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MICROBIAL ENZYME ACTIVITY IN THE WEKIVA RIVER

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MICROBIAL ENZYME ACTIVITY IN THE WEKIVA RIVER

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1. EXECUTIVE SUMMARY

It is well known that microorganisms produce enzymes in response to the surrounding environment and that microbial enzyme activity can reveal the metabolic status of an ecosystem's microbial communities. This finding has also allowed use of microbial enzyme activity as potential indicators for nutrient limitation and substrate quality in aquatic ecosystems.

Several studies conducted in the Wekiva river system have addressed the significance and discussed the implications of changing nutrient concentrations in the springs, but this study is the first to investigate the possible influence of changing nutrient concentrations and nutrient ratios on microbial enzyme activities in the Wekiva River. The objectives of this study were to determine microbial enzyme activities longitudinally along the Wekiva River and evaluate potential relationships with the water physico-chemical properties. In addition, the potential of nitrate loss via microbially mediated denitrification in the Wekiva River was investigated.

This preliminary study consisted of a one-time sampling effort of four locations along the length of the Wekiva River. From upstream to downstream, these were the Wekiwa headsprings, the Wekiva Marina, Katie's Landing, and near the confluence with Blackwater Creek. To determine microbial enzyme activities, algal biofilms (algal-microbial consortia) from sediments, and other substrata (including wood, rock, submerged vegetation etc.) were collected, along with samples for water quality measurements.

Enzyme activities were determined on 42 microbial biofilms collected from the four sites. The dominant instream substrata varied between sites, therefore attached biofilms were collected from all available substrata. Four enzyme activities were measured; nitrate reductase, alkaline phosphatase, leucine aminopeptidase and denitrogenase. Water quality measures included the important forms of nitrogen, phosphorus and organic carbon.

The results of this investigation demonstrated that:

- Enzyme activities in biofilms were higher than those observed in sediments.
- There was little variation between sites in the total or soluble reactive phosphorus concentrations. However the ratios, total nitrogen: total

- Higher alkaline phosphatase activity at the marina site supported the phosphorus limitation, but not at the headspring site. However, there was no significant overall relationship observed between any measured water quality parameter and alkaline phosphatase activity.
- Potential denitrification was measurable at all sites with relatively higher rates closer to the headsprings where the nitrate concentration was higher. Activity was highest in biofilms collected from the rock wall and the floating green mats at the headsprings. Although there was no significant trend observed with distance from the headsprings, there was a significant negative correlation between nitrate reductase activity and the ammonium concentration (r=-0.9, p= 0.09) and there was a significant positive correlation between activity and the nitrate: ammonium ratio (r= 0.92, p= 0.08).
- Some microbial components of the biofilms in the headspring appeared to be carbon limited as suggested by increased leucine aminopeptidase activity. Since leucine aminopeptidase is produced by heterotrophic bacteria, it may be possible that heterotrophic organisms at the headspring site were carbon limited by the low dissolved organic carbon concentration. However, there was no significant overall relationship observed between any measured water quality parameter and the leucine aminopeptidase activity.
- Carbon limitation in headspring biofilms may also be influencing the production of alkaline phosphatase. Therefore, it is possible that different heterotrophic components of the headspring microbial community experience carbon limitation, while the autotrophic organisms are phosphorus limited.

Results of this preliminary study suggest differences in enzyme activities of algal-microbial consortia in biofilms occur at different nutrient concentrations and ratios along the Wekiva River. While conclusions cannot be drawn from a single sampling event, enzyme activities suggest that different components of consortia may be limited by different nutrients at the same location and that longitudinal gradients of limitation are

likely. This may be a reflection of differing degrees of heterotrophic vs. autotrophic production along the river. Differences in enzyme activities can have implications on the ecology downstream by affecting the nutrient cycling and nutrient transformations. Whether the differences in enzymatic activities of the algal-microbial assemblages from different sites are a response to nutrient availability or because of different structural communities is unclear. Addressing these questions can explain the resilience of microbial community structure in the Wekiva River, to the changing nutrient concentrations and perhaps provide an indication to the threshold limit of nutrient loading before the microbial community structure permanently changes.

2. INTRODUCTION

In Wekiwa Springs, elevated concentrations (greater than 0.2 mgL^{-1}) of nitrate have been observed for more than a decade (Toth, 1999). Sources of nitrate-N, based on studies with stable isotopes, are likely from animal waste, sewage and fertilizer (Toth, 1999). Major concerns with elevated nitrate concentrations, stem from its direct and indirect influences on native ecology of Wekiwa Springs especially because high nitrate concentrations have been associated with increased green algae in the springs. This possible shift in native ecology also affects the aesthetics of Wekiwa Springs that has high recreational value. Elevated nitrogen (N) concentrations can disturb the ecological balance of a system, lead to eutrophication, and cause a shift in native ecological microbial and algal communities. Several studies in the past have shown a relationship between eutrophication and increased N and phosphorus (P) concentrations (Smith, 2003). Ecological balance of a system also depends on the cycling of nutrients, which is regulated by the microbial communities that occur in the system. Most microbial communities have the ability to accommodate temporary changes in the environment without major changes in the structural component. However, if the changes are permanent, the structural component can change. Therefore, determining the functional aspect of the microbial component allows us to assess the health of a system, to study the fate of nutrients in a system and to determine the impact of nutrient/environmental changes in a system.

Potential of enzymes to be used as microbial indicators has been used in the past in several environment systems including lakes (Steinhart et al, 2002), wetlands (Jackson et al, 1995), marine (Hoppe, 2003), etc. Microbial enzyme activities have been used to understand the fate of organic matter via decomposition, and significance of heterotrophic microbial activities in aquatic ecosystems (Hoppe et al., 1988; Sinsabaugh et al., 1994; Jackson et al., 1995).

Enzyme activities involved in microbial N cycling have been used to study the fate of N in aquatic systems where nitrate and ammonium are two inorganic forms of nitrogen that are available to the microbial groups. Ammonium can be taken up and metabolized directly, however, nitrate has to be reduced to ammonium by nitrate reductase before NO_3-N is incorporated. Therefore, nitrate reductase (NR) activity has

been used as an indicator and a measure for nitrate uptake and nitrogen assimilation in microorganisms and plants, and for studying the fate of nitrate in an environment (Eppley et al., 1970; Jiang and Hull, 1998).

Activities of some inducible enzymes have been used to determine potential nutrient limitations in aquatic systems. The enzyme synthesis and function are influenced by a variety of environmental factors that affect the microbial cells/communities. Some microorganisms have the ability to turn 'on' and turn 'off' the enzyme synthesis by regulating the gene expression in the presence and absence of enzyme specific substrates and/or products and these enzymes are termed as 'inducible enzymes'. Therefore, most of the inducible enzyme activities have been used as microbial indicators for potential nutrient limitation in the environment.

Activity of commonly studied inducible enzyme alkaline phosphatase (AP) has been found to be correlated with concentrations of soluble inorganic phosphorus and therefore, it has been used as an indicator of P–limitation. Induction of alkaline phosphatase enzyme is inhibited and its activity is suppressed in presence of bioavailable inorganic P (Chrost, 1991). Another inducible enzyme that is widely used is involved in N cycling in aquatic systems is leucine aminopeptidase (LAP). This enzyme is mainly produced by heterotrophic microorganisms and its synthesis is reported to be associated with the breakdown of organic N sources as the proteins or other cellular by products.

The goal of this study was to understand the microbial enzyme activities in Wekiwa Springs with respect to the aquatic nutrient concentrations. Specific objectives of this study were 1) to determine activities of nitrate reductase, alkaline phosphatase, and leucine aminopeptidase in Wekiwa Springs and 2) to determine their relationship with water physico-chemical properties.

In an attempt to investigate the role of denitrification in N loss in Wekiwa Springs, we determined denitrification potential in sediment and biofilms. This process is a significant mechanism for N loss in streams (Swank and Caskey, 1982; Bradley et al., 1995). In this microbially mediated process, facultative organisms reduce nitrate-N as a terminal electron acceptor (Roswall, 1981) to produce nitrous oxide and dintrogen gas, while oxidizing a carbon source. In contrast to the assimilation pathway in which nitrate-N is reduced to NH_4 to be assimilated, denitrification permanently removes N from the

system. Knowledge of potential areas where this process occurs may be significant as this process may improve the surface-water quality and thereby help offset the negative effects of N enrichment in streams. Although, the water column in the Wekiwa Spring is highly oxygenated, potential for denitrification may exist in the biofilms occurring on different substrata.

3. MATERIAL AND METHODS

3.1 Study Site

The Wekiva River is located in east central Florida north of Orlando. The river's name is derived from Seminole Indian origin. Human use of the river and adjacent lands dates back about 12,000 years (Weisman, 1993). The headwaters of Wekiva River is Wekiwa Springs, a second magnitude spring (mean annual discharge 68 cfs) located within Wekiwa Springs State Park (Figure 1). Currently the river and its headsprings are used for a variety of recreational opportunities.

Figure 1. Location of sampling sites in Wekiva River. Values in the parentheses represent the distance of sampling points from the Wekiwa spring boil. HS: near head springs; MA, Marina; KL, Katie's Landing; BC, Blackwater creek

The Wekiva River basin and the springshed lie in a karst landscape, therefore there is a high degree of interconnection between the surface and ground water. The temperatures in this subtropical region range from an average monthly high of 90-95 degrees F during August/September to an average low of 59-60 degrees F in December/January. Most of the rainfall in the basin occurs between June and September and across the region it averages 51.5 inches annually. The concentration of dissolved nitrate-N in Wekiwa Springs has varied from 0.61 mg N L^{-1} in 1961, to 2 mg N L^{-1} in 1995 and 1.3 mg N L^{-1} in 1999. A decline in nitrate-N concentration with distance from the head springs has been reported by Toth (2002)

3.2 Field Sampling and Sample Preparation

We sampled the Wekiva River at four stations near the Head springs (HS), Marina (MA), Katie's Landing (KL) and near Blackwater creek (BWC) in the month of October, 2006 (Table 1).

Table 1. Geographical location of the sampling plots in the Wekiwa Springs

Sampling station	Latitude	Longitude	Distance from headsprings (miles)
near head springs	N28.71256	W81.4599	0.05
Marina	N28.72025	W81.4373	1.80
Katie's Landing	N28.82924	W81.4127	10.20
near Blackwater Creek	N28.86522	W81.3759	14.64

Upon arrival at each sampling station, the following parameters were measured using a YSI multi-parameter probe: pH, temperature, dissolved oxygen, and water depth. We collected, unfiltered (for total phosphorus-TP and total Kjeldahl nitrogen- TKN), and

filtered (0.45μm) (for nitrite and nitrate-NOx-N, ammonium-NH_4-N, dissolved organic carbon-DOC, total dissolved phosphorus-TDP, and soluble reactive phosphorus-SRP) water samples. Samples were collected in acid washed bottles, pre-rinsed with site water, appropriately preserved (selectively acidified) and stored on ice in the field and during transportation. Upon arrival at the wetland biogeochemistry laboratory, samples were kept at 4ºC until analysis.

At each station, algal biofilms attached to various substrata (e.g., wood, rock, submerged vegetation, etc; Figures 2 - 4) were collected as bulk samples by scraping the surface (Table 2). Epiphytic biofilms attached to submerged aquatic vegetation were difficult to remove, therefore, the leaf samples were collected and once in the laboratory, the biofilms were separated from the leaves by rinsing them with sterile distilled water. We also collected three sediment cores 2.5 cm in diameter and 10-15 cm deep at each sampling site.

Table 2. List of sediment and biofilm samples collected at 4 sampling stations.

Stations	**Samples collected**
Near head springs	(S) Sediment cores (RW) Biofilm attached to rock wall (BF) Brown filamentous algae in spring bed (FG) Floating green mat (FB) Biofilm on floating bark surface (E) Biofilm attached to submerged aquatic vegetation
Marina	(S) Sediment cores (BF) Brown filamentous algae in spring bed (FB) Floating bark surface (E) Biofilm attached to submerged aquatic vegetation
Katie's Landing	(S) Sediment cores (BF) Brown filamentous algae in spring bed
Near Blackwater Creek	(S) Sediment cores (SB) Biofilm on submerged bark surface (FB) Biofilm on floating bark surface (D) Detrital material

Figure 2. a) Prolific algal growth near the Wekiwa Springs boil; b) Benthic algae near Wekiwa headsprings.

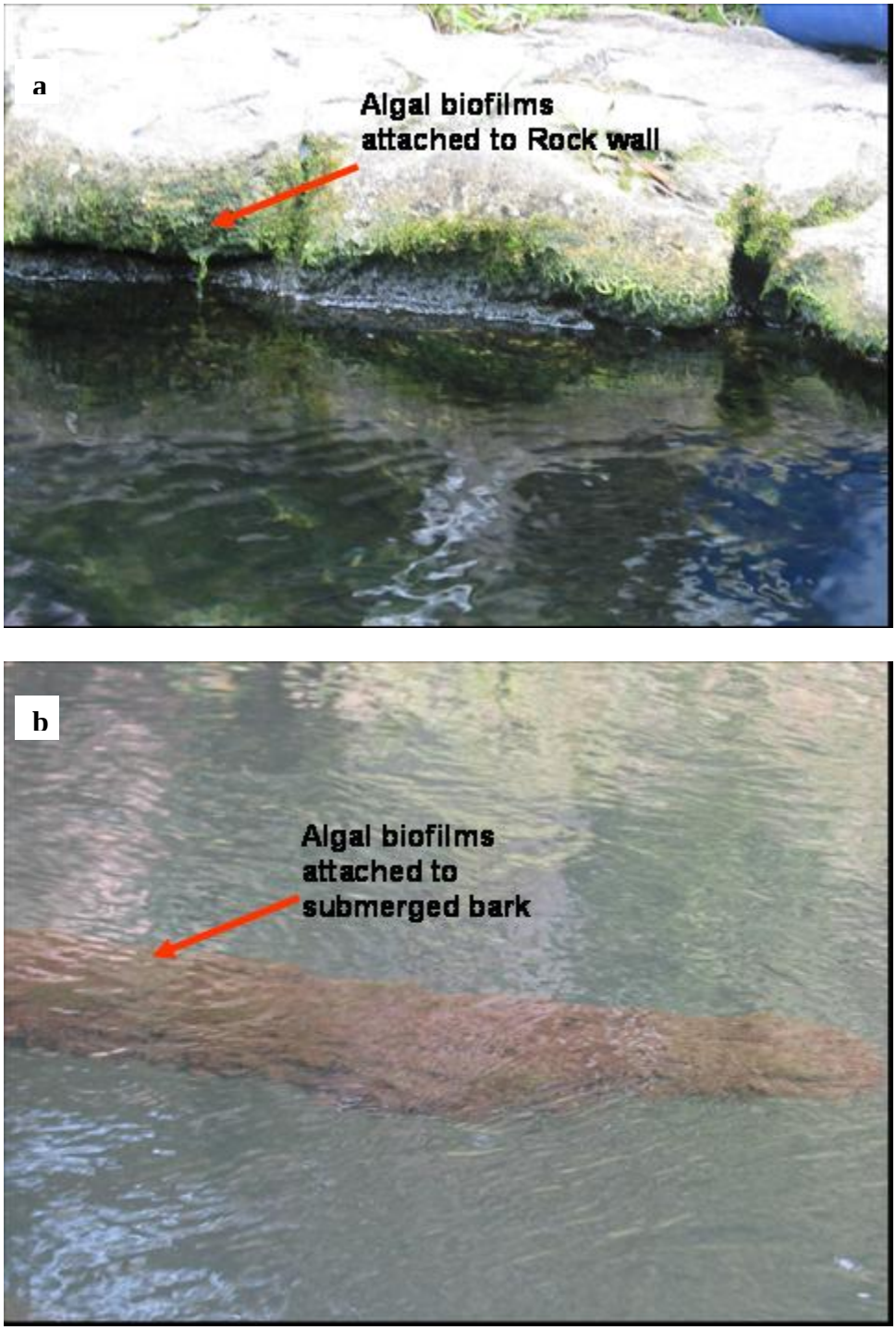

Figure 3. a) Biofilms on rock walls near Wekiwa headsprings; b) Biofilm on submerged bark near the Blackwater Creek site.

Figure 4. a) Benthic brown filamentous algae at Katie's Landing; b) Biofilm on floating bark near Blackwater Creek site.

Samples were collected in sterile plastic tubes and kept under ice during collection and during transportation back to the Wetland Biogeochemistry Laboratory at the University of Florida, Gainesville, FL. At the laboratory, samples were kept at 4ºC until analyzed. All enzyme analyses were performed within a week of sample collection.

3.3. Water Chemistry

3.3.1 Analytical Methods

Water samples collected were analyzed for dissolved organic carbon (DOC) by high temperature oxidation of DOM coupled with IR detection of CO_2 on a Shimadzu TOC 5050 (Shimadzu Inc, Columbia, MD). Total Kjeldahl nitrogen (TKN) and dissolved Kjeldahl nitrogen (TDKN) was determined in water samples colorimetrically (Method 351.2, USEPA, 1993). Nitrate/nitrite (NOx) in water samples was analyzed per methods stated as EPA 353.2. Total phosphorus (TP) and total dissolved phosphorus (TDP) in water samples was determined using 5.5 M H_2SO_4 autoclave water digestion of unfiltered and filtered water samples respectively, followed by ascorbic acid analysis technique (Method 365.1, USEPA, 1993). Soluble reactive phosphorus (SRP) was measured by method EPA 365.1(USEPA, 1993). Total dissolved organic phosphorus (DOP) was estimated by subtracting SRP from TDP. Dissolved inorganic nitrogen (DIN) was estimated as a sum of NH_4-N, and NOx-N. Dissolved organic nitrogen was determined as the difference between TDKN and NH_4-N. Similarly, DOP was determined as a difference between TDP and SRP. Total nitrogen was estimated as sum of NOx and TKN

3.4 Microbial Enzyme Activity

Enzyme activities in biofilms (complex structure of algal and bacterial cells) collected from various substrata were analyzed within a few days of sample collection. With the exception of Leucine aminopeptidase, enzyme activities determined are representative of response by both algae and bacteria that constitute the biofilms attached to different substrates (for instance, wood, rock wall, and submerged vegetation etc.). Leucine aminopeptidase is mainly produced by heterotrophic bacteria (Chrost, 1991). Samples collected from all substrata were dried at 70ºC for 3 days to obtain dry weight.

3.4.1 Nitrate Reductase (NR) Activity

Biofilm samples (30-70 mg fresh wt) collected from the 4 different sites were weighed in two separate tubes containing 5 ml of nitrate reductase assay medium containing: a buffer (0.2 M H_2PO_4, 1mM NADH; pH 8), a compound to permeabilize the membranes (0.1%1-propanol v/v), nitrate in excess (50mM $NaNO_3$), and a source of reducing power (10µM of glucose). The assay medium was bubbled with N_2 gas for 3-4 minutes before and after placing the algal sample to remove dissolved oxygen that impedes the reduction of nitrate to nitrite. Immediately after addition of sample, the tube was closed and incubated in dark for 60 min at 28ºC. The incubation was performed in darkness to prevent further reduction of NO_2^- to NH_4^+. After incubation, nitrite concentration in the assay medium was determined colorimetrically (Gordillo et al, 1997; Corzo and Neill, 1991) by adding 0.5 ml of 0.1% (w:v) naphthyl ethylene diamine in 1 N HCl, and 2.0 ml distilled water to 1 ml of media sample for a total of 4 ml. Absorbance was measured in both control and incubated sample by reading the optical density at 540nm on a Shimadzu UV-visible spectrophotometer model UV-160 (Kyoto, Japan) equipped with a 1 cm cell. Absorbance readings were calibrated against a nitrite standard calibration curve. The NR activity is reported as µmol NO_2 formed per gram dry weight sample per hour.

3.4.2 Extracellular Enzyme Assays

Extracellular enzyme activities for two enzymes, alkaline phosphatase and leucine aminopeptidase (AP and LAP), were determined in 42 samples of sediments and biofilms collected from 4 locations in the Wekiva River. Double distilled sterile water (4 mL) was added to 1 g fresh weight of biofilm subsample and the sample was homogenized with a Tissue Tearor (Model 398; Biospec Products, Bartlesville, OK). Each sample was then incubated in 200 µM of a model substrate, which upon enzymatic hydrolysis yields a fluorescent product. Substrates were 4-methylumbelliferone-phosphate (MUF-P), and L-leucine 7-amido-4-methylcoumarin. Formation of fluorescent product 4-methylumbelliferone (at excitation/emission of 360/460nm) or 7-amino-4-methylcoumarin (at excitation/emission of 380nm/440nm) were measured every 30 minutes for 3 hours in a fluorometer (Model FL600,Bio-Tek Instruments, Inc. Winooski,

VT). Fluorescence was quantified using linear calibration curves prepared from buffered standard made up with autoclaved samples.

Negative controls with no substrate and blanks with no samples were included in each run to account for any background fluorescence. All treatments were carried out in triplicates. Enzyme activity was expressed as µmol of fluorescence produced every hour and was normalized with dry weight of sample (µmol of enzyme activity $g^{-1}dwh^{-1}$)

3.4.3 Denitrifying Enzyme Activity

Potential denitrification rates were measured by acetylene (C_2H_2) block assay (Teidje et al.,1989) by measuring accumulation of N_2O. Addition of C_2H_2 blocks the formation of dinitrogen gas, the final product of denitrification, allowing accumulation of nitrous oxide (N_2O). Approximately 3-4 g wet weight of algae and sediment samples were transferred into 60 ml serum bottles and 5 ml of sterile DDI water was added before they were capped and sealed with Al crimps. Bottles were purged with N_2 gas to achieve anaerobic conditions. Acetylene was generated by adding water to calcium carbide in a sealed bottle. Within each sampling bottle, 10% (v/v) of headspace gas sample were removed and replaced by acetylene gas. Bottles were shaken longitudinally for 1 hour to allow the acetylene gas to evenly distribute through out the samples. This was followed by addition of assay medium containing glucose, nitrate and an antibiotic chloroamphenicol. Final concentrations of glucose-C, Nitrate-N, and antibiotic were 288 mg C L^{-1}, 56 mg N L^{-1}, and 2 mg L^{-1}. Samples were incubated in dark at 25ºC and gas samples were collected from the headspace at 30, 60 and 90 minutes. Prior to gas sampling, samples were shaken well to equilibrate N_2O between water and bottle headspace.

Gas samples were analyzed using a Gas Chromatograph (Shimadzu, CA, USA) equipped with a poropak Q column, and detected with ^{63}Ni electron capture detector. Total concentration of N_2O at each sampling period was calculated using Bunsen adsorption coefficient to determine the amount of gas dissolved in the liquid portion at a given headspace concentration (Knowles, 1979). N_2O production rate was expressed as N_2O-N produced per hour and normalized by sample dry weight (µg N g^{-1} dw h^{-1}).

Because these results were determined by providing optimum substrates to the sample, these numbers are the potential denitrification rates.

4. RESULTS AND DISCUSSION

4.1 Water Quality

Dissolved oxygen in Wekiwa Springs near the headsprings ranged from 0.84 to 1 mg L-1 and was 16 to 20% lower than that observed at other sampling sites (Table 3). Mean water depth at head springs was also lower than the other three sites. All water measurements were made between late morning to midday and so the sites with higher shading due to vegetation showed lower water temperature. pH values were close to neutral and did not vary much within the sampling locations.

Table 3. Water characteristics in Wekiva River at four sampling locations

Sites	Temperature ºC	DO mg/l	Water depth cm	pH
near Headsprings	23.52	1.04	45	7.72
	23.44	1.0	8	8.18
	23.45	0.84	18	8.13
Marina	19.76	5.53	50	6.93
	19.95	6.05	58	7.5
	19.97	6.03	52	7.84
Katie's Landing	20.75	6.43	60	7.7
	20.86	6.08	55	7.82
	20.9	6.83	60	8.08
near Blackwater Creek	18.2	6.4	110	6.5
	nd	nd	nd	nd
	nd	nd	nd	nd

*nd=not determined

4.2 Nutrient Concentrations

Nitrate concentrations in water declined with distance away from Wekiwa headsprings (Figure 5). Nitrate concentrations at two sites that were closer to the headsprings, HS (0.05 miles) and MA (1.8 miles), ranged from 0.95-1.0 mgL^{-1} almost two times higher than that measured at KL and BC, sites that were 10 and 14.6 miles from the head springs, respectively. This decline in nitrate concentrations with distance

from headsprings may be a result of either nitrate removal from the system by uptake or transformations, or due to dilution effect from receiving other low nitrate water inputs.

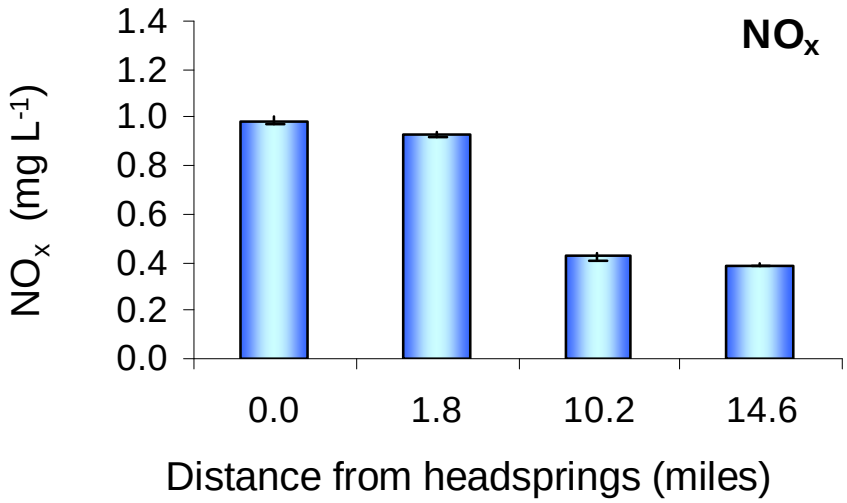

Figure 5. Nitrate concentrations in the Wekiva River downstream from headsprings. [Need to know what is being plotted in all the figures, means, medians, SDs, SEs etc etc]

Total nitrogen (TN) in water was determined as sum of nitrate and total Kjeldahl nitrogen (estimate of organic N and NH_4^+) and it ranged from 0.78 to 1.3 mg L^{-1} with MA and HS showing higher concentrations than KL and BC sites (Figure 6). Change in TN values with distance away from headsprings was similar to that observed for nitrate concentrations.

Ammonium concentrations in water were ten times lower than nitrate concentrations and ranged from 0.013 to 0.025 mg L^{-1} (Figure 7). Also, the observed trend in ammonium concentrations with distance from headsprings was not similar to nitrate and TN concentrations. These results suggest that the majority of the N in water was in nitrate form and conforms to what has been reported earlier (Wetlands Solution Inc., 2005)

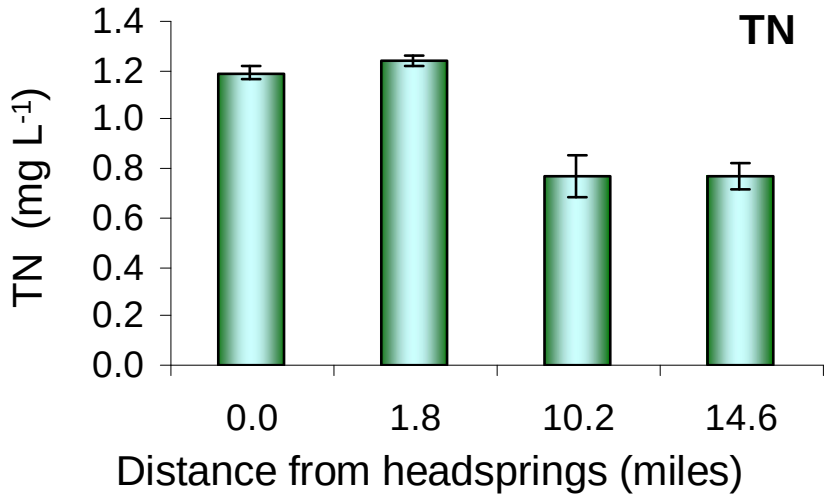

Figure 6. Total nitrogen in the Wekiva River at 4 downstream from the headsprings.

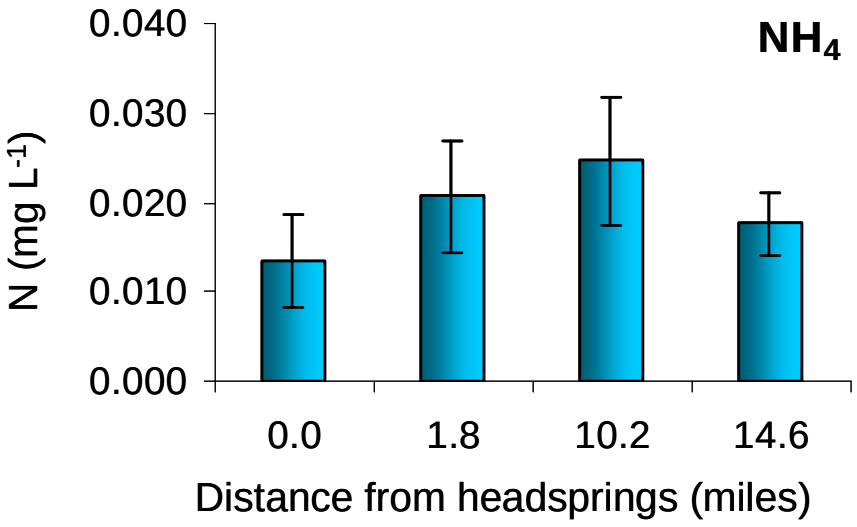

Figure 7. Ammonium concentrations in the Wekiva River at 4 sites downstream from the headsprings.

Although the nitrate content did not vary much within the two sites (HS and MA) that were close to the headsprings, the relative proportions of N forms were different (Figure 8).

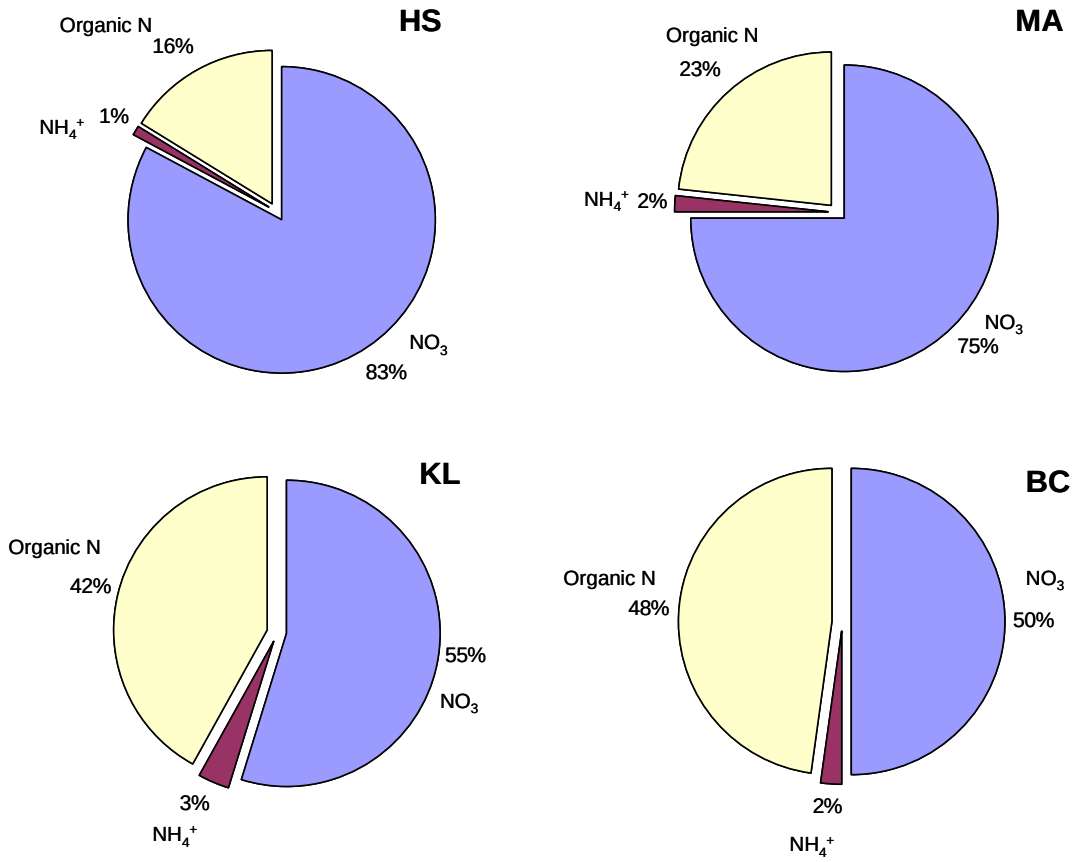

Figure 8. Relative proportions of N forms present at four sampling sites located at increasing distance from head springs.

Observed increase in relative proportion of organic N may be due to removal of NO_3-N from the water column.

Total P in the Wekiva River did not vary much at the four sampling sites HS (0.14±0.01 mg P L^{-1}), MA (0.11 ±0.01 mg P L^{-1}), KL (0.11 mg P L^{-1}), and BC (0.11±0.01 mg P L^{-1}). Most of the total P was present in its dissolved fraction (Figure 9).

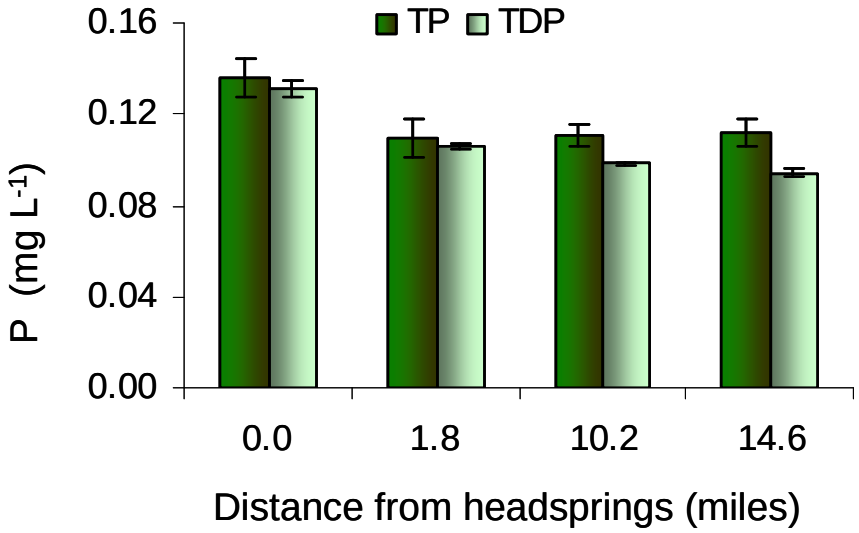

Figure 9. Total and dissolved phosphorus concentrations in the Wekiva River with increasing distance from the headsprings. TP, total phosphorus; TDP, total dissolved phosphorus.

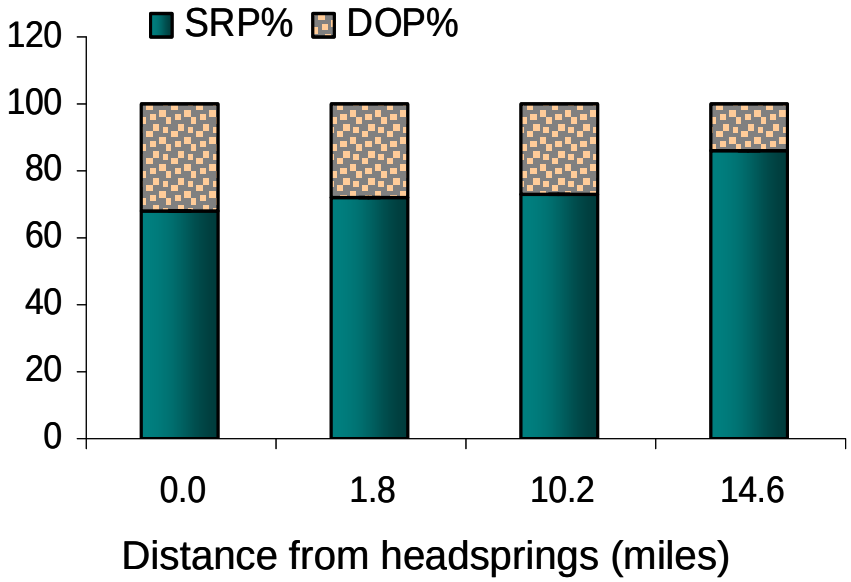

Figure 10. Percentage fraction of dissolved phosphorus concentrations in Wekiwa Spring with increasing distance from the head springs. SRP, soluble reactive phosphorus ; DOP, dissolved organic phosphorus.

Fractions of P in the dissolved form were measured as soluble reactive phosphorus (SRP) and dissolved organic P (DOP). Relative proportions of the inorganic P increased with increasing distance from headsprings (Figure 10).

Dissolved organic carbon (DOC) content increased with distance away from Wekiwa headsprings (Figure 11). Average DOC content in Wekiwa Springs ranged from 0.8 to 3.8 mg L^{-1} with higher DOC of 3.8 mg L^{-1} and 1.9 mg L^{-1} in KL and BC sites respectively.

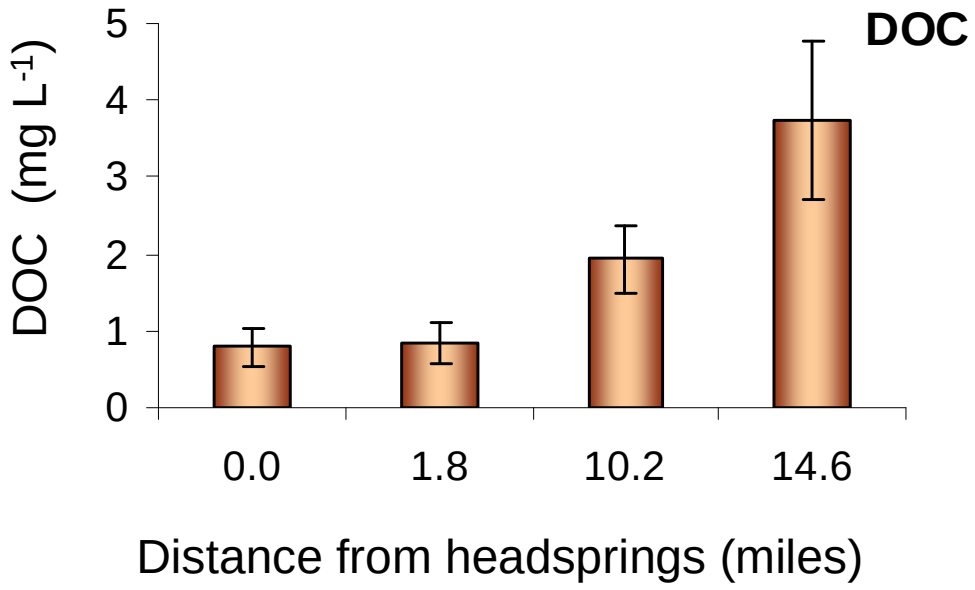

Figure 11. Dissolved organic carbon concentrations in the Wekiva River at 4 sites downstream from the head springs.

4.3 Enzyme Activity

4.3.1 Leucine Aminopeptidase (LAP) Activity

Leucine aminopeptidase (LAP) activity is widely distributed in aquatic systems and is mainly associated with heterotrophic microorganisms (Rego et al., 1985; Jacobsen and Rai, 1988). Utilizable dissolved organic matter constituents have shown to repress and inhibit this enzyme activity (Chrost, 1991). This is mainly because in the presence of low weight organic matter substrates, production of enzymes to degrade proteins and peptides is not necessary therefore their production is not induced. Low LAP activity, as measured at BC and KL sites may be explained by presence of higher organic matter in

water (Figure 12). Conversely, higher LAP activity in HS site may be induced to acquire carbon substrates for bacterial metabolism (Harbott and Grace, 2005). Although it has also been shown that proteinase production by bacteria is maximal under nitrogen limiting conditions (Wouters and Bieysman, 1977) and presence of inorganic N (nitrate or ammonium) repress the LAP enzyme, this does not appear to be the case here.

Increased LAP enzyme activity in presence of high nitrate concentrations suggests that other factors may be regulating the production of this enzyme. Low DOC levels at HS site further supports the plausible explanation that LAP activity in HS site may be for acquiring C instead of N. Although the LAP assay provides only potential information on proteolytic activity, increased presence of this enzyme stresses its importance in C-cycling by heterotrophs in this system or /and the non specific extracellular hydrolytic activity. Results have to be interpreted cautiously because these enzyme activities have been dry weight normalized which does not imply that the microbial numbers are the same in every sample. It is very likely that if specific enzyme activities were normalized by the number of bacteria a different pattern of enzymatic response would be found. However, for the purpose of this study, it is more relevant to report the results as per the activity per gram dry weight because it implies the activity of mixed microbial consortia in bulk sample as it exists in environment.

4.3.2 Nitrate Reductase Activity

In this study, NR activity was detected at all four sites and the variability within biofilms from different substrata within each site was high. Under identical conditions, different species can exhibit different NR activities (Berges et al., 1995) and therefore species composition can greatly influence the spatial distribution of NR activity (Martinez et al., 1987). High variability of NR activity within samples from same site may be due to the difference in dominant species present. Mean values for NR activity were 0.70±0.5 µmol N g^{-1}dw h^{-1}(n=15), 0.16 ±0.08 µmol N g^{-1} dw h^{-1}(n=6), 0.03 µmol N g^{-1}dw h^{-1} (n=3), and 0.18±0.2 µmol N g^{-1}dw h^{-1} (n=9), in algal biofilms from different strata at HS, MA, KL, and BC sites respectively (Figure 13). NR activity in sediments at

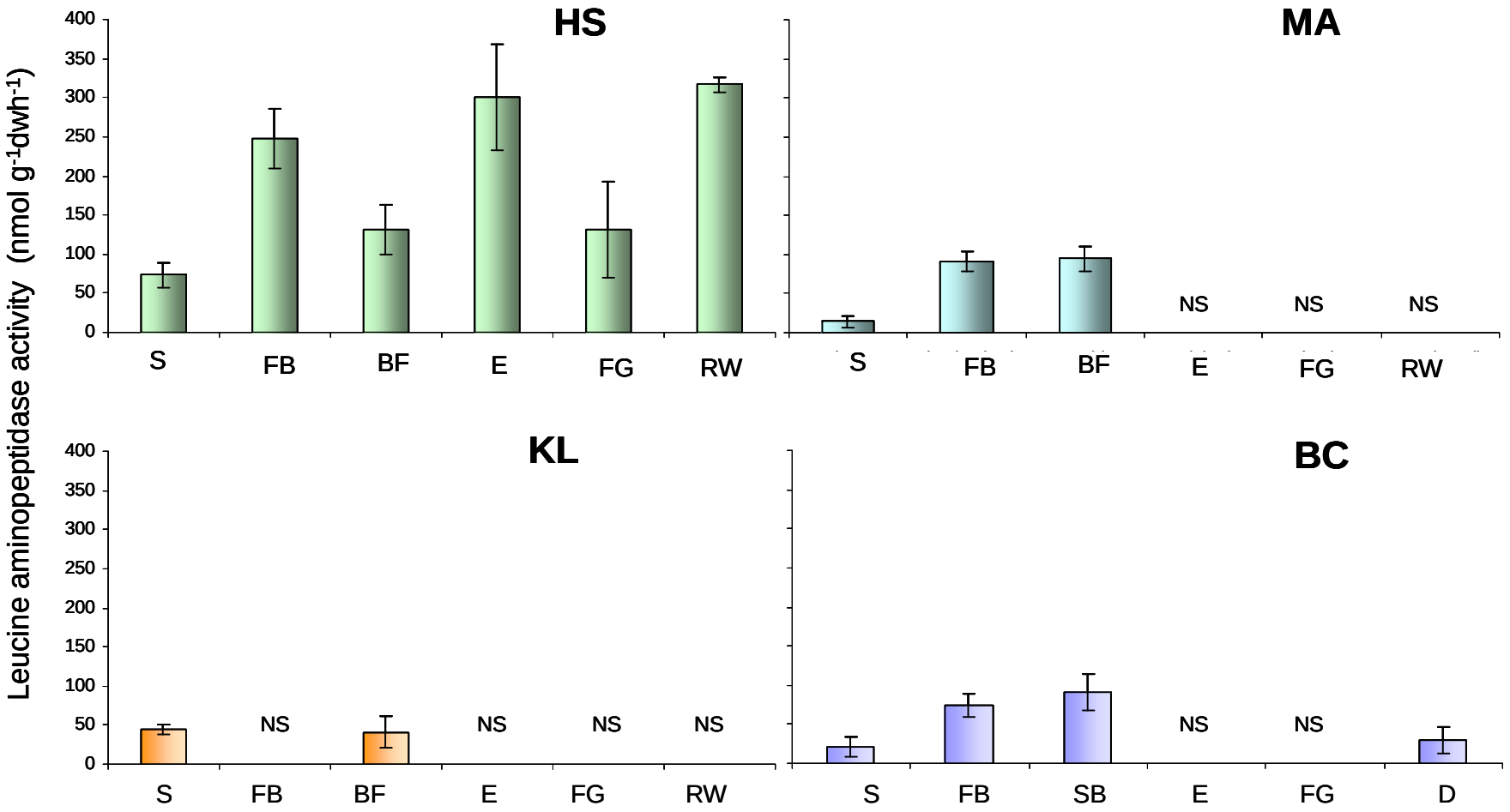

Figure 12. Leucine aminopeptidase activity in sediment, and algal biofilms collected from various substrata. S, sediment; FB, filamentous benthic algae; BF, Brown filamentous algae; E, epiphytic biofilms; FG, floating green algae-microbial biofilms; RW, Biofilms attached to rock wall; D, detrital material; SB, algal biofilm on submerged bark

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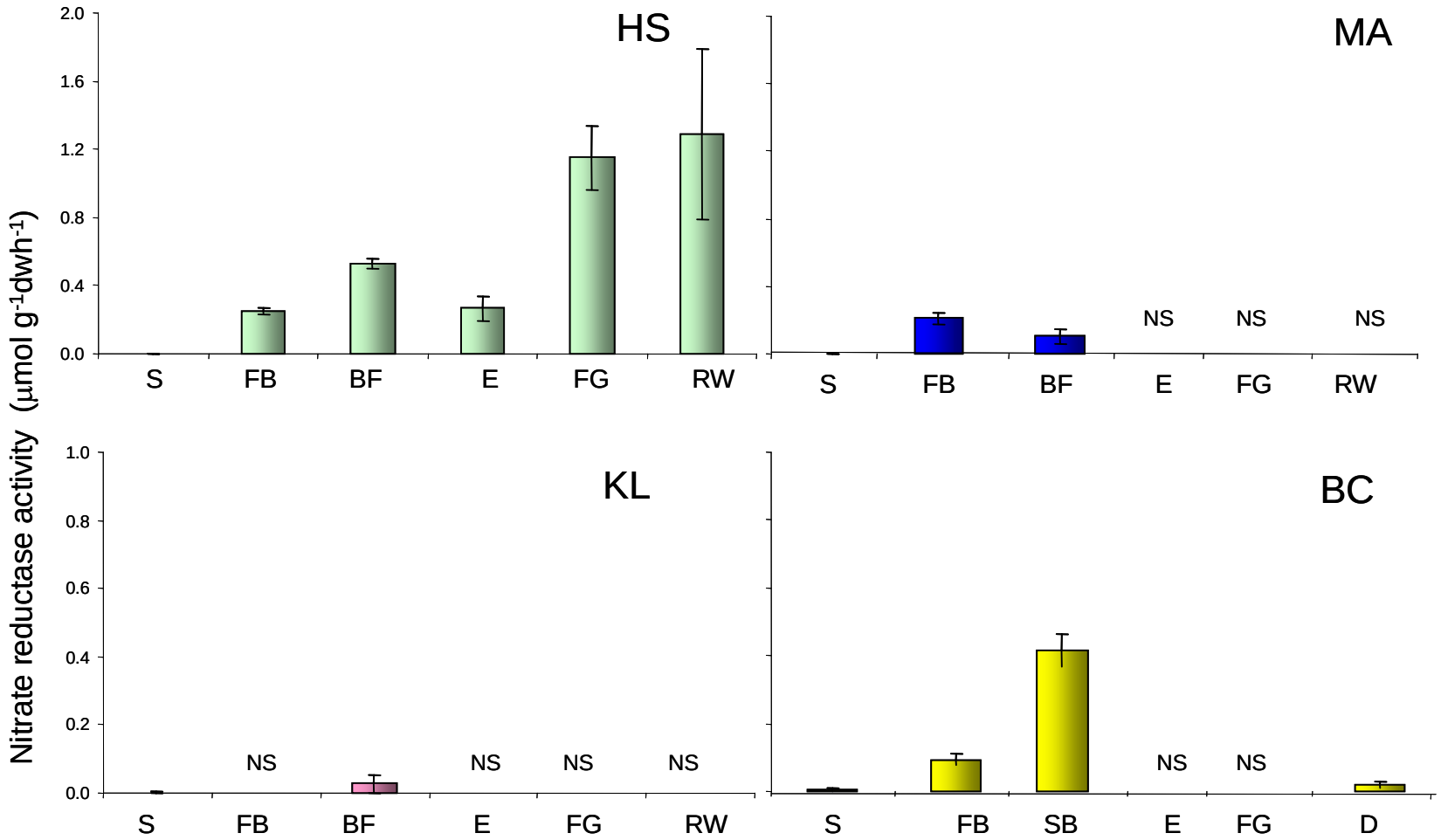

Figure 13. Nitrate reductase activity (µmol NO2 produced g-1dwh-1)in sediment, and algal biofilms collected from various substrata. S, sediment; FB, filamentous benthic algae; BF, Brown filamentous algae; E, epiphytic biofilms; FG, floating green algae-microbial biofilms; RW, Biofilms attached to rock wall; D, detrital material; SB, algal biofilm on submerged bark

.

all four sites was very low (mean values of 2 nmol N g^{-1}dw h^{-1}). Within HS site, biofilms on rock wall and that floating on water surface showed higher NR activity (1.3 µmol N g^{-1}dw h^{-1}).

Several studies have shown that the presence of nitrate induces NR activity while ammonium can act as a suppressor (Eppley et al., 1970; Berges and Harrison, 1995). NR activity in algal biofilms at HS site was higher than KL and BC sites and nitrate-N concentrations were also higher in HS (0.98±0.2 mg L^{-1}) when compared to those in KL (0.42±0.2 mg L^{-1}) and BC (0.39±0.01 mg L^{-1}) sites. It is possible that nitrate concentration influences NR activities; however, low NR activities in MA site cannot be explained by nitrate concentrations alone. High nitrate concentrations at MA would support higher NR activity, however these results showed lower activity.

It is possible that ammonium concentrations may be inhibiting the NR activity at this site. Alternatively, preference of ammonium as N source by these organisms may also be affecting the NR activity. Packard and Blasco (1974) demonstrated that a concentration as low as 2.8 µg L^{-1} of NH_4-N can inhibit the NR activity. In this study, we measured NR activity in presence of ammonium concentration as high as 25 µg NH_4-N L^{-1}. Due to high variability in NR activity in biofilms collected from different substrata, within a site, we correlated the average means of NR activity from each site to the ammonium concentrations present at the site. Correlation between the NR activity and various forms of N content are shown in Table 4.

Table 4: Correlation coefficients (r), and probabilities (p) between NR activity* and nitrate concentrations, ammonium concentrations, and NO3/total N, NH4/ total N, and NO3/NH4 ratios from 4 sites in the Wekiva River.

	Correlation (r)	*P*
NO_3	0.67	>0.1
NH_4	-0.90	0.09
NO_3/TN	0.74	>0.1
NH_4/TN	-0.82	>0.1
NO_3/NH_4	0.92	0.08

*Mean NR activity of all samples was taken to represent the NR activity of each site.

Although our results suggest that ammonium concentrations may be regulating the nitrate reductase activity in the Wekiva River, factors other than ammonium and nitrate may be influencing the uptake of nitrate and the NR activity (Wynne and Berman, 1990).

Results from this study suggest that the organisms in HS site are activity utilizing nitrate as N source. It is possible that the biofilms that exist in this part of the system have nitrate preferring organisms as dominant species and differ from those that exist in the MA site.

4.3.3 Alkaline Phosphatase Activity

Although inorganic P concentrations were high at all four sites (HS, 0.09±.01 mg P L^{-1}; MA, 0.08±.01 mg P L^{-1}; KL, 0.07±.01 mg P L^{-1}; and BC, 0.08 ±.001 mg P L^{-1}) AP activity was detected at sites (Figure 14). In most aquatic systems, bioavailable phosphorus (P) is considered as the most limiting nutrient for both algae and microorganisms. In order to meet their demands for P, organisms can produce the phosphatase enzyme to acquire bioavailable P from organic P sources. Several studies have used AP activity as an indicator for P limitation in aquatic systems (Newman and Reddy, 1993) where there is generally a negative correlation between SRP levels and the AP activity. In this study, however, we did not observe any correlations between AP activity and any measured P parameters.

Within all sites, average AP activity (mean value from AP activities expressed by all biofilm types from each site) was observed to be higher in MA (71.4 ±32 nmol $Pg^{-1}dw\ h^{-1}$) when compared with those in HS (18.8 ±9.6 nmol P $g^{-1}dw\ h^{-1}$), KL (17 ±7.7 nmol P $g^{-1}dw\ h^{-1}$) and BC (25 ±11 nmol P $g^{-1}dw\ h^{-1}$). High SRP levels in MA indicated presence of bioavailable P, but high AP activity suggested that either organisms were still P-limited or there were other factors controlling the AP production.

In the past nutrient ratios have been used to indicate nutrient limitation in aquatic systems, for instance N:P ratios (total and/or dissolved) have been used in many studies to indicate the potential limitations of these nutrients (Dodds, 2002). Either total or dissolved nutrient ratios can indicate the potential limitation of nutrients. In our study, TN :TP ratio (by weight) was <10 for all three sites, HS, KL, and BC except in MA

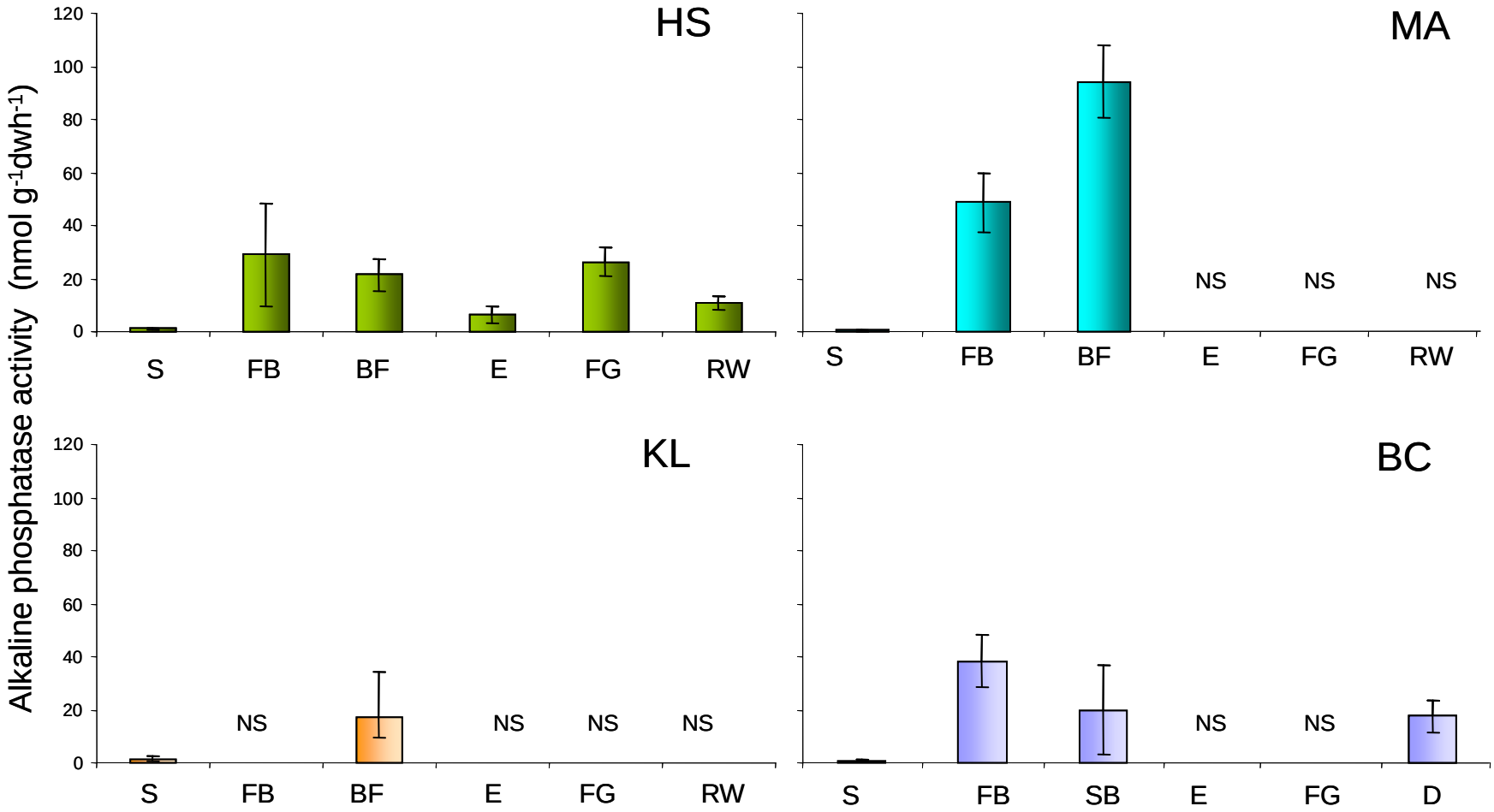

Figure 14. Alkaline phosphatase activity in sediment, and algal biofilms collected from various substrata. S, sediment; FB, filamentous benthic algae; BF, Brown filamentous algae; E, epiphytic biofilms; FG, floating green algae-microbial biofilms; RW, Biofilms attached to rock wall; D, detrital material; SB, algal biofilms on submerged bark.

(11.4± 0.9) (Figure 15). These values suggest that there is N limitation in sites except MA, however with such high nitrate concentrations as those that exist in the Wekiva River, it is highly unlikely. However, the DIN: DIP ratio (molar) in HS and MA (>24) and those in KL and BC (<14) suggests that there is potential P-limitation at HS and MA sites.

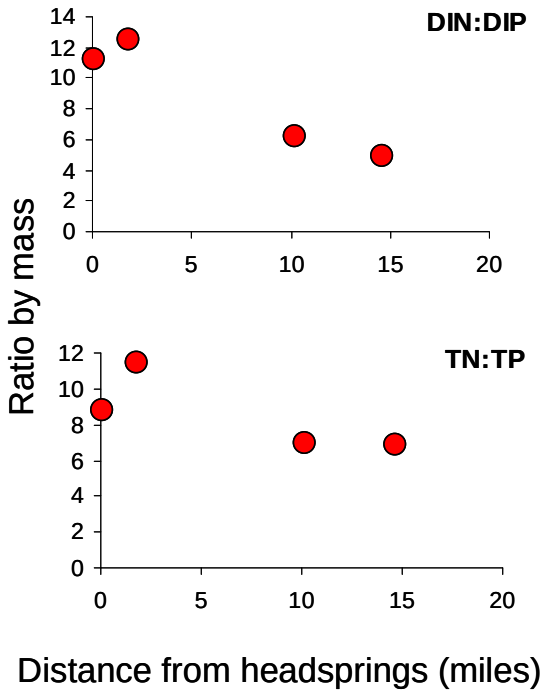

Figure 15. Nutrient ratios (by weight and by moles) in the Wekiva River with increasing distance from the headsprings. DIN, dissolved inorganic nitrogen; DIP, dissolved inorganic phosphorus.

High AP activities in biofilms at MA site supports the potential P-limitation. A possible explanation of lower AP activity at HS site is low available carbon (DOC). If organisms are limited by carbon, they will not produce AP as enzyme production is a high energy consuming process. These results also suggest that the organisms producing AP at HS site may be mostly heterotrophic. AP production at the two sites (KL and BC) that have a lower N:P ratio may be regulated by other factors than P availability.

Fate of nitrogen in an aquatic system involves biological transformations. Removal of nitrate from water column can occur through assimilation into microbial or algal biomass, or through respiratory denitrification by bacteria. This latter process is particularly important because denitrification permanently removes N from the system. Potential denitrification rates in Wekiva ecosystem were measured in sediments and in biofilms (Figure 16). Based on these results, it is difficult to conclude if there was any relationship between the denitrification rate and the distance from the headsprings. Nevertheless it is possible to state that denitrification appears to be one of the pathways for N removal from this system. All sites, showed the potential of denitrification with higher denitrification rates observed in biofilm samples on floating bark surface at HS site. Rates of denitrification ranged from 10 to 170 nmol N_2O produced g^{-1} dw h^{-1}. It may be interesting to note that although the HS site appeared to be C limited, denitrification potential was high. One possible explanation for these results may be that the denitrifying bacteria are autotrophic or are dependent on autotrophic bacteria for C source. Rates of denitrification reported in wetlands range from 0.5 to 20 mg N_2O kg^{-1} soil h^{-1} in soils within depth of 0-30 cm (White and Reddy, 2003), 15-130 nmol N cm^{-3} d^{-1} in estuarine sediments (Joye et al., 1996), and 1.9 mmol N_2O m^{-2} h^{-1} in periphyton mats (Triska and Oremland, 1981).

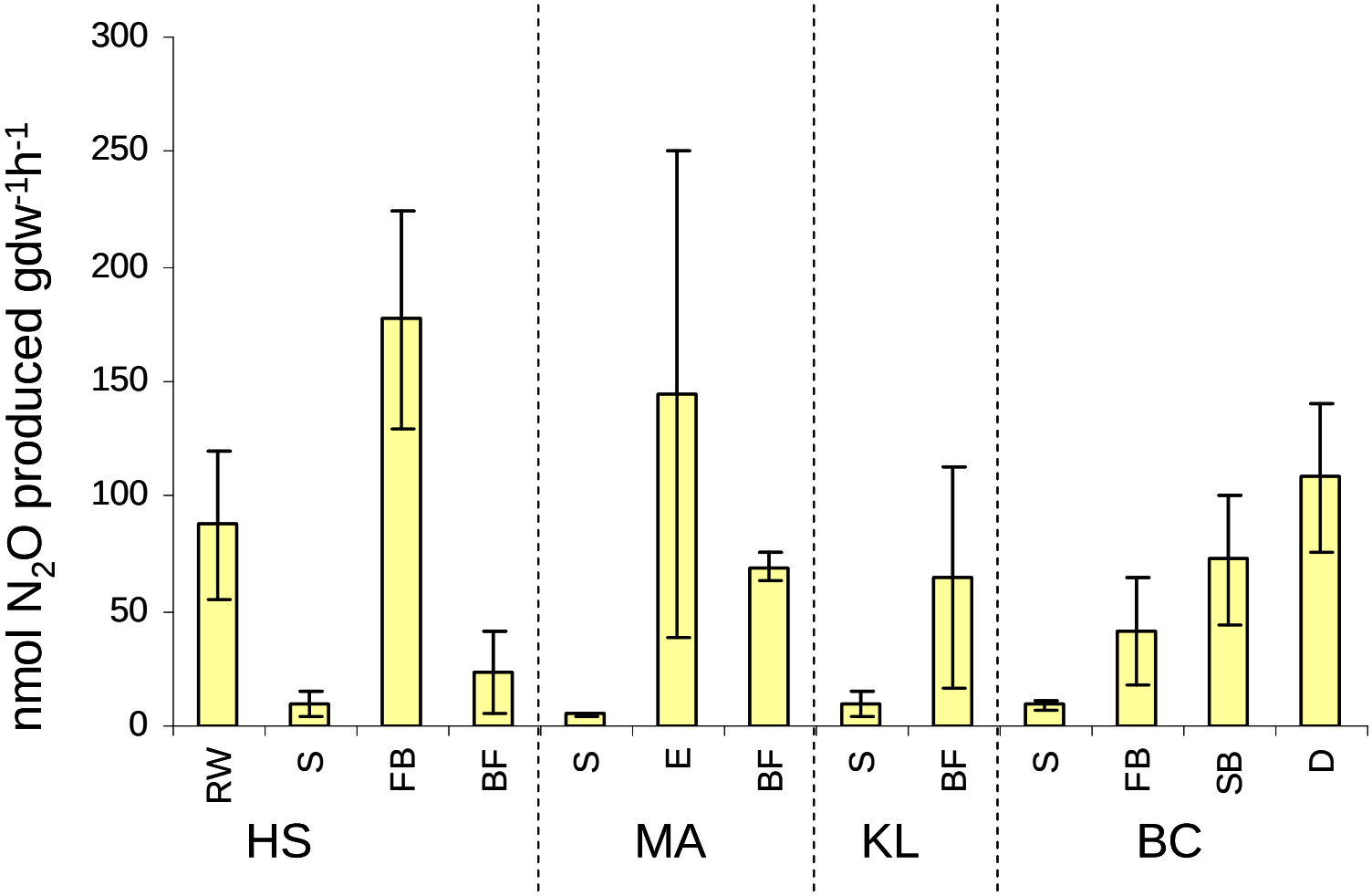

Figure 16. Denitrifying potential in sediments, and algal biofilms collected from various substrata. S, sediment; FB, filamentous benthic algae; BF, Brown filamentous algae; E, epiphytic biofilms; RW, Biofilms attached to rock wall; D, detrital material; SB, algal biofilm on submerged bark.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Results from this preliminary study are based on a one time sampling event conducted October 2006. Enzyme assays were based on grab samples collected from four locations (in Wekiva River) that exist at increasing distance from the Wekiwa headsprings. Water nutrient measures indicated that nitrate concentrations decline by more than 50% within 15 miles from the head springs. Among the microbial processes that appear to contribute to NO_3 removal from an aquatic system is nitrate assimilation by biota and nitrate reduction to dinitrogen via denitrification. By measuring NR activity we showed the potential of NO_3 assimilation was much higher near the headsprings.

Although dissolved organic nitrogen content did not vary at four sampling sites in the Wekiva River, the aminopeptidase activity was distinctly higher near the headsprings and this suggests that heterotrophic organisms produced this enzyme for acquiring C, in response to low dissolved organic content. It this were true, the HS site may be C-limited.

Relative proportions of N forms changed with distance from the headsprings thereby suggesting that the forms of N may be influencing the functional role of microorganisms that exist in that environment.

Unlike HS, organisms in biofilms at the other high nitrate-N site, MA appeared to be P-limited with higher alkaline phosphatase production. This study resulted in some interesting observations that warrant further studies.

Based on the information obtained from this study, addressing the following research questions may provide an insight to factors contributing to the change in ecology in Wekiva ecosystem. This information it will allow us to make decisions concerning the restoration and maintaining the Wekiwa ecology.

1. To understand the biogeochemical cycling of nutrients:
    - Controlled experiments with nutrient (C, N, P) additions to study the changes in microbial structure and function.
    - Role of heterotrophic and autotrophic bacteria in fates of C, N, P.
2. Understand the fate of nitrate in the Wekiva River.
3. To study factor interactions:
    - Effect of dissolved organic matter (high plant DOM, high organic material)
    - Effect of spring run flow rates on the above mentioned processes.

6. ACKNOWLEDGEMENTS

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WBL#	Distance from Wekiwa headsprings	Field ID	TKN	TDKN	TP	TDP	NOx-N	NH4-N	SRP	DOC	DON	TN	DOP	DOC:DON	DON:DOP	DOC:DOP	TN:TP	DIN:DIP	DIN
	miles		mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
1	0.05	HS-1	0.20	0.20	0.15	0.13	0.97	0.019	0.096	1.02	0.18	1.16	0.03	5.73	5.80	33.27	7.99	10.23	0.98
2	0.05	HS-2	0.20	0.25	0.13	0.13	0.99	0.012	0.080	0.84	0.24	1.19	0.05	3.46	4.66	16.10	8.84	12.60	1.00
3	0.05	HS-3	0.23	0.25	0.13	0.13	1.00	0.009	0.092	0.51	0.24	1.22	0.04	2.08	5.89	12.23	9.55	10.88	1.01
4	1.80	MA-1	0.28	0.25	0.11	0.11	0.94	0.018	0.069	1.13	0.24	1.22	0.04	4.77	6.35	30.31	11.51	13.92	0.96
5	1.80	MA-2	0.34	0.28	0.10	0.10	0.92	0.016	0.078	0.67	0.27	1.26	0.03	2.50	10.28	25.69	12.29	11.94	0.93
6	1.80	MA-3	0.31	0.28	0.12	0.11	0.93	0.028	0.083	0.70	0.25	1.24	0.02	2.77	10.41	28.81	10.44	11.54	0.95
7	10.20	KL-1	0.42	0.34	0.11	0.10	0.44	0.020	0.078	1.95	0.32	0.86	0.02	6.10	15.05	91.84	7.65	5.90	0.46
8	10.20	KL-2	0.34	0.34	0.11	0.10	0.41	0.033	0.074	1.48	0.31	0.75	0.02	4.83	12.34	59.58	6.59	6.06	0.45
9	10.20	KL-3	0.28	0.28	0.11	0.10	0.41	0.021	0.064	2.38	0.26	0.70	0.03	9.10	7.83	71.21	6.59	6.78	0.43
10	14.64	BC-1	0.45	0.28	0.11	0.09	0.38	0.016	0.080	4.08	0.27	0.83	0.01	15.32	17.86	273.55	7.49	4.97	0.40
11	14.64	BC-2	0.37	0.31	0.12	0.10	0.39	0.015	0.082	4.55	0.30	0.76	0.01	15.39	22.31	343.32	6.37	4.89	0.40
12	14.64	BC-3	0.34	0.34	0.11	0.09	0.39	0.022	0.082	2.60	0.32	0.73	0.01	8.19	30.78	252.11	6.82	5.00	0.41

WBL#	Distance from Wekiwa headsprings	Field ID	Sample	APA	NR	LAP
	miles			nM MUF g^{-1}DW h^{-1}	uM NO2 g^{-1}DW h^{-1}	nM MCA g^{-1}DW h^{-1}
1	0.05	HS-1	RW	11.0	1.277	306.26
2	0.05	HS-2	RW	8.8	1.761	322.56
3	0.05	HS-3	RW	13.2	0.827	321.01
4	0.05	HS-4	S	0.8	UDL	88.84
5	0.05	HS-5	S	0.9	UDL	68.72
6	0.05	HS-6	S	1.5	UDL	61.89
7	0.05	HS-7	BF	23.7	0.558	154.98
8	0.05	HS-8	BF	16.1	0.521	96.85
9	0.05	HS-9	BF	25.6	0.480	140.82
10	0.05	HS-10	FG	22.8	1.230	188.72
11	0.05	HS-11	FG	24.7	0.930	115.15
12	0.05	HS-12	FG	31.8	1.268	90.02
13	0.05	HS-13	FB	43.3	0.254	274.39
14	0.05	HS-14	FB	15.3	0.226	209.89
15	0.05	HS-15	FB	29.0	0.276	262.03
16	0.05	HS-16	E	4.8	0.269	344.00
17	0.05	HS-17	E	10.1	0.340	222.82
18	0.05	HS-18	E	4.3	0.198	336.87
19	1.80	MA-1	S	0.8	UDL	18.91
20	1.80	MA-2	S	0.3	UDL	6.74
21	1.80	MA-3	S	0.6	UDL	14.61
22	1.80	MA-4	BF	51.7	0.149	76.83
23	1.80	MA-5	BF	38.1	0.086	108.50
24	1.80	MA-6	BF	54.9	0.073	97.98
25	1.80	MA-7	FB	104.8	0.207	102.01
26	1.80	MA-8	FB	80.0	0.188	94.11
27	1.80	MA-9	FB	98.3	0.232	78.50
28	1.80	MA-10	E	NES	NES	NES
29	1.80	MA-11	E	NES	NES	NES
30	1.80	MA-12	E	NES	NES	NES
31	10.20	KL-1	S	2.0	UDL	48.41
32	10.20	KL-2	S	2.1	UDL	37.27
33	10.20	KL-3	S	0.8	UDL	46.74
34	10.20	KL-4	BF	11.2	0.017	28.07
35	10.20	KL-5	BF	14.9	0.048	31.81
36	10.20	KL-6	BF	25.5	0.017	63.34

WBL#	Distance from Wekiwa headsprings	Field ID	Sample	APA	NR	LAP
	miles			nM MUF g^{-1}DW h^{-1}	uM NO2 g^{-1}DW h^{-1}	nM MCA g^{-1}DW h^{-1}
37	14.64	BC-1	S	0.6	UDL	18.97
38	14.64	BC-2	S	1.0	UDL	35.27
39	14.64	BC-3	S	1.2	UDL	10.61
40	14.64	BC-4	SB	32.8	0.458	65.94
41	14.64	BC-5	SB	6.5	0.420	108.96
42	14.64	BC-6	SB	18.3	0.369	97.96
43	14.64	BC-7	FB	28.0	46.018	90.88
44	14.64	BC-8	FB	48.4	46.721	70.79
45	14.64	BC-9	FB	39.5	91.085	62.95
46	14.64	BC-10	D	12.7	0.022	21.32
47	14.64	BC-11	D	17.1	0.014	18.70
48	14.64	BC-12	D	24.1	0.021	48.30

UDL*	under detection limit
NES	not enough sample