Using Cyanobacteria and Macroalgae Stable Isotopes as Anthropogenic Point and Non-Point Source Nutrient Indicators

Southeast Florida Coral Reef Initiative
Land Based Sources of Pollution
Local Action Strategy Project 32

Using Cyanobacteria and Macroalgae Stable Isotopes as Anthropogenic Point and Non-Point Source Nutrient Indicators

Final Report

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expressed herein are those of the authors and do not necessarily reflect the views of the State of Florida, U.S. Department of Commerce, NOAA or any of its subagencies.

Table of Contents

List of Figures

List of Tables

List of Acronyms

ANOVA	Analysis of Variance
BCWO	Broward County Wastewater Outfall
CRCP	Coral Reef Conservation Program
DIN	Dissolved Inorganic Nitrogen
EPA	Everglades National Park
FDEP	Florida Department of Environmental Protection
FRRP	Florida Reef Resilience Program
HI	Hillsboro Inlet
HWO	Hollywood Sewage Outfall
IAEA	International Atomic Energy Agency
IRMS	Isotope Ratio Mass Spectrometers
LBSP	Land-Based sources of Pollution
NMDS	Non-Metric Multidimensional Scaling
PE	Port Everglades Inlet
SERC	Southeast Environmental Research Center
SIL	Stable Isotope Laboratory
SEFCRI	Southeast Florida Coral Reef Initiative

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Executive Summary

In order to better understand and manage the benthic resources of southeast Florida, links between sources and targets of land-based pollution need to be established through cause-effect demonstrations. To date, the different influences of anthropogenic point source nutrients, anthropogenic non-point source nutrients, and natural nutrient sources contributing to cyanobacteria and macroalgae blooms occurring in reefs along the eastern coast of Florida remain unresolved. Past studies have demonstrated that macroalgae and seagrass $\delta^{15}N$ and $\delta^{13}C$ values have been shown to closely reflect the isotopic composition of nitrogen and carbon sources, and thus can serve as tracers for source nutrients within the coastal system. The use of isotopic "proxies" can potentially allow for more accurate identification of the nutrient sources available to primary producers, specifically the filamentous cyanobacteria *Lyngbya*, which are capable of assimilating nitrogenous compounds, as well as fixing atmospheric N_2 and $CO_2(aq)$ and HCO_3^-. This work with *Lyngbya* spp. focused sampling efforts along the Broward County outer reef. The main objective of this project was to determine if the stable isotopic composition of *Lyngbya* spp. can be used as a reliable indicator of nitrogen derived nutrients from waste water effluent; from wastewater outfalls and storm-water runoff from inlets along the Intracoastal Waterway.

Field collections of cyanobacteria and algal samples were conducted at three different times during the summer peak productivity period of *Lyngbya* spp. from 2010 to 2011. Samples were recovered from both theoretically perturbed and nutrient enriched sites near wastewater outfalls and major inlets, and compared to control locations. Site selection was based on the Southeast Florida Coral Reef Initiative (SEFCRI) Biomarker Sites in conjunction with Broward County (Futch et al., 2011). Perturbed sites included the Hollywood sewage outfall (HWO2 & HWO3), Port Everglades Inlet (PE2 & PE3), Hillsboro Inlet (HI2 & HI3), which is adjacent to the Broward County Wastewater Outfall, BCWO. Two control sites were also sampled, which include: C2 & FTL3, which are located midway between the Port Everglades and Hillsboro Inlets; and two sites 20 km north of the Hillsboro Inlet (FRRP 1508 and 1517).

Isotopic results were variable for all the cyanobacteria and algal samples and did not correlate with nutrient levels measured in the water column. *L. polychroa* had the highest amount of isotopic variability throughout the study with averages varying more than 3‰. *L. confervoides* had isotopic values very similar to those of the non N_2 fixing brown macroalgae *Dictyota* spp. The nitrogen isotopic values measured in this study are not outside previously measured natural sources. There is a clear indication that *L. polychroa* switches between N-fixation and DIN

assimilation caused by the variability in values when comparing June and July sampling to August (2010). The nitrogen isotopic values are most depleted in June 2011, with the majority of samples between 0‰ to 2‰. However, our approach to measure N-fixation showed both species in July 2011 were not fixing N. Thus the species are variable depending on temporal and potentially spatial (environmental) conditions.

Statistical analyses of the isotopic data were conducted with non-metric multidimensional scaling (NMDS) analysis. This analysis used both $\delta^{15}N$ and %N as factors to look for patterns between individual species and between sites. These data were also analyzed using one-way ANOVA for the effects of site and species on each factor ($\delta^{15}N$ and %N). Species had a significant effect on both $\delta^{15}N$ and %N ($p<0.001$ for both). Site also had a significant effect on both $\delta^{15}N$ and %N values ($p = 0.026$ and $p < 0.001$, respectively). However, the NMDS plot of samples collected at different sites shows no grouping according to site. The effect of site on $\delta^{15}N$ and %N was likely driven by the different species which were collected at each site.

Growth experiments with *L. confervoides* and *L. polychroa* with different treatment water from near the Hollywood Outfall, Port Everglades Inlet and an offshore control site indicated only one species responded with increased growth, *L. confervoides.* When the two species were analyzed separately by one-way ANOVA, there was no effect of treatment water on growth of *L. polychroa*, but there was a significant effect of the different treatment waters on growth of *L. confervoides.* However, the growth of cyanobacteria in control water did not differ significantly from that grown in either the Hollywood Outfall or PE Inlet water. This result could be caused by the fact the selected control water for this experiment had higher DIN values then either the Hollywood Outfall or PE Inlet water. These results suggest some intriguing responses in the growth of different *Lyngbya* spp. in seawater from different sites and should be repeated.

This preliminary study shows that *Lyngbya* and the other primary producers collected for this project were not recording primary sewage wastewater outfalls, or non-point sources from the inlets. Although samples collected from different sites were significantly different in their $\delta^{15}N$ and %N values, this difference was likely driven by the collection of different algae and cyanobacteria from different sites. *Lyngbya* spp. appear to be highly opportunistic and are capable of using different nutrient sources. *L. confervoides* and *L. polychroa* $\delta^{15}N$ values not only show variability, but also the potential for N-fixation. This variability changes temporally, as the same species appear to be fixing N during one month, but not the next. Future isotopic biogeochemical work with *Lyngbya spp.* should focus on longer temporal studies to further document how variable these cyanobacteria

are across the Broward County coastal system. In general, sessile benthic organisms, and especially primary producers, are excellent recorders of environmental waters, however *Lyngbya spp*. has proven to be a complex organism to study.

1.0 Introduction

In order to better understand and manage the benthic resources of southeast Florida, links between sources and targets of land-based pollution need to be established through cause-effect demonstrations. To date, the differential influences of anthropogenic point source nutrients (sewage outfalls), anthropogenic non-point source nutrients (ground-water discharge through sediments and surface-water run-off into coastal inlets) and natural nutrient sources (primary productivity and seasonal, wind-driven upwelling events) contributing to cyanobacteria and macroalgae blooms occurring in reefs along the southeastern coast of Florida remain unresolved. Macroalgae and seagrass $\delta^{15}N$ and $\delta^{13}C$ ratios have been shown to closely reflect the isotopic composition of nitrogen and carbon sources, and thus serve as tracer for nutrients within some systems and organisms (Raven et al. 2002, Anderson and Fourqurean 2003, Fourqurean et al., 2005). The employment of isotopic "proxies" will potentially allow for more accurate identification of the nutrient sources available to primary producers, specifically the filamentous cyanobacteria *Lyngbya*, which are capable of assimilating nitrogenous compounds, as well as fixing atmospheric N_2 (Paul et al. 2005). This work with *Lyngbya* spp. focused sampling efforts along the Broward County outer reef and followed from the work of Paul et al. (2009). The main objective of this project was to determine if the stable isotopic composition of *Lyngbya* spp. can be used as a reliable indicator of nitrogen derived nutrients from waste water effluent; from secondary treated wastewater outfalls and storm-water runoff from inlets along the southeast coast of Florida.

1.1 Stable isotopic composition of marine plants, algae and cyanobacteria

The $\delta^{15}N$ values of cyanobacteria, algae, seagrasses, benthic organisms, particulate organic material, and dissolved inorganic nitrogen in South Florida have been well documented (Barile, 2004; Chasar et al., 2005; Corbett et al., 1999; Fourqurean et al., 2005; Lamb, 2007; Lamb and Swart, 2008; Lapointe et al., 2004; Leichter et al., 2007; Paul et al, 2009). Yet little isotopic work has been completed on *Lyngbya* spp. $\delta^{15}N$ values can be used in combination with other tracers to provide information on the origin and process of nitrogen cycling. Some of these studies attempted to use $\delta^{15}N$ ratios to make the conclusion that there has been input of anthropogenic nitrogen into the coastal ocean, while others have taken a more conservative approach and attempted to explain the patterns observed by other processes. For example, in isotopic studies in Florida Bay, highly enriched $\delta^{15}N$ values were found in the northeastern portion of the bay (Corbett et al., 1999). Past investigations suggested that the elevated values might be a result of the input of ^{15}N enriched groundwater (Corbett et al., 1999). However, to test this

hypothesis, samples of ground water must be collected and measured for the isotopic composition of dissolved inorganic nitrogen (DIN). Further studies by some of the same authors appear to discount this idea (Fourqurean et al., 2005), as nutrient limitation can also cause isotopic enrichment from one region relative to another (or period of time). In contrast, McClelland and Valiela (1998) demonstrated that estuarine sub-aquatic vegetation $\delta^{15}N$ values were correlated to nitrogen derived from wastewater within the local watershed, which was relatively isotopically enriched (>8‰ typically).

A major issue with using $\delta^{15}N$ as a tracer for the origin of nitrogen is that the $\delta^{15}N$ values can be affected by a wide number of processes during assimilation, nitrification, and denitrification. Nevertheless a great majority of papers reporting $\delta^{15}N$ data ignore the fractionation factors involved in these processes. However, these fractionation factors are rather poorly constrained due to the difficulty in measuring within experimental conditions, which often do not replicate the natural environment. For example, consider the fractionation of NH_4^+ and NO_3^- during assimilation; published values for the assimilation of NO_3^- range from 1.000 to 1.012 and for NH_4^+ from 0.99 to 1.008 (Lajtha and Michener, 1994). Based on these data it would be difficult to say whether there is any definitive isotopic effect during assimilation. When considering a nutrient limited natural setting there should be an inverse relationship between $\delta^{15}N$ of a primary producer (e.g., algae) and the concentration of DIN. Yet primary producers also record their source of nutrients isotopically, and do not always display an inverse isotopic change to nutrient limitation, in fact the opposite has been observed (Corbett et al., 1999). Lamb and Swart (2008) proved that the nitrogen on the Florida Keys reef tract was not derived from anthropogenic sources. Additionally, cyanobacteria may fix N directly from dissolved N_2, N-fixation results in $\delta^{15}N$ values near 0‰, the value of atmospheric N_2 (Hamme and Emerson, 2004; Montoya et al., 1996). The question remains as to whether marine algae and other primary producers' isotopic values reflect nutrient source and/or nutrient limited conditions; environmentally controlled, or affected by productivity.

2.0 Study Site

The southeast Florida reef tract extends from coastal Martin, Palm Beach, Broward, and Miami-Dade counties through the Florida Keys (Monroe County). In contrast to the Florida Keys reef tract, which has an average stony coral coverage of 5.9% (CREMP, 2011), the reef communities of the more northern portions of the reef in Broward County are composed of sponges, gorgonian soft corals with limited stony corals with cover ranges between 0.5% to 2.5% (NOAA, 2008). Nutrient levels and water quality of the Broward reef communities, the

focus of this study, are affected by pollution from multiple point and non-point sources (Futch et al., 2011). Point sources include secondarily treated wastewater from ocean outfalls at the northern (Broward outfall) and southern (Hollywood outfall) portions of the county (Fig. 1), which accounts for ~43% of the Broward County's treated wastewater disposal (USEPA, 2006). Non-point sources of pollution (e.g. nutrients) include all the run-off from the urban area adjacent to the coastal system, which move into the Intracoastal Waterway via rivers and canals (Futch et al., 2011). These waters contain a variety of pollutants such as fertilizers, leaks from septic systems, herbicides and pesticides, which then are exchanged with Atlantic waters at a series of ocean inlets e.g., Port Everglades and the Hillsboro inlet. Lapointe et al. (2005) have shown that these types of nutrient inputs have affected the southeastern Florida coastal system, possibly causing serious blooms of macroalgae, which have affected the reef communities. In order to better understand these perturbations based on sources (point and non-point) we have selected monitoring stations adjacent to the outfalls, inlets and non-perturbed sites (Fig. 1).

3.0 Methods

3.1 Sampling

Field collections of cyanobacteria and algal samples were conducted at three different times during the summer peak productivity period of *Lyngbya* spp. from 2010 to 2011. The goal was to collect samples from theoretically perturbed and nutrient enriched sites near wastewater outfalls and major inlets, and compare these sites to control locations. Our sites were chosen (Figs. 1 & 2) based on the Southeast Florida Coral Reef Initiative (SEFCRI) Biomarker Sites in conjunction with Broward County (Fauth et al., 2006; Futch et al., 2011). Perturbed sites included the Hollywood sewage outfall (HWO2 & HWO3), Port Everglades Inlet (PE2 & PE3), Hillsboro Inlet (HI2 & HI3) (which is adjacent to the Broward County Wastewater Outfall, BCWO). Two control sites were also sampled depending on conditions, which include: C2 & FTL3, which are located midway between the Port Everglades and Hillsboro Inlets; and two sites 20 km north of the Hillsboro Inlet. The first sampling in August 18, 2010 focused on the more northern sites around the Hillsboro Inlet with one site, FRRP 1506 near BCWO and two controls to the north (FRRP 1508 & 1517) (Fig. 2). The second set of samplings occurred on June 17, 2011 and July 21, 2011. These sampling trips concentrated on the southern sites near Port Everglades (PE2 & PE3) and the Hollywood outfall (HWO2 & HWO3), with control sites 7 km north of Port everglades, sites FTL3 & C2 (Fig. 1).

3.2 Field sampling

Surveys were sampled in conjunction with scheduled Southeast Florida Coral Reef Initiative (SEFCRI) Biomarker trips with Broward County. Replicate samples of selected macroalgae and cyanobacteria were collected at each site (Table 1) as based on the sampling date. These samples were transported back to the Smithsonian Marine Station in natural seawater and then rinsed in DI-water and oven dried. These samples were then sent to the Southeast Environmental Research Center (SERC) Stable Isotope Laboratory for $\delta^{15}N$ and $\delta^{13}C$ analyses. Samples of all common cyanobacteria present at each site were collected. Macroalgae of the genus *Dictyota* were also collected for comparisons with primary producers that do not fix N_2. Tufts from the ends of the *Lyngbya* spp. were collected. For the other cyanobacteria and *Dictyota* that were smaller in size, the whole tufts were collected. Water samples were also collected at each site, at the surface and at depth, immediately above the benthic communities for nutrient concentrations including; DIN (NO_2^-+NO_3^-, NH_4^+), PO_4^{3-}, and dissolved organic carbon (DOC)). Additionally, a sub-set of the collected *Lyngbya polychroa* and *Lyngbya confervoides* from the July 2011 sampling were selected for two different incubation studies.

Table 1. Species collected on different field sampling date (stations varied by sampling period).

Species	**August10**	**June 11**	**July 11**
Dictyota sp.	X	X	X
Lyngbya confervoides		X	X
Lyngbya polychroa	X	X	X
Black *Lyngbya* - mat		X	
Red-black *Phormidium*		X	X
Symploca hydnoides	X	X	

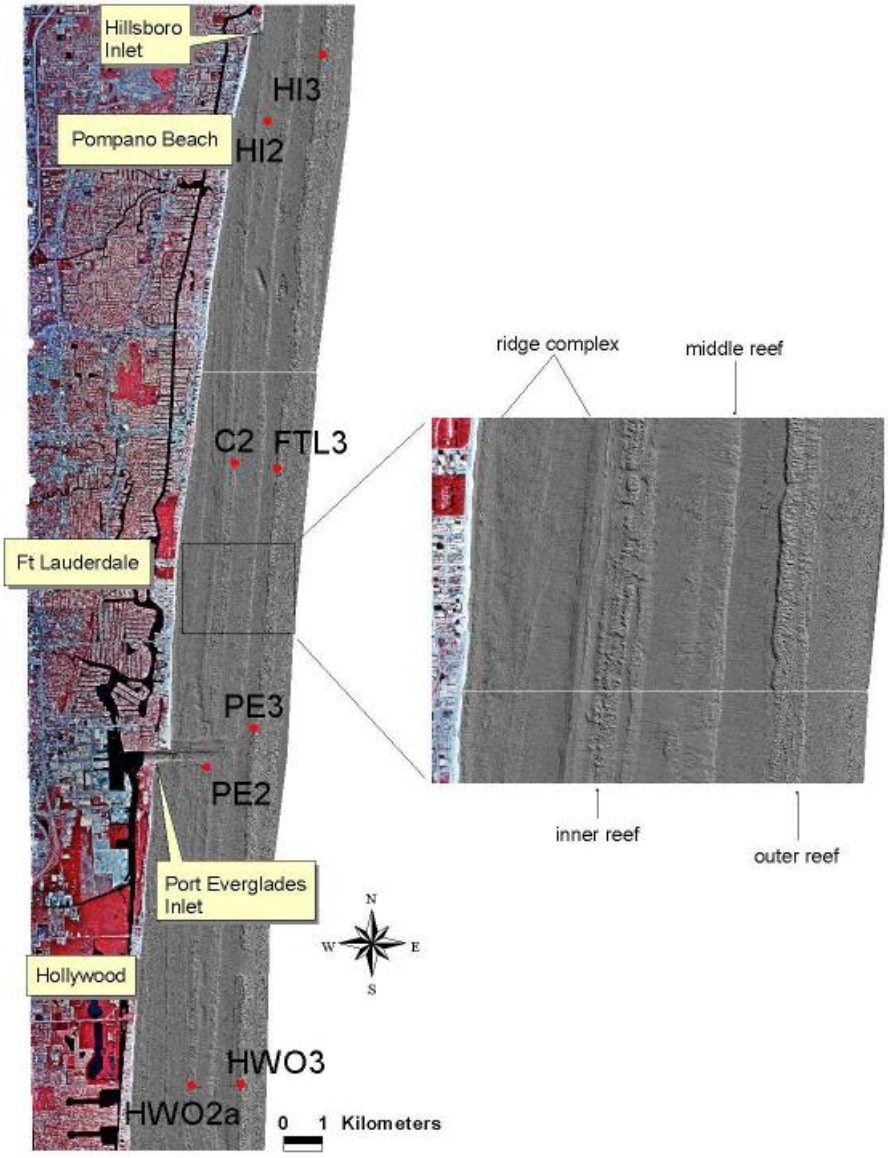

Figure 1. Offshore sampling locations along Broward County. Perturbed sites include HWO2a, HWO3, PE2, PE3, HI2 and HI3. Control sites include: C2 and FTL3. These sites were sampled in June and July of 2011.

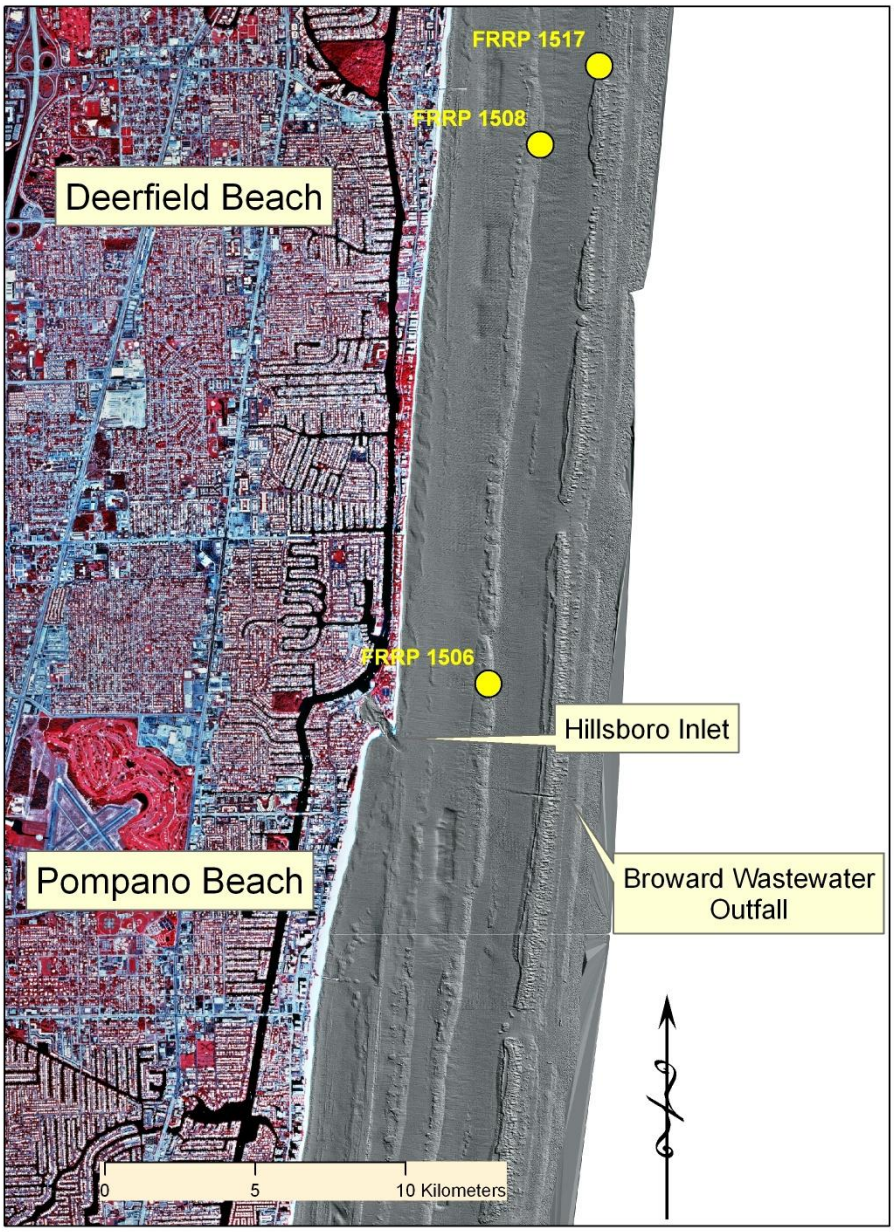

Figure 2. Initial offshore sampling locations along Broward County, north of originally intended sites. These sites were sampled initially in August 2010 and were used due to the presence of *L. polychroa.* FRRP 1506 is a disturbed site adjacent to the BWO and Hillsboro Inlet and FRRP 1517 and 1508 are control sites.

3.3 Analytical methods

Isotope analyses were conducted in the SERC Isotope Laboratory, Florida International University, by standard elemental analyzer isotope ratio mass spectrometer procedures (Anderson and Fourqurean, 2003). The elemental analyzer combusts all organic material and subsequently reduces the formed gasses into N_2 and CO_2, which are measured with Finnigan MAT CF-IRMS (continuous flow isotope ratio mass spectrometry) methodology. Each sample was measured twice, once for ^{15}N, and the second time on a decarbonated sample for ^{13}C. A sub-sample of each macroalgae and cyanobacteria was decarbonated in 1N HCl in order to remove any marine carbonate, which would affect ^{13}C values. Additionally, two IAEA international reference materials are analyzed in every sample run for cross calibration. Sample isotopic ratios (R) are reported in the standard delta notation: δ (‰) = [(Rsample/Rstandard)–1] × 1000, and will be presented with respect to the international standards of atmospheric nitrogen (N_2) and Vienna Pee Dee belemnite (V-PDB) for carbon.

Watersamples collected at each site were analyzed at the SERC Nutrient Laboratory. All samples for dissolved nutrients were pre-filtered in the field. These samples were measured for DIN, soluble reactive phosphate, and total phosphorus (TP). Ammonium, nitrate and nitrite, were analyzed following EPA methods 350.1 and 353.2. Phosphate was measured as TP and soluble reactive phosphorus using EPA method 365.1.

3.4 Incubations

Two sub-samples of *Lyngbya polychroa* and *Lyngbya confervoides* were incubated with two different approaches in order to study: a) N-fixation and b) growth rate with/under different nutrient levels.

3.4.1 ^{15}N-fixation

Incubations were conducted at one study site in order to determine N-fixation rates with "natural" light conditions and at night. N_2 fixation was measured by the ^{15}N tracer approach as based on Montoya et al. (1996). Using a triple collector isotope ratio mass spectrometers (IRMS), the sensitivity for ^{15}N in trace amounts is excellent, and requires low volume samples. Seawater collected from where the cyanobacteria was growing was filtered through an GF/F filter to avoid zooplankton, POM, and detritus and placed in an over flowing 250ml Pyrex bottle in which a cyanobacteria sample was placed and then sealed with a Teflon faced septum cap. 0.5 ml of $^{15}N_2$ (98%, Cambridge Isotope Labs) gas was injected via a gas-tight syringe, and the same volume of water was removed to equalize pressure in the bottle. These samples were incubated at the dock (floating in the

water) on the same day as collection at the Nova Southeastern Oceanographic Center for approx. 4 hours with 98% ^{15}N to calculate N-fixation rates (night time experiment were conducted back at the lab later that night). Cyanobacteria were removed from each bottle at the end of the experiment through filtering onto pre-weighed (and cleaned) GF/F filter (0.1 mm) which, once dried to a constant weight, was analyzed on an EA-IRMS system at the SERC Stable Isotope Laboratory (SIL). Each sample was measured in triplicate (for both species of cyanobacteria), for both day and night incubations. Isotope abundances were used to measure fixation of N_2 by comparing the isotopic composition change of the cyanobacteria before and after the addition of the $^{15}N_2$ tracer gas, over the specified incubation period (Montoya et al., 1996). This approach is very straight forward and modern IRMS systems are very sensitive for this type of isotopic measurement. The initial ^{15}N percentage of the tracer is a function of temperature and salinity, which was measured before and during each incubation, depending on the amount introduced into the known water sample (Hamme and Emerson, 2004; Montoya et al., 1996).

3.4.2 Incubations with different source waters

In order to determine if excess nitrogen in the water released from the Hollywood sewage outfall (HWO) or from terrestrial run-off via the Port Everglades Inlet (PE Inlet) has the potential to increase the growth of cyanobacteria off the coast of Ft. Lauderdale, Broward County, we incubated two species of *Lyngbya* in water collected from each of these sources, and monitored changes in growth rate.

The water was collected, transported and stored in plastic carboys that were first rinsed three times with water from the site. Within 24 hours the water was filtered through a 25µm bag filter into five gallon buckets, and for each treatment (HWO, PE Inlet, and Control) 150 ml was added to ten 200 ml glass jam jars (Weck). The cyanobacteria were blotted dry with a paper towel and ~ 1.00 g/wet wt was added to each jar so that each species was incubated in each of the three water treatments (n=5). The initial wet weight for each replicate was recorded. Jars were placed in a climate control chamber set at 28 °C with a 12/12 light dark cycle. Jars were placed in random order on a shelf in the center of the chamber. Every day the water in the jars was exchanged with unused water from the respective original stock and the position of the jars was changed based on a new set of random numbers. After six days, the cyanobacteria were removed from their treatments, blotted dry, and weighed.

4.0 Results

Isotopic results were variable for all the cyanobacteria and algal samples and did not correlate with nutrient levels measured in the water column (Table 2-4). Table 2 shows the variability in $\delta^{15}N$ values of three species which form the main focus of this work including: *Lyngbya confervoides*, *Lyngbya polychroa*, and *Dictyota* spp. *L. polychroa* had the highest amount of variability throughout the study with averages varying more than 3‰. *L. confervoides* has isotopic values very similar to those of the non N_2 fixing brown macroalga *Dictyota* spp. Changes in $\delta^{15}N$ values and the spatial relations for each sampling are presented in Figures 3-5. A comparison between $\delta^{15}N$, $\delta^{13}C$ and C:N ratios are presented in Figures 6-10.

Table 2. Nitrogen isotope results for all field collections of key cyanobacteria and algae.

Month	**Aug. 2010**		**June 2011**		**July 2011**	
Species	**$\delta^{15}N$ ‰ (±)**	**n**	**$\delta^{15}N$ ‰ (±)**	**n**	**$\delta^{15}N$ ‰ (±)**	**n**
Lyngbya polychroa	3.93 (±0.54)	6	0.89 (±0.67)	9	2.01 (±0.47)	6
Lyngbya confervoides			3.65 (±0.26)	9	3.55 (±0.76)	6
Dictyota spp.	3.36 (±1.05)	5	3.16 (±0.15)	6	3.73 (±0.41)	4

Table 3. June 2011 Dissolved seawater nutrient values of stations sampled. Below detection limit is listed as: b.d.l.

Site	DIN (μM)	SRP (μM)	DOC (μM)
PE3	1.95	0.06	96.5
PE2	0.37	b.d.l.	79.32
HWO3	0.72	b.d.l.	106.25
HWO2	0.52	0.06	105.33
Control Site			
FTL3	0.37	b.d.l.	89.00

Table 4. July 2011 Dissolved seawater nutrient values of stations sampled. Below detection limit is listed as: b.d.l.

sites	DIN (μM)	SRP (μM)	DOC (μM)
PE2	1.19	b.d.l.	149.15
HWO2	0.82	b.d.l.	96.25
Control Site			
C2	0.62	b.d.l.	107.63
Offshore FS	2.07	b.d.l.	175.55

^{15}N-fixation incubation data for the July 2011 analyses did not show any change in value from the ^{15}N enriched flasks and the controls for both *L. confervoides,* and *L. polychroa.* This lack of ^{15}N uptake was observed in both day time and night time incubations. Incubation samples' values ranged between 3‰ to 5‰, which was the same range for the samples collected on that day. However, measurements of *L. polychroa* from June 2011 had values between 0‰ to 1‰, indicating N-fixation. Yet, the samples of both *Lyngbya* species did not show any fixation in July 2011.

Statistical analyses of the isotopic data are presented in Figures 11 & 12. The non-metric multidimensional scaling (NMDS) analysis (Hammer et al. 2001) carried out on the 2011 samples at these sites are the focus of this report. Only a few species and samples were collected in 2010 (Table 1), which did not allow for statistical comparisons of those samples. This analysis used both δ^{15}N and %N as factors to look for patterns between individual species (Fig. 11) and between sites (Fig. 12). These data were also analyzed using one-way ANOVA for the effects of site and species on each factor (δ^{15}N and %N). Data were rank transformed when they did not meet the assumption of normality. Species had a significant effect on both δ^{15}N and %N ($p<0.001$ for both). This is illustrated in the NMDS plot that shows samples grouping by species based on their δ^{15}N and %N values (Figs. 11 & 12). Site also had a significant effect on both δ^{15}N and %N values ($p = 0.026$ and $p < 0.001$, respectively). However, the NMDS plot of samples collected at different sites shows no grouping according to site (Figs. 11 & 12). The effect of site on δ^{15}N and %N was likely driven by the fact that different species were collected at different sites.

The two species of *Lyngbya* (*L. confervoides* and *L. polychroa*) were incubated/treated with the different environmental waters and growth was

measured over the incubation period. Growth was calculated as the percent change in wet weight. Data were arcsin transformed and compared using a 2-way Analysis of Variance (ANOVA) using Systat 10. Results were considered significant when $p < 0.05$. Water collected from a sewage outfall area (HWO) and from an area that is subject to high amounts of terrestrial run-off (PE Inlet) had no significant effect on the growth of the two cyanobacterial species studied here when compared to control water collected off shore ($p=0.827$, Fig. 13). *L. polychroa* incubated in HWO, PE Inlet, and control water increased in wet weight by an average of 28.3 ± 17.2%, 36.7 ± 4.9%, and 36.7 ± 7.9% (mean ± SE), respectively. While *L. confervoides* incubated in water collected from near the sewage outfall did have a higher amount of growth (32.4 ± 4.9%) compared to specimens incubated in PE Inlet (14.4 ± 3.3%) and Control water (18.1 ± 4.7%), these differences were not significant according to ANOVA. There was no significant difference in the average growth of the two species ($p=0.086$) or in their response to the treatments ($p=0.331$, Fig. 13).

When the two species were analyzed separately by one-way ANOVA, there was no effect of treatment water on growth of *L. polychroa*, but there was a significant effect of the different treatment waters on growth of *L. confervoides*. However, the growth of cyanobacteria in control water did not differ significantly from that grown in either the Hollywood Outfall or PE Inlet water.

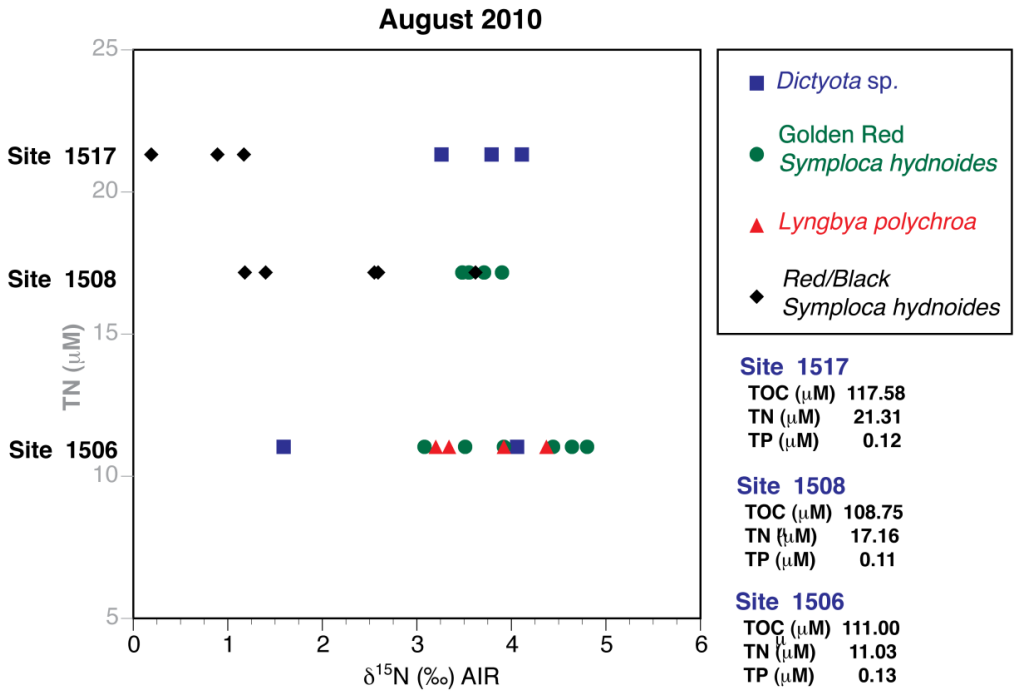

Figure 3. $\delta^{15}N$ values of cyanobacteria and algae from the initial August 2010 sampling. The vertical axis displays location and TN values (dissolved nutrients were not analyzed on this day, only totals were collected). Additional nutrient data is present for each site.

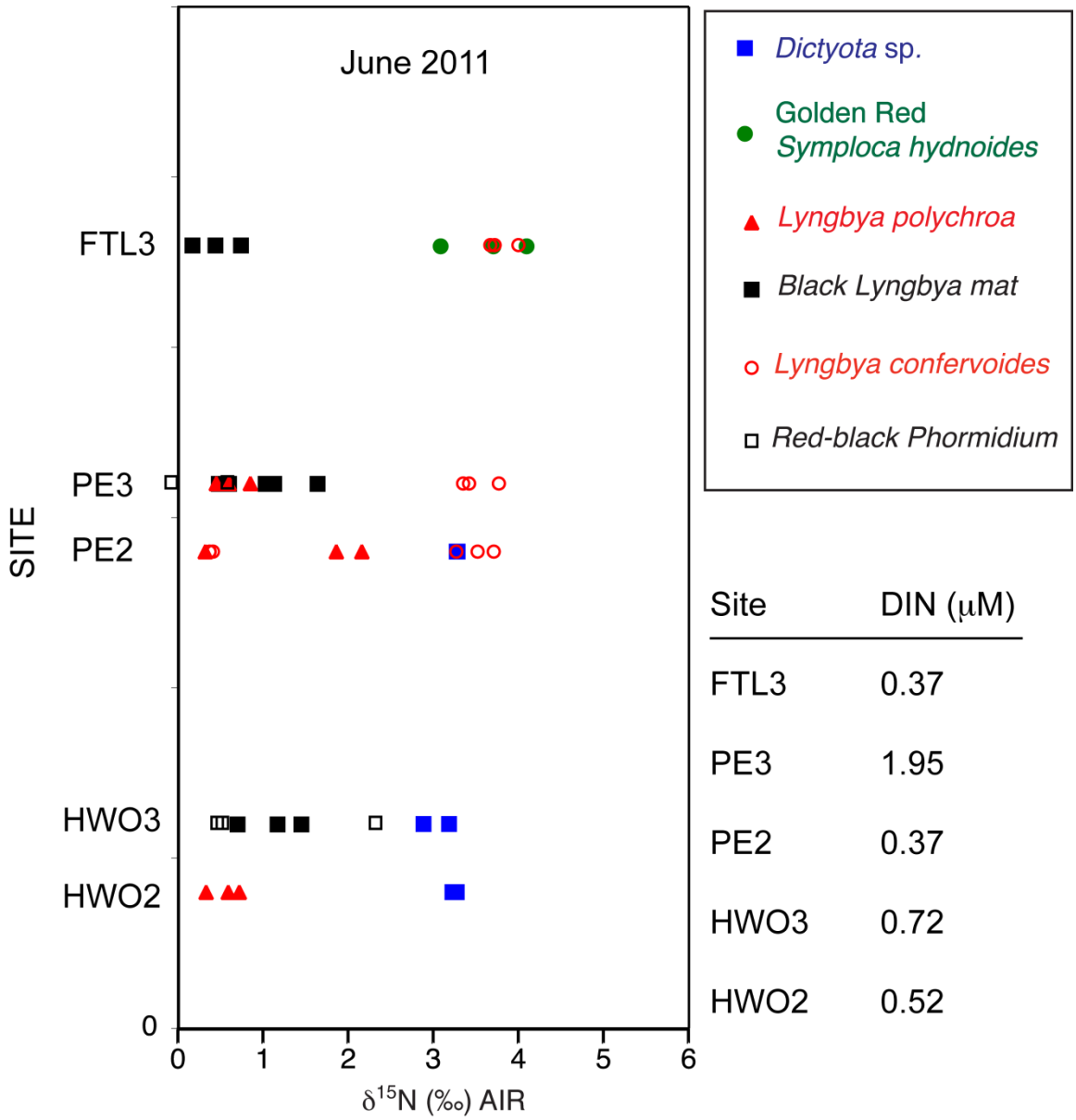

Figure 4. $\delta^{15}N$ values of cyanobacteria and algae from June 2011 sampling. Additional nutrient data is present for each site.

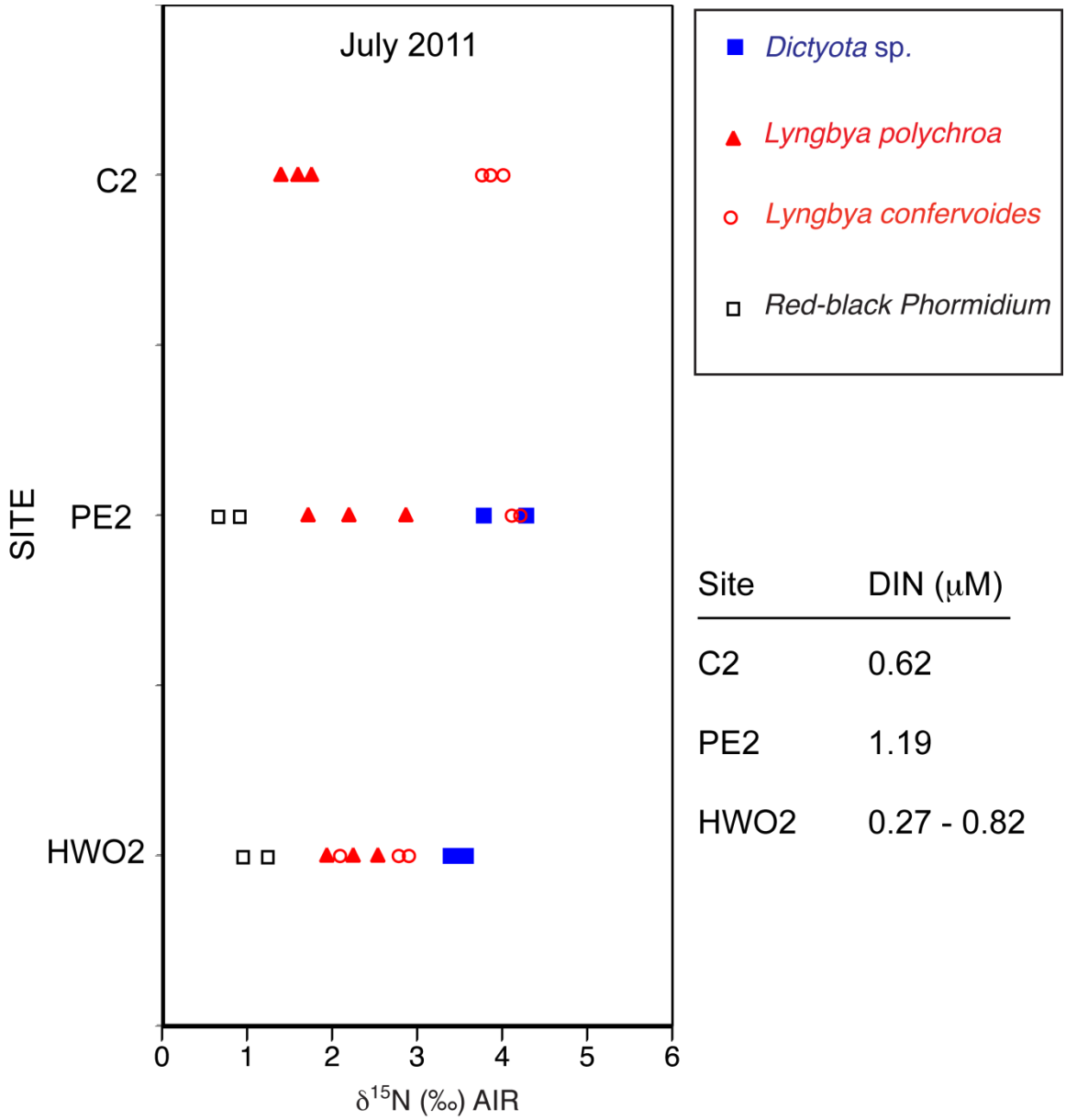

Figure 5. $\delta^{15}N$ values of cyanobacteria and algae from July 2011 sampling. Additional nutrient data is present for each site. DIN values from HWO2 show the variability at the site.

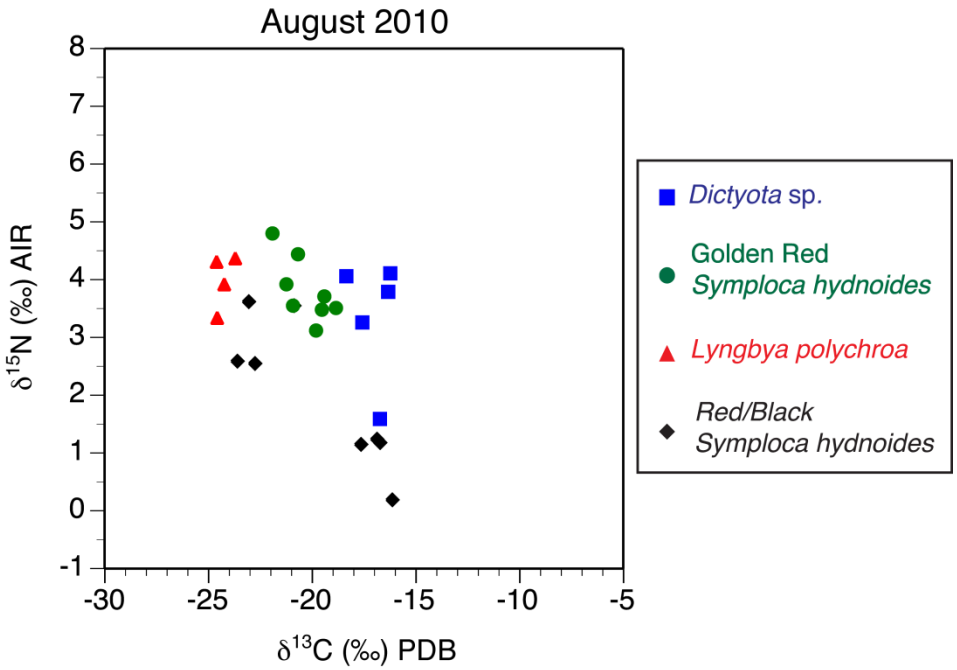

Figure 6. Bivariate plot of $\delta^{15}N$ vs $\delta^{13}C$ values of cyanobacteria and algae from August 2010 sampling.

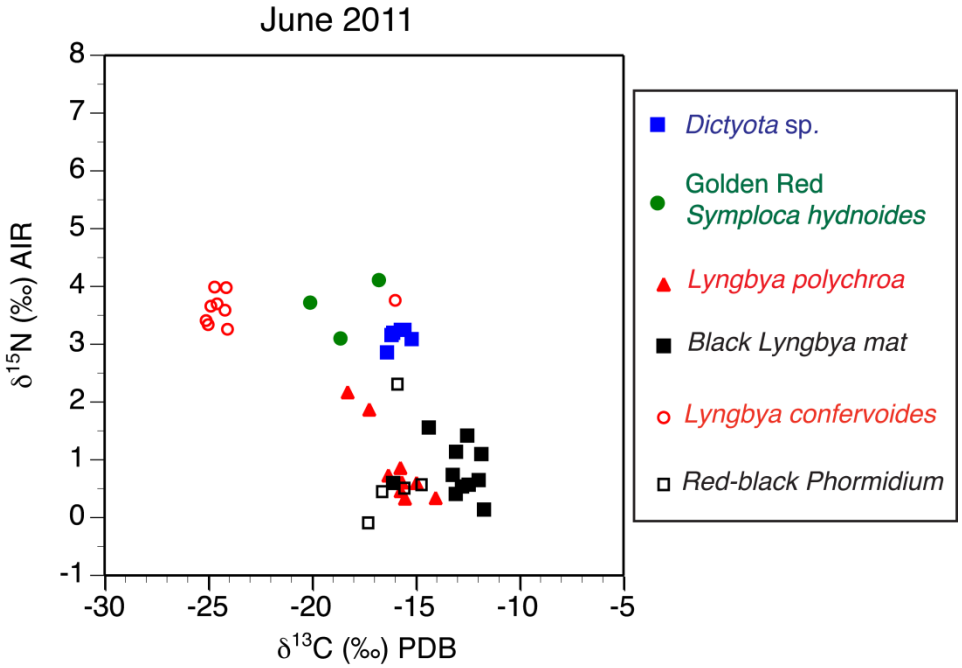

Figure 7. Bivariate plot of $\delta^{15}N$ vs $\delta^{13}C$ values of cyanobacteria and algae from June 2011 sampling.

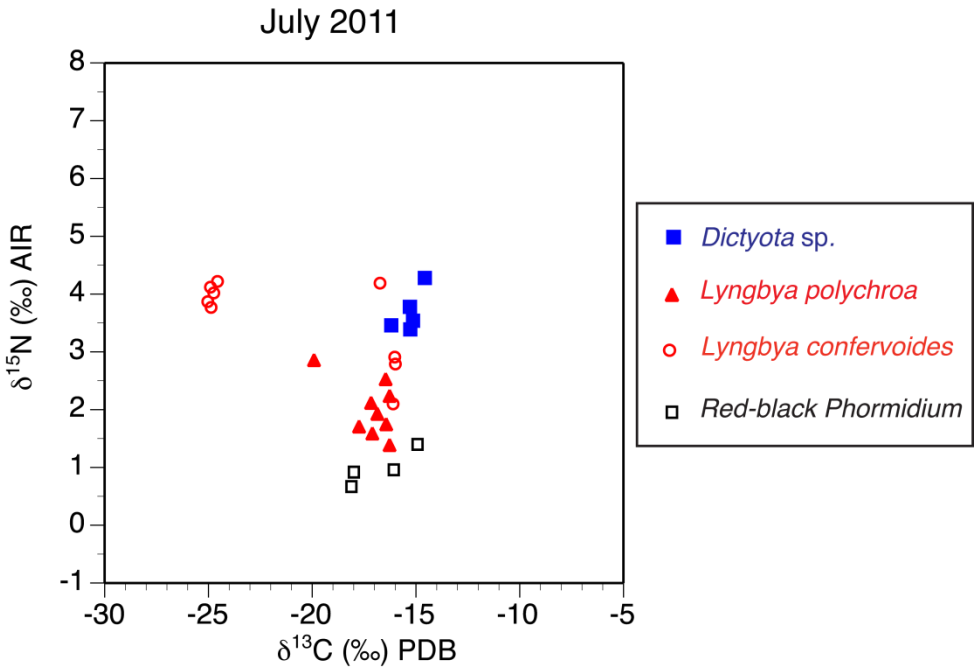

Figure 8. Bivariate plot of $\delta^{15}N$ vs $\delta^{13}C$ values of cyanobacteria and algae from July 2011 sampling.

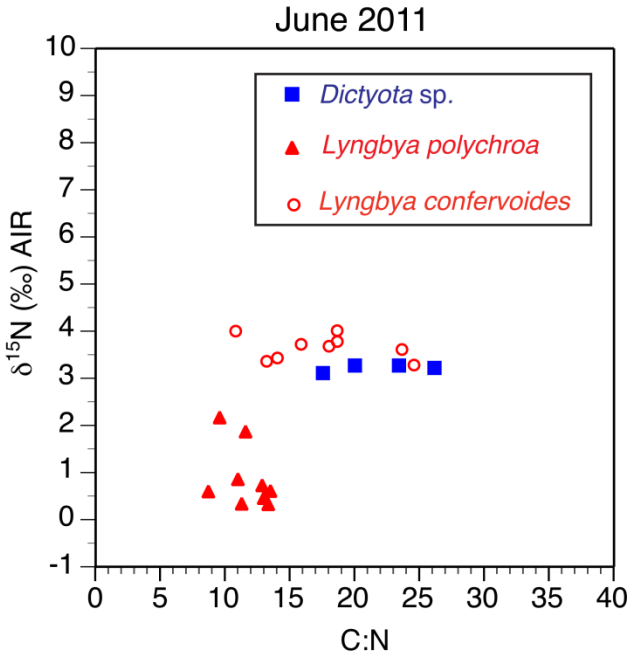

Figure 9. A comparison of $\delta^{15}N$ values with C:N (atomic ratio) of cyanobacteria and algae from June 2011 sampling.

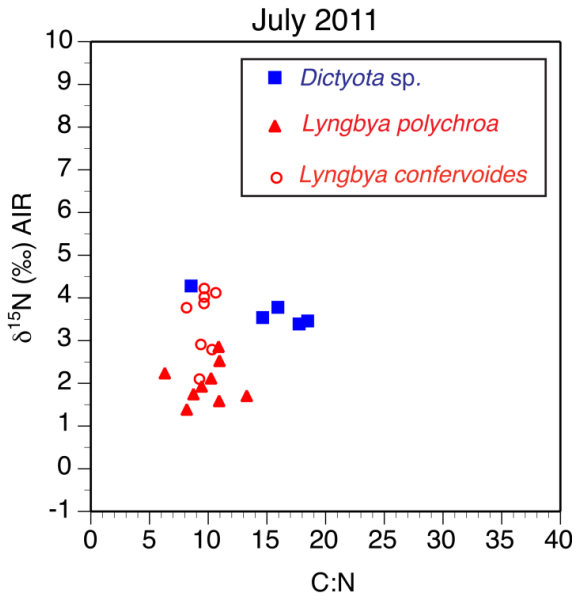

Figure 10. A comparison of $\delta^{15}N$ values with C:N (atomic ratio) of cyanobacteria and algae from July 2011 sampling.

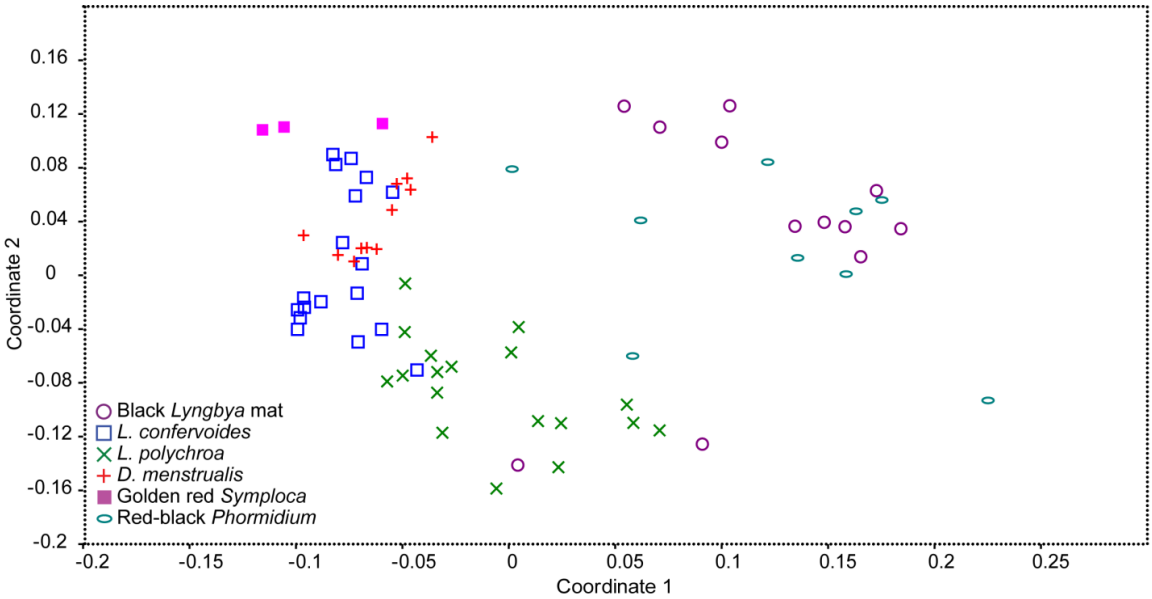

Figure 11. Non-metric multidimensional scaling (NMDS) plot of samples by species using δN and %N as factors. Plot prepared with PAST (PAlaeontological Statistics) (Hammer et al. 2001).

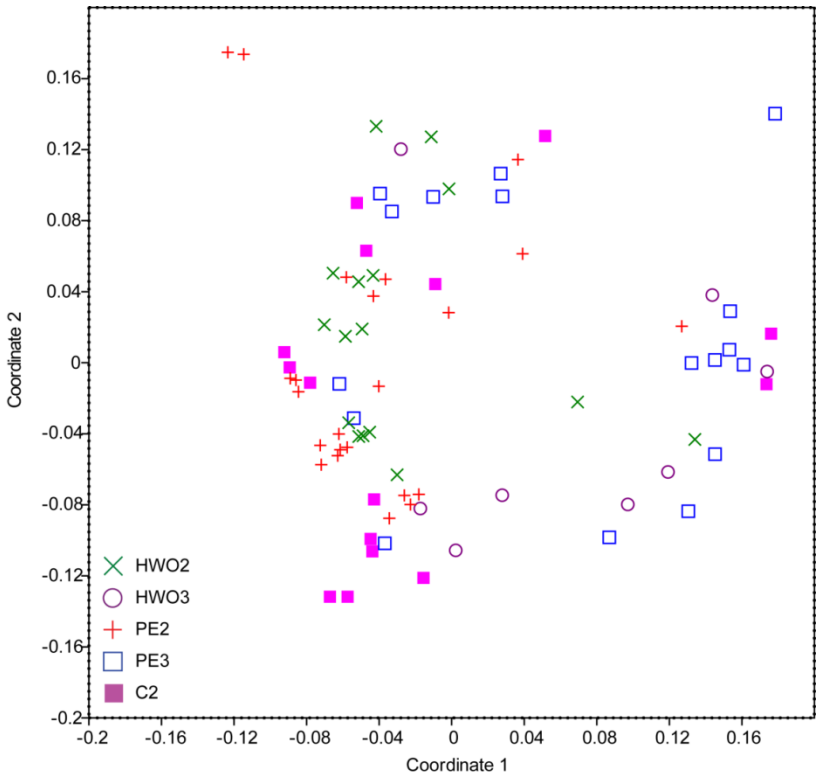

Figure 12. Non-metric multidimensional scaling (NMDS) plot of samples by collection location using $\delta^{15}N$ and %N as factors. Plot prepared with PAST (PAlaeontological Statistics) (Hammer et al. 2001).

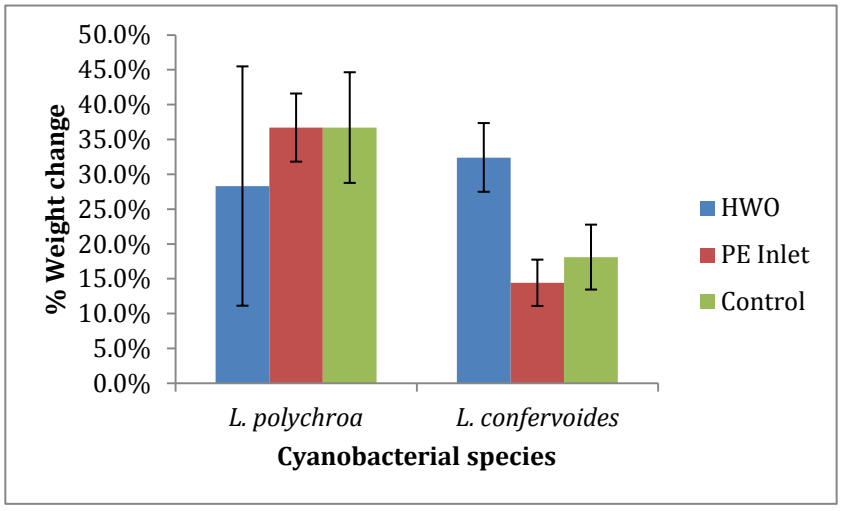

Figure 13. Growth in terms of percent weight change of two cyanobacterial species incubated in water collected from near a sewage outfall (HWO), within the Port Everglades inlet (PE Inlet) and offshore (Control). Bars represent the mean ± SE, n=5.

5.0 Discussion

The main focus of this study was to determine if the nitrogen isotopic composition of *L. confervoides* and *L. polychroa* could be used to understand the spatial effect of point source and non-point source nutrients affecting the coastal zone of Broward County. Additional samples of other cyanobacteria and brown algae were also measured for stable isotopic composition. Our result shows that *Lyngbya* and the other primary producers collected for this study were not recording primary sewage wastewater outfalls, or non-point sources from the inlets. Although samples collected from different sites were significantly different in their $\delta^{15}N$ and %N values, this difference was likely driven by the collection of different algae from different sites and different years (Fig. 1).

Typically, primary producers that are growing in nutrient discharges from (e.g., sewage treatment plants) have relatively enriched $\delta^{15}N$ values >8‰ (Savage and Elmgren 2004). All values measured in the study were <5‰. Values <1‰ indicate N-fixation (Montyoa et al. 1996) or non N-limited systems (DIN concentrations are sufficient) where discrimination is high (Mahaffey et al. 2004). Previous work by Lapointe et al. (2004) from a wastewater impacted site (further north of this study) showed macroalgae samples with $\delta^{15}N$ values between 7.5‰ to 11‰. Sewage treatment causes ^{15}N enrichments as the volatilization of NH_4^+ occurs (Kendall et al. 2008), leading to values of 10‰ (Heaton, 1986). In contrast upwelled North Atlanic NO_3^- has been measured at 4.8‰ (Sigman et al. 2000), and the subtropical Atlantic thermocline has NO_3^- values between 2‰ to 4‰ (Knapp et al. 2008). The nitrogen isotopic values measured in this study are not outside the range of previously measured natural sources. There is a clear indication that *L. polychroa* switches between N-fixation and DIN assimilation due to the variability in values when comparing the differences observed in the June and July 2011 sampling to August (2010) (Fig. 3-5). The nitrogen isotopic values are most depleted in June 2011, with the majority of samples between 0‰ to 2‰ (Fig. 4). However, our approach to measure N-fixation showed both species in July 2011 were not fixing N. Thus the species are variable depending on temporal and potentially spatial (environmental) conditions.

Comparison of $\delta^{15}N$ values with $\delta^{13}C$ values, showed that *L. confervoides* and *L. polychroa* plotted as different groups due to the degree of N-fixation ($\delta^{15}N$ values between 0‰ to 2‰) and potentially different carbon sources, as observed in Paul et al. (2009) (Fig. 6-8). If primary producers are utilizing $CO_{2(aq)}$ then $\delta^{13}C$ values will typically range between -30‰ to -20‰, where as if the HCO_3^- is the major carbon source, values will be <-20‰ (Hemminga and Mateo 1996; Raven et al. 1995). *L. confervoides* with values between -26‰ to -23‰ appears to be using one

carbon source, where *L. polychroa* is variable with values between -26‰ to -13‰. However, it has been demonstrated that changes in boundary layers conditions of macroalgae (as controlled by baffling of flow by nearby marine macrophytes) also causes change in $\delta^{13}C$ values within the same species, similar to the magnitude as those observed here in our study (France and Holmquist, 1997). Changes in hydrodynamic conditions (protected vs. more open or exposed locations) will affect CO_2 diffusion into the algae or cyanobacteria, thus changing its respective ^{13}C isotopic composition (Raven et al. 1995).

C:N ratios were also compared to $\delta^{15}N$ values for both *L. confervoides* and *L. polychroa* (*Dictyota* spp. was also plotted for comparison purposed) (Fig. 9 & 10). There was no observed relationship between N-limitation and $\delta^{15}N$. If N becomes limited, then the isotopic discrimination would decrease resulting in a relative isotopic enrichment (Anderson and Fourqurean 2003). However, in this present study, there was no statistically significant relationship observed for C:N and water column nutrients.

Growth experiments with *L. confervoides* and *L. polychroa* with different treatment water indicated only one species responded with increased growth, *L. confervoides* (Figure 13*).* When the two species were analyzed separately by one-way ANOVA, there was no effect of treatment water on growth of *L. polychroa*, but there was a significant effect of the different treatment waters on growth of *L. confervoides.* However, the growth of cyanobacteria in control water did not differ significantly from that grown in either the Hollywood Outfall or PE Inlet water. This result could be caused by the fact that the selected control water for this experiment had higher DIN values then either the Hollywood Outfall or PE Inlet water (Table 4). This experiment suggests some intriguing responses in the growth of different *Lyngbya* spp. in seawater from different sites and should be repeated.

6.0 Conclusion

Lyngbya spp. are toxic bloom-forming cyanobacteria that negatively impact coral reefs and other benthic organisms (Paul et al. 2005). *Lyngbya* appear to be highly opportunistic and are capable of using different nutrient sources (Paul et al, 2009). Our isotopic data supports this later observation. *L. confervoides* and *L. polychroa* $\delta^{15}N$ values not only show variability, but also the potential for N-fixation. However, this variability changes temporally, as the same species appear to be fixing N during one month, but not the next. Perhaps, the ability to switch nutrient sources limits the use of *Lyngbya spp*. to trace nutrient laden wastewater effluent with isotopic approaches, which past work with macroalgae

has proven to be possible. Another possibility for the lack of a coherent signal in our data was perhaps our sampling stations were too far from sources and a higher spatial concentration of samples along a transect directly adjacent to the source would be better such as conducted by Futch et al. (2011). A coast to seaward transect with tight spatial sampling is recommended rather than just focusing on previously established sites (which are optimal for some studies, but perhaps not the optimal approach for as realized now). Future isotopic biogeochemical work with *Lyngbya spp.* should focus on longer temporal studies (multi-year and with quarterly sampling to improve seasonal resolution) to document further how variable these cyanobacteria are across the Broward County coastal system. In general, sessile benthic organisms, and especially primary producers, are excellent recorders of environmental waters, however *Lyngbya spp.* has proven to be a complex organism to study.

7.0 References

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