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# Changing Bacterial Growth Efficiencies across a Natural Nutrient Gradient in an Oligotrophic Estuary

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

CHANGING BACTERIAL GROWTH EFFICIENCES ACROSS A NATURAL

NUTRIENT GRADIENT IN AN OLIGOTROPHIC ESTUARY

A thesis submitted in partial fulfillment of the

requirements for the degree of

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in

GEOSCIENCES

by

Amber Alexis Kiger

2015

To: Dean Michael Heithaus
    College of Arts and Sciences

This thesis, written by Amber Alexis Kiger, and entitled Changing Bacterial Growth Efficiencies across a Natural Nutrient Gradient in an Oligotrophic Estuary, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

_____
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Joseph N. Boyer, Co-Major Professor

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William Anderson, Co-Major Professor

Date of Defense: March 27, 2015

The thesis of Amber Alexis Kiger is approved.

_____
Dean Michael Heithaus
College of Arts and Sciences

_____
Dean Lakshmi N. Reddi
University Graduate School

Florida International University, 2015

DEDICATION

This thesis is dedicated to my family for encouraging me to reach this point in my academic and scientific career.

ACKNOWLEDGMENTS

ABSTRACT OF THE THESIS

CHANGING BACTERIAL GROWTH EFFICIENCIES ACROSS A NATURAL

NUTRIENT GRADIENT IN AN OLIGOTROPHIC ESTUARY

by Amber Alexis Kiger

Florida International University, 2015

Miami, Florida

Professor William Anderson, Co-Major Professor

Professor Joseph N. Boyer, Co-Major Professor

Recent studies have characterized coastal estuarine systems as important
components of the global carbon cycle.  This study investigated carbon cycling through
the microbial loop of Florida Bay by use of bacterial growth efficiency calculations.
Bacterial production, bacterial respiration, and other environmental parameters were
measured at three sites located along a historic phosphorus-limitation gradient in Florida
Bay and compared to a relatively nutrient enriched site in Biscayne Bay.  A new method
for measuring bacterial respiration in oligotrophic waters involving tracing respiration of
$^{13}$C-glucose was developed.  The results of the study indicate that $^{13}$C tracer assays may
provide a better means of measuring bacterial respiration in low nutrient environments
than traditional dissolved oxygen consumption-based methods on the basis of strong
correlations between incubation length and $\delta^{13}$C values.  Results also suggest that overall
bacterial growth efficiency may be lower at the most nutrient limited sites.

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1. INTRODUCTION

In light of increasing greenhouse gas concentrations and the threat of climate change, recent studies designed for quantifying potential carbon sinks have indicated that coastal marine systems are capable of storing carbon on a globally significant scale (Boullion et al. 2011, McLeod et al. 2011). A study by Donato et al. (2011) quantified carbon storage within mangrove systems, which are common along tropical and subtropical coastlines. The study indicated that mangrove systems on average store 1,023 megagrams carbon per hectacre (Mg C ha$^{-1}$), storing significantly more carbon on average than upland tropical forests (300 Mg C ha$^{-1}$) that are typically considered in carbon sequestration efforts. Another study by Fourqurean et al. (2012a) quantified carbon storage within seagrass beds. The study concluded that seagrass meadows globally can store up to 829 Mg C ha$^{-1}$. As research continues in this field, more detailed quantification of coastal carbon budgets will be necessary in order to better understand the role of coastal communities in the global carbon cycle.

Current research is characterizing carbon budgets across different habitats and communities within the Everglades and Florida Bay. Fourqurean et al. (2012b) examined carbon storage under seagrass beds in Florida Bay, and showed that on average, Florida Bay seagrass beds store 163.5 Mg C ha$^{-1}$. The highest percentage of organic carbon (8%) was found in sediment adjacent to the shoreline in Central Florida Bay. Smoak et al. (2013) measured carbon storage at two mangrove sites in the southwestern Everglades located on the Shark River and Harney River. The overall carbon burial rate, measured by $^{210}$Pb dating of sediment cores, was 151-168 g C m$^{-2}$ yr$^{-1}$. A study by Engel et al. (2011), however, suggests that most carbon uptake in these mangrove forests is exported

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through tides as dissolved inorganic or organic carbon and does not accumulate in sediment. Although these components of the coastal Everglades carbon budget have been determined, more research is needed, particularly for carbon cycling within the Florida Bay water column.

Previous work has characterized air-water $CO_2$ exchange in different parts of Florida Bay. DuFore (2012) measured $CO_2$ exchange through floating chambers at 24 sites throughout Florida Bay. At Eastern Florida Bay sampling stations, water was found to be oversaturated with $CO_2$. DuFore (2012) hypothesized that this oversaturation was a consequence of freshwater input from Taylor Slough. In Central Florida Bay, water was generally undersaturated with $CO_2$ and acted as an atmospheric $CO_2$ sink. DuFore (2012) did not quantify microbial data in this study; thus, the role of microbes in the sink or source status of Florida Bay is still unknown.

Water column microbial communities can often be a significant component of coastal and estuarine carbon cycles (Azam et al. 1983). Bacteria, phytoplankton and zooplankton can move C and nutrients across air-land-water boundaries and transform C into different organic and inorganic forms (Arrigo 2005). One particularly important energy and nutrient pathway utilized by bacteria is the microbial loop, which is considered separate from the classical marine food chain (Azam et al. 1983). Within the microbial loop, water column bacteria take up dissolved organic carbon (DOC) produced by other tropic levels and either respire the carbon as $CO_2$ or convert it into bacterial biomass that moves back into the traditional food chain (Figure 1).
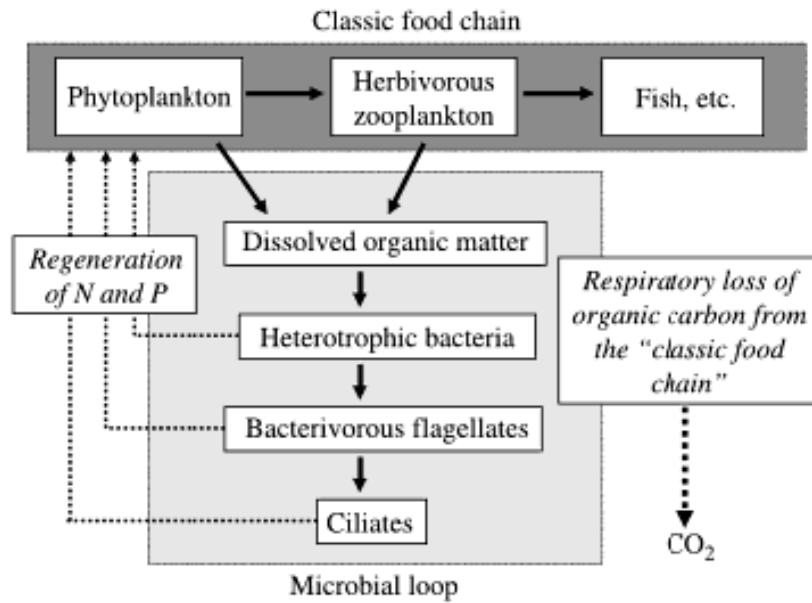
Figure 1: Diagram of the classical marine food chain and the microbial loop (modified from Robinson 2008).

Microbial loop carbon cycling processes can be quantified through bacterial growth efficiency measurements. Bacterial growth efficiency (BGE) is a ratio that indicates the proportion of bacterial carbon uptake that is used to produce biomass relative to the amount of carbon respired (del Giorgio et al. 1997). Bacterial growth efficiency is represented by the equation BGE = BP/(BP + BR), in which BP is bacterial production and BR is bacterial respiration (del Giorgio and Cole 1998). Both BP and BR are reported in $\mu$g C L$^{-1}$ hr$^{-1}$. Numerous factors influence BGE, which ultimately influences the sequestration of carbon into biomass or loss of carbon to respiration in an aquatic or marine system. According to a review of global BGE calculations by del Giorgio and Cole (1998), BGE varies in different aquatic environments and increases along productivity gradients (Figure 2). Low BGE is expected in oligotrophic environments as a result of the energy cost of maintaining active transport systems and

basic metabolic machinery. Mesotrophic and eutrophic areas, however, do not need to

allocate as much energy for utilization of nutrients, so BGE tends to be higher in these

waters.
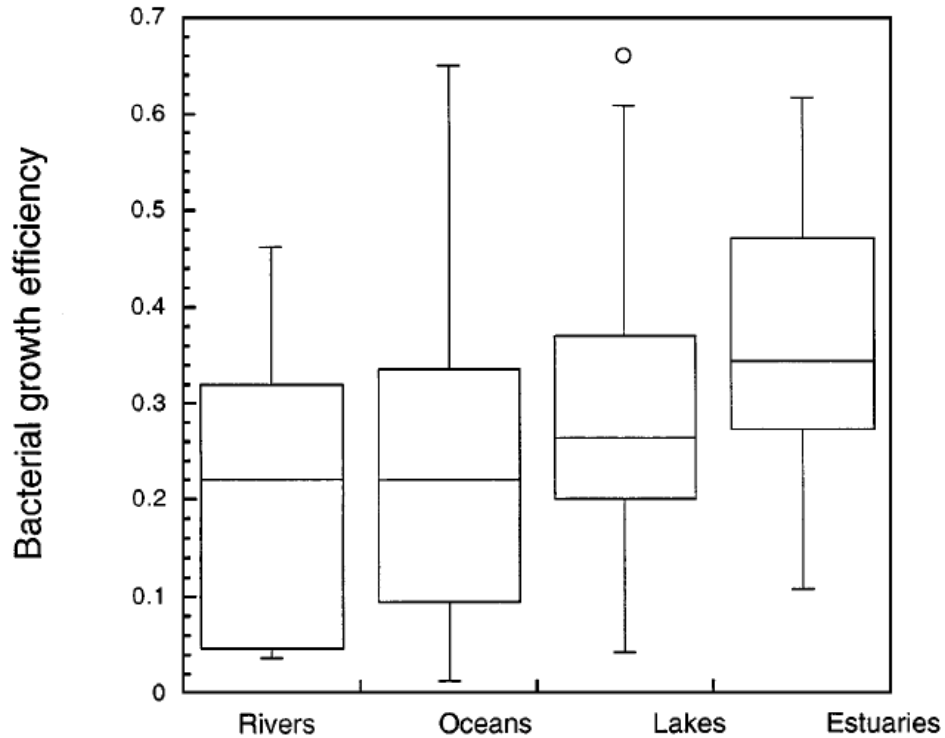


Figure 2: Range of BGE reported in previous literature in rivers, oceans, lakes, and
estuaries (modified from del Giorgio and Cole 1998).


Although the del Giorgio and Cole (1998) study indicated that trophic state index

was a main factor influencing BGE, other studies have investigated additional potential

controls of BGE. Eiler et al. (2003) found that DOC concentration correlates with BGE

in an asymptotic pattern, suggesting that DOC concentration affects BGE only in the

most oligotrophic conditions. A study by Apple and del Giorgio (2007) showed that

BGE positively correlated with long-term DOC lability in a temperate estuary, indicating

that dissolved organic matter (DOM) source (quality) may impact BGE. A study

conducted by Lennon and Pfaff (2005) showed that DOM with low C:P ratios (less than 160:1 by mol) from different sources was associated with high bacterial productivity, indicating that P availability can affect BGE values. A weak negative correlation between BGE and salinity was found in a study by Griffiths et al. (1984), but this correlation may have been an effect of productivity gradients rather than an effect of salinity gradients (del Giorgio and Cole 1998). Other potential controls on BGE, such as N, are also examined in the del Giorgio and Cole (1998) review.

Methods to analyze BGE often involve measurement of BP via tritiated thymidine or leucine incorporation (Fuhrman and Azam 1982, Kirchman et al. 1985, Simon and Azam 1989) and measurement of BR through $O_2$ consumption (Biddanda et al. 1994, Chin-Leo and Benner 1992). Samples collected for BR measurements are usually filtered to separate bacteria from larger organisms and then incubated in dark conditions to measure initial and final dissolved oxygen (DO) levels. Although the vast majority of BR measurements are made in this way (Robinson 2008), this method may not be ideal, especially for oligotrophic systems. Bacterial respiration measurements in oligotrophic waters often require long incubations (24 hours or greater) to obtain a detectable change in DO levels (del Giorgio and Cole 1998). A study by Aranguren-Gassis et al. (2012) concluded that DO consumption measurements lead to overestimation of BR rates in oligotrophic waters because of long incubation times altering the natural microbial community structure. In addition to long incubation times altering community structure, contamination of samples with atmospheric oxygen may impact results. Earth's atmosphere contains 21% $O_2$ while water contains DO at a concentration several orders of magnitude smaller (approximately 1-10 parts per million); thus, careful methodology

must be used to avoid aeration of samples and atmospheric contamination (Friederich et al. 1991). Because DO consumption measurements may not be representative of natural BR in an oligotrophic environment, alternative methods should be developed to better assess BR and hence, BGE.

Carbon isotope tracer experiments may serve as an alternative method to determine BR. Early work on quantifying uptake and respiration of C focused on bacterial consumption of radiolabeled compounds, such as $^{14}$C-labeled glucose (Parsons and Strickland 1961, Hobbie and Crawford 1969). Although these studies were sensitive enough to measure respiration in low productivity systems (del Giorgio and Cole 1998), these measurements led to overestimates of BGE because of the simplicity of the compounds compared to natural food sources as well as isotopic dilution within the intracellular carbon pool (King and Berman 1984, Bjørnson 1986, Kirchman 2003). Measurement of natural stable isotope ratios before and after incubation rather than use of radioactive tracers may provide a way to measure BR in oligotrophic systems while overcoming concerns about use of simple tracer compounds. Natural stable isotope ratio measurement would involve bacterial consumption of a variety of simple and complex compounds, producing BR values more representative of field conditions. Ratios of $^{12}$C and $^{13}$C are utilized frequently in ecological studies (Fry 2006) and are usually reported as $\delta^{13}$C values (Craig 1957). Recent developments in isotope ratio mass spectrometry have created more precise methods for measuring $\delta^{13}$C in dissolved inorganic carbon (DIC) (St. Jean 2003, Torres et al. 2005). Tracking changes in $\delta^{13}$C-DIC before and after dark incubation may serve as an alternative to both DO consumption methods and radioisotope methods for measuring BR.

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Past studies have shown that DIC concentration, as well as $\delta^{13}$C-DIC values, can be impacted by microbial activity (Torres et al. 2005); thus, known microbial impacts to $\delta^{13}$C-DIC can be used to produce respiration measurements in the current study. Preferential uptake of $^{12}$C by phytoplankton during photosynthesis can lead to $\delta^{13}$C-DIC increases. Decay and bacterial processing of organic matter impacted by this biological fractionation, conversely, tends to decrease $\delta^{13}$C-DIC values (Li and Liu 2011). Thus, size-filtered BR samples containing only natural sources of DOM (previously impacted by phytoplankton and higher trophic levels) would decrease in $\delta^{13}$C-DIC over time. Samples with an isotopically labeled substrate, however, would have increasing $\delta^{13}$C-DIC values over time as the labeled DIC is consumed and respired.

Studies that assess $\delta^{13}$C-DIC values typically involve addition of a preservative to a water sample in order to prevent this microbial activity from occurring (Doctor et al. 2008, Li and Liu 2011). A study by Taipale and Sonninen (2009) specifically addressed the effects of collection vial type and preservation method on $\delta^{13}$C-DIC values and found significant changes when samples were not properly stored or preserved. Other methods used to keep $\delta^{13}$C-DIC values unchanged include filtration of samples, storage of samples under refrigeration, and complete filling of sample vials to prevent fractionation with air (Taipale and Sonninen 2009). In this study, the inhibition methods described above were used to stop biological activity at specific incubation intervals.

The primary objective of this study was to calculate baseline bacterial growth efficiency values at three historically oligotrophic sites (Childers et al. 2006) located in Florida Bay from bacterial production and bacterial respiration measurements. A secondary goal of this study was to develop an alternative method of measuring bacterial

respiration using changes in $\delta^{13}$C-DIC values.  On the basis of previous research, BGE

values were expected to positively correlate with historic trophic state index (TSI) and

were expected to be lowest at the most oligotrophic site.  In mesotrophic waters,

traditional DO consumption methods were expected to be effective for measuring

bacterial respiration and were expected to not differ significantly from the $\delta^{13}$C-DIC

method.  In oligotrophic waters, however, the DO method was expected to be ineffective.

Under these conditions, the $\delta^{13}$C-DIC method was expected to detect respiration after less

than 24 hours of incubation, while the DO method was expected to require incubations of

24 hours or longer to detect respiration.  In summary, this study was expected to address

the following three objectives and hypotheses:

> Objective 1: To determine bacterial growth efficiencies across a natural nutrient
>
> gradient in Florida Bay.
>
> H1: Bacterial growth efficiencies will positively correlate with historic trophic
>
> state index and nutrient stoichiometry.
>
> Objective 2: To develop a method that detects BR in oligotrophic waters which is
>
> more sensitive and requires a shorter incubation time than traditional DO
>
> consumption-based measurements.
>
> H2:  In oligotrophic waters, $\delta^{13}$C-DIC changes and DIC increases will be
>
> detectable after less than 24 hours of incubation.  DO consumption, however, will
>
> not be detectable within 24 hours.
>
> Objective 3: To determine whether measurements of $\delta^{13}$C-DIC before and after
>
> dark incubation can serve as an alternative to traditional DO consumption
>
> measurements when determining bacterial respiration in a marine water sample.

<u>H3</u>: Bacterial respiration values calculated by $\delta^{13}$C-DIC measurements will not be significantly different than standard oxygen-based methods under mesotrophic or eutrophic conditions.

## 2. METHODOLOGY

### 2.1 Study Site Description

Florida Bay is a 1,600 km$^2$ lagoonal estuary located between the southern tip of mainland Florida and the Florida Keys. Carbonate mud banks that restrict water circulation divide Florida Bay into shallow basins (Fourqurean and Robblee 1999). Taylor Slough, located north of the eastern side of the Bay, is the main terrestrial surface water source and provides over 90% of the freshwater in Eastern Florida Bay (Swart and Price 2002). Conversely, Western Florida Bay lacks direct terrestrial surface water input resulting in rainfall being the primary freshwater input. Seasons are primarily defined by wet-dry precipitation cycles rather than air temperature. Approximately 60% of rainfall occurs from June-September, while 25% of rainfall occurs between November-April (Obeysekera et al. 1999). Florida Bay is characterized by occasional periods of hypersalinity, ranging up to 70 psu, when freshwater input from the Everglades is low (Boyer and Jones 2001, Robblee et al. 2001).

The geology of Florida Bay was briefly reviewed by Obeysekera et al. (1999). Oolitic Miami Limestone forms the bedrock foundation of Florida Bay. The Lower Florida Keys are composed of higher relief portions of oolitic Miami Limestone, while the Upper Florida Keys are composed of fossilized coral (Key Largo Limestone). The geologic foundations of the Florida Keys and Florida Bay were formed approximately

100,000 years ago during the Sangamon interglacial (Hoffmeister 1974, Obeysekera et al. 1999).  During this time, warm shallow seas covered the present-day Everglades and Florida Keys, creating conditions for both inorganic limestone formation (Miami Limestone- Oolitic) and biologic limestone formation (Key Largo Limestone, Miami Limestone- Bryozoan).  The limestone foundation of Florida Bay is fairly flat; however, calcareous sediments have produced a network of banks and shallow basins throughout Florida Bay.  The beginning of this sediment deposition began approximately 4000 years ago as sea level rise began to flood present-day Florida Bay.  The bank and basin system is hypothesized to be a product of converging currents during flooding of Florida Bay, drowned marsh rills or minor bedrock depressions (Wanless and Tagett 1989, Obeysekera et al. 1999).

Physical, chemical, and nutrient limitation characteristics have been used to divide the bay into three biogeochemically distinct regions: Eastern Florida Bay, Central Florida Bay and Western Florida Bay (Boyer et al. 1997).  Eastern Florida Bay, which is influenced by freshwater input from Taylor Slough, is strongly P-limited and is considered the most oligotrophic part of the Bay. Western Florida Bay is less P-limited and is more strongly influenced by oceanic connections with the Gulf of Mexico (Boyer et al. 1997).  Central Florida Bay lies in the middle of this nutrient gradient and is characterized by high DOM, long water residence time, and periodic cyanobacterial blooms dominated by *Synechococcus* (Phlips et al. 1999, Boyer et al. 2006).  This nutrient limitation pattern has led to Florida Bay being described as an "upside-down" estuary, meaning that limiting nutrients are derived from marine sources rather than terrestrial sources (Childers et. al 2006).

Samples for this study were collected from three sites in Florida Bay that belong

to the Florida Coastal Everglades Long-Term Ecological Research (FCE-LTER)

network: TS-9, TS-10 and TS-11 (Figure 3). Each of these sites is representative of

different biogeochemical conditions that exist in Florida Bay. TS-9 is a shallow estuarine

site and is most representative of conditions in Eastern Florida Bay (Boyer et al. 2006).

TS-10 and TS-11 are both representative of Central Florida Bay, but TS-11 experiences

more exchange with the Gulf of Mexico and is located farther west along the nutrient

gradient.
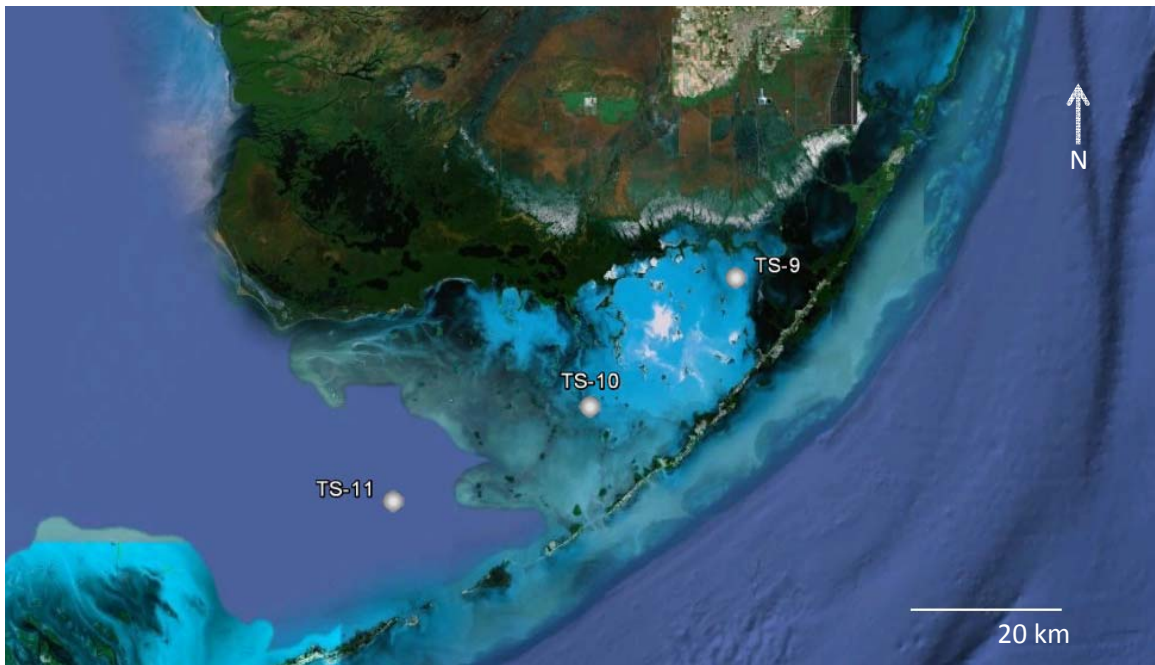


Figure 3: Aerial image of Florida Bay and field collection sites (modified from Google Earth).

In addition to the Florida Bay sites, a historically more nutrient enriched site in

Biscayne Bay was selected for method development purposes (Figure 4). This site

corresponds to site #134 (Oleta River Park: 25.90500˚N, 80.13333˚W) of the former

SERC Water Quality Monitoring Network. This site, as well as others in northern

Biscayne Bay, has been previously characterized as having elevated total P (up to 0.18 mg $L^{-1}$), high chlorophyll a (0.96 µg $L^{-1}$), and low DO (<4ppm) in comparison to the rest of the Bay. These conditions have been attributed to nutrient input from canals, surrounding urban land use, and possible influence from the Munisport landfill (Caccia and Boyer 2005, Caccia and Boyer 2007). As a consequence of the mesotrophic nature of the site, changes in DO should be detectable over relatively short incubation times. Therefore, water samples collected from this site were expected to have matching bacterial respiration values whether measured by DO consumption or through ^{13}C respiration assays.
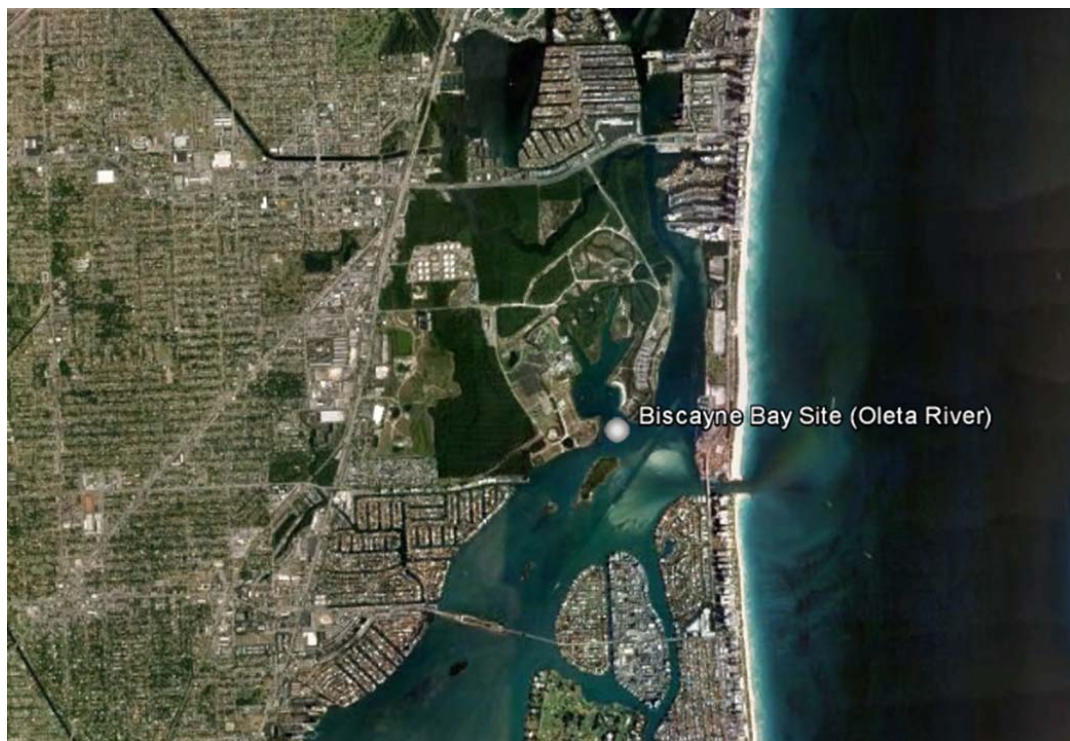


Figure 4: Aerial image of northern Biscayne Bay and the Oleta River Park sampling site (modified from Google Earth).

2.2 Sample Collection

Initial samples for this study were collected on May 23, 2013 from the Biscayne Bay site starting at 9:50 AM.  Thirty two samples for ^{13}C-DIC analysis and twelve samples for DO analysis were collected.  All samples collected during the initial sampling event were unfiltered, allowing the detection of maximum community respiration.  Dissolved oxygen samples were collected first via peristaltic pump (E/S Environmental Sampler) from a depth of approximately 0.3 m into 1 liter amber narrow-mouth HDPE bottles.  Bottles were rinsed three times with sample water then gently filled from the bottom to prevent introduction of atmospheric $O_2$ into the sample.  Sample bottles were permitted to overflow by approximately one bottle volume before tubing was removed.  The DO bottles were then sealed without headspace with parafilm (as a secondary gas barrier), capped (as a primary gas barrier), and placed into a box.

Three of the DO samples were collected directly into 125 ml glass flasks for initial measurements ($t_0 = 0$ h).  Flasks were also filled from the bottom via silicone tubing until the flask overflowed.  One ml of 3 M manganous chloride solution and 1 ml of 4 M sodium iodate/8 M sodium hydroxide solution were then added to the flasks (Carpenter 1966, Friederich et al. 1991).  The flasks were then capped with glass stoppers and inverted several times until a precipitate was distributed throughout each sample.  At 8, 12, and 24 h after collection (initiation of dark incubations), water was transferred via peristaltic pump from the amber bottles into 125 ml glass flasks.  The procedure described for the initial samples ($t_0 = 0$ h) was repeated for the 8, 12 and 24 h DO samples.

Samples for $^{13}$C-DIC analysis were collected into 40 ml amber glass vials. Silicone septa provided with the vials were replaced with butyl rubber septa to prevent atmospheric $CO_2$ exchange (Taipale and Sonninen 2009). Fifteen of the vials contained 1 ml of $^{13}$C-glucose solution at a concentration of 6 μg $L^{-1}$. Sample vials were filled from the bottom via peristaltic pump and silicone tubing. These vials were filled until a positive meniscus formed to eliminate headspace. Sample vials containing $^{13}$C-glucose were not permitted to overflow in order to keep $^{13}$C-glucose concentrations similar in all vials. Six vials labeled as initial (0 h) vials were treated with one drop of saturated $HgCl_2$ solution to stop biological activity and were then sealed with parafilm, capped and placed on ice in a cooler. All other vials were sealed with parafilm and capped without $HgCl_2$ treatment. These samples were placed into a box and kept out of light to prevent photosynthetic processes. After 4, 8, 12 and 24 h of dark incubation at ambient temperatures (25°C), six samples were treated with 1 drop of saturated $HgCl_2$ solution, resealed and placed in a refrigerator at 4°C. Remaining samples included a control that was not treated with $HgCl_2$ and a control that contained approximately 2 cm of headspace to determine if the $HgCl_2$ effectively stopped respiration and to determine if atmospheric air contamination impacted samples (Figure 5).

The second sampling event occurred on June 11, 2013 at a shoreline location adjacent to the Biscayne Bay site. Samples collected during this event were size filtered so that bacterial respiration rather than community respiration could be determined. This filtration was accomplished by placing an in-line filter holder (47 mm filter diameter, Millipore) containing 1 μm pore filters (Whatman GF/B) between two sections of silicone tubing. This filtration pore size falls within the 3 to 0.6 μm range used in other

BGE studies to separate bacteria from larger organisms (Aranguren-Gassis 2012). All other sample treatments remained identical to the initial collection event. Incubation times were changed to 24, 48 and 72 hours for both $\delta^{13}$C-DIC and DO samples after a review of previous results.

The third sampling event occurred on June 26, 2013 at the TS-9 site in Florida Bay. Samples were size filtered as previously described. Incubation times were reduced to 12, 24 and 48 hours after analysis of June 11th results. The overall goal of the third sampling event was to determine the shortest incubation time that yielded significant results for oligotrophic Florida Bay samples. The fourth sampling event occurred on July 24th, 2013 and involved sample collection from all 3 Florida Bay sites. Initial samples and samples for 24 hour incubations were collected at each site.

During all sampling events, salinity and water temperature were measured for water density calculations and for use as general environmental parameters. An additional 1 liter water sample was collected during all sampling events for BP and bacterial abundance (BA) measurements. The bottles for BP/BA analysis were rinsed three times, filled via peristaltic pump, and placed on ice in a cooler.
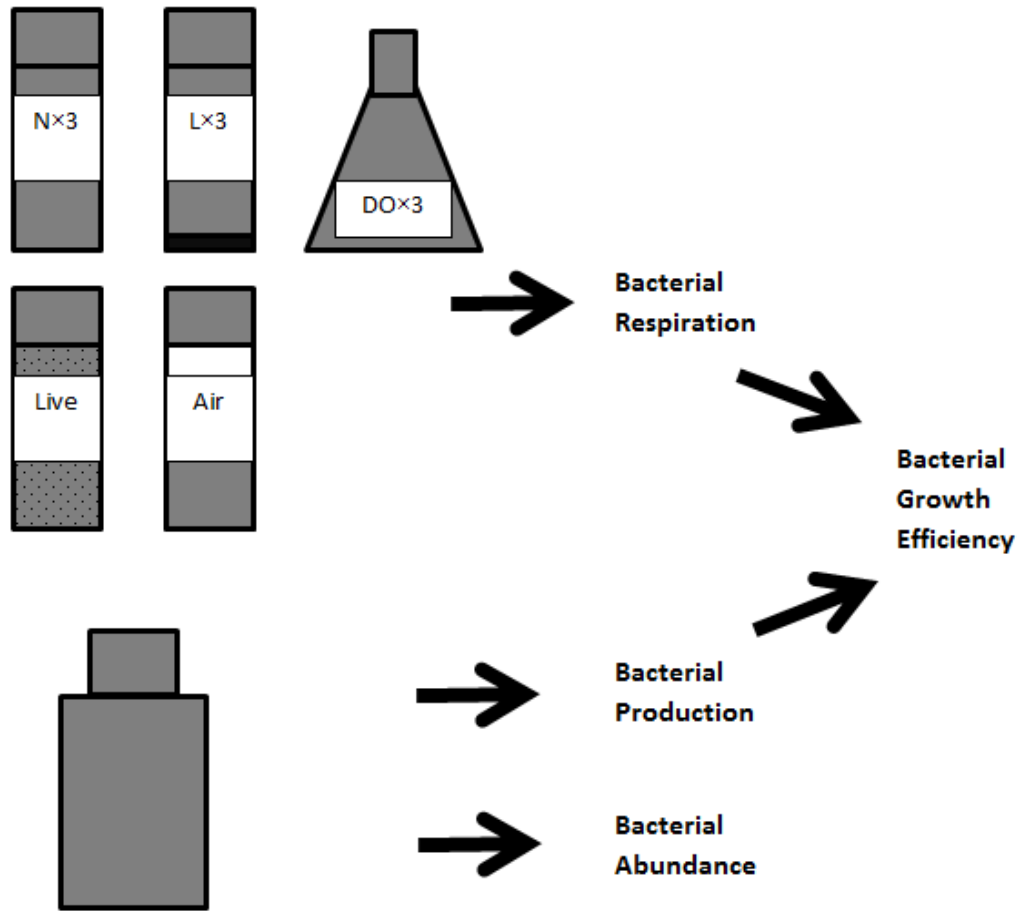
Figure 5: Process diagram showing sample collection scheme and sample treatments.

2.3 Sample Analysis

The DO samples were analyzed using an automated Winkler titration system (modified from Friederich et al. 1991).  Within one week of sample collection, flasks were opened and treated with 1 ml of 5 M sulfuric acid.  Sodium thiosulfate solution (0.14N) was then titrated into each sample, and the endpoint of each titration was detected via amperometric methods.  Values were initially reported in $\mu$mol $O_2$ $kg^{-1}$ $H_2O$ and were converted into $\mu$g C respired $L^{-1}$ using water density calculations and a respiration quotient of 1 for conversion of $O_2$ consumed to $CO_2$ produced (del Giorgio and Cole 1998).

Samples collected for $\delta^{13}C$-DIC measurements were analyzed using a Gas Bench II coupled to a Thermo Delta V GC-IRMS at the University of Miami Stable Isotope Laboratory (modified from Torres et al. 2005).  Excess acid was added to each sample, allowing $CO_2$ to accumulate in a headspace purged with helium above each sample.  The accumulated $CO_2$ was then processed by the GC-IRMS.  $\delta^{13}C$ values produced by the GC-IRMS were reported in reference to the Vienna Peedee Belemnite (VPDB) standard.

$\delta^{13}C$-DIC values were further analyzed and converted into $\mu$g C respired.  First, $\delta^{13}C$ values were converted into atom percent $^{13}C$ via the following equation:

$$\text{atom \%} = 100 \times AR \times (\delta^{13}C/1000 + 1)/ 1 + AR \times (\delta^{13}C/1000 + 1)$$

in which AR is the absolute molar ratio of $^{13}C$ to $^{12}C$ (0.0111796).  Next, peak amplitudes measured by GC-IRMS for $^{12}CO_2$ were converted into parts per million by plotting a linear regression between standards of known DIC concentration prepared for each sample run and measured peak amplitudes.  The equation produced by the linear regression was then applied to convert peak amplitudes for each sample into $^{12}C$-DIC

ppm. The resulting values were then multiplied by atom % $^{13}$C and ultimately converted to $\mu g \ C \ L^{-1}$.

Both bacterial abundance (BA) and bacterial production (BP) were determined using methods described by Boyer et al. (2006). Bacterial abundance assays were performed using the DAPI staining technique (Coleman 1980, Porter and Feig 1980). Samples were fixed by treating 10 ml of unfiltered water with 2 ml of 20% formalin solution. After storage, samples were incubated for 20 minutes in dark conditions with DAPI stain at a final concentration of 25 $\mu g \ ml^{-1}$. These samples were then filtered onto black 0.2 $\mu$m polycarbonate filters and subsequently mounted onto slides. The slides were examined with an epifluorescence microscope illuminated with a 100 W Hg bulb. The number of fluorescent bacteria within 10 different sampling fields of a known size per slide were counted. BA as cells $ml^{-1}$ was then determined through use of the following formula:

$$BA = (filtration \ area/field \ area)*N/D$$

in which N is the total number of cells counted divided by the number of fields, and D is the volume of sample stained divided by the total volume of sample available.

Bacterial production was measured in unfiltered water samples via $^{3}$H-thymidine incorporation incubations (Bell 1993, Boyer et al. 2006). In summary, 10 ml triplicates of each sample were placed into 50 ml centrifuge tubes in addition to a 4% formalin blank sample. Samples were then treated with 100 $\mu$l of $^{3}$H-thymidine solution and incubated for 1 hour. After incubation, samples were filtered, dried overnight, and treated with 10 ml of scintillation cocktail. Samples were then placed into a liquid

scintillation counter. Disintegrations per minute (dpm) were converted into moles thymidine $L^{-1}$ $hr^{-1}$ using the following equation:

$$\text{Moles thymidine } L^{-1} \text{ hr}^{-1} = [(\text{dpm}_{\text{sample}} - \text{dpm}_{\text{blank}})(C)/SA \times t \times v] \times 10^{-3} \times f$$

in which C is the number of curies per dpm, SA is the specific activity of the $^{3}$H-thymidine solution, t is the incubation time in hours, v is the filtered volume in liters, and f is a correction factor for addition of formaldehyde.

The result of the preceding equation was then converted into µg C $L^{-1}$ $hr^{-1}$ using the following formula:

$$\mu\text{g C } L^{-1} \text{ hr}^{-1} = (\text{moles thymidine } L^{-1} \text{ hr}^{-1}) \times (2 \times 10^{18}) \times (1 \times 10^{-14})$$

in which $2 \times 10^{18}$ is the number of cells per mole thymidine and $1 \times 10^{-14}$ is the number of grams C per cell.

2.4 Statistical Methods

One way analysis of variance (ANOVA) was used to determine whether BR results were significantly different among incubation times for each sampling event, with significance set at $p < 0.05$. Sets of incubation times that were found to be significantly different were then analyzed by Tukey's post hoc test to determine which pairs of incubation times were significantly different from each other. Welch's t-test was also used to compare pairs of incubation times with unequal variance among replicates. Incubation time averages, as well as BP results, were graphed with error bars representing a one sigma error unless otherwise noted. Tests of statistical significance were not used for salinity, water temperature, and BA.

3.  RESULTS

3.1 Environmental Parameters

As a result of the shallow estuarine nature of the study sites, salinity and water temperature varied between sampling events (Table 1).  Salinity ranged from 17.0 to 34.7 practical salinity units, with the lowest salinity measured during the June 11th Biscayne Bay sampling event and the highest measured at TS-10 on July 24th.  Water temperature ranged from 27.3°C on May 23rd in Biscayne Bay to 30.7°C on July 24th at TS-10.  Light rainfall occurred during the June 11th sampling event, while all other sampling occurred under dry, sunny conditions.  Salinity on June 11th was likely impacted by a tropical storm that left up to 13 inches of rain in localized parts of northern Miami-Dade County on June 7th (Rainfall data from S29, South Florida Water Management District).

Table 1: Salinity and water temperature during each sampling event

| Sampling Location | Sampling Date | Sampling Time | Salinity | Water Temperature (°C) |
|---|---|---|---|---|
| Biscayne Bay-1 | May 23, 2013 | 9:55 AM | 27 | 27.3 |
| Biscayne Bay-2 | June 11, 2013 | 11:50 AM | 17 | 28.4 |
| TS-9 | June 26, 2013 | 10:00 AM | 33.4 | 28.9 |
| TS-9 | July 24, 2013 | 9:25 AM | 21.1 | 29.2 |
| TS-10 | July 24, 2013 | 10:40 AM | 34.7 | 30.7 |
| TS-11 | July 24, 2013 | 12:05 PM | 34.6 | 30.3 |

3.2 Bacterial Abundance and Bacterial Production

BA ranged from 4.7 to $9.5 \times 10^6$ cells ml$^{-1}$ (Table 2). The highest counts were observed on June 11$^{th}$ in Biscayne Bay, while the lowest valid counts were observed on July 24$^{th}$ at TS-9. The May 23$^{rd}$ counts fell below 300 cells per 10 visual fields (estimated total of $1.8 \times 10^6$ cells ml$^{-1}$); thus, these results are not valid (Boyer et al. 2006) and are not considered with the rest of the data set.

Table 2: Bacterial abundance at each sampling site

| Sampling Location | Sampling Date | Bacterial Abundance (cells ml$^{-1}$) |
|---|---|---|
| Biscayne Bay-1 | May 23, 2013 | 1.68E+06 |
| Biscayne Bay-2 | June 11, 2013 | 9.49E+06 |
| TS-9 | June 26, 2013 | 5.69E+06 |
| TS-9 | July 24, 2013 | 4.72E+06 |
| TS-10 | July 24, 2013 | 4.80E+06 |
| TS-11 | July 24, 2013 | 4.94E+06 |

Bacterial production triplicate measurements for each sampling event were averaged without removing values that fell below the blank dpm measurement. Average BP measurements for Biscayne Bay on May 23$^{rd}$ were below zero. This is a common result when BP is below method detection and does not truly denote negative production. The remaining BP averages ranged from 0.29 to 45.22 µg C L$^{-1}$ day$^{-1}$. The highest average was observed on June 11$^{th}$ in Biscayne Bay and was an order of magnitude higher than all other averages. The two lowest averages, observed at TS-9 on June 26$^{th}$

and TS-10 on July 24th, include triplicate values below the blank dpm measurement (Figure 6).
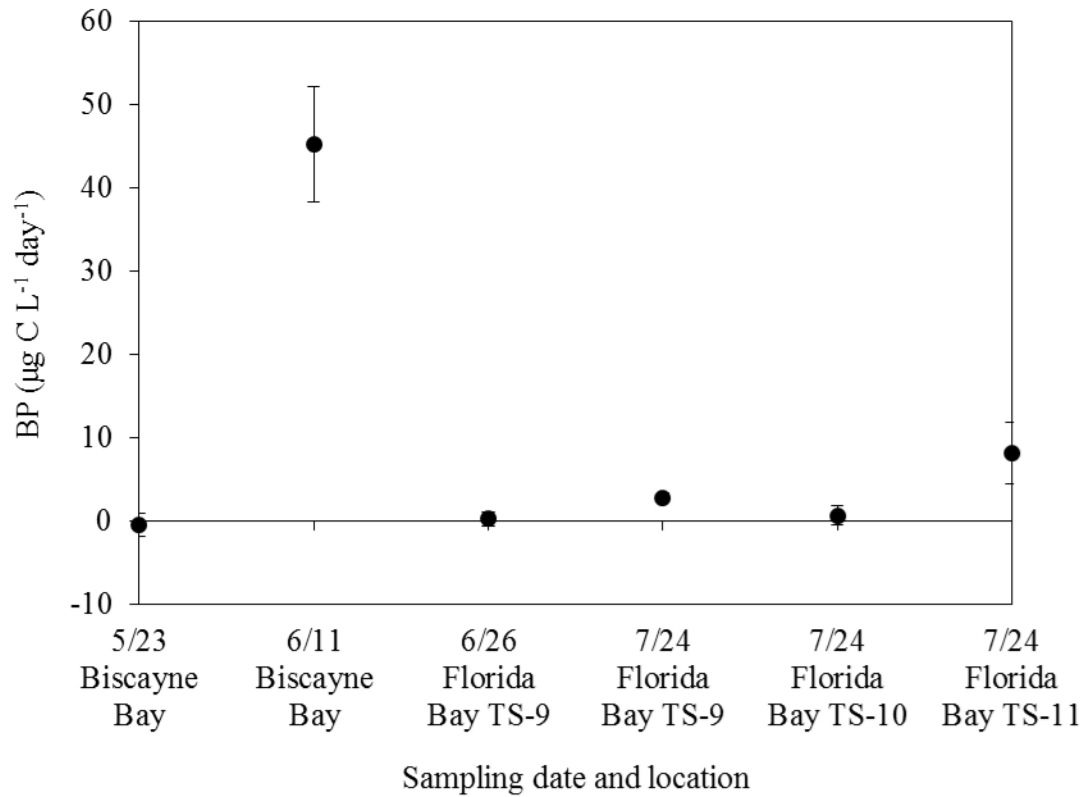


Figure 6: Average bacterial production values for each sampling event.

3.3 Bacterial Respiration

Dissolved oxygen levels measured by Winkler titrations were expected to decrease with increasing incubation time. During every sampling event, however, DO levels and corresponding C production calculations showed no statistically valid relationship with incubation length. DO levels measured for the first sampling event ranged from 185.6 to 199.5 μmol $O_2$ kg$^{-1}$ $H_2O$. During the first sampling event, DO levels at each incubation length were found to not significantly differ from each other

(ANOVA, p = 0.39). After DO values were converted to C respired over time, a significant correlation between incubation length and carbon increase was not found (Pearson r = 0.75, p = 0.18, Figure 7).
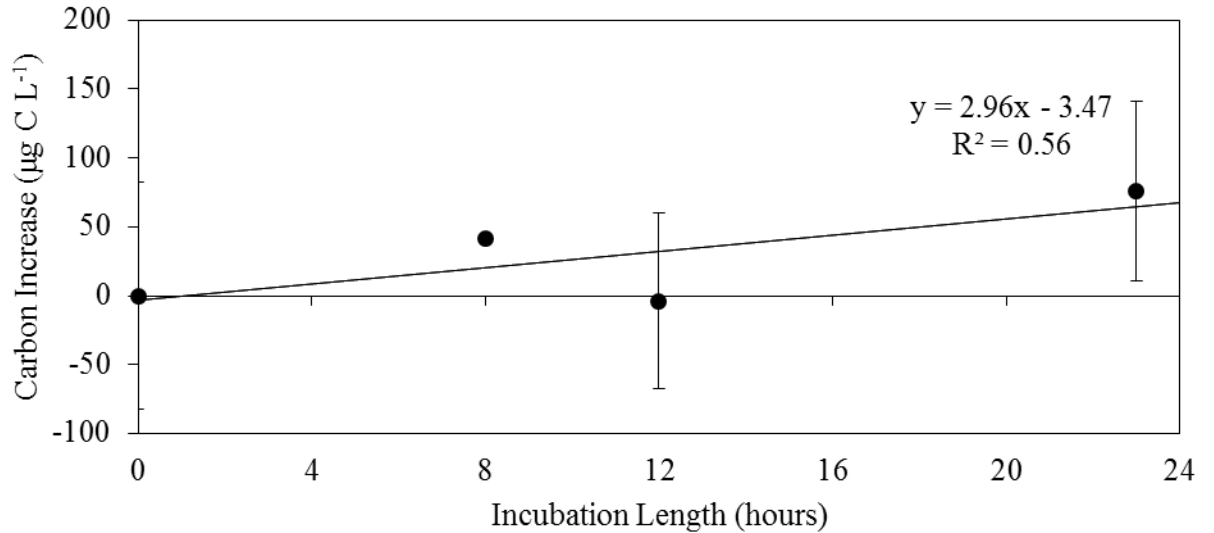


Figure 7: Carbon respired over time measured by Winkler titration for Sampling Event 1.

High replicate sample variability affected June 11th DO results. DO levels measured for the June 11th sampling event ranged from 171.5 to 193.7 μmol $O_2$ kg$^{-1}$ $H_2O$. When converted to C respired over time, a significant negative correlation was found between incubation time and C concentration (r = −0.89, p = 0.049, Figure 8). Because this correlation was negative rather than positive, respiration of carbon cannot be determined from this data set. Although the correlation seems to indicate that DO concentrations increased during the incubation, DO values for each incubation time were found to not significantly differ from each other (ANOVA, p = 0.73).

Figure 8: Carbon respired over time measured by Winkler titration for samples collected on June 11th, 2013 (Sampling Event 2).

Winkler titration results for June 26th were also not reliable for determination of C respiration. Raw DO values ranged from 183.6 to 194.8 $\mu mol\ O_2\ kg^{-1}\ H_2O$. Unlike Winkler titration data from May 23rd and June 11th, significant differences were found between incubation times (ANOVA, $p = 0.03$). Post-hoc analysis indicated that only the 12 hour and 48 hour $O_2$ concentrations were significantly different. Similar to the June 11th results, a negative correlation was found between carbon concentration and incubation time (Figure 9). This negative correlation, however, was statically insignificant ($r = -0.81$, $p = 0.13$), indicating replicate sample variability may have produced a false decreasing trend.
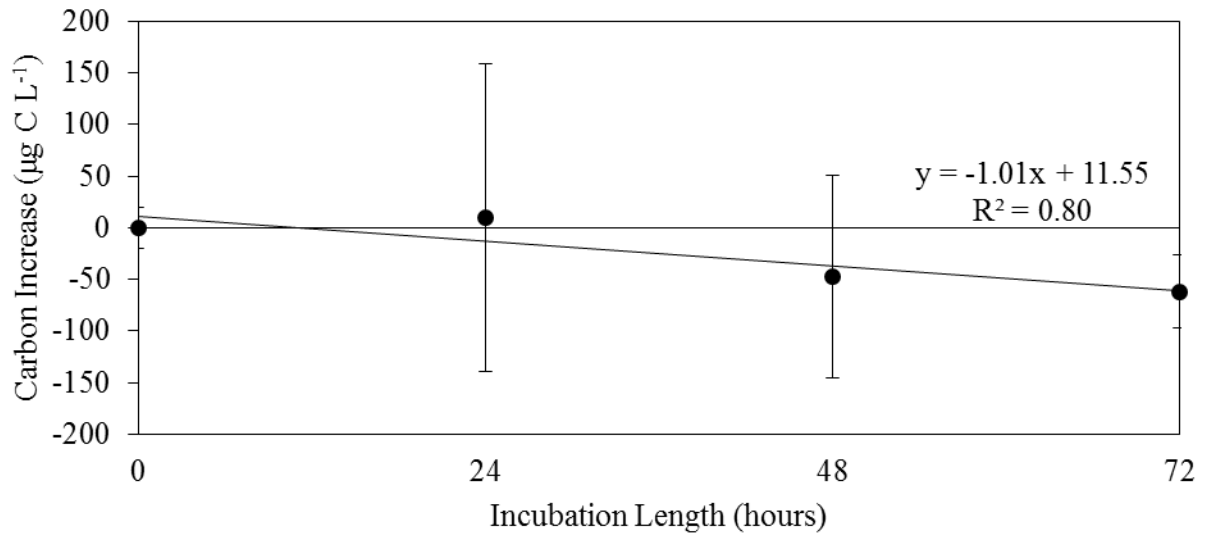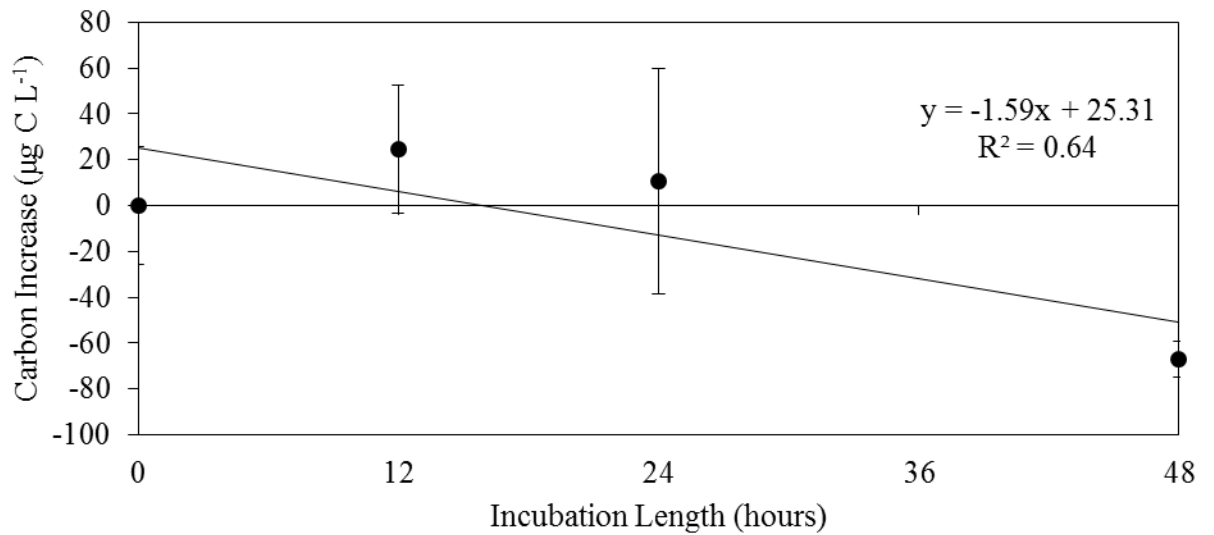
Figure 9: Carbon respired over time measured by Winkler titration for samples collected on June 26th, 2013 (Sampling Event 3).

Samples collected on July 24th had an overall higher range of initial and final DO concentrations. The highest DO concentrations were observed at TS-10 (221.5 to 234.1 $\mu$mol kg$^{-1}$), while the lowest overall concentrations were observed at TS-11 (187.2 to 211.9 $\mu$mol O$_2$ kg$^{-1}$). Initial DO concentrations at each site showed high variability. The average initial DO concentration at TS-9 was $217.3 \pm 5.8$ $\mu$mol O$_2$ kg$^{-1}$ (reported with standard deviation), the average initial concentration at TS-10 was $229.9 \pm 7.3$ $\mu$mol O$_2$ kg$^{-1}$, and the average initial concentration at TS-11 was $196.9 \pm 13.2$ $\mu$mol O$_2$ kg$^{-1}$. A series of one-tailed Welch's t-tests performed on the DO concentrations at each site indicated that there were no significant differences between initial DO concentrations and final DO concentrations ($p = 0.11$, 0.15, 0.41 for TS-9, TS-10 and TS-11, respectively). Although the difference between the initial and final DO values was not statistically significant, BR was still estimated for each site after conversion of O$_2$ consumed to C

25

produced (Figure 10).  Results calculated from this conversion were 69.5 µg C L$^{-1}$ day$^{-1}$

for TS-9, 71.7 µg C L$^{-1}$ day$^{-1}$ for TS-10, and 23.5 µg C L$^{-1}$ day$^{-1}$ for TS-11.
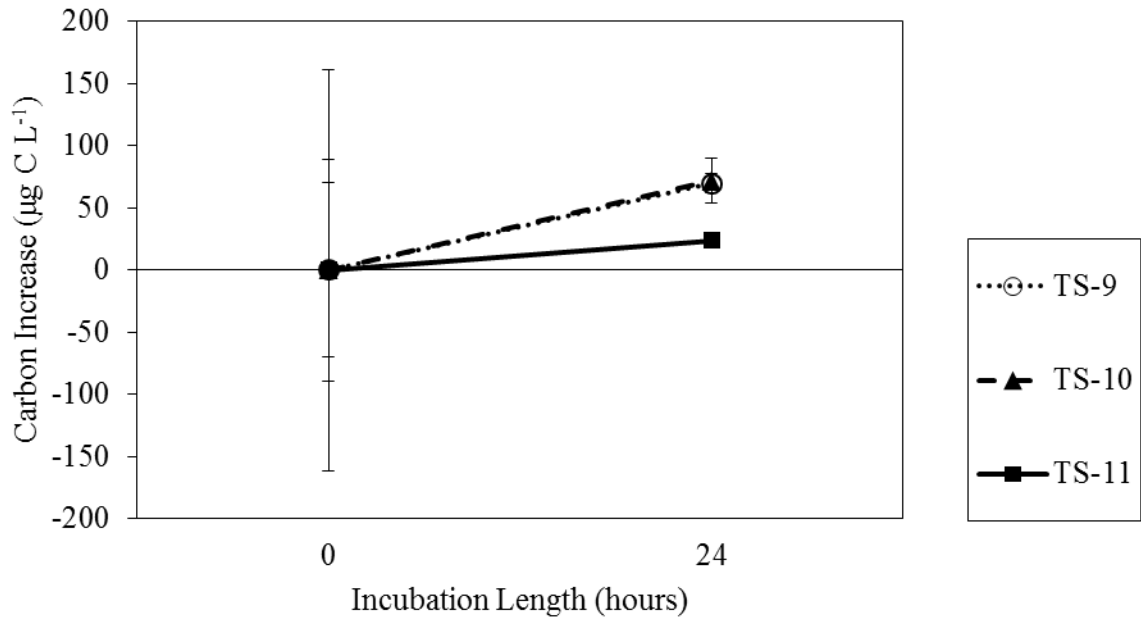


Figure 10: Carbon production determined by Winkler titration at TS-9, TS-10 and TS-11 on July 24th, 2013 (Sampling Event 4).

Use of $^{13}$C isotope ratios to determine BR produced better results overall than the

Winkler titration method.  The labeled tracer method in comparison to the natural isotope

ratio method produced stronger changes in $\delta^{13}$C-DIC between incubation times.  Clear

BR results, however, were not observed for May 23rd samples as a consequence of the

very low $^{13}$C-glucose concentrations added to these samples (1 ml of 6 µg L$^{-1}$ $^{13}$C glucose

solution).  $\delta^{13}$C values for samples collected on May 23rd both before and after incubation

ranged from −3.67‰ to −3.04‰.  The sample $\delta^{13}$C range did not differ from either the

air contamination control sample ($\delta^{13}$C = −3.32‰) or the live control sample (sample not

treated with HgCl$_2$, $\delta^{13}$C $= -3.24‰$).  Other than possible air contamination or ineffective

HgCl$_2$ treatment, these results indicated that incubation time was not sufficient to produce

measurable respiration, or insufficient $^{13}$C-glucose was added to labeled samples.  A two-

tailed t-test indicated that $\delta^{13}$C values in labeled samples were significantly higher than

$\delta^{13}$C values in natural isotope samples (p $= 0.005$).  However, labeled samples showed a

general decreasing trend in $\delta^{13}$C values rather than the expected increase over time

(Figure 11).  Additionally, $\delta^{13}$C values in natural isotope samples appeared to increase

over time rather than decrease as expected (Figure 12).  As a result of variability among

replicate samples, incubation time did not show a significant relationship with $\delta^{13}$C

values in either natural isotope samples or labeled samples (p $= 0.73, 0.06$ for natural and

labeled samples, respectively).
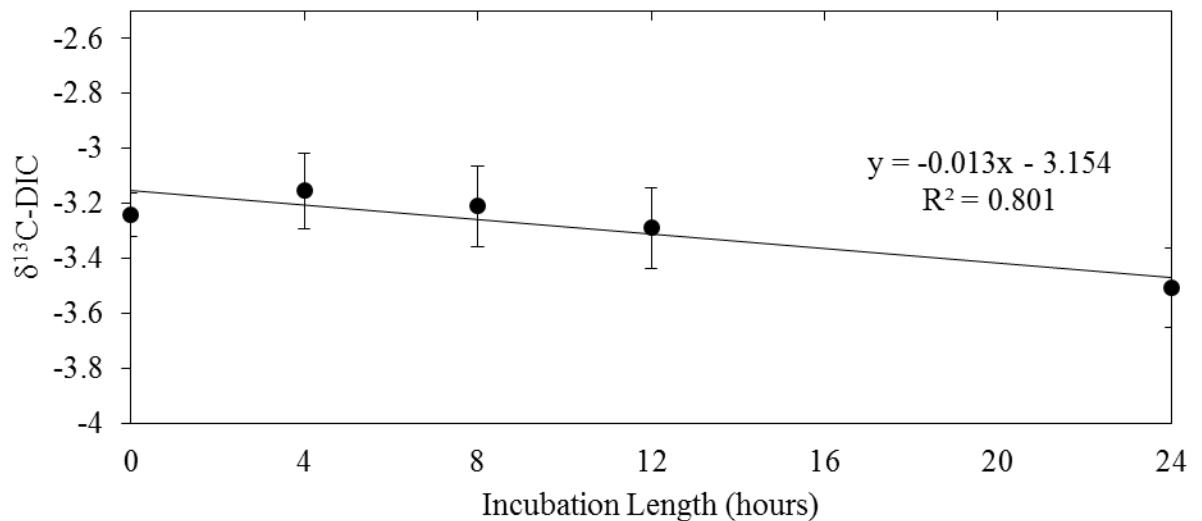


Figure 11: $\delta^{13}$C-DIC values at varying incubation times in samples labeled with $^{13}$C-glucose collected on May 23$^{rd}$ 2013.
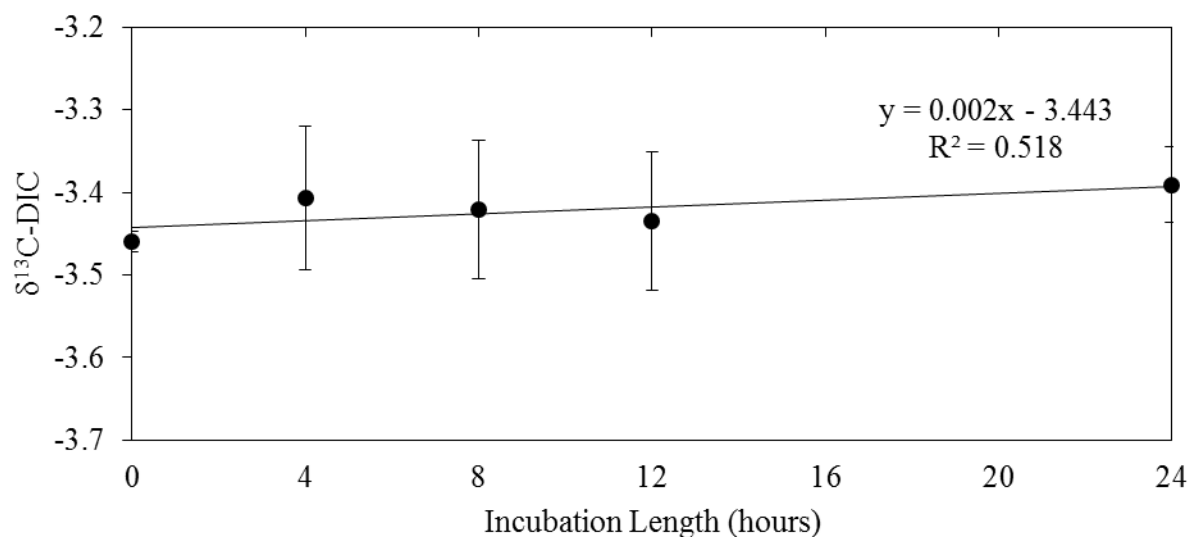
Figure 12: $\delta^{13}$C-DIC values at varying incubation times in unlabeled samples collected on May 23$^{rd}$ 2013.

Although May 23$^{rd}$ results showed a significant difference between labeled samples and unlabeled samples, more $^{13}$C-glucose was added to June 11$^{th}$ labeled vials with the intention of increasing signal to noise ratios and to ensure $^{13}$C-glucose was being used as the primary bacterial food source. Concentration of $^{13}$C-glucose was increased to 0.6 mg L$^{-1}$ in the stock $^{13}$C-glucose solution. 1 ml of the stock solution was then added to each labeled vial before sample collection. Additionally, incubation times were increased from a maximum of 24 hours to a maximum of 72 hours.

$\delta^{13}$C values observed in samples collected on June 11$^{th}$ showed stronger terrestrial influence, with initial $\delta^{13}$C-DIC values ranging from −8.28‰ to −8.10‰ in both labeled and unlabeled samples. As a result of the increased concentration of $^{13}$C-glucose as a bacterial food source, a stronger correlation between incubation time and $\delta^{13}$C values was observed, and statistical differences were found among differing incubation times

(ANOVA, $p = 2.40 \times 10^{-6}$). The most significant change in $\delta^{13}$C-DIC was observed between the initial sample and the 24 hour labeled sample, with $\delta^{13}$C-DIC values increasing from an average of $-8.15‰ \pm 0.08$ to $-4.32‰ \pm 0.13$ (Figure 13, $q > q_{critical}$). The air contaminated control sample and the non-preserved sample also differed from the 24 hour labeled sample (air control $\delta^{13}$C $= -7.78‰$, live control $\delta^{13}$C $= -8.52‰$). Beyond the 24 hour incubation point, $\delta^{13}$C-DIC values did not significantly increase (Tukey's HSD, $q < q_{critical}$ for 24hr vs. 48 hr, 24hr vs. 72hr, 48hr vs. 72hr), indicating that maximum biological uptake of $^{13}$C-glucose occurred at or before 24 hours (represented by dotted line in Figure 13). Unlabeled samples showed a general decrease in $\delta^{13}$C-DIC over time (Figure 14). However, this decreasing trend in $\delta^{13}$C-DIC was not statistically significant (ANOVA, $p = 0.09$).



linear: $y = 0.05x - 7.04$
$R^2 = 0.63$

Figure 13: $\delta^{13}$C-DIC values at varying incubation times in labeled samples collected from Biscayne Bay on June 11th, 2013.
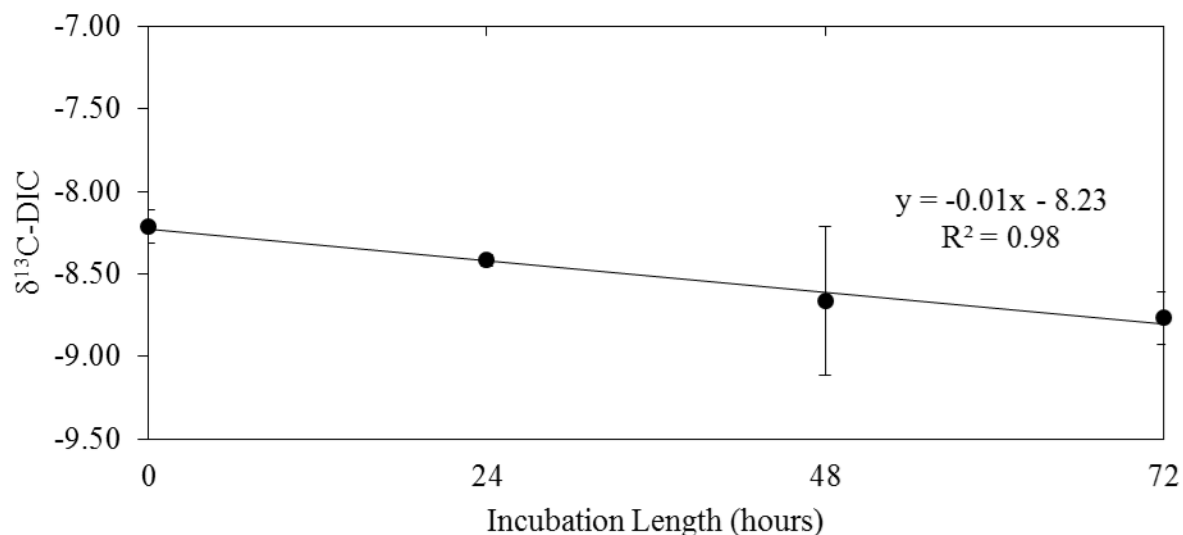
Figure 14: $\delta^{13}$C-DIC values at varying incubation times in unlabeled samples collected from Biscayne Bay on June 11th, 2013.

After examination of June 11th results, incubation times were shortened to a maximum of 48 hours on June 26th. $\delta^{13}$C-DIC values in initial samples showed stronger marine influence than Biscayne Bay samples, with $\delta^{13}$C-DIC values of $-2.28‰ \pm 0.18$ and $-2.30‰ \pm 0.27$ in unlabeled and labeled samples, respectively. Labeled samples showed a strong correlation between $\delta^{13}$C-DIC values and incubation time (Figure 15). $\delta^{13}$C-DIC values increased from $-2.30‰ \pm 0.27$ to $7.46‰ \pm 0.88$ over the course of 48 hours. Significant differences were found among incubation times (ANOVA, $p < 0.001$), and post-hoc analysis indicated $\delta^{13}$C-DIC values for each incubation time significantly differed from all others. In unlabeled samples, a weak decrease in $\delta^{13}$C-DIC values was observed (Figure 16). Although a high Pearson correlation coefficient was found ($r = -0.98$), analysis of unlabeled sample results indicated that $\delta^{13}$C-DIC values for each

incubation time did not significantly differ from each other because of high replicate
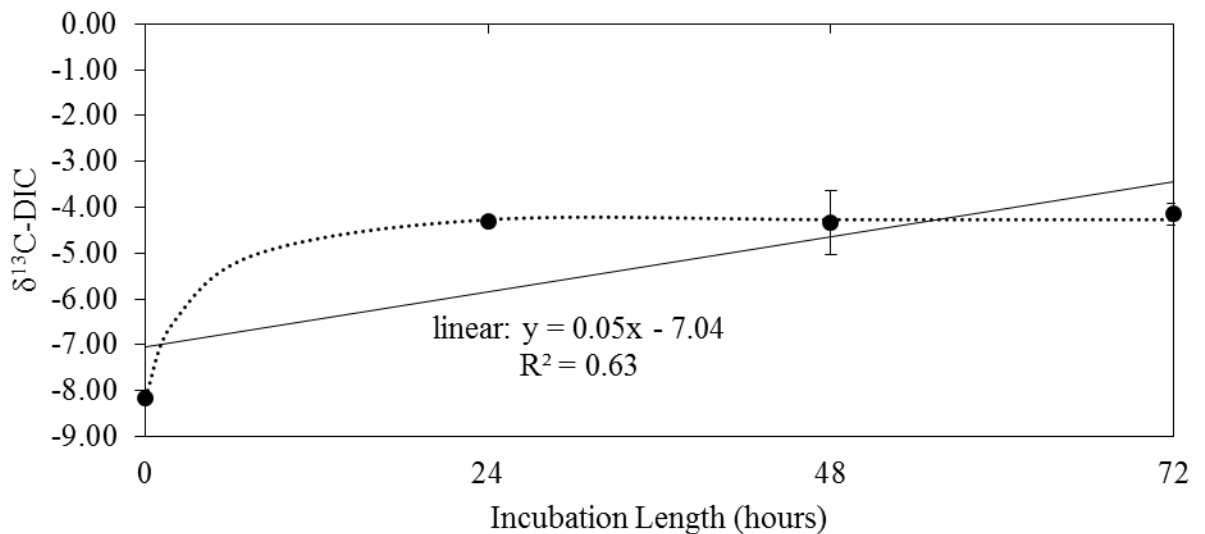
sample variability (ANOVA, p = 0.55).
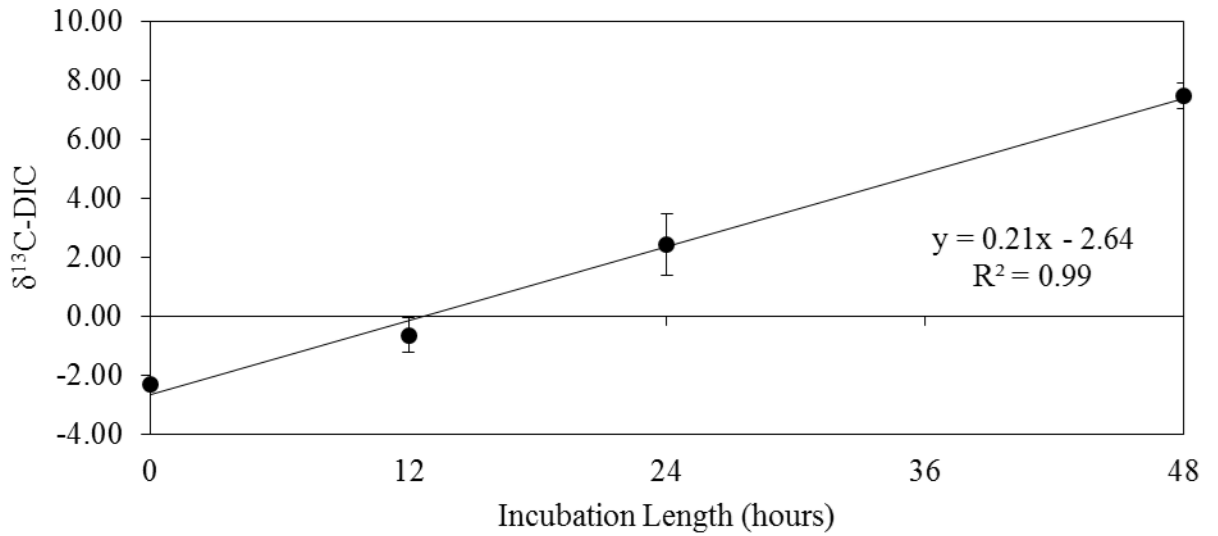


Figure 15: $\delta^{13}$C-DIC values at varying incubation times in labeled samples collected from Florida Bay site TS-9 on June 26th, 2013.
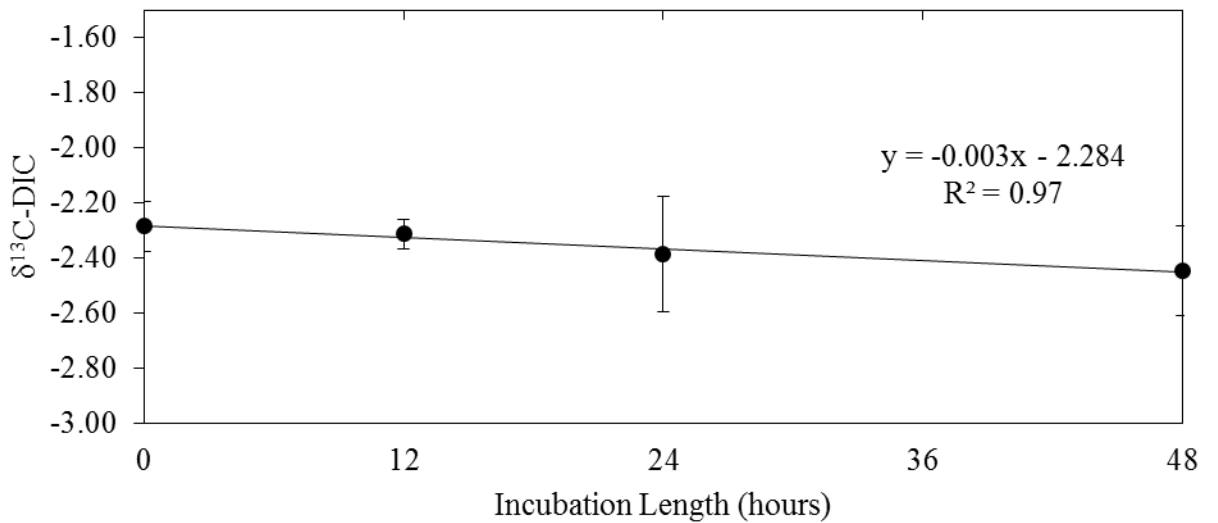


Figure 16: $\delta^{13}$C-DIC values at varying incubation times in unlabeled samples collected from Florida Bay site TS-9 on June 26th, 2013.

Initial $\delta^{13}$C-DIC values from samples collected on July 24$^{th}$ varied among sample sites. TS-9 had initial $\delta^{13}$C-DIC values ranging from −3.52‰ to −3.33‰ in labeled and unlabeled samples. $\delta^{13}$C-DIC initial values in TS-10 samples ranged from −1.44‰ to −1.13‰, and initial $\delta^{13}$C-DIC values at TS-11 ranged from −1.89‰ to −1.42‰. Further analysis indicated that all 3 sample sites had significantly different initial $\delta^{13}$C-DIC values from each other (ANOVA, $p = 8.89 \times 10^{-14}$; Tukey's HSD, $q > q_{critical}$ for all site combinations). In labeled samples incubated for 24 hours, $\delta^{13}$C-DIC increased for TS-9 and TS-11, but not for TS-10 (Figure 17). TS-11 showed the largest change in $\delta^{13}$C-DIC, increasing from an average of −1.56‰ to +6.28‰ over the 24 hour incubation period. Samples from TS-9 had a smaller increase from an average of −3.35‰ to −0.90‰. Statistical analysis confirmed that the increases in $\delta^{13}$C-DIC over time at TS-9 and TS-11 were significant (Welch's one-tailed t-test; TS-9, $p = 0.03$; TS-11, $p = 0.01$). Labeled samples from TS-10, however, showed an apparent decrease in $\delta^{13}$C-DIC values over time. The initial samples from TS-10 had an average $\delta^{13}$C-DIC value of −1.39‰, while final samples had an average value of −1.43‰. Further analysis indicated that this decrease was not significant ($p = 0.11$).
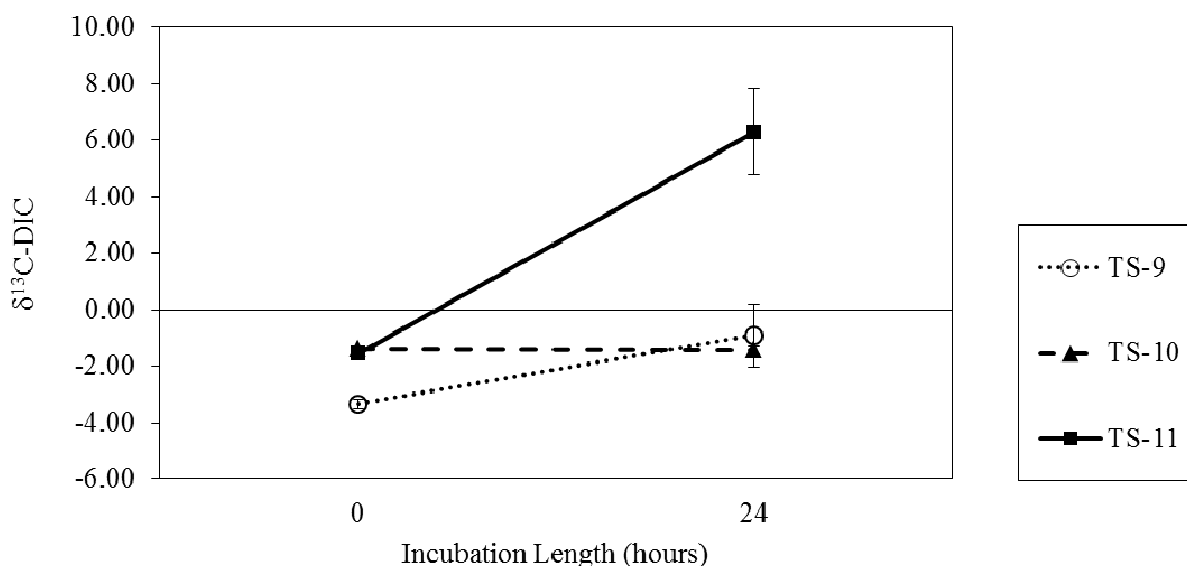
Figure 17: Changes in $\delta^{13}$C-DIC in incubated $^{13}$C-labeled samples collected from TS-9, TS-10, and TS-11 on July 24th, 2013.

Similar to samples collected on other dates, unlabeled samples from July 24th overall did not considerably change in $\delta^{13}$C-DIC during the incubation period (Figure 18).  In samples from TS-10, $\delta^{13}$C-DIC values decreased from −1.30‰ to −1.64‰.  High sample variance caused this apparent decrease to be statistically invalid ($p = 0.11$).  In samples from TS-11, $\delta^{13}$C-DIC values increased from an average of −1.60‰ to −1.56‰ during the incubation; again, this change was not supported statistically ($p = 0.36$).  TS-9 samples, however, did undergo a significant decrease from −3.40‰ to −3.55‰ ($p = 0.02$).
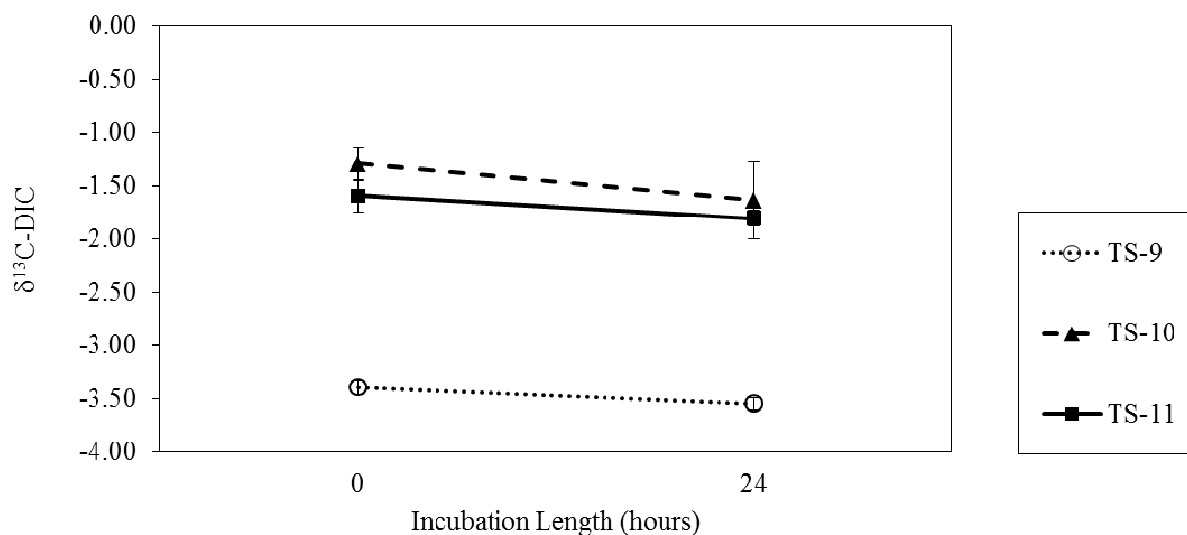
Figure 18: Changes in $\delta^{13}$C-DIC in incubated unlabeled samples collected from TS-9, TS-10, and TS-11 on July 24$^{th}$, 2013.

$\delta^{13}$C-DIC values were converted into $\mu$g C L$^{-1}$ using a linear regression between DIC standards and peak amplitudes measured by the GC-IRMS (Figure 19), as well as additional calculations previously described in the methods section. Conversion of $\delta^{13}$C-DIC to $\mu$g C L$^{-1}$ generally weakened correlations with incubation time. This resulted from highly variable peak amplitudes playing a larger role than $\delta^{13}$C values in calculations. Significant differences between incubation times were not found for unlabeled samples from May 23$^{rd}$ and June 26$^{th}$ (p = 0.35, 0.20) or for labeled samples from June 11$^{th}$ (p = 0.45). A significant difference among incubation times was found for labeled May 23$^{rd}$ samples (p = 0.02). However, an apparent decrease in $\mu$g C L$^{-1}$ occurred rather than an increase over time (Figure 20). Similar results were found for unlabeled June 11$^{th}$ samples. Although differences were found between 24 hour and 48 hour samples (p = 0.02, q > q$_{critical}$), calculated C concentrations decreased from an

average of 532 to an average of 393 μg C L$^{-1}$ during those hours.  A significant

difference was also found between initial and 48 hour samples for June 26$^{th}$ samples (p =

0.03, q > q$_{critical}$).  Although this significant difference was found, the relationship

between μg C L$^{-1}$ and incubation time on June 26$^{th}$ (r = 0.82, Figure 21) was much

weaker than the relationship previously shown between unconverted δ$^{13}$C and incubation

time (r = 0.99).
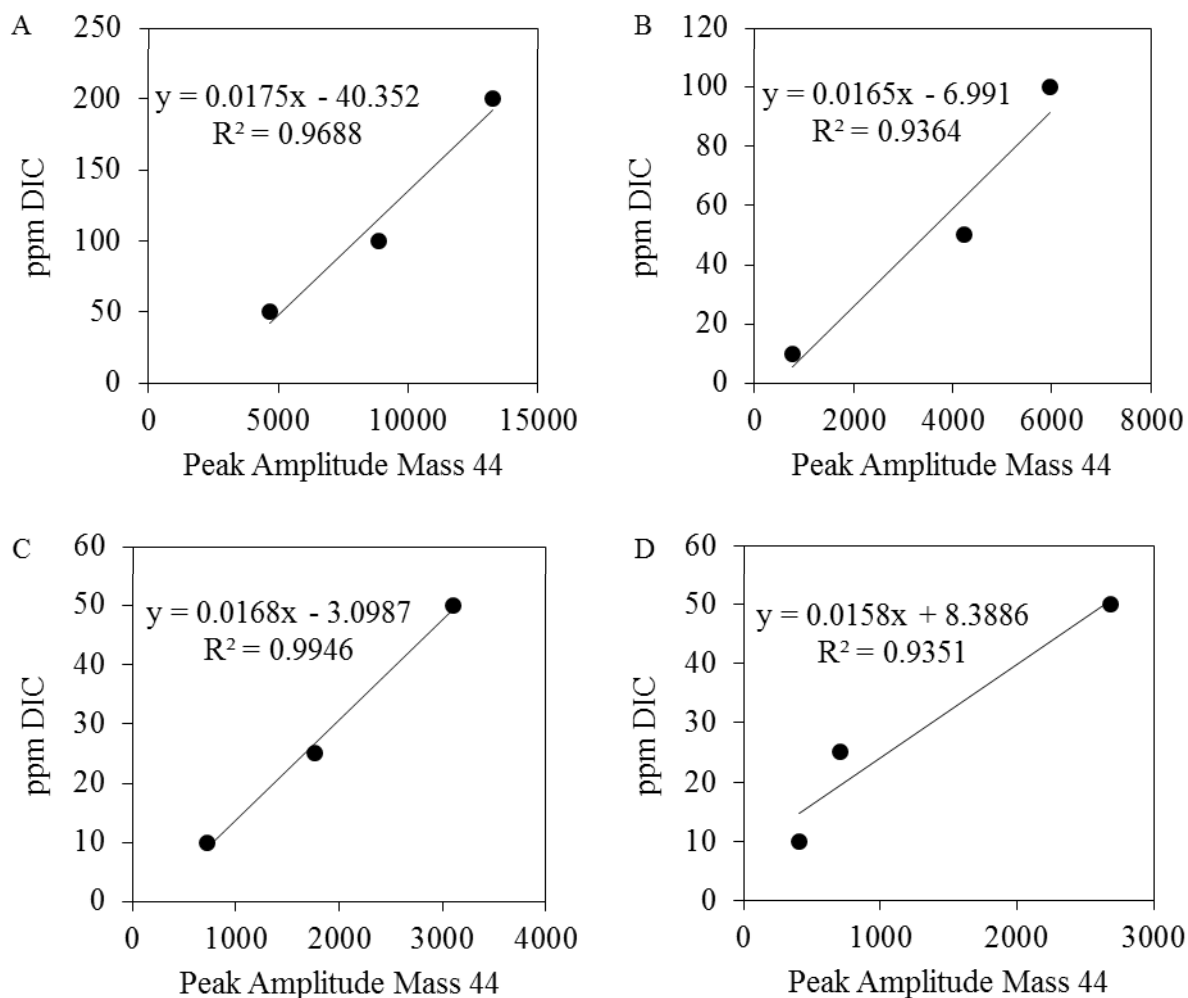
Figure 19: Linear regression between mass 44 peak amplitude measured via GC-IRMS and DIC measured in parts per million for each sampling event. Graph A was generated for May 23rd data, graph B for June 11th data, graph C for June 26th data and graph D for July 24th data.

Conversion from $\delta^{13}$C-DIC to μg C L$^{-1}$ also weakened trends observed for July 24th samples (Figure 22, Figure 23). Although significant changes in $\delta^{13}$C-DIC were found for labeled TS-9 and TS-11 samples, no significant change was observed during incubation for all three sites when data were converted to μg C L$^{-1}$ (p = 0.15, 0.09 and

0.18 for TS-9, TS-10 and TS-11, respectively). Significant changes were also not
observed for unlabeled samples collected on July 24th after data conversion to µg C L$^{-1}$ (p
= 0.12, 0.16 and 0.11 for TS-9, TS-10 and TS-11, respectively).
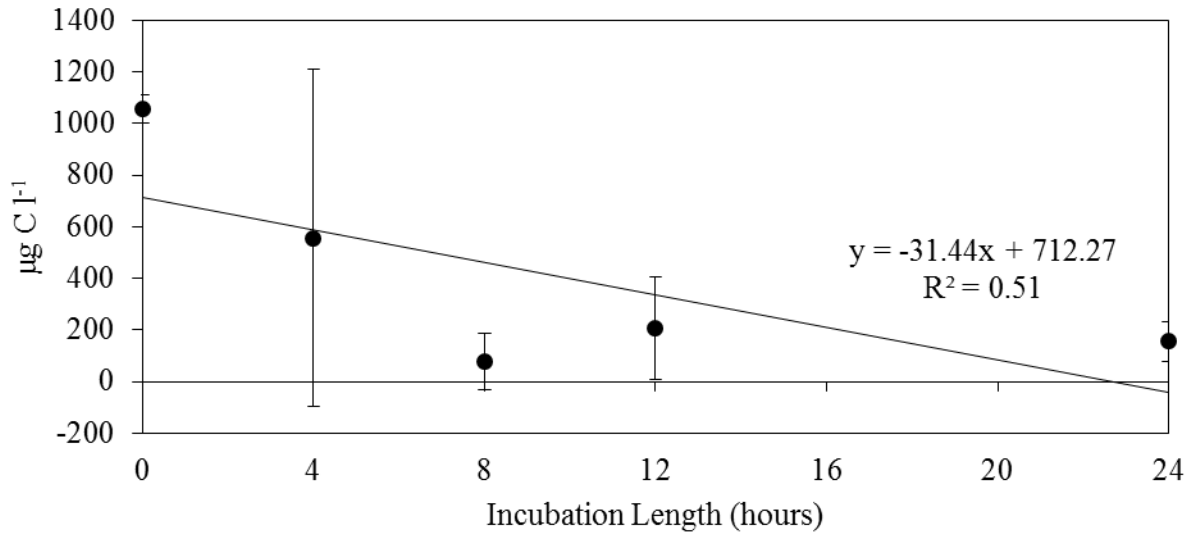


Figure 20: Relationship between $\delta^{13}$C-DIC values converted into µg C L$^{-1}$ and incubation
time for samples collected on May 23rd, 2013.
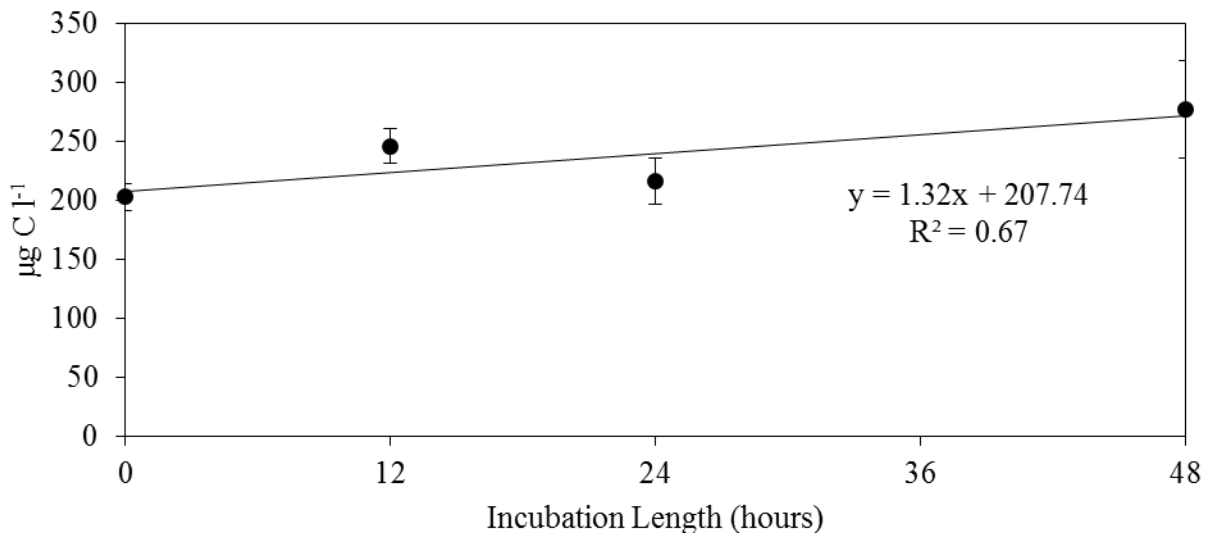


Figure 21: Relationship between $\delta^{13}$C-DIC values converted into µg C L$^{-1}$ and incubation
time for samples collected on June 26th, 2013.
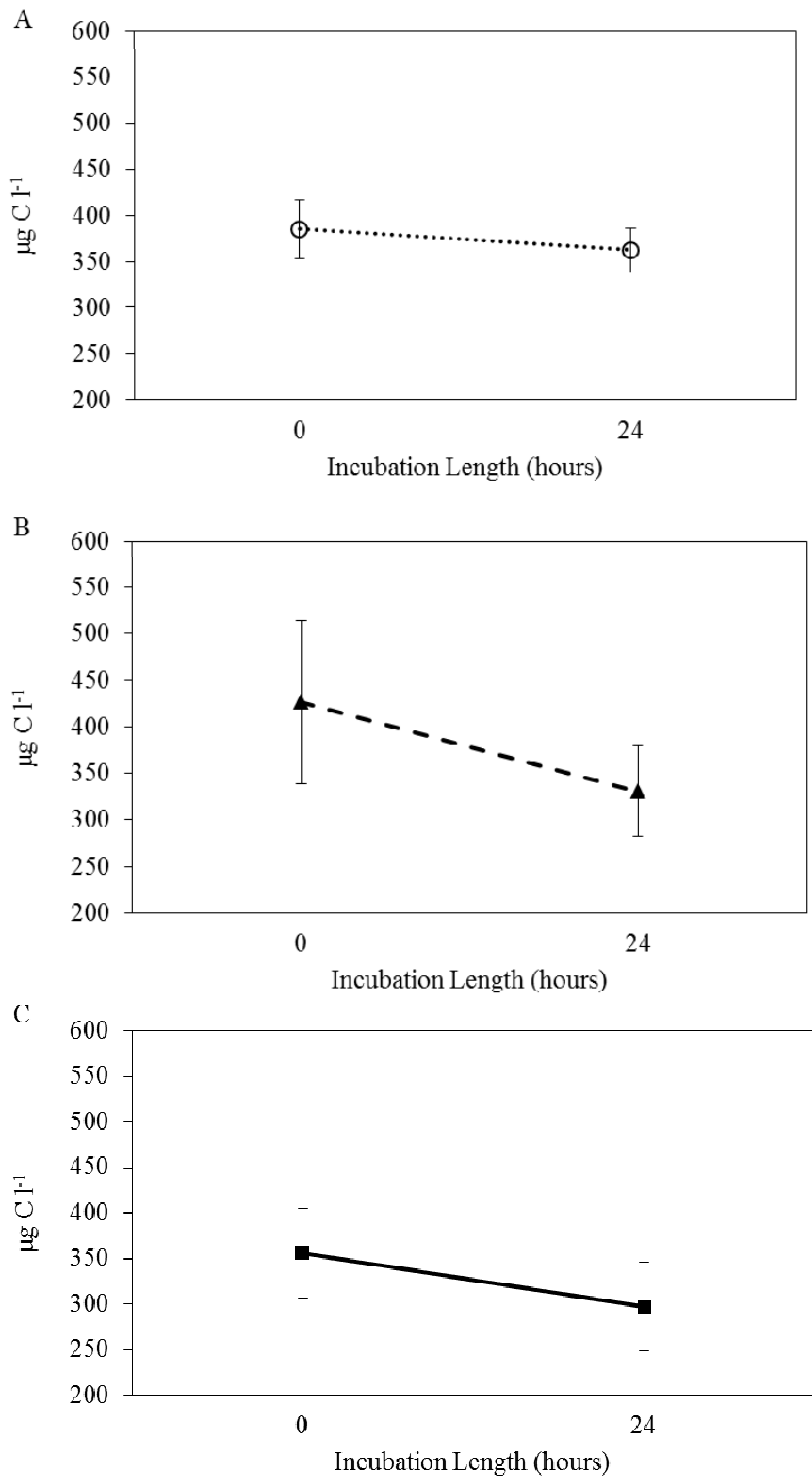
Figure 22: Relationship between $\delta^{13}$C-DIC values converted into μg C L$^{-1}$ and incubation time for unlabeled samples collected on July 24$^{th}$, 2013. Graph A represents TS-9 data, graph B represents TS-10 data, and graph C represents TS-11 data.
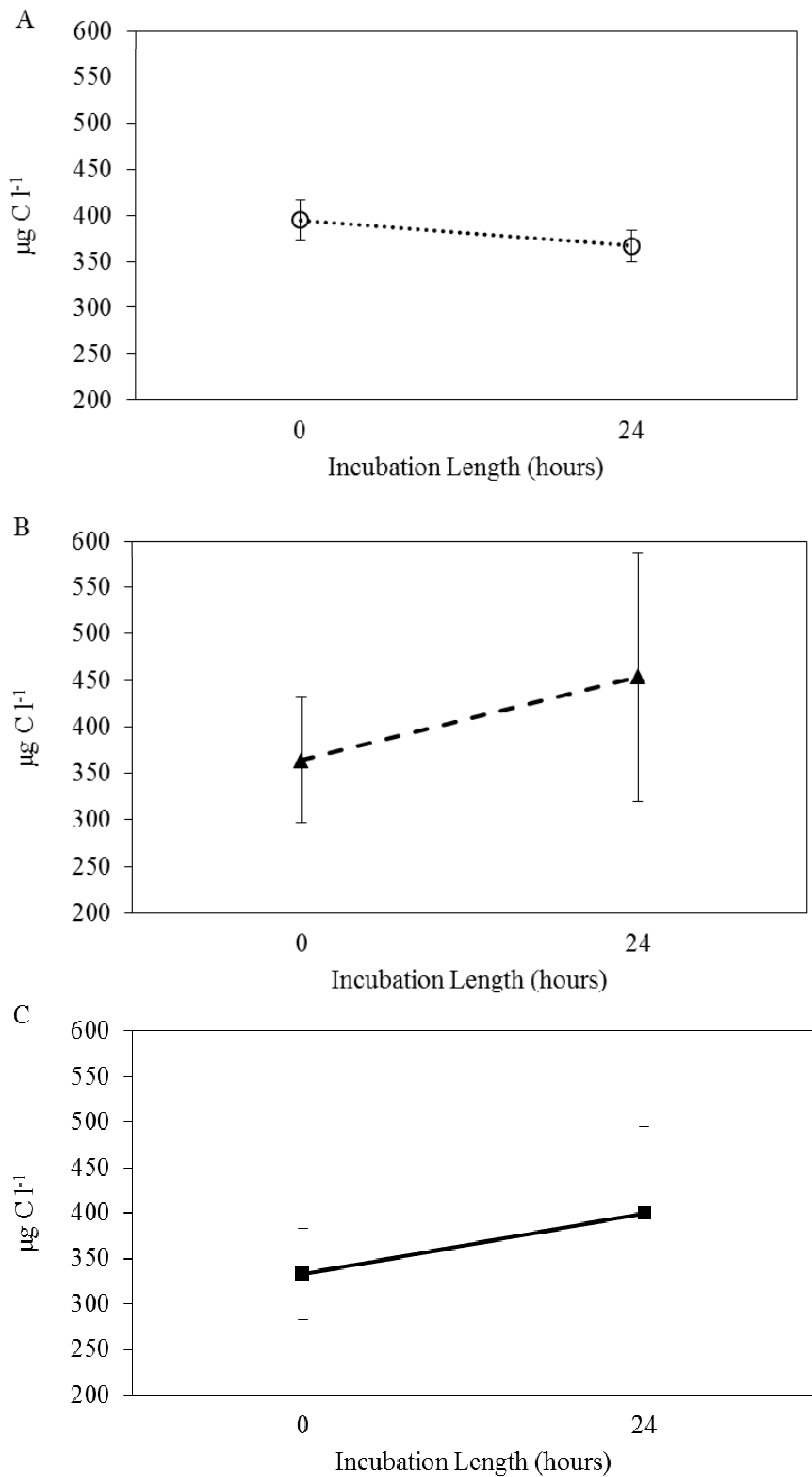
Figure 23: Relationship between $\delta^{13}$C-DIC values converted into μg C L$^{-1}$ and incubation time for $^{13}$C-labeled samples collected on July 24$^{th}$, 2013. Graph A represents TS-9 data, graph B represents TS-10 data, and graph C represents TS-11 data.

Respiration rates calculated from both Winkler titrations and $\delta^{13}$C-DIC were used

to create estimates of baseline bacterial growth efficiency for each sampling site and

location (Table 3). As reviewed previously, a large portion of the BR results were

negative because of calculated decreases in DIC rather than increases expected during the

respiration process (these are represented as <0.001 in Table 3). Additionally, conversion

of raw respiration data to $\mu$g C L$^{-1}$ resulted in labeled TS-9 results from June being the

only statistically significant positive result. Although data conversion weakened

statistical significance of the results, general trends can still be noted in relation to the

raw data. For example, raw labeled $^{13}$C-DIC results for TS-9 and TS-11 in July were

statistically significant. Respiration calculated from the labeled $^{13}$C method as well as BP

were higher at TS-11 than TS-9. When BGE is calculated from these results, TS-11

shows a higher BGE (0.108) than TS-9 (<0.001). The same relationship is observed in

BGE calculations from the traditional Winkler titration method (BGE TS-9 = 0.039, BGE

TS-11 = 0.258).

Table 3: Bacterial production, bacterial respiration (in ug C L$^{-1}$ hr$^{-1}$) and bacterial growth efficiency calculations for all sampling events. Asterisks represent statistically signficant differences during incubation after conversion of raw data to $\mu$g C L$^{-1}$.

| Sampling Location | BP | BR Unlabeled $^{13}$C | BR Labeled $^{13}$C | BR Winkler | BGE Unlabeled $^{13}$C | BGE Labeled $^{13}$C | BGE Winkler |
|---|---|---|---|---|---|---|---|
| Biscayne Bay 1 | <0.001 | 2.440 | <0.001* | 2.960 | <0.001 | 0.001 | <0.001 |
| Biscayne Bay 2 | 1.884 | 0.090 | <0.001* | <0.001 | 0.954 | ≥1.000 | ≥1.000 |
| TS-9 (June) | 0.012 | <0.001 | 1.325* | <0.001* | <0.001 | 0.009 | <0.001 |
| TS-9 (July) | 0.118 | <0.001 | <0.001* | 2.890 | <0.001 | <0.001 | 0.039 |
| TS-10 (July) | 0.029 | <0.001 | 3.738 | 2.990 | <0.001 | 0.008 | 0.010 |
| TS-11 (July) | 0.340 | <0.001 | 2.795 | 0.980 | <0.001 | 0.108 | 0.258 |

4. DISCUSSION

Previous characterization of BA, BP, BR and BGE in Florida Bay is highly limited. Bacterial abundance and BP have been examined by Boyer et al. (2006) at TS-9 and TS-10. Data from that study were gathered monthly between July 2001 and July 2002. Median BA values reported in the Boyer et al. (2006) study at TS-9 and TS-10 were $2 \times 10^6$ cells ml$^{-1}$ and $3 \times 10^6$ cells ml$^{-1}$, respectively. Bacterial abundance values in Florida Bay produced from the current study range from 4.7 to $5.7 \times 10^6$ cells ml$^{-1}$. Though these results are higher than median values reported by Boyer et al. (2006), these values are still within range of the range of BA reported in the previous study. July 2013 BA results were similar across all three Florida Bay sites (4.7 to $4.9 \times 10^6$ cells ml$^{-1}$) and were higher at TS-9 in June ($5.7 \times 10^6$ cells ml$^{-1}$), suggesting that temporal variation rather than spatial variation was an influence on BA in the current study.

Bacterial production was also examined in the Boyer et al. (2006) study at TS-9 and TS-10. The BP median was approximately 1 μg C L day$^{-1}$ at TS-9, while the median at TS-10 was approximately 0.75 μg C L day$^{-1}$. Average BP values in the current study were 0.29 μg C L day$^{-1}$ in June at TS-9, 2.84 μg C L day$^{-1}$ in July at TS-9, 0.70 in July at TS-10, and 8.16 μg C L day$^{-1}$ in July at TS-11. Results from July in the current study show TS-9 having a higher BP rate than TS-10, similar to results in the Boyer et al. (2006) study. June TS-9 results, however, were lower than those found at TS-10. These BP results suggest that seasonal variation, such as incoming Everglades runoff at TS-9 during the wet season, may impact microbial parameters. Additionally, comparatively higher BP results at TS-11 suggest that BP may also be influenced by nutrient limitation gradients.

The challenge of measuring BR in oligotrophic environments has limited BR research in Florida Bay. Budgen et al. (1998) examined heterotrophic activity through bacterial $^{14}CO_2$ uptake and respiration. Bugden et al. (1998) found that heterotrophic activity was generally higher in western Florida Bay (0.5 μg C h$^{-1}$) than in eastern Florida Bay (0.25 μg C h$^{-1}$). The Bugden et al. (1998) study linked differences in heterotrophic activity among sample sites to seagrass health rather than nutrient limitation status. Clear BR differences among sites were not apparent in the current study as a result of methodological challenges. Bacterial respiration calculations using all three methods ranged from negative to 2.89 μg C h$^{-1}$ at TS-9, negative to 3.74 μg C h$^{-1}$ at TS-10, and negative to 2.80 μg C h$^{-1}$ at TS-11. Raw $\delta^{13}$C-DIC data, however, suggest that respiration may be highest at TS-11. TS-11 showed the greatest change in $\delta^{13}$C in $^{13}$C-labeled samples (−1.56‰ to +6.28‰) in comparison to TS-9 and TS-10 in July. Although Winkler titration data suggests that TS-11 had the lowest BR among the Florida Bay sites, the Winkler titration results from all three sites were found to be nonsignificant.

The $^{13}$C-labeling method of measuring BR may be a viable alternative to Winkler titrations on the basis of strong correlations between $\delta^{13}$C-DIC and incubation time. Conversion of $\delta^{13}$C-DIC to μg C L$^{-1}$ using the methods previously described weakened correlations. In future studies employing $\delta^{13}$C-DIC as a way to measure BR, a more complete tracing of carbon pathways could result in better correlations. For example, both DIC and DOC concentrations could be measured, in addition to $\delta^{13}$C-DIC and $\delta^{13}$C-DOC measurements using GC-IRMS. Additionally, more work will be needed for applying the method to eutrophic systems. BR results from June 11$^{th}$ suggest that a shorter incubation length is needed in mesotrophic and eutrophic systems in order to

observe a linear uptake of $^{13}$C-glucose. Although a significant change in $\delta^{13}$C-DIC was observed between 0 and 24 hours of incubation during the June 11$^{th}$ sampling event, respiration of consumed $^{13}$C-glucose may have plateaued before 24 hours of incubation. Thus, inclusion of shorter incubation times in these environments may produce better data to describe BR.

Typical values for BGE in the literature range from 0.01 to 0.50, with a maximum literature value of 5 (del Giorgio and Cole 1998). When excluding negative BGE calculations, most the results from this study fall into the low range when compared to literature values. The resulting BGE calculations matched overall expectations of low BGE being present in historically low nutrient environments.

Evidence of terrestrial runoff impacting microbial results is present in the current data set and is supported by previous studies. Previous work has shown that freshwater runoff into estuaries lowers both salinity and $\delta^{13}$C-DIC values (Mook and Tan 1991, Fry 2002, Atekwana et al. 2003). Additionally, previous studies have also indicated that terrestrial runoff can impact bacterial parameters. Cotner et al. (2000) found that site proximity in Florida Bay to Everglades runoff can influence BP and also found that BP is up to an order of magnitude higher during the wet season than during the dry season. Lavrentyev et al. (1998) found that nutrient input and freshwater inflow in Florida Bay impacted microbial community structure, resulting in three distinct microbial community structures dependent upon location within Florida Bay. In the current data set, impacts of terrestrial runoff may be present in June Biscayne Bay samples and July TS-9 samples. June Biscayne Bay samples had lower salinity (17 psu) and lower initial $\delta^{13}$C-DIC values (−8.28‰ to −8.10‰) than all other samples collected. The June Biscayne Bay samples

also had the highest BP and BA values. Although May Biscayne Bay samples had BP and BR results more similar to oligotrophic Florida Bay samples, differences between May and June samples can be attributed to minor differences in sampling location (center of navigation channel in May vs. shoreline location in June) and rainfall influence (recent tropical storm for June samples). Similar comparisons can be made between June TS-9 and July TS-9 samples. June TS-9 samples had a higher salinity (33.4 psu) and higher initial $\delta^{13}$C-DIC values ($-2.45$‰ to $-2.48$‰) than July TS-9 samples (21.1 psu, $-3.52$‰ to $-3.33$‰), indicating July TS-9 samples may have been influenced by freshwater input. July TS-9 samples also had higher BP rates, though BA was higher in June at TS-9 than in July. Stronger similarities were present between June TS-9 and July TS-10 results, particularly in regard to salinity (33.4 vs. 34.7) and BP rates (0.012 vs. 0.029 $\mu$g C L$^{-1}$ h$^{-1}$). These comparisons suggest that freshwater influence at TS-9 increased in July after the start of the wet season. Although the Florida Bay sampling events were separated by only one month, potential seasonal influences are present in the data.

## 5. CONCLUSION

The results of this study indicated that tracing of $^{13}$C may serve as a better method of measuring BR in oligotrophic systems than traditional Winkler titration-based DO consumption measurements. The original hypothesis that the Winkler titration method could be used in a mesotrophic environment to calibrate the $^{13}$C-based methods was incorrect. In both Florida Bay and Biscayne Bay, changes in DO before and after dark incubation were found to be either insignificant or highly variable, making the Winkler titration an invalid method of determining BR in these environments. Bacterial

abundance and BP results indicated that Biscayne Bay was a higher productivity site than the Florida Bay sites; however, BR was still not measureable by Winkler titration. In future studies, the labeled and natural $^{13}$C BR methods can be compared to BR measured by Winkler titration in a more eutrophic environment.

In comparison to the tracing of natural $^{13}$C ratios, the $^{13}$C-glucose consumption method, also referred to as the $^{13}$C labeling method, produced better BR results. Changes in natural $\delta^{13}$C-DIC ratios before and after dark incubation were largely insignificant throughout the study. Samples spiked with $^{13}$C-glucose, however, produced strong correlations between $\delta^{13}$C-DIC and incubation time during 3 of the 4 sampling events. While the tracing of natural $^{13}$C ratios would have been more ideal for determining realistic BR values on the basis of bacterial consumption of natural DOC sources, the correlations produced by this method were too weak to be used reliably for BR measurements.

The correlations produced by the labeled $^{13}$C method suggest that this method could be used to measure BR and BGE in oligotrophic systems. When $\delta^{13}$C-DIC values were converted into µg C respired, correlations were greatly weakened. Peak amplitudes measured by GC-IRMS were used to produce DIC concentrations during this conversion. While concentrations of DIC can be produced from GC-IRMS data, future studies could use more specialized instrumentation specifically designed for DIC and DOC measurements, such as a total organic carbon analyzer. In order to address potential concerns about isotopic dilution, future experiments can also examine $^{13}$C-glucose additions at varying concentrations, similar to methods used in radiotracer studies (Simon

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and Azam 1989). These additional measurements may refine bacterial energy pathways and improve conversion of $\delta^{13}$C-DIC into respiration values.

This study was designed to measure BR, BGE and other carbon pathways specifically within the Florida Bay ecosystem. Beyond determination of Florida Bay carbon cycling components, the $^{13}$C tracing method may be a way to determine BR and BGE in other environments where these measurements are difficult to make, such as low-productivity pelagic marine environments. Currently, techniques are being developed to measure $\delta^{13}$C-DIC using lower cost and more portable cavity ring-down mass spectroscopy (CRDS) systems (Hartland et al. 2012). While GC-IRMS would be impractical to utilize in field studies, CRDS has the potential to be used outside of traditional laboratories. The GC-IRMS method described in this study can ultimately be adapted to CRDS systems, allowing a field-based alternative to traditional techniques of quantifying microbial respiration in open oceanic waters.

The BR methodologies described in this study will need further refinement before reliable BGE values can be calculated from Florida Bay. Overall, data gathered in this study suggest that BGE is lower in highly nutrient limited Eastern Florida Bay than in Western Florida Bay. Further application of the methods described in this study can be used to explore other factors in Florida Bay that could affect BGE. Differences between June and July results suggest that seasonal variation, particularly in relation to water input from the Everglades, could impact BR, BP and BGE in Florida Bay. Additional long-term studies could explore the influence of this potential variable on BGE. Further laboratory and mesocosm studies could isolate other variables that may impact Florida Bay BGE, such as salinity and nutrient concentrations. With the addition of these

suggested long-term BGE studies, the role of Florida Bay water column microbes can be better understood in relation to carbon cycles in Florida Bay and the southern Everglades. Because the southern Everglades and Florida Bay may serve as an important carbon sink, further assessment of BGE throughout this system is warranted.

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