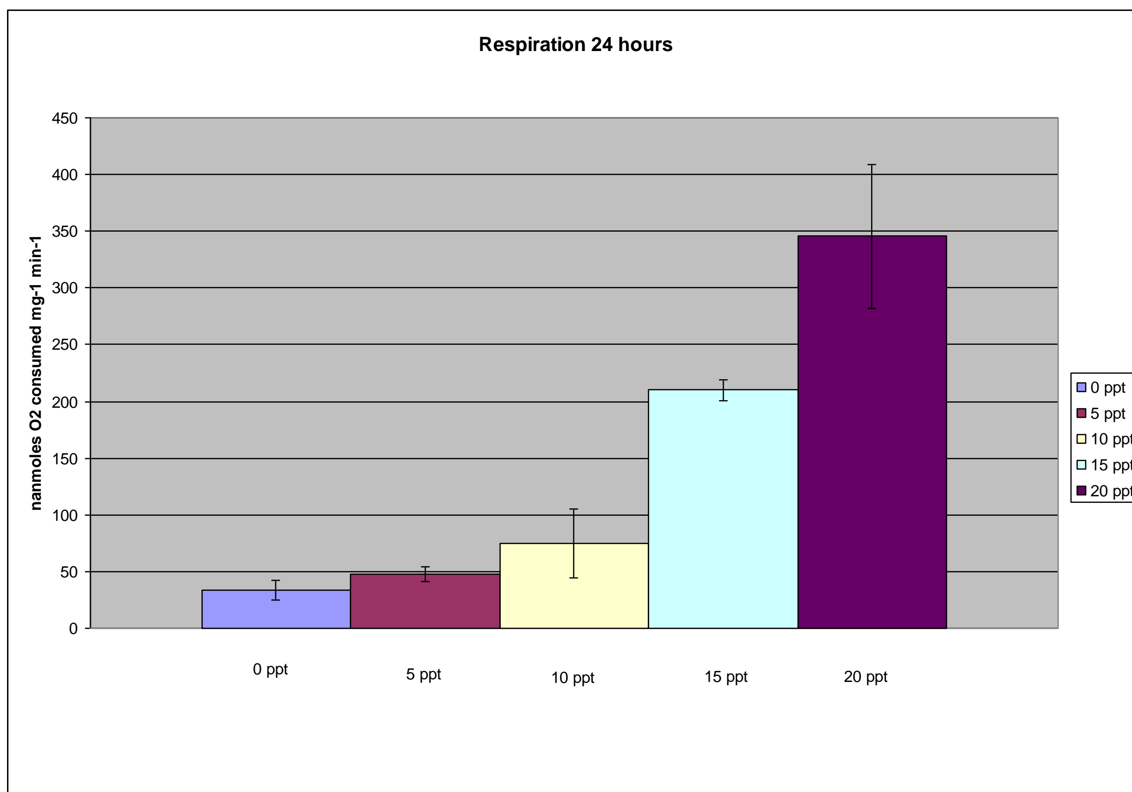

Figure 2. Twenty four hour incubation at salinities ranging from 0-20 ppt

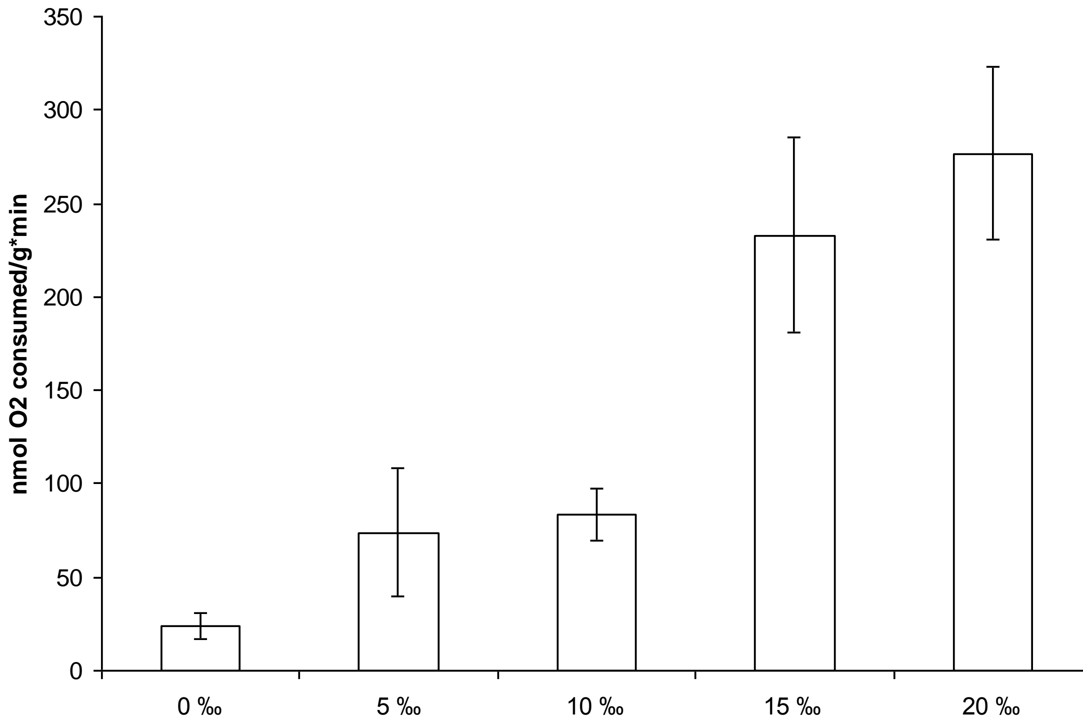

Figure 3. Seven day incubation at salinities ranging from 0-20 ppt.

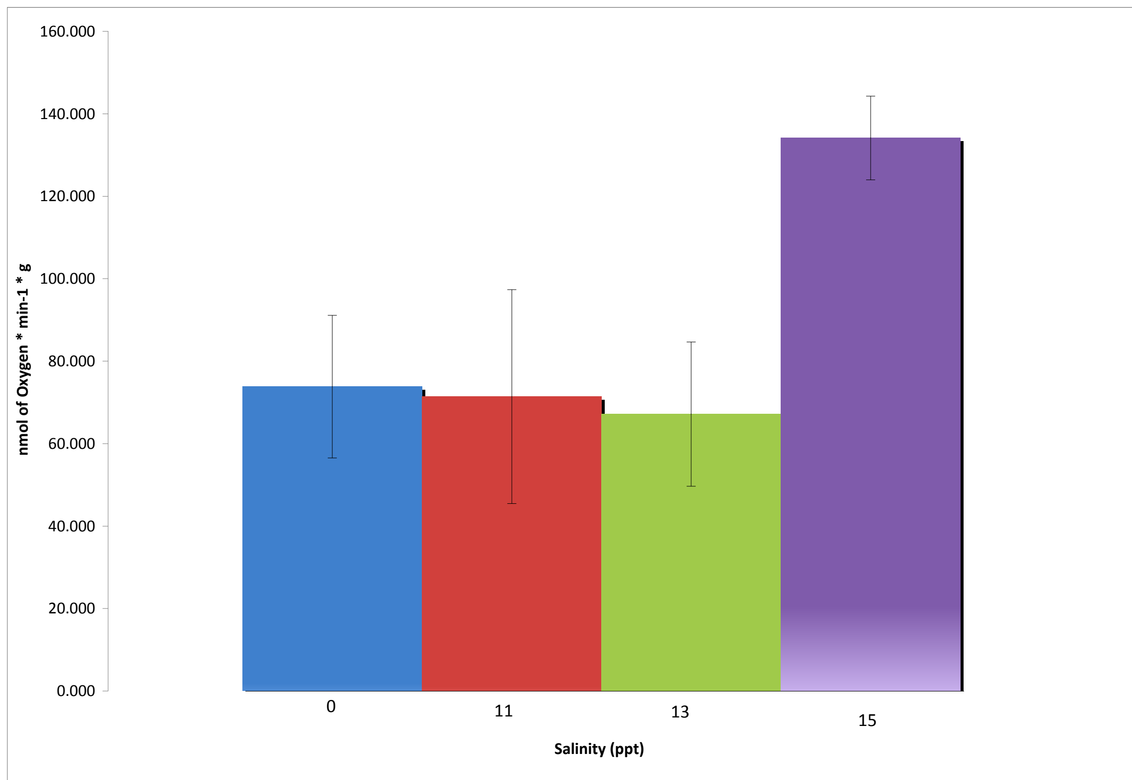

Figure 4. Twenty four hour incubation at salinities ranging from 11-15 ppt (0ppt used as control)

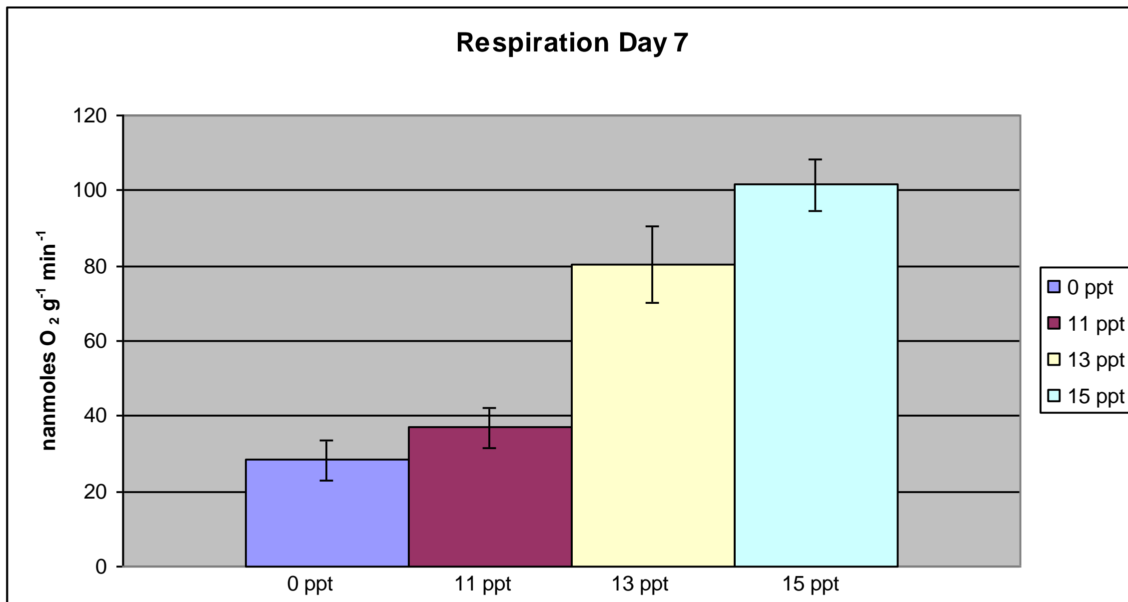

Figure 5. Seven day incubation at salinities ranging from 11-15 ppt (0ppt used as control)

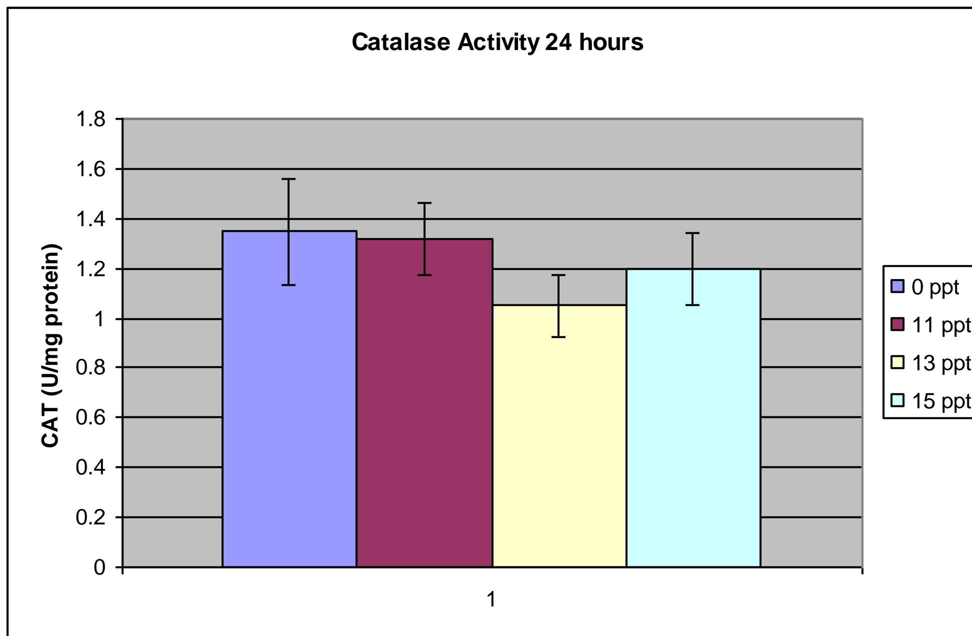

Figure 6) Catalase activity after 24 hour incubation

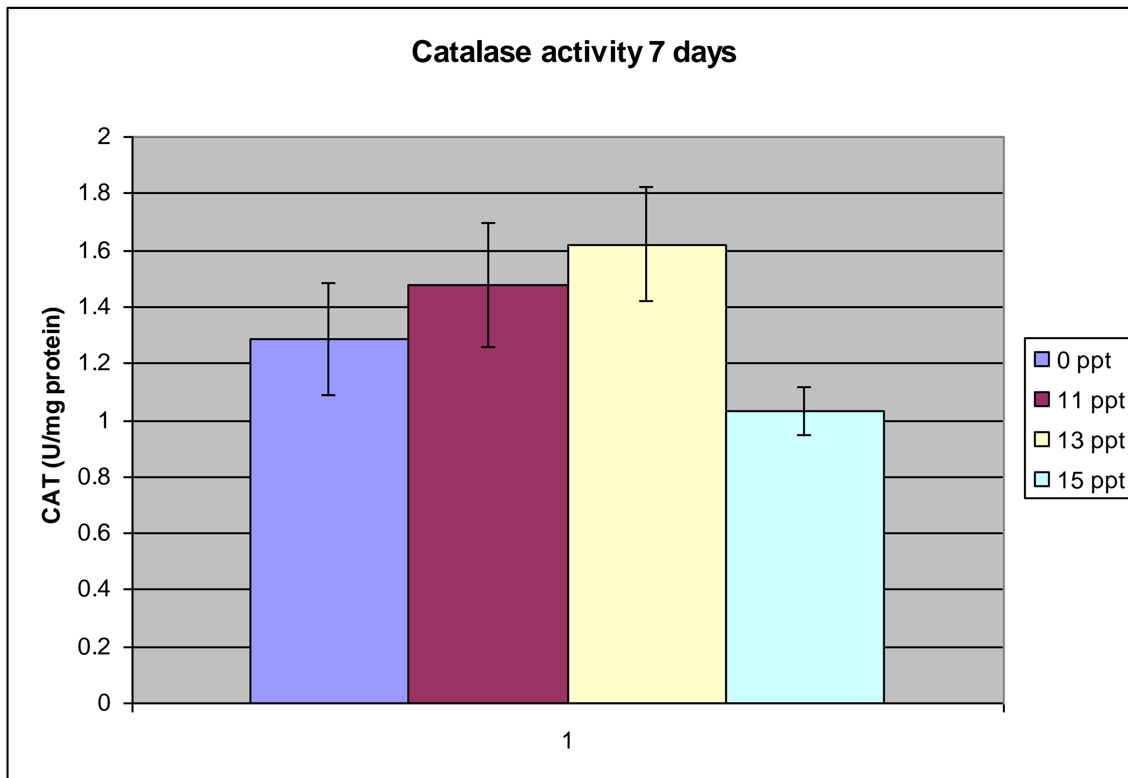

Figure 7. Catalase activity after the 7-day incubation

Superoxide Dismutase activity (anti-oxidant enzyme)

As shown in figures 8 and 9a there does not appear to be much significant change in SOD activity as a function of salinity or time. However, in an additional experiment (Fig. 9a) plants did show a unique response at 15 ppt indicating that variability is certainly observed.

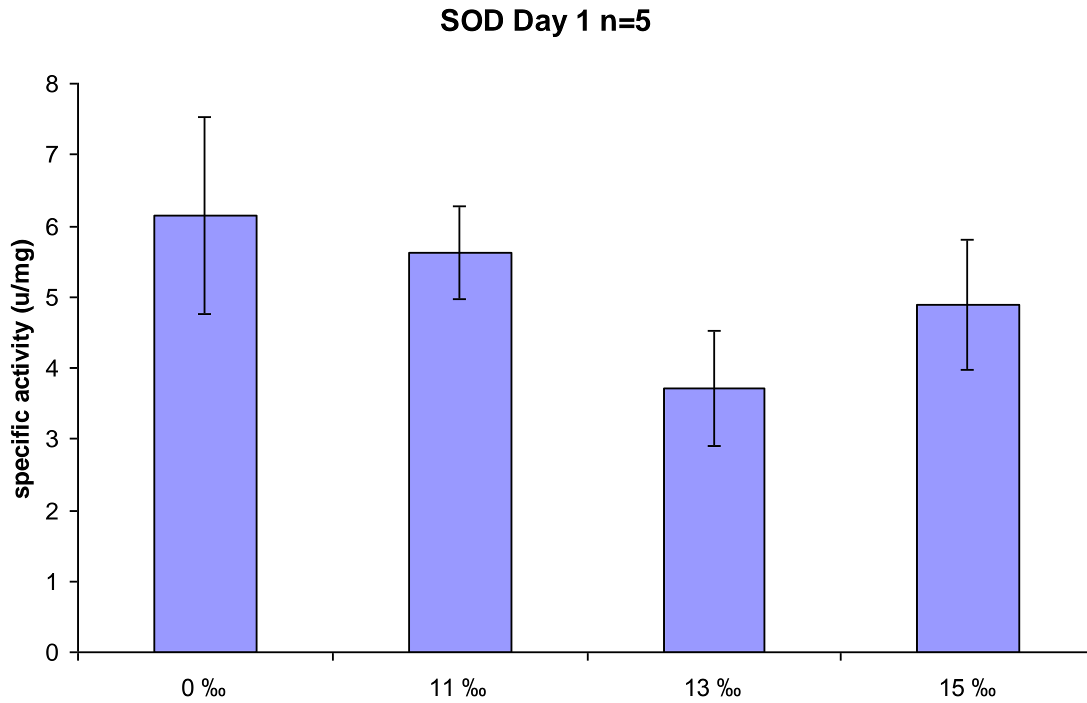

Figure 8. SOD activity after 24 hour incubation

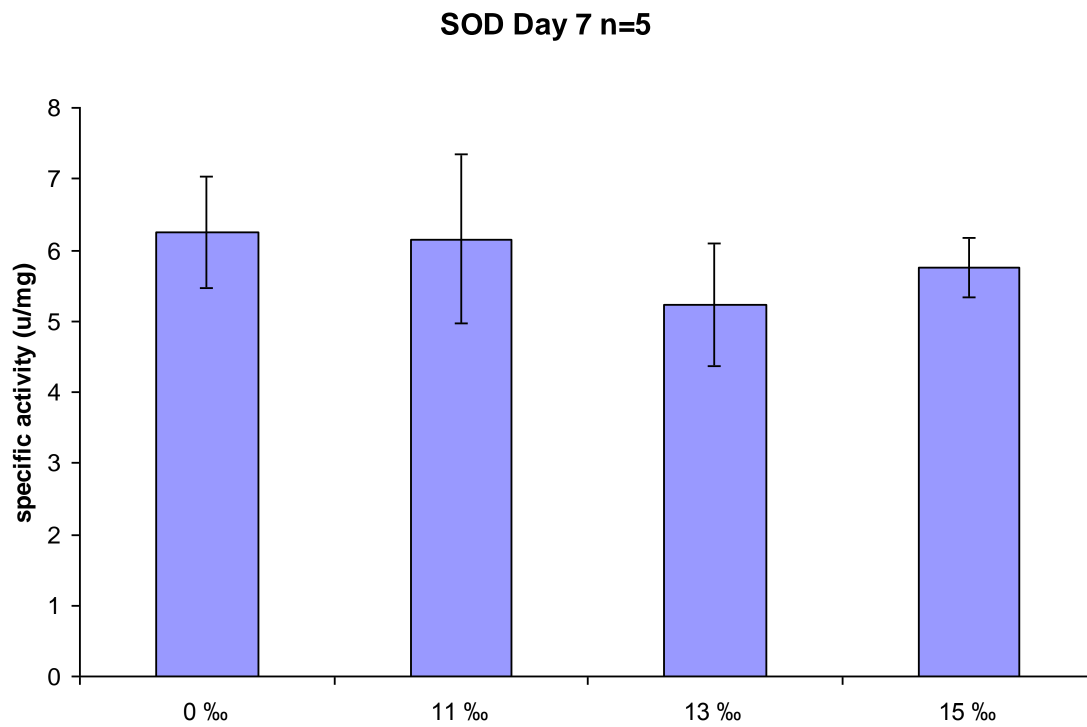

Figure 9a. SOD activity after a 7-day incubation (11-15ppt)

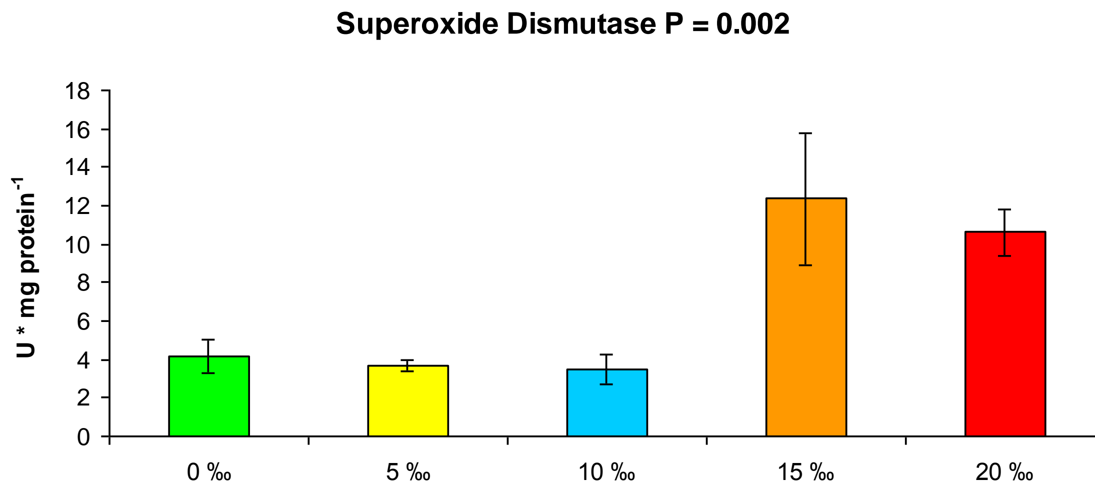

Figure 9b. SOD activity after a 7-day incubation (0-20ppt)

Lipid Peroxidation (sign of damage)

Lipid peroxidation was shown to increase early at the 15ppt exposure (Fig. 10). After incubation for 7 days the 13ppt treatment demonstrated an increase in lipid peroxidation content as well (Fig. 11).

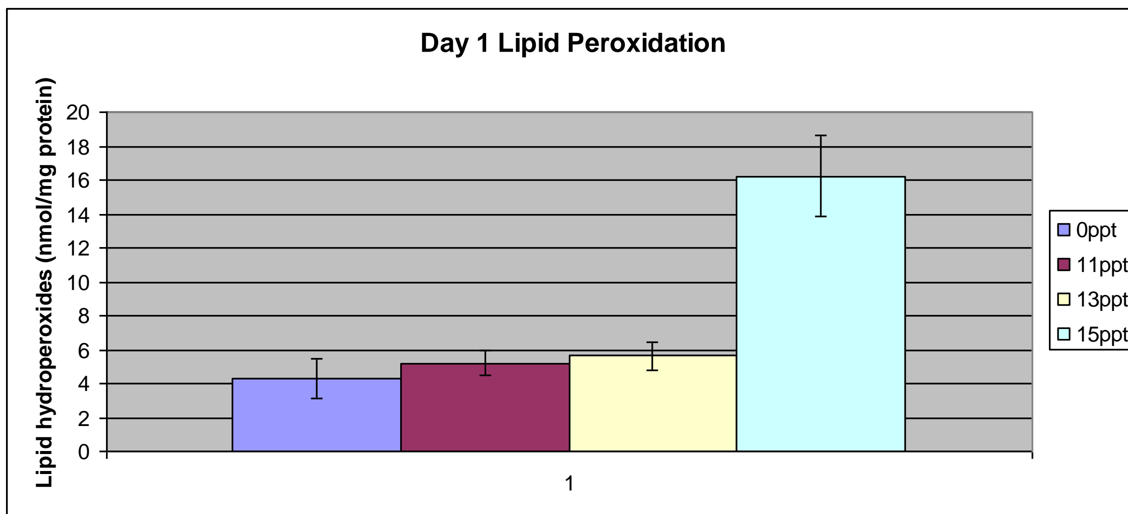

Figure 10. Lipid peroxidation after 24 hour incubation

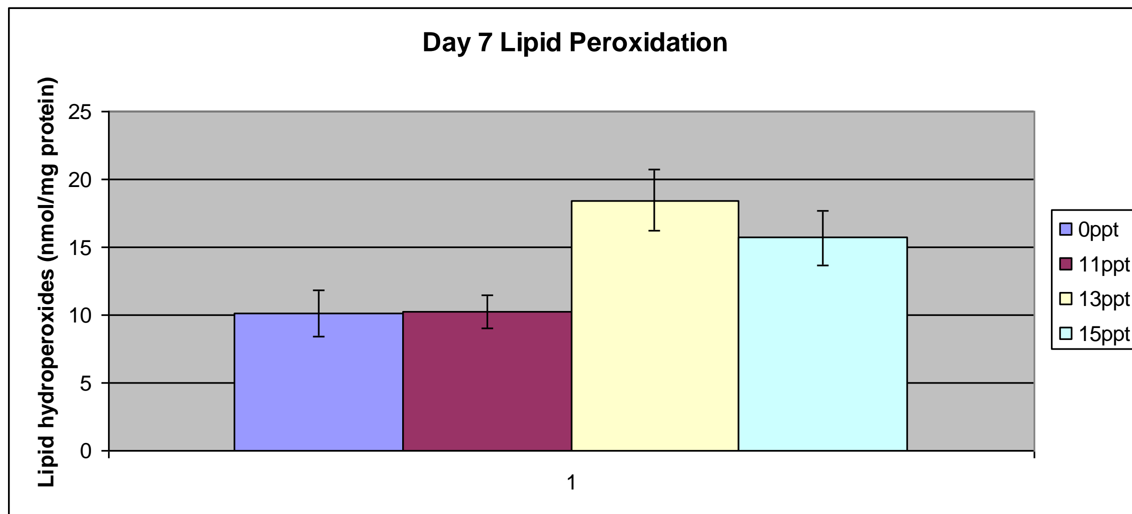

Figure 11. Lipid peroxidation after a 7-day incubation

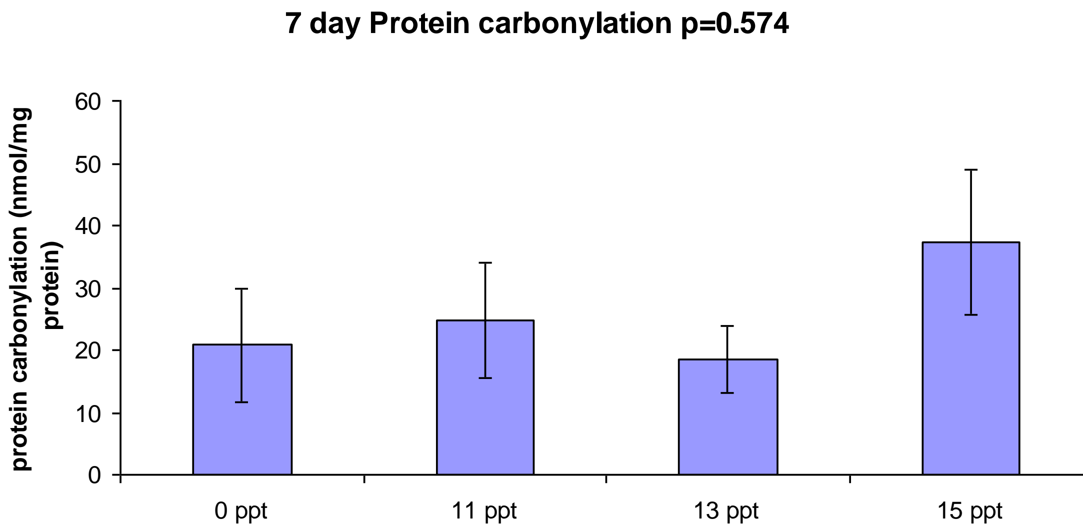

Figure 12. Protein Carbonylation after a 7-day incubation

Protein Carbonylation (sign of damage)**—*Only run at 7 day incubation (not at 24 hours due to limited supplies—expensive kit)**

In summary, no change in protein carbonylation as a function of any salinity treatment or time exposure.

Field study

Both Buckman and Orangedale sites showed salinity fluctuations throughout the field season. Buckman values ranged from 0.5-8.5ppt (Fig 13) while Orangedale's ranged from 0.5-2.2ppt (Fig 14). May 17th clearly had the highest salinity values for both sites.

Protein concentrations in the *V. americana* samples collected from Buckman (Fig. 15) showed no significant change as a function of collection date. Similar observations were noted in Orangedale samples (Fig. 16). One correlative observation was that when a jump in salinity occurred (May 17th) the following sampling date displayed a slight drop in protein concentration. Figure 17 illustrates the average protein content over the course of the collection period. Orangedale had a higher value.

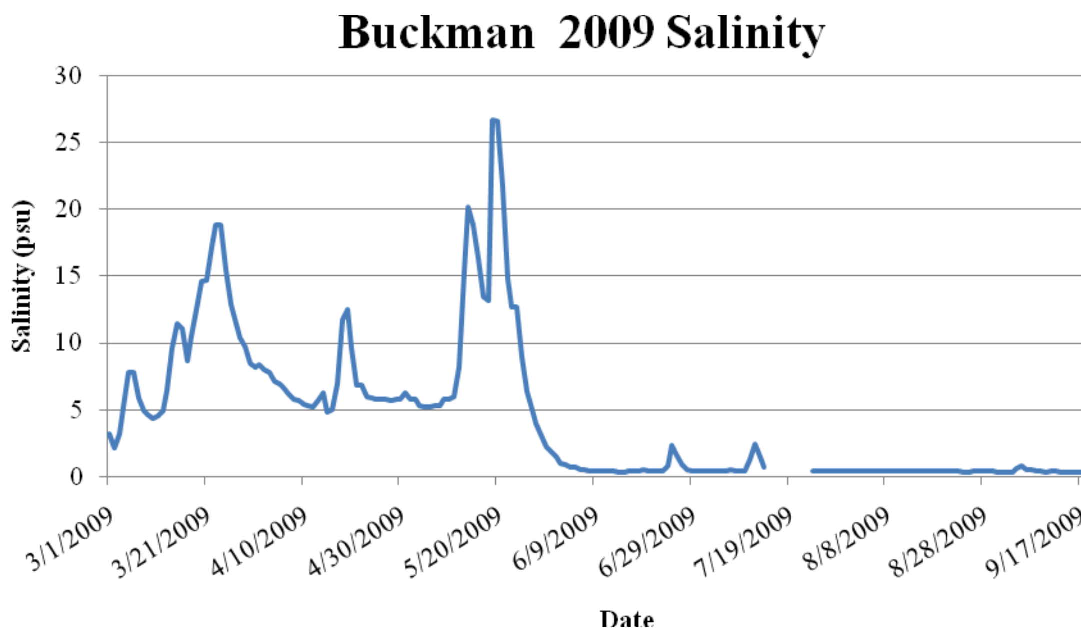

Figure 13. Salinity at Buckman site over course of study

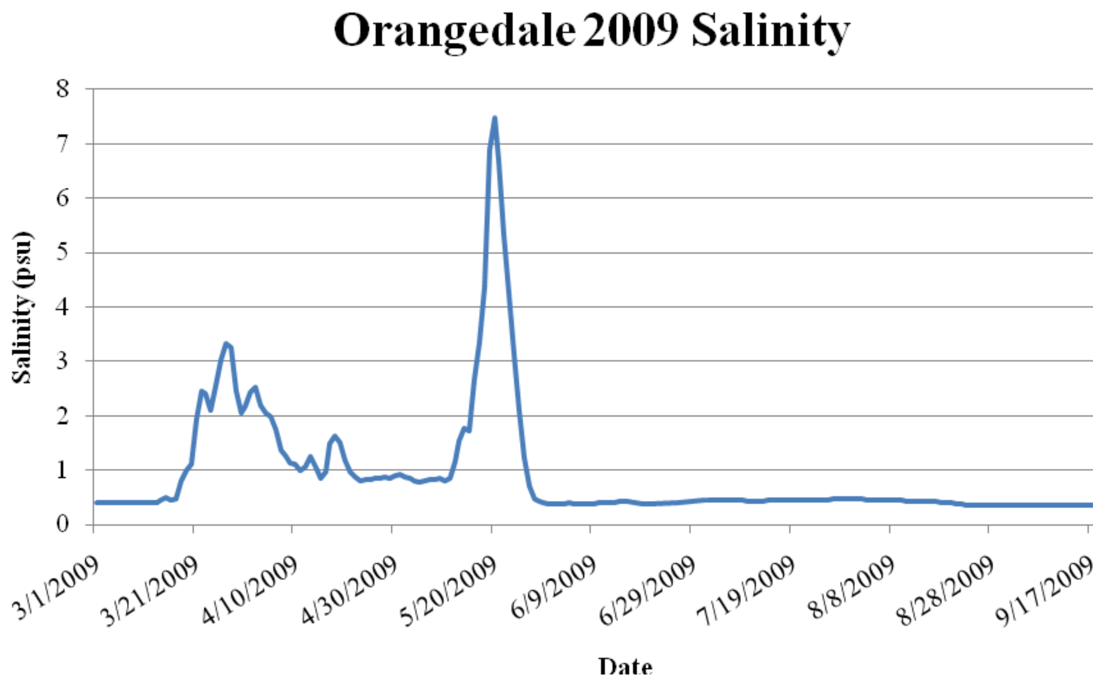

Figure 14. Salinity at Orangedale site over course of study

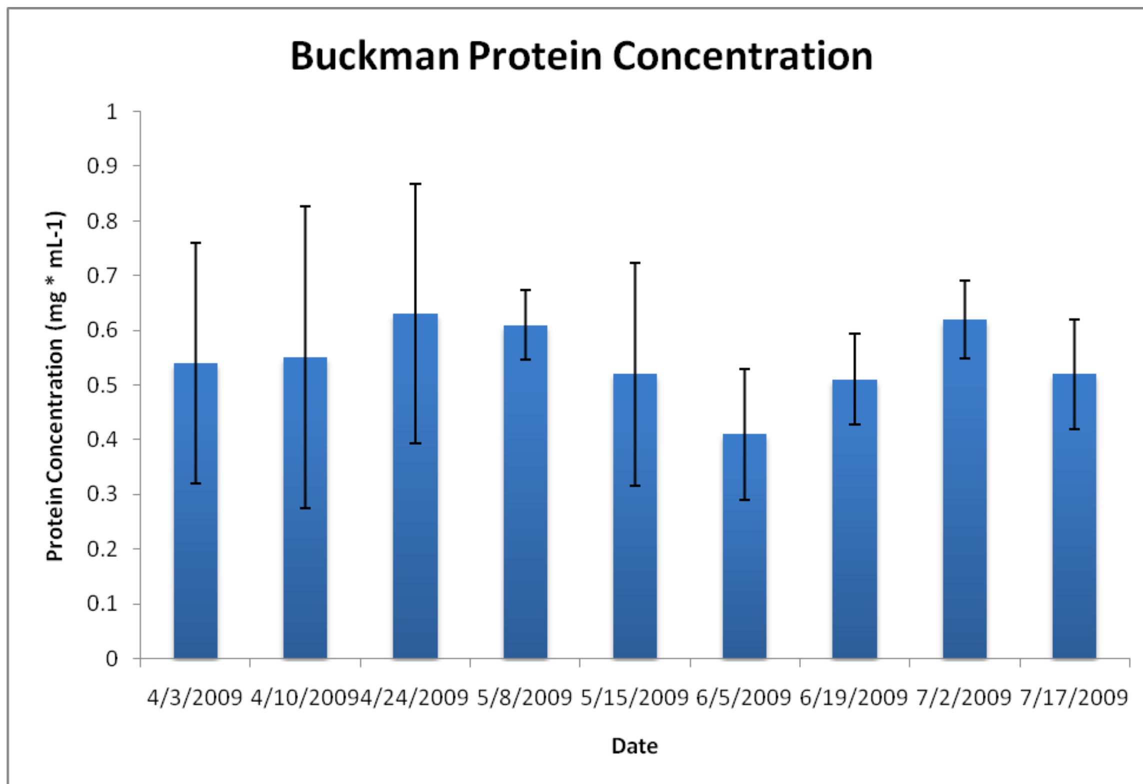

Fig. 15. Protein content of samples collected at Buckman

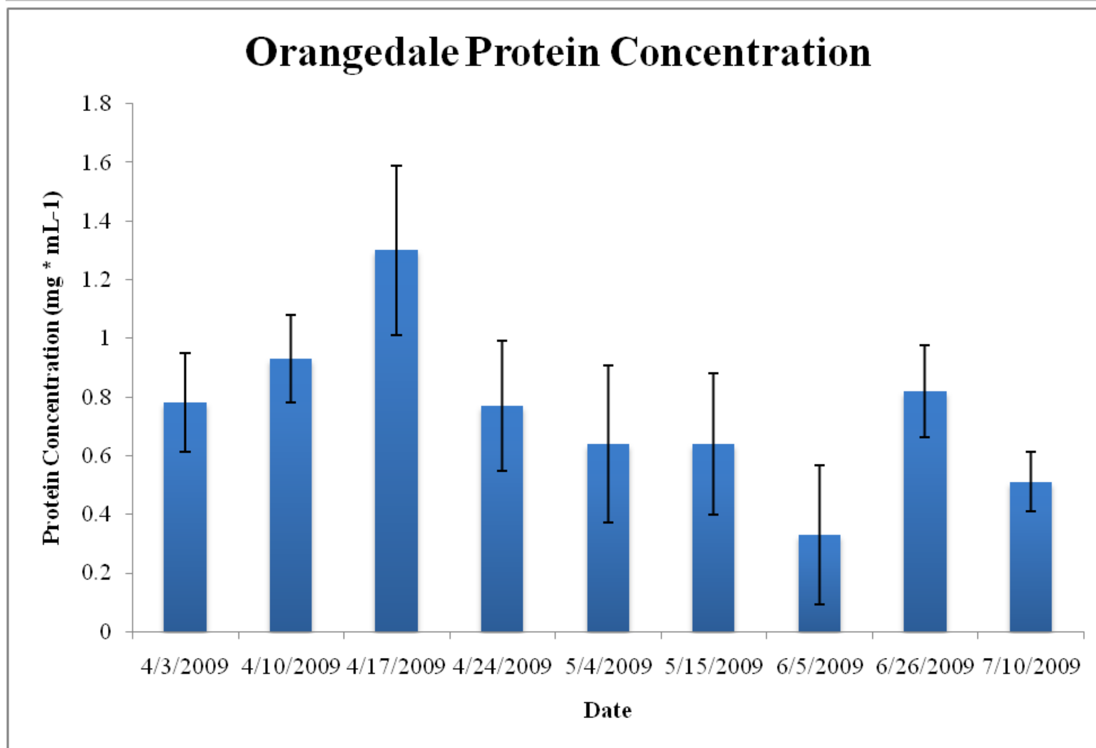

Fig. 16. Protein content of samples collected at Orangedale

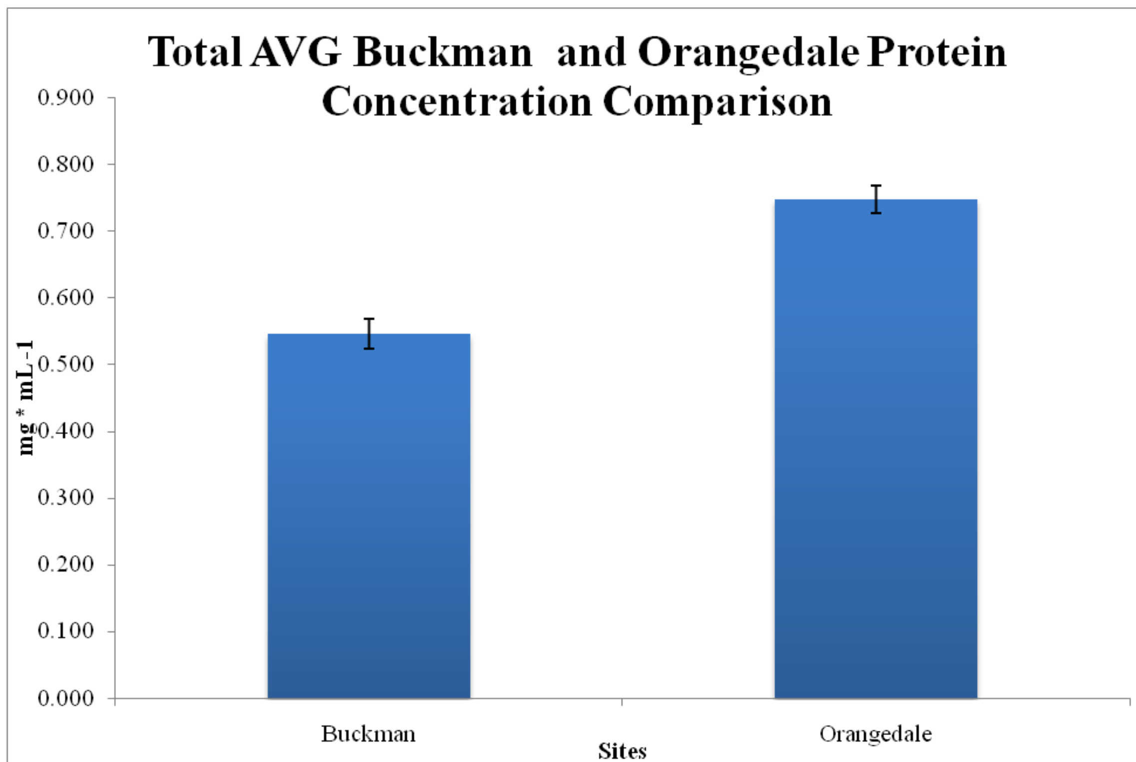

Fig 17. Average protein values for Buckman and Orangedale for the course of the season sampled.

Catalase activity did not undergo a notable change over the course of the growing season for either site (Figs. 18 &19). It was interesting to note that CAT values from Buckman were always higher than the values obtained from Orangedale. This may imply that oligohaline waters cause *V. americana* to have baseline CAT levels that are constitutively higher than freshwater congeners. Average CAT values over the sampling period are shown in Fig. 20.

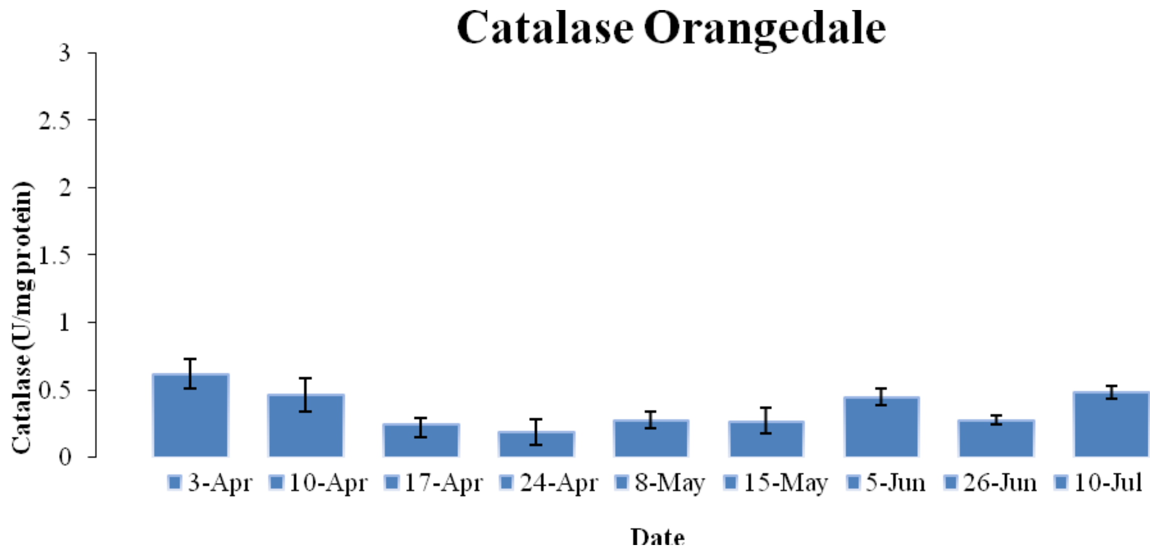

Figure 18. CAT activity over the course of the growing season at Orangedale.

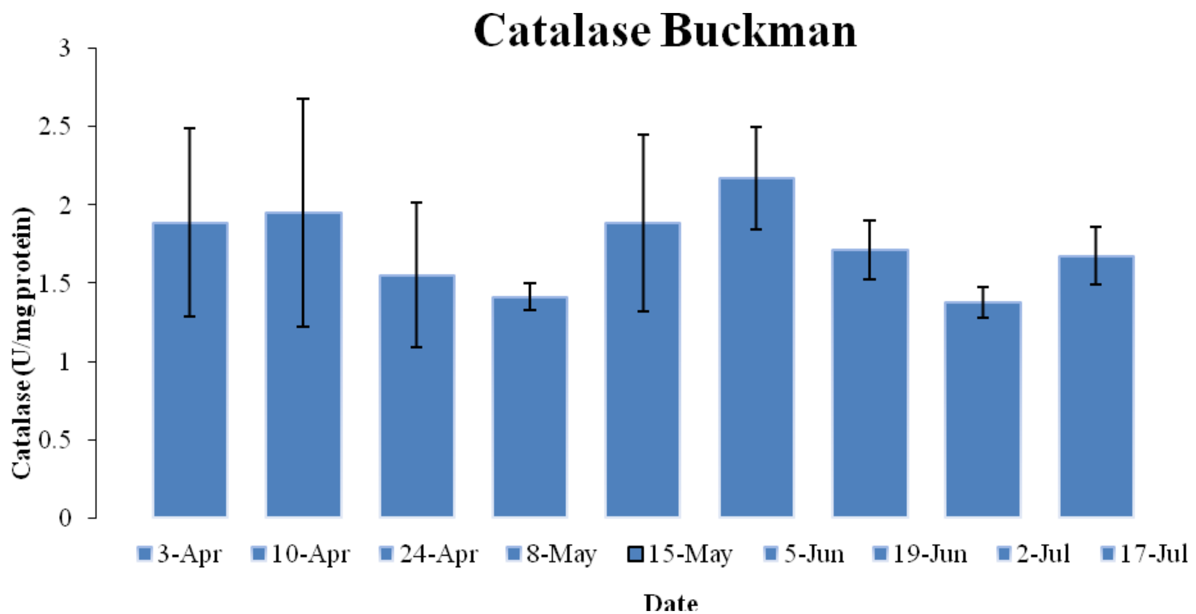

Figure 19. CAT activity over the course of the growing season at Buckman.

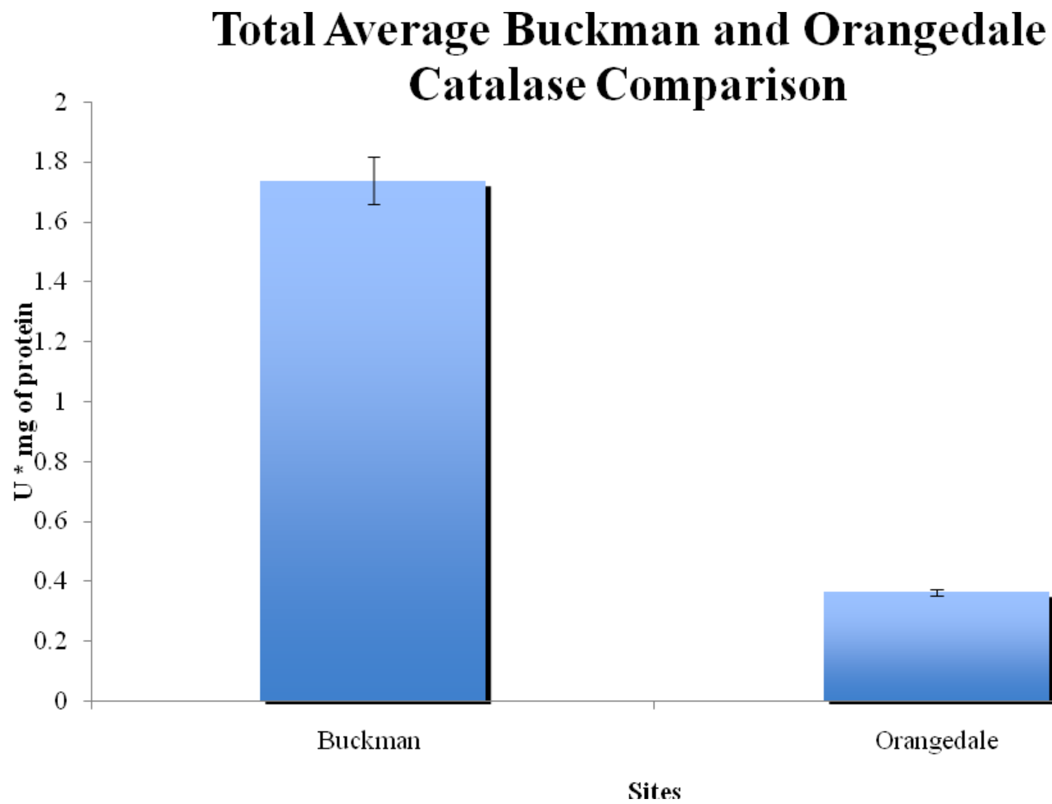

Figure 20. Average CAT for the course of the season sampled

No significant differences were found in either lipid peroxidation values (Figs. 21-23) or SOD activity (Figs. 24-26) over the course of the season with respect to both sites, irrespective of salinity.

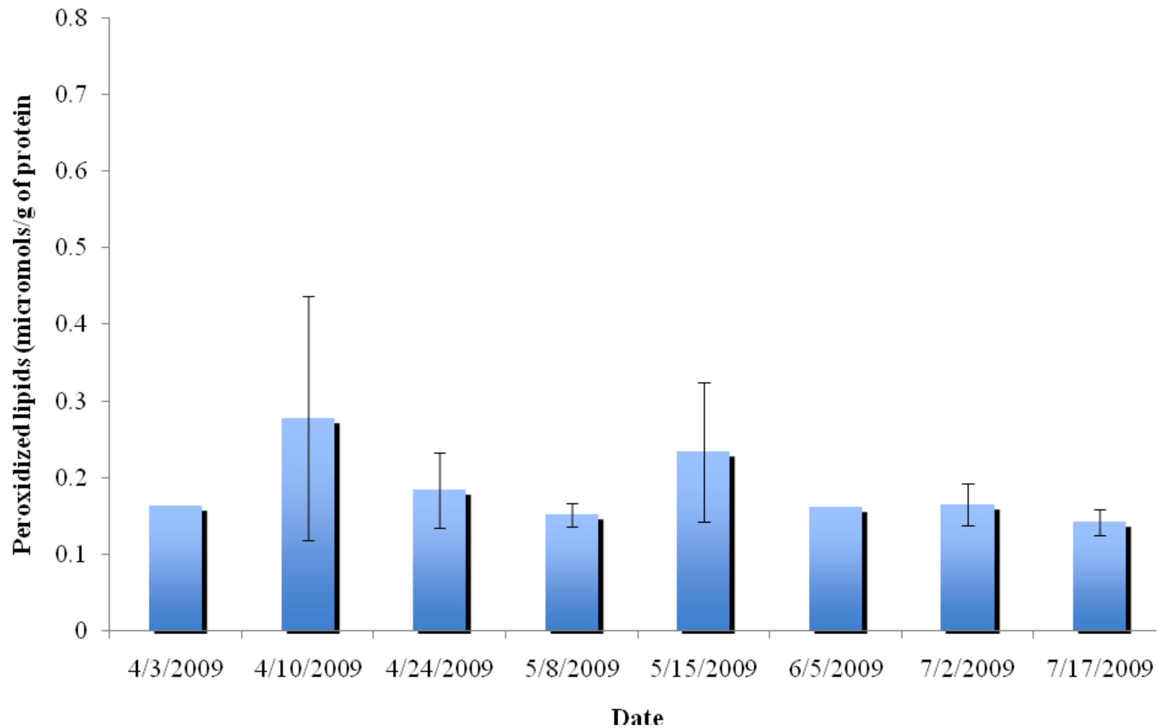

Figure 21. Lipid peroxidation over the course of the growing season at Buckman.

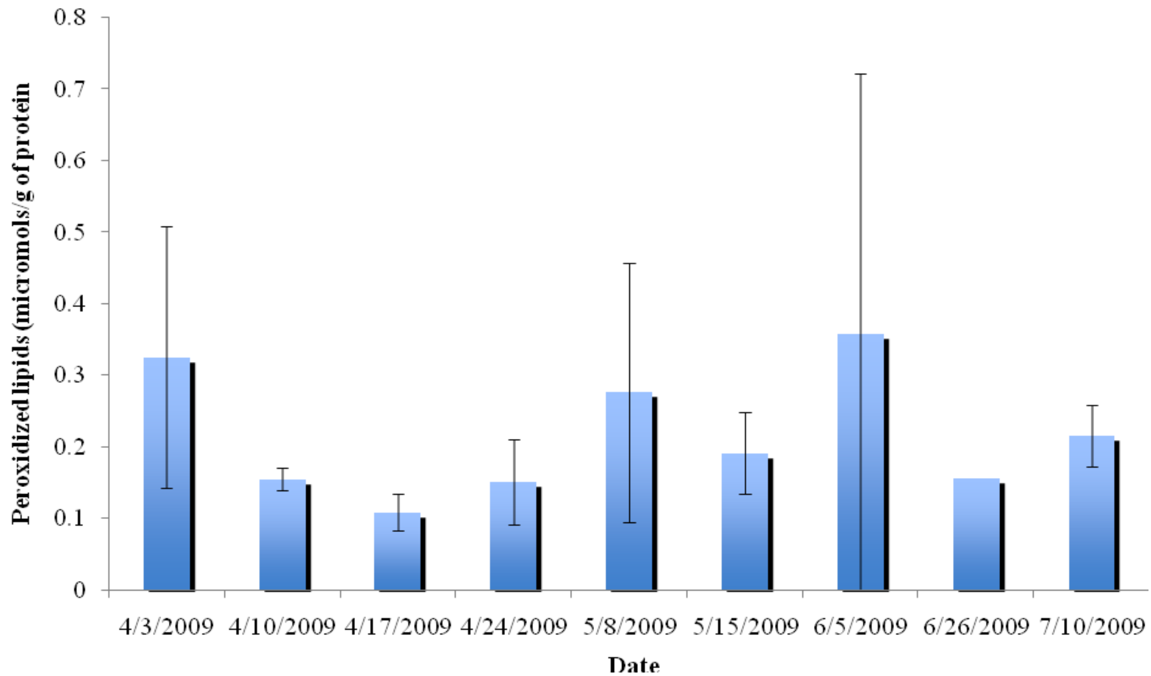

Figure 22. Lipid peroxidation over the course of the growing season at Orangedale.

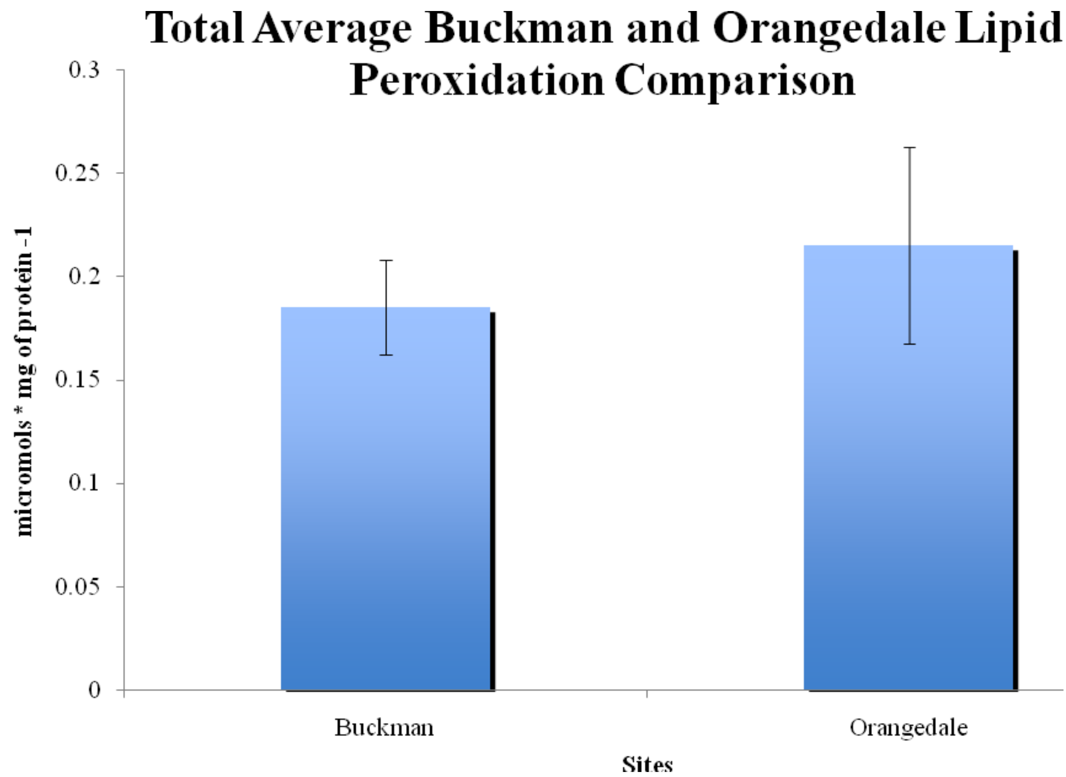

Figure 23. Average Lipid peroxidation values for the course of the season sampled

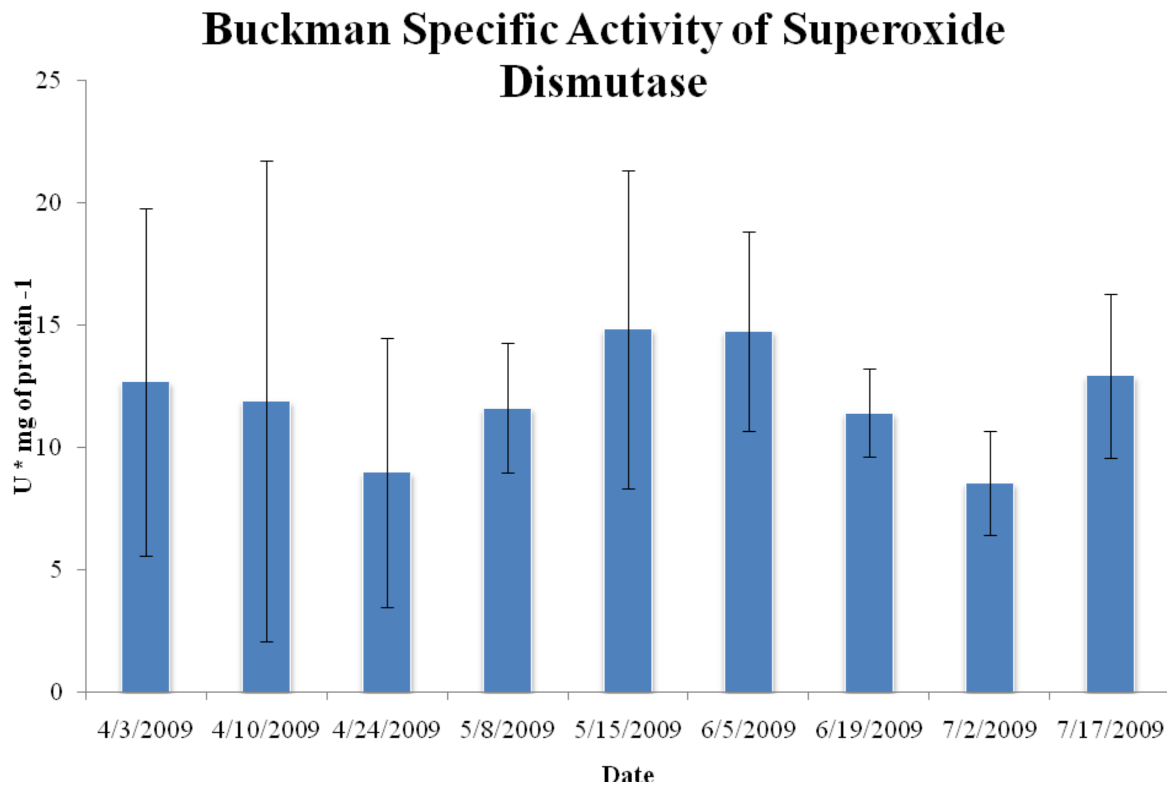

Figure 24. SOD activity over the course of the growing season at Buckman.

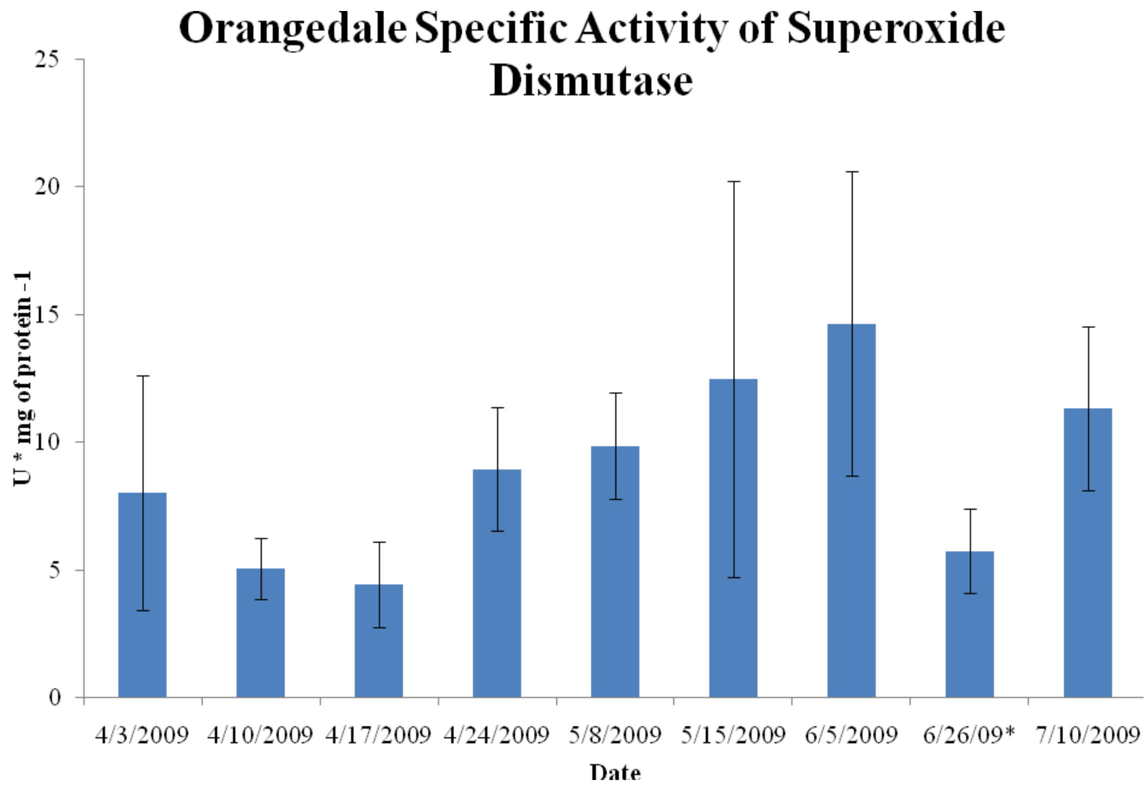

Figure 25. SOD activity over the course of the growing season at Orangedale.

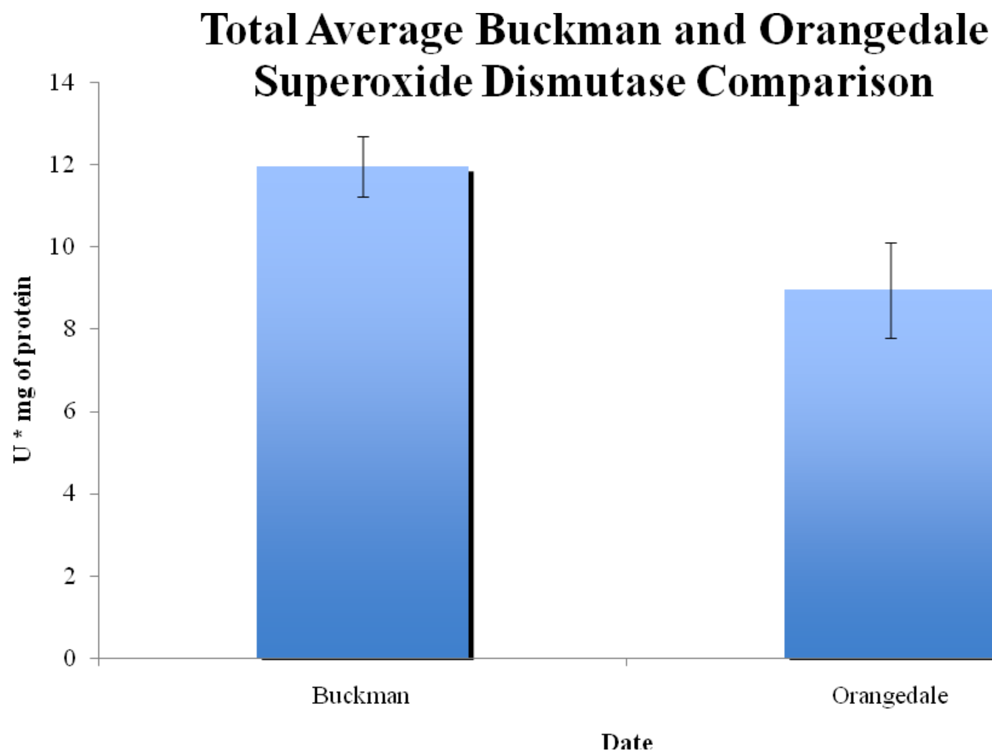

Figure 26. Average SOD for the course of the season sampled

For mesocosm-based work it's certainly possible to control all parameters. However, even under these circumstances variability still becomes an issue. Over the course of a growing season any number of natural stressors can influence plant health and overall metabolism (e.g. salinity, temperature, photosynthetic active radiation). This makes if difficult to pinpoint the effects of any particular stressor.

When comparing salinity profiles at the two sites it is clear that Buckman has access to water 7 times the salinity of Orangedale. It was anticipated that Buckman samples would have higher protein content as a reflection of the plant's upregulation of it's osmoregulatory machinery. While neither field site demonstrated any significant change in protein content across the field season it was observed that Buckman plants had, on average, lower protein content. Perhaps this was due to the negative impact of oligohaline conditions on plant metabolism. It was noted that plants were much smaller at the Buckman site. Work by Kraemer et al. (1999) noted that *V. americana* from the west coast of Florida displayed a decline in protein content prior to plant death. This suggests that protein concentration alone may be a proficient biomarker to assess the physiological stress of *V. americana* when exposed to elevated salinity.

With respect to the field samples, neither SOD nor CAT demonstrated any significant change during the time period analyzed despite clear changes in salinity. There was a large amount of variability within replicates which makes it difficult to assess results. However, the most striking observation was that Buckman samples had CAT values that on average were ~4 times higher than Orangedale samples. It appears as though the Buckman samples were constitutively expressing higher levels of CAT as a response to ongoing oxidative stress associated with the increased salinity of that geographic site.

In conclusion, our preliminary data suggests that CAT activity may be a useful tool for detecting oxidative stress in plants grown under oligohaline conditions. However, one must use caution when interpreting any results associated with natural field conditions as any combination of natural stressors may skew results making it difficult to pin point the specific cause of stress. Future work will be focused on exploring other potential biomarkers of cell stress such as heat shock proteins (Hsp 70 & 90) and ubiquitin to determine if a more reliable tool can be used. In addition it would be of interest to focus on biochemical markers of plant recovery associated with past bouts of salt stress.

References

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