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Phytoplankton Ecology and Biogeochemistry: Methodology

The Influence of *Juncus*-rhizosphere Dissolved Organic Matter on Coastal Plankton Communities

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Abstract:

Many aquatic plants produce copious amounts of dissolved organic matter (DOM) which enters surrounding waters and potentially stimulates planktonic activity. In the northern Gulf of Mexico, *Juncus roemarianus* (i.e. black needle rush) is a dominant marsh grass species residing in coastal zones and barrier islands. The below-ground biomass i.e. rhizosphere, can be consistently submerged, serving as a potential source of DOM to the surrounding waters. The lability and possible stimulatory effect of *J. roemarianus* DOM was examined for three plankton communities collected within the discharge region of Mobile Bay and adjacent waters of Gulf Shores, Alabama (less affected by Mobile Bay). DOM within the pore water surrounding the *J. roemarianus* was extracted, concentrated, and added to the field communities along with positive (i.e. addition of labile organic matter) and negative (i.e. no additions) controls. In the Mobile Bay experiment, the DOM addition stimulated increased autotrophic biomass and heterotrophic activity well above that observed in the negative controls. However, experiments utilizing Gulf Shores water showed little to no stimulation. Our results suggest that *J. roemarianus* DOM addition may stimulate planktonic activity; however, the degree of enhancement is likely controlled by the community composition and water properties (e.g. nutrient availability).

Additional conduction of this research is required in order to determine if these results are significant in characterizing the optimal environment of toxic phytoplankton species such as *Pseudo nitzschia*. This marine planktonic diatom genus is capable of producing the neurotoxin domoic acid when it is subjected to environmental stressors. This acid causes the neurological disorder amnesic shellfish poisoning, which poses as significant ecological and anthropogenic problems.

This research was conducted in the joint efforts of the Dauphin Island Sea Lab (University of Southern Alabama, University of Alabama, and Bowling Green State University.)


**Introduction:**

Juncus roemeri anus releases significant dissolved organic matter (DOM) within its rhizosphere. Due to subsurface water movement (e.g. submarine groundwater discharge, tidal flux) and sediment benthic flux, these concentrations of rhizosphere DOM are transported into the water column, proliferating the ambient waters where phytoplankton communities thrive. We predict this DOM is labile, and will increase net phytoplankton production and bacterial production. The following metrics will be utilized to test this hypothesis: Chlorophyll a (proxy of photoautotrophic community) and oxygen consumption (proxy of heterotrophic community).

The aim of this experiment is to quantify the biodegradability and bioavailability of groundwater dissolved organic carbon and nitrogen entering coastal waters, and analyze the influence of these chemical components upon ambient phytoplankton communities. DON often dominates the nutrient pool in aquatic systems, but this nutrient source remains poorly understood (Bronk et. al. 2007). In 1996 the US EPA recognized groundwater as a potential source for nutrient loading to watersheds. However, the role of DON and DOC in submarine groundwater discharge (SGD) as source of excess nitrogen to marine ecosystems has been overlooked because traditional dogma generally considers DON as refractory and unavailable (Kroeger et al. 2006). However, the bioaccumulation of DON and DOC in the rhizosphere DOM of coastal native vegetation and its inherent influences upon phytoplankton communities have yet to be widely studied, and may be influencing the dormancy stage (absence of production of domoic acid) of *pseudo nitzschia* species in this region.

**Groundwater**

Groundwater has been categorized as the world’s largest resource for freshwater (Aeschbach-Hertig and Gleeson 2012). With global populations on the rise, water resources are running low and escalating amounts of pressure are being put on groundwater supplies. The more humans begin to rely on groundwater resources, aquifer systems become more vulnerable to various contaminations and depletion. Anthropogenic activities are increasingly enriching total dissolved nitrogen concentrations in groundwater sources around the world and in Alabama (Murgulet and Tick 2008), which has serious implications for the quality of drinking water supply. Often, SGD nutrients far outweigh those of the overlying coastal waters. Annually, 2400 km$^3$ of freshwater discharges into the world’s oceans in the form of SGD (Kroeger et. al. 2008, Cable et al., 1996; Moore, 1996; Moore 2010), and is increasingly being implicated in the eutrophication of nearshore marine ecosystems (Zhang et. al. 2012).

SGD can be an important source of both freshwater and recirculated seawater inputs into marine environments, as it can carry high levels of inorganic nutrients and other elements (Slomp and Van Cappellen, 2004; Santos et al., 2009). For example, groundwater seepage is the primary source of inorganic nitrogen in many estuaries along the coastal areas of the United States (Seitzinger and Sanders 1997). Nitrogen loading from groundwater seepage has been shown to greatly impact harmful algal blooms and entire ecosystems (LaRoche et al. 1997).

As an important source of nutrients, SGD has the potential to impact the chemical budget of coastal waters. Although many previous and current SGD studies have been carried out worldwide, knowledge of the significance and impact on our oceans is still limited (Taniguchi et
al. 2002; Burnett et al. 2003; Johannes 1980). Nutrient rich groundwater resources are not just high in dissolved inorganic nitrogen (DIN) species, such as nitrate, but a large pool of DON and DOC exists as well (Kroeger et. al. 2006). There are many unanswered questions about how humans have altered the natural processes within the nitrogen cycle, especially with increasing nitrogen loads. It is imperative we develop a more thorough understanding regarding how DON and DOC are being processed upon their entrance into freshwater and marine communities. Hence, this research focuses primarily on the role of DOC and DON upon the phytoplankton communities within the ambient marine water column.

**DOC and DON in Groundwater**

While many investigations have studied the transport and reactivity of inorganics within groundwater (Cai et al. 2003; Murgulet and Tick 2009; Murphy and Zachara 1995), considerably less is known about the sources and concentrations of organic matter in groundwater, particularly DOC and DON. Bacteria and some phytoplankton rapidly assimilate very low molecular weight compounds, such as amino acids and urea (Glibert et al. 2004). These low molecular weight compounds are generally considered to comprise 10% or less of the DOC and DON transported by SGD to estuaries, with the bulk of the DON consisting primarily of complex, high molecular weight (HMW) compounds, such as humic and fulvic acids (Paerl 1997). Most DOC and DON inputs to estuaries have been considered limited in biological availability based on the prevalence of HMW compounds (Weigner et al. 2006). However, the utilization of nitrogen contained in the HMW compounds that make up the bulk of DOC and DON has not been substantially addressed by experimental measurements.

In contrast to previous conclusions about the bioavailability of HMW DOC and DON, studies demonstrate that a considerable portion of HMW DON from freshwater inputs is readily used by bacteria (Amon & Benner 1996). In order to fully understand the contribution of SGD to coastal biogeochemical processes, we need to know whether the DOC and DON transported by the influx of groundwater is biologically available. DON can be incorporated into the biological cycle in estuaries when microbial populations assimilate the N into bacterial biomass and/or remineralize the DON, often as ammonia (Caron & Goldman 1990). DON may also become biologically available due to the release of ammonia, a preferable N nutrient form, following photochemical oxidation of DON (Bushaw et al 1996), degradation of DON by microbiota (Glibert et al. 2010) and direct microbiota uptake (Bronk et al 2007). As a result, a detailed investigation is necessary to quantify the availability of DOC and DON in groundwater intruding into Alabama coastal waters, and to examine the resulting ecological shifts of microbiota communities (phytoplankton communities).
Materials and Methodology:

Field Sites of Collection

The *Juncus roemarianus* rhizosphere DOM content was collected in the form of pore water from the airport salt marsh located on the Dauphin Island Sea Laboratory (DISL) premises. This pore water was extracted and concentrated (Dittmer et.al.) to serve as a DOM-rich treatment group which was added to phytoplankton communities later collected.

The proposed locations of phytoplankton community sample collection were Gulf Shores Pier: 30.247°N, 87.668°W (Sample 1,2) and Mobile Bay: 30.440°N, 88.998° W (Sample 3). The Gulf Shores Pier is located in an area removed from the flume of river water and SGD outflow into the Mobile Bay. The water in this area is noticeably clearer and contains less nutrients. In comparison, the Mobile Bay is situated directly in the flume, and appears cloudy and contains a large concentration of nutrients and potentially DOC and DON.

Location of Phytoplankton Community Sampling Sites:

Figure 1: Google Maps satellite image of the Mobile Bay region. Samples 1 and 2 were collected from the Gulf Shores Pier (Far right red marker) while Sample 3 was collected at the mouth of the bay (middle-centered red dot). The *Juncus roemarianus* rhizosphere DOM content was collected at the airport saltwater marsh at DISL (far-left orange dot).
Methodology of *Juncus roemarianus* rhizosphere DOM treatment synthesis:

**Sampling of Pore Water**

The first step of this experiment was synthesizing the DOM into a concentrated form that could be easily applied to the collected water samples (phytoplankton communities). The DOM was collected in the form of pore water from the salt marsh at DISL. The materials required for the sampling of the pore water include: Nitrogen (N2) tank, 1 Three-way adapter gaseous fill bag (equipped with valve), 1 Polycarbonate Syringe (75mL), (2) 1 Liter Polycarbonate containers equipped with water tight lid, 10 sippers established in the marsh, each consisting of 2 plastic tubules protruding from partially submerged sipper, each fitted with a three-way adapter and valve (Sippers spaced on average. 3ft apart, located from approx. 10-100ft away from land into the marsh).

The pore water slowly accumulates with the daily influx of tides, and is retained in the air-tight sippers until collection. Sampling can be performed twice per day in correspondence with the tidal fluxes, amounting to approximately 2-4L of pore water.

Upon arrival at sampling location, carefully and securely fit three-way adapter gaseous fill bag onto N2 tank. Switch blue valve to face the open third air passage of the adapter. Ensure it is tightly attached before filling bag with N2 until fully inflated. Shut off N2, switch the blue valve to face the gas bag, detach. Enter marsh and locate the first sipper. Find the tubule with the zip-tie, indicating this tubule is intended for the N2 bag attachment (All sippers have a zip-tie indicating which of the two tubules are for N2 attachment. Firmly screw N2 bag onto the adapter, switch the blue valve into position to face the exposed air passage, and the second blue valve on the sipper tube to air passage. Slight deflation of gas bag may occur upon the switching of the valves. Screw polycarbonate syringe onto second, naked tubule. Begin pulling the syringe plunge towards you slowly, ensuring not to disturb the vacuum that has been established within the apparatus. Fill syringe completely.

When syringe has been filled, switch the blue valve to face the tubule, disconnect syringe, dispense sample into 1 Liter Polycarbonate container, screw cap on, attach syringe to tubule, switch blue valve to face the air passage, and continue drawing sample from sipper. When one observes the rapid disappearance of the vacuum (physical observable by a release of the plunger and bubbling/hissing) dethatch syringe once again and deposit water. Switch the N2 tubule blue valves to face the tubule and the gas bag. Disconnect the gas bag from tubule. Attach syringe, place blue valve facing the air passage, and draw air from sipper, switch blue valve so it is facing the tubule, push air out of syringe to re-establish vacuum to increase quantity of water.

![Figure 2: Nitrogen tank and Three-way adapter gaseous fill bag. Blue valve of the Three-way adapter is facing the air passage, this is the open position. Turn the green valve of the Nitrogen tank on to allow gas fill bag to inflate, then turn the Nitrogen valve off and turn the blue valve off.](image-url)
intake within the sipper. Repeat twice. Switch blue valve to face the tubule, detach syringe. Repeat process until all sippers have been harvested. One should obtain approximately 1 L sample from the sippers cumulative. Ensure caps of 1L containers are tightly fitted, then place samples in trunk or equivalent dark bag (Long lengths of exposure to sunlight will promote primary productivity amongst photoautotrophs in sample, potential source of error in results).

Return to laboratory to filter and acidify samples immediately (time-sensitive). The sample water is then stable for the duration of one month. Within the month, perform Solid-Phase Extraction (According to Dittmer et.al.). Store the DOM extract in methanol solution in freezer at -20 Celsius (stable for 6-12 months/).

**Figure 3**: Two sipper tubules each fitted with a Three-way adapter (stop cock) and valve. The N2 Adapter is marked with a zip-tie. The syringe is screwed into the other adapter, which is unmarked.

**Evaporation and Reconstitution of DOM**

The purpose of this protocol is to perform the elution(evaporation) of the methanol from the Solid Phase Extracted Dissolved Organic Matter Extracts (SPE-DOM) from the previously collected pore water samples. The final product SPE-DOM should be stored in -20 degree Celsius conditions in a combusted glass ampoule with a large surface area to volume ratio. The intent of this protocol is to rid the extracts of the methanol solvent to produce the isolated DOM dry extracts.

Following the elution(evaporation) process of the methanol solvent, the next step is the reconstitution of the isolated DOM in artificial seawater (ASW) or Milli-Q water.

The Elution and Reconstitution Protocol for the Extracted DOM is fractioned into two sub-processes which encompass the entirety of the protocol:

1. **Elution (Evaporation) of methanol**
2. **Reconstitution of isolated DOM**

**Elution**

The materials required for the evaporation of the organic solvent: Evaporation of methanol: Precision Vacuum Oven Model 19 (and 2 valves, pressure meter, and washers), Plastic tubing to connect to vacuum oven valve and vacuum, Alcohol thermometer, Access to fume hood, Combusted ampoule containing DOM Extracts and methanol

Assemble the vacuum oven, and connect to a vacuum gas pump, and place outflow tube in hood. Tighten both bolts on the top of the vacuum oven until they are completely shut. Place
alcohol thermometer on top shelf in oven so you can read the temperature when the oven is closed. Close oven door, secure with shoe strings to prevent it from popping open. Turn on vacuum to approx. 10 Hg and Temp to 3 setting on the knob to establish the high heat, low pressure system. Combine the first filling DOM elution and second filling DOM elution’s by pouring the second fillings into the first fillings. Mix the DOM and methanol (swirl and rotate the bottle efficiently). Eluting DOM with 1 filling methanol every 3L then a second filling of methanol to remove remaining DOM from ABE will produce 100mL DOM extract in methanol total.

Combust a glass vial at 400 degrees Celsius for 4 hours. Pipette 10% of the DOM extract in methanol into the combusted glass vial for DOC analysis if wanted/required. In this experiment, 10mL is 10% of the 100mL total DOM in methanol. Store vial at -20 degrees Celsius. Evaporate the DOM methanol extract in three separate portions (3) 30 mL portions. With a sterile pipette, transfer 30mL DOM extract in methanol into the second filling of methanol container, already rinsed with Milli-Q water.

Store remaining 2/3rds of the DOM extract in methanol at -20 Celsius until you begin the second and third replicate of the experiment. Place glass jar filled with the first 30mL portion of DOM extract in methanol in the vacuum oven which has already been heating to the stabilized temperature of 50 degrees Celsius. Evaporate until the methanol solvent is entirely removed, turn vacuum off at night but oven can be left on.
**Reconstitution**

Measure out 10mL fresh Milli-Q water straight from the tap into combusted glass graduated cylinder not ever in contact with WAF. Pour 10 mL Milli-Q water into the glass jar of 30mL dried DOM extract and shake vigorously until the DOM is completely dissolved in the solution. Pipette the 10mL reconstituted DOM in Milli-Q into a combusted sampling vial and store at -20 degrees Celsius.

Once the DOM has been reconstituted, it is ready to be added as a treatment variable to the sample collections. This should occur on the day the samples are collected, as the experiment becomes time sensitive upon the collection of the samples.

**Treatment Additions and Incubations**

Once the DOM has been reconstituted, it is ready to be added as a treatment variable to the sample collections. This should occur on the day the samples are collected, as the experiment becomes time sensitive upon the collection of the samples. This experiment was performed three separate times and performed in a triplicate manner to minimize statistical and experimental error.

3 Treatments (triplicate bottles): Control, DOM, Labile (\(C_6H_{12}O_6+ [(NH_4)_2SO_4]\))

<table>
<thead>
<tr>
<th>Quantity/Magnitudes of Treatments:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DOM Treatment:</strong></td>
</tr>
<tr>
<td>+19.8 8 µM DOC addition</td>
</tr>
<tr>
<td>+ 0.5 µM DON addition</td>
</tr>
<tr>
<td><strong>Labile Treatment (i.e. Positive Control):</strong></td>
</tr>
<tr>
<td>+1,000 µM Glucose (C) addition</td>
</tr>
<tr>
<td>+20 µM Ammonium (N) addition</td>
</tr>
</tbody>
</table>

Once the treatments have been applied to the water sample, the replicates are placed into the incubation chamber, which simulates the natural ambient water column. The incubation chamber is to be located in the dock in approximately 4 feet of water. 200mL samples of water will be taken and tested every 12, 24, 48, and 72 hrs. These samples will be analyzed utilizing standardized tests for Chlorophyll a (acetone extraction), Dissolved Oxygen (optical method), and Fluorescent particle imaging (FlowCam).

![Figure7: Incubation apparatus.](image)
Data Analysis and Assessment:

Analytical tests Conducted:
- Chlorophyll a (acetone extraction)
- Dissolved Oxygen (optical method)
- Fluorescent Particle Imaging (FlowCam)

Treatment Chlorophyll Concentration (±Stdev)

Over the course of the 72-hour incubation periods, the chlorophyll a concentration increases in all three treatment groups. However, in samples 1 and 2 (Gulf shores water samples), there is not a distinguishable difference in the control vs. the DOM treatment groups chlorophyll a biomass, suggesting there was no photoautotrophic stimulation as a result of DOM treatment in the phytoplankton community. However, in the sample 3 (Mobile Bay) chlorophyll data, the DOM appears to experience a rapid increase in chlorophyll a concentration between the 48-72-hour time interval. Although there is a high standard error bar for this data, it was determined to be statistically significant. Hence, photoautotrophic activity was stimulated by the DOM treatment in the Sample 3 phytoplankton community.

These results indicate that the community itself (in regard to its properties, population, and diversity) may be responsible for the extent of influence DOM has on the phytoplankton community. This is based on the chlorophyll a data and the qualitative observation of the differences in turbidity, location, and environment of the samples 1, 2, and 3 which would cause a variance in ambient phytoplankton community composition.
The concentration of dissolved oxygen over time in the samples served as a proxy for the quantitative analysis of the presence and activity of the heterotrophic community present in each sample. As indicated by the graph, heterotrophic respiration was the highest over time for the labile treatment group. Heterotrophic respiration appears to be approximately the same rate for the control and DOM treatment groups. However, upon running a linear regression and Statistical analysis of the results, it was found that the slope of the DOM is 18% steeper than the slope of the control. This 18% was found to be statistically significant. This difference in slope indicates that the DOM heterotrophic community was more active than the control. Additionally, this data was only recorded until the 48-hour time interval. If this trendline was to be extrapolated to the 72-hour time, which appeared to be the most active time interval for the photoautotrophic community in the chlorophyll results, then the difference in slope between the control and DOM treatments would be expected to increase drastically.

Linear Regressions of Heterotrophic Activity Data:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Linear Equation</th>
<th>R^2 Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Y= -1.29x + 194.09</td>
<td>0.91</td>
</tr>
<tr>
<td>DOM</td>
<td>Y= -1.52x + 190.01</td>
<td>0.99</td>
</tr>
<tr>
<td>Labile</td>
<td>Y= -3.80x +193.80</td>
<td>0.99</td>
</tr>
</tbody>
</table>
The FlowCam apparatus was utilized to characterize the phytoplankton communities present in the samples over time. The characterization of the phytoplankton community was performed utilizing the quantitative parameters: number of particles per mL, circularity, diameter, and width. As indicated by the graph, all three treatments do not show any change in the phytoplankton community over time, suggesting the chlorophyll a and dissolved oxygen results are not due to a shift in the phytoplankton community. This inference leaves only one other explanation for the results seen thus far; the DOM is altering the behavior of the phytoplankton rather than the community structure.
Discussion:

Before analyzing the influence of the DOM treat in respect to the phytoplankton community, we had to determine how concentrated the Solid Phase Extraction DOM was in respect to the DOC and DON levels. This was accomplished utilizing general respiration stoichiometry (Eq.1) in respect to the initial volumes of pore water processed. It was deduced that the DOM treatment was an addition of 19.8µmol/L DOC. This value was compared to literature values of DOC and DON within the area that had been compiled over decades (Pennock et. al). Utilizing the water sample salinities of sample 1, 2, and 3 (31.02 & 32.45 psu) it was found that the additions of DOM treatment in respect to the standard values of DOC and DON within the water was barely significant relative to the data. The addition of 19.8µmol/L DOC to a water sample already containing approximately 300.0µmol/L DOC is a substantially low amount of DOC added.

However, it was determined through respiration stoichiometry that 18µmol/L DOC more was consumed in the DOM groups compared to the control group. Overall, this suggests that 23µmol/L DOC was consumed by the DOM treatment group community, which is over 100% of the DOC added due to the DOM treatment. Hence, this indicates the community that received the DOM treatment consumed all of the DOC supplied, while being induced to utilize DOC concentrations within the water sample.

General Respiration Stoichiometry (Eq.1):

\[(\text{CH}_2\text{O})_{106}(\text{NH}_3)_{16}(\text{H}_3\text{PO}_4) + 138\text{O}_2 \rightarrow 106\text{CO}_2 + 16\text{HNO}_3 + \text{H}_3\text{PO}_4 + 122\text{H}_2\text{O}\]
Conclusions:

In conclusion, the DOM treatment that was prepared utilizing Solid Phase Extraction (Dittmer et.al.) contained a concentration of 19.8 µmol/L DOC. Hence, the DOM treatment phytoplanktonic and heterotrophic communities received an additional 19.8 µmol/L DOC compared to the control and labile groups. Utilizing respiration stoichiometry, it was deduced that the DOM treatment group consumed 18.0 µmol/L DOC more than the control group, in total consuming 23 µmol/L DOC throughout the duration of the incubation. This data suggests that the DOM treatment group consumed over 100% of the DOM received from the DOM addition, and was induced to utilize DOC in the water sample. This suggests a significant spike in phytoplanktonic activity, stimulated by the DOM treatment.

Furthermore, this data is of particular interest because the natural levels of DOC in the water column for the Mobile Bay region are approximately 300 µmol/L DOC (Pennock et. al.). This data suggests that the DOM addition received by the treatment group was an extremely low amount (19.8 µmol/L DOC in comparison to 300 µmol/L DOC in the water column). Therefore, the smallest quantity of DOM still had a large impact on the phytoplankton and heterotrophic community. As a result of the quantitative analysis of the data, it was revealed in Sample 3 exclusively, chlorophyll increase was observed after 48hr period. Also, there was a significantly steeper decline of dissolved oxygen over time in the DOM group, insinuating the heterotrophic community respiration was increased by the presence of DOM. The FlowCam data revealed there was no change in phytoplankton community throughout the experiment, supporting the postulation that the DOM influences the phytoplankton community’s behavior rather than composition.

These results suggest *Juncus roemerianus* rhizosphere DOM appears to be very labile, i.e. rapidly consumed by the heterotrophic and phytoplanktonic community. The results may be interpreted to support the explanation DOM is directly labile to phytoplankton community or made accessible after remineralization by the heterotrophic community (supported by the high respiration rates of the DOM heterotrophic community and the large increase in chlorophyll a in the DOM treatment group between 48-72 hours). In conclusion, additional research is required to determine which of these two explanations is valid in regards to this experiment.
References


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