# C-43 Water Quality Treatment and Testing Project: Phase 1 Demonstration – Bioassays December 2014-October 2015

Final Report to the South Florida Water Management District
(Contract #4600003105)

**Prepared by:**

Rudolf Jaffé, Ph.D.
Oliva Pisani, Ph.D.
Joseph N. Boyer, Ph.D.

January, 2016

Southeast Environmental Research Center
Florida International University
Miami, FL 33199
http://serc.fiu.edu/

Technical Contribution #773T of the Southeast Environmental Research Center at Florida International University

# EXECUTIVE SUMMARY

The Caloosahatchee River and Estuary (CRE) is an important component of the aquatic landscape of South Florida. During the past 135 years, the CRE has been altered by agricultural and urban development. Its hydrology and ecology have been impacted by a combination of these anthropogenic activities including altered salinity pattern and extent of reach, elevated nutrients, and shift in seasonal flow regime. Nitrogen inputs from these activities to the CRE are of significant concern to the environmental health of the system and are one of the primary drivers for its being put on the 303(d) list as an impaired water body since 1998. The reduction of nutrient concentrations and loads to CRE was required by the Northern Everglades and Estuaries Protection Program, passed by the Florida Legislature and signed into law in 2009 and by CRE Total Maximum Daily Loads (TMDL) published by the Florida Department of Environmental Protection in 2009 [Rule 62-304.800, Florida Administrative Code (F.A.C.)]. The CRE TMDL requires a 23% reduction in total nitrogen (TN) loads from the baseline conditions. However, since most of the TN in this system is in the form of dissolved organic nitrogen (DON), a complex mixture of mainly molecularly uncharacterized compounds with varying degrees of bioavailability, the environmental dynamics and reactivity of this biogeochemical component need to be assessed. Better determination of DON bioavailability will inform demonstrations the SFWMD is undertaking to optimize wetland-based strategies for removal of TN, particularly the DON fraction, in the Caloosahatchee River (C-43).

Here we studied the composition and reactivity of DON along the C-43 over both spatial (stations: S-77, S-78 and S-79) and temporal scales (Dec. 2014, Feb. 2015, Apr. 2015, Jun. 2015, Aug. 2015, and Oct. 2015). The biological reactivity of particulate N was not addressed in this study. Samples were collected bimonthly (every 2 months) over one year at the three stations and analyzed for ambient DON concentrations (T0) and after 28 day incubation (light exposed and dark; T28). DON composition was also categorized as total hydrolysable amino acids (THAA); urea; and the uncharacterized pool. The bioavailable DON (BDON) was determined by difference after 28 days and reported as % loss from the DON pool (%BDON). Inorganic N ($NO_2^-$, $NO_3^-$, and $NH_4^+$), bacterial numbers, and leucine aminopeptidase activity (LAPA) were also measured. DON quantification was performed using the conventional methods by subtracting total dissolved inorganic nitrogen (DIN) from total dissolved N (TDN).

Ambient TDN composition showed a high degree of variability on seasonal scales but was consistent in being dominated by DON (ca. 80%), where THAA and urea represented only a minor fraction of the DON. No significant variations or seasonal/spatial trends were observed for the %DON. In contrast, %BDON time series showed a general decrease in the values between Dec. 2014 and Oct. 2015, but again with no clear spatial patterns. The variability in %BDON suggests variations in DON composition (and thus quality) combined with variations in bacterial community structure significantly affect DON bioavailability. The incubation data suggest that DON was more bioavailable during the dry season. No relationship between BDON and C-43 flow (as date of collection, 15-day average, or 30 day average) was observed. Samples for April, August and October 2015 featured negative %BDON values, suggesting a production of DON during microbial incubation. Salinity incubations, particularly for the 30 ppt experiments, had %BDON values that were significantly negative, probably due to a combination of initial DON flocculation as a result of increased complexation, and possibly a difference in the microbial community structure (i.e. more prone to efficient consumption of DIN and generation of DON for this sample, inoculated with estuarine bacteria). In general, bacterial counts were consistent with ambient expectations for South Florida aquatic systems, and while no clear differences between wet and dry season could be determined, they were enhanced in the light incubations. In general terms however, while BDON was observed to be highly variable seasonally, it was fairly consistent along the Caloosahatchee River transect. The mean %BDON was 39% but with a large range (0-61%). This behavior seems consistent with literature-reported BDON values in other terrestrially-influenced aquatic systems worldwide (mean = 35%; range 0-73% supplied from land). In agreement with other such reports, and because of the large component of relatively recalcitrant DON, reductions in TN loads as a means to reduce estuarine eutrophication and associated harmful algal blooms would not necessarily guarantee a reduction in estuarine eutrophication and the associated ecological impacts.

The findings from this study will guide the development of and be compared to the next phase of the C-43 Water Quality Treatment and Testing Project, a mesocosm demonstration. While the current phase focused on water column microbial processes, the mesocosm demonstration will also include plant and soil processes that may alter the BAN concentration and affect TN removal from the system. This next phase is critical for understanding wetland

potential as a treatment technology for removing nitrogen for the Caloosahatchee River and other freshwater systems sensitive to nitrogen loading.

# Table of Contents

1. INTRODUCTION ........ 6
2. MATERIALS AND METHODS ........ 8
    2.1. Site Description and Sample Collection ........ 8
    2.2. Bioassays ........ 8
    2.3. Standard Nutrient Analyses ........ 9
    2.4. Urea Analysis ........ 10
    2.5. Total Hydrolysable Amino Acids Analysis ........ 11
    2.6. Optical Properties of DOM ........ 11
    2.7. Leucine Aminopeptidase Assay (LAP) ........ 12
    2.8. Bacterial Enumeration ........ 12
    2.9. Data Analysis ........ 13
3. RESULTS AND DISCUSSION ........ 13
    3.1. Ambient Time Series and System Characterization ........ 13
    3.2. Bioavailability and Microbial Dynamics (Dark Incubations) ........ 15
    3.3. Seasonality and Light Effects ........ 17
    3.4. DON Composition ........ 18
    3.5. Principal Component Analysis (PCA) ........ 19
4. SUMMARY ........ 20
5. REFERENCES ........ 22
APPENDICES ........ **Error! Bookmark not defined.**

# 1. INTRODUCTION

The Caloosahatchee River and Estuary (CRE) is an important component of the aquatic landscape located on the lower west coast of Florida. Three water control structures (S-77 at Moore Haven, S-78 east of LaBelle and S-79 at Olga) control freshwater flow in the C-43 to the estuary, (Fig. 1) which runs in a westerly direction for approximately 100 km from Lake Okeechobee to the Gulf of Mexico (SFWMD, 2012). This ecosystem has been impacted by a combination of managed freshwater inflows and loss of aquatic habitat (e.g., submersed plants and oysters) through watershed modification in support of agriculture and urbanization.

As a consequence of potential environmental impacts, the Basin Management Action Plan associated with the Total Maximum Daily Load (TMDL) require reductions in nutrient loads and concentrations for the CRE ( Wetland Solutions, 2012). Nitrogen inputs from these activities to the CRE are of significant concern to the environmental health of the system and are one of the primary drivers for its being put on the 303(d) list as an impaired water body since 1998. The reduction of nutrient concentrations and loads to CRE was required by the Northern Everglades and Estuaries Protection Program, passed by the Florida Legislature and signed into law in 2009 and by CRE Total Maximum Daily Loads (TMDL) published by the Florida Department of Environmental Protection in 2009 [Rule 62-304.800, Florida Administrative Code (F.A.C.)]. The TMDL for the CRE calls for a 23% reduction in loading of TN (Bailey et al., 2009).

Total nitrogen (TN) inputs to the estuary are significant as the estuary is sensitive to N loading, which can result in algal blooms. However much of the TN is in the form of dissolved organic N (DON). For example, the water entering the C-43 canal from Lake Okeechobee from 1981 to 2011 at S-77 had an average total nitrogen(TN) concentration of 1.76 mg/L of which89% was DON (Wetland Solutions, 2012). Since TN is known tocontain a mix of labile and recalcitrant compounds, setting a TMDL based on the TN pool overestimates potential eutrophication because not all TN is bioavailable. Jørgensen et al. (2014) argue that regulating the pool of bioavailable N (BAN = BDON + DIN) is more important than using TN to assess potential marine eutrophication.

While it remains unknown how bioavailable the organic N is to phytoplankton and bacteria, the percentage of total organic nitrogen (as % of TN) has been reported to decrease from 85% at S-78 to 78% at S-79 (Wetland Solutions, 2012), suggesting that it might potentially be available to phytoplankton and bacteria, and might therefore be removed from the water column using biological treatment systems. To this end, the South Florida Water Management District

(SFWMD) and Lee County are partners in the development of the C-43 Water Quality Treatment and Testing Project (C-43 WQTTP) to reduce material loads to improve downstream water quality in the CRE (Figure 1). The findings from this study will guide the development of and be compared to the next phase of the C-43 Water Quality Treatment and Testing Project, a mesocosm demonstration. While the current phase focused on water column microbial processes, the mesocosm demonstration will also include plant and soil processes that may alter the BAN concentration and affect TN removal from the system. This next phase is critical for understanding wetland potential as a treatment technology for removing nitrogen for the Caloosahatchee River and other freshwater systems sensitive to nitrogen loading.

While the SFWMD has implemented Stormwater Treatment Areas (STAs) designed to reduce total phosphorus (TP) loads and concentrations using wetland systems, N removal, especially in organic form, has not been formally evaluated (Wetland Solutions, 2012). The cycling of TN among the sediments, water, and atmosphere is complicated by the comparatively large fraction of dissolved organic N (DON), the contributions of N fixation and denitrification to the TN pool, and the rapid exchanges among inorganic and organic pools through algal uptake and microbial remineralization (Bronk et al., 2007; Eyre et al., 2011; Wetland Solutions, 2012). Assuming that STAs can be effective at removing dissolved inorganic N (DIN) from inflow before outflow, it is important to evaluate and quantify the bioavailable DON (BDON) for planktonic biota in the Caloosahatchee River.

Previous work in Florida Bay conducted and/or supported by the SFWMD has improved our understanding of the importance of DON in nutrient availability for biota (Boyer et al. 2004). The management significance, scientific question, and experimental design of preceding work are relevant to the main goals of the proposed study, which is to determine to what extent the DON in the freshwater Caloosahatchee River is bioavailable for microbial consumption and if this differs on spatial and temporal scales. The overall objective of this project is to conduct experimental bioassays that evaluate the bioavailability of ambient DON in the Caloosahatchee River. Biological reactivity of particulate N was not addressed in this study. The specific objectives are to measure differences and variations in BDON between DON source water (S-77, S-78 and S-79), source of microbial inoculum, and salinity (freshwater and estuarine), and doing so on a seasonal basis (wet and dry), and with and without photo-exposure.

# 2. MATERIALS AND METHODS

## 2.1. Site Description and Sample Collection

The Caloosahatchee River (C-43 Canal) is located on the southwest coast of Florida and has been altered by human activities starting in the 1880s when the river was straightened and deepened. Three water control structures (S-77, S-78 and S-79) determine freshwater flow to the estuary which begins at S-79 (Fig. 1). Source water and inoculum samples were field collected three times during the dry season (December 2014, February 2015 and April 2015) and three times during the wet season (June, August and October 2015). Water samples (6 L) were collected just upstream (~500 m) of the indicated water control structure in dark, pre-rinsed (acid and base) 6 liter plastic bottles. The inoculum (500 ml) was collected just downstream (~500 m) of the water control structure in dark, pre-rinsed, 500 ml plastic bottles.

## 2.2. Bioassays

In the laboratory, water samples were filtered through Whatman™ GF/F filters to remove particulate material (see methodological flow chart in Fig. 2). Five laboratory incubations were set-up to determine BDON at different locations along the river (S-77, S-78 and S-79) under different physical and chemical conditions (Table 1). All filtered ambient samples were amended with labile carbon (glucose) and inorganic phosphorus ($NaH_2PO_4$) to obviate carbon and phosphorus limitation. For incubations 1-3, the source water was collected just upstream (~500 m) and the inoculum just downstream (~500 m) of the indicated water control structure. To determine if estuarine microbes are more or less adapted to using DON, and to test the DON "shuttle" hypothesis (Stepanauskas et al., 1999), the salinity of the source water (S-79) for incubations 4 and 5 was adjusted to 5 and 30 ppt respectively, using artificial sea salt. The inoculum for incubations 4 and 5 was collected closer to the estuary, at the SR-31 Bridge where the microbial community is likely different from the one at S-79.

A parallel set of incubations was run to address the potential for photolytic breakdown of DON and to assess the effect of light exposure on bioavailability. Split samples were filtered (0.7 µm GF/F and 0.22 µm membrane) prior to exposure to artificial sun light under controlled laboratory conditions, followed by dark incubations as described below. Samples were incubated for 24 hours in 250 mL quartz Erlenmeyer flasks with quartz lids and placed in a solar simulator

(Suntest XLS+, Atlas Material Testing Technology, LLC) set at 765 W $m^{-2}$. These conditions are equivalent to mid-day sunlight on a typical sunny summer day in South Florida and correspond to about 4-day sunlight dose in this environment (Chen and Jaffé, 2014). Extensive light exposure would result in DOM 'bleaching' and thus not be representative for processes occurring in natural systems were shading by aquatic macrophytes, periphyton and light absorption by colored DOM is evident. The samples were placed in a water bath kept at ~26 °C.

The inoculum was added to the water sample after exposure to artificial sunlight. Three replicate incubations were performed for each sampling period. Bottles were incubated in the dark at 25 °C for 28 days, a long enough period to ensure measureable changes in the DON pools under the various experimental conditions (Seitzinger and Sanders, 1997; Boyer et al., 2004; Wiegner et al., 2006). Measurements were made at time 0 to establish pre-existing concentrations and after 28 days incubation time.

## 2.3. Standard Nutrient Analyses

The analyses for dissolved inorganic nitrogen (DIN; $NO_2$, $NO_3$ and $NH_4$) were performed using an OI four-channel Rapid Flow Analyzer following standard procedures (EPA 353.2 and EPA 350.1). Nitrite was determined as an azo dye formed by the reaction of nitrite with sulfanilamide and subsequent coupling with N-1-naphthylethylenediamine (NEDA). Nitrate was determined by the quantitative reduction of nitrate to nitrite using an activated cadmium column and the determination of nitrite as described above. Nitrate concentration was calculated by subtracting the nitrite concentration before reduction from the nitrite concentration after reduction. Ammonium was determined using the indophenol blue method.

Total dissolved nitrogen (TDN) analysis was performed following the ASTM D 5176-91 Standard Test Method for Total Chemically Bound Nitrogen in Water by Pyrolysis and Chemiluminescence Detection using an ANTEK Instruments Inc. Model 9000 Nitrogen Analyzer. This procedure is a modification of the classical Dumas (1831) method of determining nitrogen by a combustion technique with the addition of chemiluminescence. The method involves converting all forms of N into nitric oxide (NO) upon combustion of a sample with oxygen at a temperature in excess of 1000 °C. The NO is reacted with ozone ($O_3$) to form a metastable form of nitrogen dioxide ($NO_2^-$). As the metastable form of $NO_2^-$ decays, a quantum of light is emitted in an amount directly proportional to the amount of N in the sample. The chemiluminescent emission is detected

by a photomultiplier tube at a specific wavelength. DON was determined by subtracting DIN from TDN.

Dissolved organic carbon (DOC) was measured following USGS SOP NU-062-1.8, which is based on Standard Method 5310B. Using a Shimadzu TOC-5000, samples are introduced into a combustion tube (hot catalyst combustion) and the total carbon of the samples (non-purgeable organic carbon) is combusted/oxidized to form $CO_2$, which is detected by infrared analysis.

Total dissolved phosphorus (TDP) was measured following EPA method 365.1 with a modification of the sample preparation method described by Solórzano and Sharp (1980). TDP was determined by oxidizing and hydrolyzing all of the phosphorus-containing compounds in a sample to soluble reactive phosphate (SRP). SRP was then determined suing a OI Rapid Flow Analyzer by reacting phosphate with molybdenum (VI) and antimony (III) in an acid medium to form a phosphoantimonylmolybdenum complex, which was then reduced with ascorbic acid to form a colored dye.

Particulate nitrogen (PN) was analyzed using the standard flash combustion method on a Carlo Erba NA 1500 Series 1 Nitrogen/Carbon analyzer. This analysis was performed post-incubation only on pre-combusted, pre-weighed 25 mm 0.7 µm GF/F filters that were used to filter the incubated samples.

## 2.4. Urea Analysis

Urea was measured using the direct method, which is based on the formation of a colored product when urea reacts with diacetylmonoxime in acid solution (Revilla et al., 2005). Three separate reagent solutions were prepared: diacetylmonoxime solution, thiosemicarbazide solution and reagent B (sulfuric acid and ferric chloride solution). These reagents along with Reagent A (25 parts of diacetylmonoxime solution with 1 part of thiosemicarbazide solution), the color developing reagent (COLDER; 1 part reagent A and 3.2 parts reagent B) and the optical turbidity blank (OTB; 1 part water and 3.2 parts reagent B) were prepared fresh prior to each analysis. A sample volume (4 ml) was dispensed in a polypropylene tube and mixed with COLDER reagent (1.2 ml). Another set of tubes was prepared by mixing 1.2 ml of OTB with 4 ml of sample. The tubes were capped, stirred by vortex and kept in the dark at room temperature. After 72 hours, the absorbance was measured at 520 nm on a Varian Cary-50 Bio spectrophotometer using a 1 cm quartz cuvette and Milli-Q water as the

blank. The absorbance of the samples treated with COLDER was corrected for their individual turbidity blanks. An external calibration was performed for each session of analysis using urea.

## 2.5. Total Hydrolysable Amino Acids Analysis

Total hydrolysable amino acids (THAA) were measured using a previously published method (Castell et al., 1979) in which the sample is subject to acid hydrolysis and reacted with fluorescamine. The sample (2 ml) was mixed with concentrated HCl (2 ml) and 11mM ascorbic acid (20 µl) in a hydrolysis tube. The air in the tube headspace was replaced with argon and the tubes were capped and heated at 110 °C for 24 hours. The hydrolysate was neutralized with 10M NaOH (2.4 ml). In a quartz fluorescence cuvette, the sample (100 µl) was mixed with borate buffer (100 µl) and 0.03% fluorescamine (100 µl) for one minute. Water was added (2.5 ml) and the fluorescence was measured with an excitation at 390 nm and an emission at 475 nm. Fluorescence was measured on a Horiba Scientific Aqualog spectrofluorometer. An external calibration was performed for each session of analysis using glycine. An analytical blank was performed and the value subtracted from the samples, respectively.

## 2.6. Optical Properties of DOM

Differences or similarities in the DOM characteristics of the samples pre- and post-incubation were assessed through the analysis of optical properties, including the Fluorescence Index (FI), Humification Index (HIX) and Slope Ratio ($S_R$). The FI was obtained by calculating the ratio of the emission intensity at a wavelength of 470 nm to that at 520 nm, with an excitation of 370 nm (McKnight et al., 2001; Cory and McKnight, 2005). The FI has been correlated to the relative contribution of microbial versus plant derived organic matter (McKnight et al., 2001). The HIX is directly proportional to the humic content of DOM and was calculated as the ratio of the peak area under each curve at emissions 435-480 nm and 300-345 nm from an excitation of 255 nm (Zsolnay et al., 1999). The Slope Ratio ($S_R$) was obtained by taking the ratio of two spectral slope regions of the absorbance spectra (275-295 nm and 350-400 nm) and is related to DOM molecular weight and to photochemically induced shifts in molecular weight (Helms et al., 2008). All fluorescence spectra were corrected following the procedures described by Jaffé et al. (2008) and references therein. Briefly, each sample excitation emission matrix (EEM) underwent spectral subtraction with a water blank to remove effects due to Raman scattering. Instrument bias related

to wavelength-dependent efficiencies of the specific instrument's optical components (gratings, mirrors, etc.) were corrected by applying multiplication factors supplied by the manufacturer. To compare intensities among samples analyzed over time, the fluorescence intensities in all sample spectra were normalized to the area under the water Raman peak collected on the day of analysis.

### 2.7. Leucine Aminopeptidase Assay (LAP)

Leucine aminopeptidase (LAP) is an exopeptidase that catalyzes the hydrolysis of polypeptide chains. LAPs are ubiquitous in nature and biologically important because of their role in protein degradation. The titer (concentration) of leucine aminopeptidase (LAP) was determined using the model substrate L-leucine-4-methyl-7-coumarinylamide hydrochloride (Leu-MCA) applied to yield the fluorescent product 7-amino-4-methylcoumarin (AMC) after hydrolysis of the substrate (Chróst and Velimirov, 1991). The substrate solution was added to the water sample (4.5 ml) and all samples were incubated at room temperature in the dark for 3 hours. Fluorescence was measured at an excitation of 380 nm and emission of 440 nm. Water sample blanks were held as the incubated samples and the substrate was added at the end of the incubation time. The fluorescence of blanks, including intensity of sample water and concentration of non–enzymatic-produced AMC, was measured immediately after substrate supplementation and subtracted from the sample fluorescence. An external calibration was performed for each session of analysis using Leu-MCA.

### 2.8. Bacterial Enumeration

Bacterial enumeration was performed by epifluorescence microscopy using the DAPI staining technique (Coleman, 1980; Porter & Feig, 1980). Pre- and post-incubation, a sample aliquot (10 ml) was transferred to a sterile vial, preserved with 1 ml of 2% formaldehyde, and stored at room temperature for no more than two weeks before preparing slides. Samples were incubated at a final concentration of 25 µg $ml^{-1}$ DAPI (Molecular Probes) in a dark filtration tower for 20 min prior to filtration onto a 02 µm black polycarbonate filter. The filter was mounted onto a slide with low fluorescent immersion oil and examined under a 100 W Hg bulb using a Leica epifluorescence microscope. Ten sampling fields of a known size per slide, with a minimum of 300 cells per slide were counted. Bacterial density was calculated using the following equation: Bacteria $ml^{-1}$ = (membrane conversion factor * ND), where the membrane conversion factor is the

filtration area divided by the area of the micrometer field, N is the total number of bacteria counted divided by the number of micrometer fields counted and D is the dilution factor (volume of sample stained divided by the total volume of sample). Note: Due to a methodological error bacterial counts for the December 2014 samples are unavailable.

### 2.9. Data Analysis

All data for the study are reported in the attached metadata file. The Mann-Whitney U-test was used to determine significant statistical differences among treatments (T-0d, T-28d Dark, and T-28d Light), site (S-77, S-78 and S-79), salinity (0, 5 and 30 ppt), and season. This nonparametric test allows treatments to be compared without assuming that values are normally distributed. In an effort to determine if a proxy for BDON could be developed using more easily measured variables, a principal component analysis (PCA) was performed on the ambient variables and BDON results. Data were standardized (Z-scores) prior to PCA to reduce variable magnitude effects and the resulting principal component solution was rotated (Varimax) to facilitate interpretation.

## 3. RESULTS AND DISCUSSION

### 3.1. Ambient Time Series and System Characterization

Basic water quality parameters, including salinity, conductivity, turbidity, temperature, chlorophyll-a (Chl-a) and dissolved oxygen (DO), for each sampling site at time of sample collection are shown in Figure 3. While salinity was indicative of freshwater throughout the sampling period, with relatively constant values for conductivity, temperature fluctuated seasonally as expected but within a range of nearly 10 degrees and very consistently for all sites. In contrast, between February and August, turbidity was highest at S-77 and similar at S-78 and S-79, peaking for all sites between April and June 2015. This turbidity seemed unrelated to planktonic primary production (PP) as the Chl-a time series did not correlate with turbidity (Fig. 3). The Chl-a levels were similar at all sites and peaked for the August 2015 samples. DO remained roughly in the 60-80% range for most samples, with S-79 peaking in June (supersaturated) and all sites showing a minimum during August and/or October 2015 sampling

(approximately 20-40%). This may be the result from high plankton PP (i.e. more organic matter oxidation) during August and October combined with high water temperatures.

The time series for the optical properties (fluorescence Index – FI, humification index – HIX, and slope ratio – $S_R$) are shown in Figure 4a. FI values were in the range of approximately. 1.39 to 1.47 suggesting a mixed terrestrial – algal/microbial DOM pool, but significantly influenced by organic matter from microbial sources (e.g., plankton). Terrestrial and microbial end-member values for FI would be about 1.2 and 1.8 respectively (McKnight et al., 2001; Jaffe et al., 2008). The slightly reduced values for April coincide with the higher turbidity values, possibly resulting in reduced plankton PP and enhanced relative contributions from higher plants during this period. Higher FI values during August and October suggest enhanced OM inputs from plankton and correlate with higher Chl-a values during this period. In general terms, however, the FI values remained within a reasonably narrow range throughout the sampling. HIX values remained relatively constant during the dry period and into early summer (June), but increased for the peak wet season samples. This is a bit surprising as HIX is usually inversely correlated with FI. We can only speculate that while higher planktonic PP during the wet season makes a significant contribution to the DOM pool, higher HIX during the same time period might indicate a higher contribution of soil-derived (versus plant-derived) DOM. Lastly, the $S_R$ values were very narrow throughout and nearly identical for all sites. They decreased during August and October, indicating that the average molecular weight of DOM increased during this period. Commonly, such changes are associated with increased inputs of soil-derived DOM, but they could also be related to the generation of biomolecules (see mass spectrometry data discussed below). Either scenario could be possible considering the FI and HIX distributions.

Nitrate ($NO_3^-$), ammonium ($NH_4^+$), DON, DOC and TDP ambient values for the six sampling events are presented in Figures 5a-e, respectively. $NO_3^-$ and TDP values were highest for October sampling, with DON showing a quite variable distribution, while DOC was also highest for August and October. With the exception of $NH_4^+$, which was most commonly lowest at S-79, no clear gradients between S-77 and S79 were observed. The temporal increase in TDP seemed to correlate with similar increments in Chl-a and FI, suggesting an enhancement in plankton PP. The distribution of both urea and THAA (Figs. 5f & 5g, respectively) showed neither clear seasonal nor spatial trends and remained reasonably similar throughout the sampling period.

The distribution of total dissolved nitrogen (TDN) for the time series samples clearly shows that DON is the dominant component (Fig. 6), representing approximately 40-70 µM of the TDN. Highest contributions of $NO_3^-$ were in October 2015 samples. Surprisingly, as DON and DOC are commonly correlated, the linear correlation coefficient for the ambient samples was quite low ($R^2$=0.339; Fig. 7), suggesting that the DON and DOC pools are decoupled (different sources and biogeochemical processing) in the Caloosahatchee River. This can also be seen using DOC:DON ratios (Fig. 8). No discernible gradients from S-77 to S-79 were observed, implying a reasonably consistent elemental makeup for these parameters along the transect. However, ratios ranged from approximately 20 to 40. The values observed are significantly above Redfield ratio predictions of 6.6:1, suggesting that organic matter degradation in the Caloosahatchee River may be N-limited.

More interestingly, the molar ratio of TDN: TDP (Fig. 9) strongly suggests that the Caloosahatchee River is P-limited in the dry season but shifts to potentially being N-limited during the wet season. It is also important to note that there is a spatial gradient of P limitation with downstream reach being higher than at the lake inlet.

### 3.2. Bioavailability and Microbial Dynamics (Dark Incubations)

The bioavailability of dissolved organic carbon (BDOC as % of initial pool) ranged from approximately 25-40% with highest values observed during the peak of the dry season (April), and a gradient showing a slight increase in bioavailability was observed for December, February and April (Fig. 10a). Such a spatial gradient was not observed for the samples between June and October. When salinity incubations (5 and 30 ppt) were included in the dataset (Fig. 10b), no clear patterns were discernable.

For the BDON (Fig. 11a) in contrast, a more obvious seasonal trend was observed, where BDON decreased from December 2014 to April 2015 and remained low thereafter. Several samples from April, August, and October showed significant negative BDON values. The observed trend suggests that bioavailability of DON is highly variable in the Caloosahatchee River system, ranging from as high as 61% of the pool to negative values. Negative values are the result of measuring higher DON after incubation than before. Aside from methodological variations, negative BDON is most probably due to microbial uptake of DIN and release of DON during bacterial growth. Numerous studies have shown that $^{15}N$-labeled inorganic N is rapidly released as $^{15}N$-DON (e.g., Bronk & Ward, 1999; Varela et al., 2003) and we expect the same may be true

for the C-43 system. This hypothesis becomes even more evident when the salt-added samples are included in the dataset (Fig. 11b), as evidenced by highly negative BDON values (see also Table 2). It is also possible that for the salinity samples, particularly for the 30 ppt samples, DOM flocculation due to the significant increase in ionic strength may have further enhanced the effect of reducing DON values prior to the incubation through precipitation.

Instead of classifying BDON by season, we tried to relate BDON to river flow. Daily flow data (cfs) for C-43 basin (S-78) and lake outlet (S-77) was provided by SFWMD. C-43 outlet flows were calculated as S-77 + S-78. BDON was graphed according to flow on day of collection for three sites (Fig. 12a), flow averaged over 15 days prior to collection (Fig. 12b), and flow averaged over 30 days prior to collection (Fig. 12c). No significant relationships between BDON and flow were observed.

The proteolytic enzymatic activity during incubations is stimulated by C and P additions. Leucine aminopeptidase activity (LAPA, as T28-T0) for all sites is roughly opposite to that of BDON, increasing from December 2014 to October 2015 (Fig. 13a); however, no significant regression was observed. In addition, while no clear spatial trends in LAPA were observed along the transect, after incubation the bacterial community responded strongly to apparent N limitation (Fig. 13a) by inducing the enzyme. The exception was S-79 in August (and marginally at S-78) which went negative, meaning that more enzyme was degraded than produced. The addition of salt did not promote LAPA but may have caused some inhibition of enzyme production especially in the wet season (Fig. 13b). Because water sampling is a snapshot of time, stimulation of LAPA may signify that: 1) bacteria are actively growing but N limited, and the ramped up gene expression has increased LAP titer in an effort to catabolize bioavailable DON; or 2) the bacterial community is in decline after an activity peak, having already degraded much of the BDON but still possessing elevated enzyme levels (LAP may persist for weeks in nature). This potential contradiction makes using LAPA somewhat problematic as a proxy for BDON.

The time series of bacterial response (Fig. 14a) is fairly consistent with levels (2-8 x $10^6$ $ml^{-1}$) similar to those commonly observed in other aquatic systems. In fact, prior sample filtration and subsequent inoculum additions did not cause an artifact in bacterial community response. No clear temporal or spatial patterns were discerned based on the time series; however, it is interesting to note that the estuarine inoculum used in the 30 ppt treatment resulted in the highest bacterial counts (Fig 14b) and most negative BDON (DON production).

### 3.3. Seasonality and Light Effects

Comparisons were performed between wet and dry season averages and between light and dark incubations to assess seasonal effects on DON composition and reactivity. Variations in the optical properties are depicted in Figures 15a-c. No significant changes were observed for the FI between ambient and dark incubated samples suggesting no significant transformations in the bulk composition of the fluorescence DOM. As expected from literature reports (Jaffé et al., 2004), lower FI values were clearly apparent for the light-exposed samples, but still these were no different between T0 and T28 (Fig. 15a). No significant differences were observed between wet and dry seasons except for S-78. The HIX (Fig. 15b) was higher for the wet season samples and higher for some of the incubated samples for the wet season samples. Higher HIX values after incubation could arise from bio-polymerization reactions. No significant differences between ambient and incubated samples were observed for the $S_R$ (Fig. 15c), suggesting that light exposure did not result in a significant change in molecular weight of the DOM. $S_R$ values were slightly lower during the wet season, indicating a slightly more elevated MW for bulk DOM during that time period.

For the freshwater incubations, there was a significant reduction in DON concentration after incubation, particularly during the dry season sampling (Fig. 16), although no significant differences were observed between light and dark incubations. This trend was not observed for the wet season samples where DON remained relatively constant. For the saline samples, a slight increase in DON was observed for the wet season samples in the 5 ppt samples, and a more significant increase for the 30 ppt samples (also wet season only). These data suggest, as mentioned above, a generation of DON through bacterial consumption of DIN and consequent release of newly generated DON into the water column. The initial removal of DON due to flocculation, particularly for the 30 ppt samples, might further aid in this effect; however, this was not the case for the 30 ppt dry season samples.

The comparative analyses for the BDON are shown in Figure 17. No significant differences were observed between light and dark incubations for the dry season samples. The wet season samples generally had a lower BDON compared to the dry season samples, suggesting a seasonal change in molecular composition (DON quality), a difference in the bacterial community structure, or both. Compositional differences for the DON could not be assessed in

the present study considering that most of the DON is molecularly uncharacterized, and that the optical properties used are for DOM and not DON-specific. Wet season BDON for the saline samples were clearly negative (see discussion above). In comparison, LAPA was also commonly higher during the wet season but only significantly higher for the light treatment for the 30 ppt samples (Fig. 18). The LAPA in August for S-79 and S-78 were strongly negative and influenced the seasonal averages disproportionately. Higher LAPA during the wet season when BDON is lower suggests more significant bacterial N-limitation and/or the presence of more refractory DON. However, when ambient LAPA values were subtracted from the incubation experiments (i.e., LAP stimulation; Fig.19), a slightly different picture emerges. With the exception of site S-77, no significant differences were observed between wet and dry seasons, while differences between dark and light were apparent only for the 5 and 30 ppt S-79 samples, with the light-exposed samples showing some positive stimulation. It is important to note that the initial LAP values include background fluorescence as well as enzyme activity, and the relatively high background may have contributed to negative LAPA.

Figure 20 shows the bacterial counts, which as mentioned above, are higher in the incubated samples for both wet and dry seasons. The light-exposed samples had significantly higher bacterial counts compared to the dark samples in all of the freshwater samples, but only for the wet season for the 5 ppt sample. Wet vs. dry sample differences were not significant except for the S-79 0 and 5 ppt dry samples being higher, compared to the wet samples. For the freshwater samples, in all but one case (S-79 Wet) the particulate nitrogen (PN) was higher in the light compared to the dark incubations (Fig. 21), suggesting a higher incorporation of N into bacterial biomass for the former. Therefore, light-exposure seemed to have an effect on the composition of the DOM by making it more bioavailable. This effect was reflected in the PN for both seasons, although BDON was higher during the dry season.

### 3.4. DON Composition

DON composition was assessed through the analysis of THAA and urea. Compositional features are detailed in Table 3 by station and seasonally as %DON. Clearly, DON in the Caloosahatchee consisted primarily of molecularly uncharacterized organic compounds, considering that this fraction was in the upper 90% in the great majority of the samples. THAA and urea usually accounted for less than 5% of the total DON. As such, THAA and urea seem

nearly negligible when assessing BDON in this system. Figure 22 shows the variations for the THAA during wet and dry seasons and for dark versus light-exposed incubations. Seasonal differences and incubation effects seem minimal, if that, for the S-77 and S-78 samples, while some degradation was observed for the S-79 samples. However, no clear trend was determined for the latter between wet and dry season. Similarly, the patterns observed for the urea (Fig. 23), show no clear seasonal differences. However, in three out of five dark incubations for the wet season (S-77, S-78 and S-79 5 ppt) the urea values increased compared to the T0 samples, suggesting a metabolic induced generation. This effect was not observed for the light-exposed samples, indicating that much of the ambient urea and existing precursors were photodegraded resulting in a lower productivity of this compound during the incubation period. While the exact reasons remain unknown and are only speculative at this point, the highest production of urea during degradation was for the October samples, likely due to specific DON compositional features for this sampling. In general terms, the contribution of urea and THAA to the overall DON pool in the Caloosahatchee River seems rather small and their bioavailability rather variable. Time series for THAAs and urea are shown in the Appendix (Appendix Figures A2 and Fig.A3).

### 3.5. Principal Component Analysis (PCA)

Results of the PCA show that a portion of BDON variability was explained by two factors. Factor-1 was composed of BDON, $NO_3^-$, TDP, FI and SC, while Factor-2 was composed by BDON, $NH_4^+$, Chl-a and urea (Table 4a). When seasonal data are separately analyzed (wet versus dry), Factor-1 for the dry season data correlates BDON with $NO_3^-$, TDP, and Chl-a, while Factor-1 for the wet season data correlates BDON with urea only (Table 4b). While some of these correlations are likely concentration-related, environmental conditions such as redox conditions, OM quality, bacterial community structure, etc. might also play critical roles in driving BDON. Explaining the observed correlations without a significantly more extensive dataset would be speculative and was not attempted at this stage. However, it is clear that the PCA results confirm that BDON is driven by a complex combination of environmental factors.

# 4. SUMMARY

The BDON assessed for the Caloosahatchee River system during the December 2014 to October 2015 sampling period resulted in a highly variable dataset, particularly on temporal scales and less so on spatial scales. One of the main hindrances in any attempt to characterize BDON and develop proxies for its spatial and temporal scale assessment is that the conditions reflect a mainly uncharacterized pool of organic nitrogen on the molecular level and, as such, it is difficult to extrapolate from one sample to the next.

Ultra-high resolution mass spectrometry (FT-ICR/MS) data for June 2015 DOM samples from S-77, S-78 and S-79 (sampled separately; study not funded through SFWMD; Appendix Figure A1a), showed that the MS-sensitive fraction isolated through solid phase extraction, contained between 3,800 and 5,700 identifiable N-containing molecular formulas (individual compounds) showing a significant abundance of DON components as well as a high molecular diversity (Appendix Table A1). Based on their elemental composition (Appendix Figure A1b), the DON was classified into approximately 7% black nitrogen (polycondensed-N), 17% N-polyphenols, 42% N-unsaturated compounds with low oxygen content, 15% N-unsaturated compounds with high oxygen content, and 19% peptides. Mass spectrometry data plotted in the format of van Krevelen diagrams (H/C vs. O/C; Appendix Figure A1c) showing the molecular formulas that were removed during incubation (dark and light) and those generated during incubation (dark and light) suggest that a significant amount of new DON molecules are actually generated during incubation and are dominated by highly aliphatic N-containing molecules of the peptide type (Appendix Fig. A1). Neither the exact molecular structure, nor the origin or the reactivity and seasonal variation of these DON compounds are presently known.

From the Wetlands Solutions report (2012), "There is currently no recognized analytical test to fractionate total organic N (TON) from biologically available N (BAN). As used in this report, BAN includes all biologically available N for ms, including organic and inorganic compounds". That said, we circle back to TMDL concerns by using the data to calculate BAN from the data reported in this study and compare to Jørgensen et al. (2014) estimates. Excluding negative BDON, BAN (calculated as (DIN + BDON) / TDN) ranged from 3.5 – 69.9%, with a mean of 32.4% of the TDN being bioavailable (Fig. 24). The components of BAN varied over time and to some spatial degree (Fig. 25) but no obvious trends were observed. Therefore, based on this limited dataset, increased TN loading without deleterious effects (e.g., eutrophication)

could be feasible, should BAN be used as a measure of loading rather than TN. However, this is not a recommendation by the authors of this study as the available dataset is quite limited and further studies would be needed to confirm this suggestion. Applying BAN can also provide insight to nutrient limitation for PP. Finally, using the BAN:TDP ratio shows that the Caloosahatchee River was actually P-limited during the entire study period except for December and February (Fig. 26). However, there is still little known about how different environmental drivers (hydrology, water quality, microbial community structure, etc.) control BDON and, thus, proxies indicative of BDON remain elusive at this time. Larger datasets and better molecular-level DON characterizations are required to advance this field. Finally, we propose a conceptual model of DON bioavailability as it relates to environmental conditions (Fig. 27). The idea is that simultaneous production and consumption of DON is influenced by both the bioavailability of DON sources, the ratio of DIN to BDON, and degree of N limitation in the ecosystem. We expect that deconvolution of these factors may help in making BDON more predictable.

Overall, this study showed that BDON dynamics are highly variable on temporal scales, but less so on spatial scales. BDON was highestin December (averaging 61% of DON), decreased through the dry season, and remained low in the wet season. No consistent spatial patterns were detected. The addition of salinity to test the DON "shuttle" hypothesis (Stepanauskas et al., 1999) actually resulted in a reduction in BDON (and possible microbial conversion of DIN to DON) instead of an increase as predicted by the "shuttle" hypothesis. Exposure to light did not significantly change BDON concentration. These results have important implications for the mesocosm phase of the C-43 WQTT project. For example, the lack of a photolytic response led to a redesign of the mesocosm treatments with the elimination of an open water treatment, which resulted in a cost savings. In addition, the seasonality of the BDON may have important implications for the nitrogen removal rates achievable in the treatment wetlands over the course of a year and may need to be factored into sampling designs and data analysis. In conclusion, this portion of the C-43 WQTT project has been invaluable for understanding the many issues associated with nitrogen removal to meet the TMDL for the Caloosahatchee River.

# 5. REFERENCES


Bailey N, Magley W, Mandrup-Poulsen J, O'Donnell K, Peets R (2009) Nutrient TMDL for the Caloosahatchee Estuary (WBIDs 3240A, 3240B, and 3240C). Florida Department of Environmental Protection. TMDL Report. 119 pp.

Boyer JN, Jaffé R, Dailey SK, Maie N (2004) Biological Availability of Organic Nitrogen in Florida Bay. South Florida Water Management District, West Palm Beach, p. 43.

Bronk DA, Castell JV, Cervera M, Marco R (1979) A convenient micromethod for the assay of primary amines and proteins with fluorescamine. A reexamination of the conditions of reaction. *Analytical Biochemistry* 99, 379-391.

Bronk DA, See JH, Bradley P, Killberg L (2007) DON as a source of bioavailable nitrogen for phytoplankton. *Biogeosciences* 4, 283-296.

Bronk DA, Ward BB (1999) Gross and net nitrogen uptake and DON release in the euphotic zone of Monterey Bay, California. *Limnology and Oceanography* 44, 573-585

Chen M, Jaffé R (2014) Photo- and bio-reactivity patterns of dissolved organic matter from biomass and soil leachates and surface waters in a subtropical wetland. *Water Research* 61, 181-190.

Chróst RJ, Velimirov B (1991) Measurement of enzyme kinetics in water samples: Effect of freezing and soluble stabilizer. *Marine Ecology Progress Series* 70, 93-100.

Coleman AW (1980) Enhanced detection of bacteria in natural environments by flourochrome staining of DNA. *Limnology and Oceanography* 25, 948–951.

Cory RM, McKnight DM (2005) Fluorescence spectroscopy reveals ubiquitous presence of oxidized and reduced quinones in dissolved organic matter. *Environmental Science and Technology* 39, 8142-8149.

Dumas JBA (1831) Procedes del'analyse organic. *Annales de Chimie et de Physique* 247, 198-213.

Eyre B, Ferguson AJP, Webb A, Maher D, Oakes JM (2011) Denitrification, N-fixation, and nitrogen and phosphorus fluxes in different benthic habitats and their contribution to the nitrogen and phosphorus budgets of a shallow oligotrophic subtropical coastal system (southern Moreton Bay, Australia). *Biogeochemistry* 102, 111-133.

Helms JR, Stubbins A, Ritchie JD, Minor EC, Ki eber DJ, Mopper K (2008) Absorption spectral slopes and slope ratios as indicators of m olecular weight, source, and photobleaching of chromophoric dissolved organic matter. *Limnology and Oceanography* 53, 955-969.

Jaffé R., Boyer J.N., Lu X., Maie N., Yang C ., Scully N. and Mock S. (2004) Sources characterization of dissolved organic matter in a mangrove-dominated estuary by fluorescence analysis. *Marine Chemistry* 84: 195-210.

Jaffé R, McKnight D, Maie N, Cory R, McDowell WH, Campbell JL (2008) Spatial and temporal variations in DOM composition in ecosystems: The importance of long-term monitoring of optical properties. *Journal of Geophysical Research* 113, G04032, doi:10.1029/2008JG000683.

Jørgensen L, Markager S, Maar M (2014) On the importance of quantifying bioavailable nitrogen instead of total nitrogen. *Biogeochemistry* 117, 455-472.

McKnight DM, Boyer EW , Westerhoff PK, Do ran PT, Kulbe T, Andersen DT (2001) Spectrofluorometric characterization of dissolved organic matter for indication of precursor organic material and aromaticity. *Limnology and Oceanography* 46, 38-48.

Porter KG, Feig Y (1980) The use of DAPI fo r identifying and counting aquatic m icroflora. *Limnology and Oceanography* 25, 934.

Revilla M, Alexander J , Glibert PM (2005) Urea analys is in coastal waters: C omparison of enzymatic and direct methods. *Limnology and Oceanography: Methods* 3, 290-299.

Seitzinger SP, Sanders RW (1997) Contribution of dissolved organi c nitrogen from rivers to estuarine eutrophication. *Marine Ecology Progress Series* 159, 1-12.

SFWMD (2012) Caloosahatchee River W atershed Protection Plan 2012 Update. 2012 South Florida Environmental Report, Volum e 1, Appendix 10-2. Available on-line at: www.sfwmd.gov/SFER.

Solorzano L, Sharp JH (1980) D etermination of total dissolved phosphorus and particulate phosphorus in natural waters. *Limnology and Oceanography* 25, 754-758.

Stepanauskas R, Leonardson L, Tranvik LJ (19 99) Bioavailability of wetland-derived DON to freshwater and marine bacterioplankton. *Limnology and Oceanography* 44, 1477-1485.

Varela MM, Barquero S, Bode A, Fernande z E, Gonzalez N, Teira E, Varial M (2003) Microplanktonic regeneration of ammonium and dissolved organic nitrogen in the upwelling

area of the NW of Spain: relationships with dissolved organic carbon production and phytoplankton size structure. *Journal Plankton Research* 25, 719–736.

Wetland Solutions (2012) Evaluation of Total Nitrogen Reduction Options for the C-43 Water Quality Treatment Area Test Facility, Final Task 2 Report. Prepared for the South Florida Water Management District, April 12, 2012. 119 pgs.

Wiegner TN, Seiztinger SP, Glibert PM, Bronk DA (2006). Bioavailability of dissolved organic nitrogen and carbon from nine rivers in the eastern United States. *Aquatic Microbial Ecology* 43, 277-287.

Zsolnay A, Baigar E, Jimenez M, Steinweg B, Saccomandi F (1999) Differentiating with fluorescence spectroscopy the sources of dissolved organic matter in soils subjected to drying. *Chemosphere* 38, 45-50.