Fall 2011

Phytoplankton Ecology in the Skidaway River Estuary

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PHYTOPLANKTON ECOLOGY IN THE SKIDAWAY RIVER ESTUARY

by

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(Under the Direction of Risa Cohen)

ABSTRACT

Short-term and seasonal variation in abiotic factors such as salinity, nutrient concentration, and light availability strongly influence total phytoplankton biomass but less is known about their effects on species-level changes due to difficulties associated with identifying and enumerating individual taxa. Understanding taxon-specific shifts is important as species often contribute differently to primary productivity and food web support for higher trophic levels. The goals of this study were to examine changes in overall phytoplankton abundance and community composition following short-term increases in freshwater input (Chapter 1), seasonal variation in abiotic conditions (Chapter 2), and manipulated nutrient concentrations within the Skidaway River Estuary (Chapter 3). Both microscopy and molecular analysis were used to examine species make-up. To characterize short-term and seasonal variation in the phytoplankton assemblage, three, two-week intensive field samplings were conducted following rain events of different magnitudes and in different seasons. During each sampling event, total phytoplankton abundance and species composition were determined twice daily at high and low tide along with abiotic measurements of salinity, temperature, light attenuation, and nitrate concentration. To examine estuarine phytoplankton community response to increased nutrients under manipulated conditions, phytoplankton from the SKE were

exposed to either nutrient enrichment (phosphorous and/or nitrogen addition) or control treatments. Within and among all sampling periods the phytoplankton community was dominated by large diatoms including *Coscinodiscus* and *Nitzschia* species, and other taxa including chlorophytes like *Nannochloropsis* and dinoflagellates like *Gymnodinium* were more common in Spring. Salinity explained most of the variation in phytoplankton abundance and species composition and there were also positive relationships between the abundance of dominant species and temperature, light attenuation, and nitrate concentration, river discharge, and rainfall.

INDEX WORDS: Phytoplankton, Community composition, Multivariate methods, Diatoms, PCR analysis, Estuary

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Bachelor of Science, Georgia Southern University, 2007

Master of Science, Georgia Southern University, 2011

A Thesis Submitted to the Graduate Faculty of Georgia Southern University in Partial Fulfillment

of the Requirements for the Degree

MASTER OF BIOLOGY

STATESBORO, GEORGIA

2011

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Electronic Version Approved:
December 2011

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CHAPTER 1

PHYTOPLANKTON COMMUNITY RESPONSES TO SHORT-TERM AND SEASONAL VARIATION IN RIVER DISCHARGE IN THE SKIDAWAY RIVER ESTUARY

Introduction

Phytoplankton biomass and species composition are important in determining rates of primary productivity and food availability to consumers in estuaries. The relationships between total phytoplankton biomass and changes in abiotic conditions are well established and increased biomass is generally associated with higher rates of production and consumption (Lehman, 2000, Wetz et al. 2006). However, examining only biomass overlooks valuable information as the species composition of these assemblages also leads to differences in overall rates of production and efficiency of energy transfer to consumers (Ramus et al. 2003, Lehman, 2007). For example, primary productivity along estuarine salinity gradients varies with species composition, and increases in diatoms like *Coscinodiscus*, *Thalassiosira,* and *Melosira* sp. often accompany peaks in productivity (Lehman, 2007, Muylaert et al. 2009). These diatoms are also associated with efficient energy transfer to copepods, mussels, and other consumers as they contain long chains of polyunsaturated fatty acids (Wichard et al. 2007), and the size and availability of different species can alter grazing rates of highly selective consumers like copepods (Frost, 1972, Sipura et al. 2003). In contrast, estuarine productivity may decrease with higher abundance of flagellates including *Gymondinium* and *Gonyaulux* sp., cyanobacteria including *Anabaena* and *Gleotricha* sp., and these taxa

are often associated with harmful algal blooms (HABs) within estuaries (Ramus et al. 2003). HABs cause many negative effects including low dissolved oxygen, increased turbidity, and fish kills and there are bloom-forming species within every major class of phytoplankton (Tang et al. 2003). Therefore, characterizing changes in phytoplankton species composition is an essential component of understanding estuarine productivity and energy transfer.

Although phytoplankton communities can be shaped by bottom-up regulation through nutrient availability and top-down regulation due to the influence of grazers (Acuna et al. 2008, Thompson et al. 2008), bottom-up regulation due to the availability of nutrients is often the strongest determinant of community structure in estuarine systems (Verity, 2002, Springer et al. 2005, Wetz et al. 2006). Since estuaries receive freshwater input from rivers and are tidally flushed, frequent, short-term changes in nutrient availability occur emphasizing the importance of examining phytoplankton assemblages over small time scales. For example elevated nutrient levels can trigger rapid (within 2-3 days) changes in phytoplankton species make-up and total biomass. (Verity, 2002, Springer et al. 2005, Wetz et al. 2006). Additional factors including light, salinity, and temperature also control short-term and seasonal differences in estuarine phytoplankton assemblages as individual species have a preferred set of physical conditions (Vrede et al. 1996, Lehman, 2000, Cloern and Dufford, 2005). Low light and high nutrient availability within estuaries, for example, has been found to favor dominance by large diatom species like *Skeletonema costatum, Coscinodiscus* sp., and *Nitzschia* sp., while rapidly increasing nutrients and high temperatures can lead to the proliferation of dinoflagellates like *Gonyaulux* sp. and cyanobacteria like *Anabaena* sp. (Bledsoe and Phlips, 2000, Ramus et

al., 2003, Cloern and Dufford, 2005). While much is known about factors promoting seasonal differences in the species make-up of estuarine phytoplankton communities, less is understood about short-term variation in these assemblages, the factors that influence them, and how short-term patterns may differ seasonally.

Consequences of short-term alteration of phytoplankton communities can include daily fluctuation in primary production and sudden onset of harmful algal blooms (Cote and Platt, 1982, Tang et al. 2003, Madhu et al. 2009) underscoring the importance of examining individual phytoplankton species in highly variable estuarine systems. The goals of this study were to examine changes in phytoplankton abundance and species composition following short-term increases in freshwater input at a fixed location within the Skidaway River Estuary (SKE) during three different seasons. I hypothesized that community composition would shift rapidly following increased river discharge. Specifically, increases in freshwater input and associated nitrate availability were expected to decrease the proportion of large diatoms like *Skeletonema costatum, Coscinodiscus* sp., and *Nitzschia* sp. in the community and promote the growth of chlorophytes and dinoflagellates including *Nannochloropsis* and *Gonyaulux* sp. Furthermore, I expected salinity, nitrate concentration and temperature to relate to phytoplankton abundance and community composition both within and among seasons.

Methods and Materials

Site Description and Sampling Periods

The Skidaway River Estuary (SKE, 32^{o} 37’ 05.64’’ N, 81^{o} 52’ 43.71’’ W, Figure 1) is a well-mixed, tidally dominated estuary receiving little freshwater input from the Savannah and Ogeechee rivers except following major rain events (Verity 2002a). To

examine phytoplankton species composition following changes in abiotic conditions within the SKE, three intensive field samplings were conducted during 2009. Two of the sampling events followed major rain events. Sampling events lasted 2 weeks to ensure that changes in phytoplankton community composition as a result of increased freshwater input could be detected (Lehman, 2000, Verity 2002, Cloern and Dufford, 2005). The March/April sampling followed the first major rain event in southeast Georgia during 2009. From March 28th – April 2nd the Savannah River watershed received 20-25 cm of rain with a single day maximum of 9.5 cm on April 2nd (NWS 2009). Discharge of the Savannah River increased from 2,298 m^3/s on March 30th and peaked at 5,060 m^3/s on April 2nd which is slightly higher than the monthly mean for March of 4,998 m^3/s. Over the 3 days prior to the May sampling the Savannah River watershed received 15-18 cm of rainfall and average rainfall during the duration of the May sampling was approximately 0.6-1.25 cm per day. This rainfall translated into an increase in discharge from 1,847 m^3/s on May 14th to 2,280 m^3/s by May 28th. However, this range was lower than the average monthly mean discharge of 3,444 m^3/s. The December sampling was not preceded by a single major rain event but discharge was highest during this period due consistent rainfall in previous months. Discharge decreased from 8,291 m^3/s on December 4th to 5,090 m^3/s on December 12th and increased through the remainder of the sampling to peak at 10,150 m^3/s. Average mean monthly discharge during December is typically 4,980 m^3/s for the Savannah River (USGS, site #02198500).

Sampling Design

Five stations around the University of Georgia floating dock at the Skidaway Institute of Oceanography (31^o 59’ 21.32’’ N, 81^o 01’ 26.33’’ W) were mapped using a

Garmin E-trex H gps, and these stations served as replicates to characterize the area. The first station was adjacent to the west end of the dock and and the other four stations were arranged 25m away, parallel to the dock, and 25m away from one another. At each station, dissolved oxygen (DO), salinity, conductivity, temperature (YSI 85 Multipurpose meter), pH (Mettler Toledo pH meter), and light intensity (Licor 192SA quantum sensor) were recorded. Light intensity measured at the surface and 0.5m below the surface was converted to extinction coefficients using the formula $k = (2.3 \times (\log I_{d1} - \log I_{d2})) / (d2 - d1)$ where I is light intensity and d1 and d2 represent the two depths where intensity was measured (Kenworthy and Fonseca, 1996). Surface water samples were collected 0.25m below the surface in acid-washed, 125 mL sample containers twice daily at high and low tides to determine nitrate concentration. Phosphate was measured during the May sampling, however, only nitrate data was considered as past evidence suggests nitrogen is the limiting nutrient in this system (Verity, 2002, J. Parris, unpublished data). Replicate 500 mL surface water samples were collected during each sampling and sub-sampled for visual examination of the phytoplankton community using microscopy and for estimation of overall abundance using chlorophyll *a*.

Sample Analysis

Nutrient Concentrations:

To analyze nitrate concentration, 125 mL surface water samples were passed through Whatman GF/F glass fiber filters and frozen until analysis at the JBC Analytical Lab, University of Georgia, Athens, Georgia. Briefly, analysis of nitrate involved the reduction of nitrate to nitrite and treatment with acid to produce a colored solution. The

solution was then measured colorimetrically using an autoanalyzer (LACHAT, U.S. EPA Method 353.2). The limit of detection was >1µM.

Chlorophyll *a* Concentration

Total phytoplankton abundance was estimated using chlorophyll *a* concentrations (Lehman, 2000, Verity 2002). Phytoplankton were concentrated from a 100 mL volume onto a Whatman GF/F glass fiber filter. Following extraction of chlorophyll from cells on each filter in 90% acetone at -20^{o}C in the dark for 24 hr., chlorophyll *a* was measured using a Turner Designs-700 flurometer (EPA Method 404, Arar and Collins, 1997).

Visual Identification of Phytoplankton

Samples for visual analysis of phytoplankton species composition were preserved by adding approximately 0.5 mL of Lugol's solution to 50mL water samples. Each sample was concentrated using centrifugation and resuspended in 1mL. Phytoplankton identification and counts were performed using a Sedgewick-Rafter counting chamber and an inverted microscope at 200x magnification (Bledsoe and Phlips, 2000, Cloern and Dufford, 2005). Counts for each sample were completed when 100 individuals of the three most numerous taxa had been tallied and only organisms that could be identified at least to the genus level were included in further analysis. (Cloern and Dufford, 2005).

Data Analysis

Indirect gradient analysis was used to determine the length of the gradient between environmental variables and abundance of each phytoplankton species for each sampling period. The length of the gradient in all cases was less than three, therefore redundancy analysis (RDA) was used to relate species composition to the measured environmental parameters. Data from each sampling event were analyzed separately to

determine effects of short-term changes in the phytoplankton community due to river input. Data included in redundancy analysis were tested for normality using Shapiro-Wilke's test and species data was log +1 transformed prior to analysis. Only species observed in greater than 50% of all samples were included (Suikkanen et al. 2007). RDA analysis was conducted using CANOCO 4.5 for Windows and forward selection was used to determine which environmental variables were significant in explaining changes in species abundance and the proportion of variation explained by each factor.

To further characterize relationships between the dominant taxa (occurring in greater than 90% of samples) and changes in abiotic conditions, multiple regressions using a stepwise selection procedure were run (Minitab). The stepwise selection procedure used backwards and forwards selection to identify only the independent variables which were significant to the dependent variable (species) being examined.

Light intensity data was not available for the March sampling, therefore this variable was only considered during the May and December samplings. Since pH did not change within sampling events, conductivity exhibited the same trend as salinity, and DO did not drop below 4.5 mg/L, these factors were not included in analyses. Daily average rainfall and discharge for each sampling were obtained (NOAA Precipitation data, USGS site #02198500) and included in the redundancy analysis.

Linear or quadratic (2^{nd} order) regressions were used to examine trends in daily average salinity, temperature, dissolved oxygen, light extinction coefficients, and overall phytoplankton biomass through time (JMP).

Similarity of the diatom community was compared among sampling periods using Whitaker's (1952) equation where a_i = percentage of species i in sample A and b_i =

percentage of species i in sample B. This formula takes into account the species common to both samples as well as their relative abundances:

$$PS_c = 100 - .5\Sigma_{i=1}^{s}|a_i - b_i| = \Sigma_{i=1}^{s}\min(a_i, b_i)$$

Results

Water Quality

Salinity decreased over each sampling (y = 24.4-0.454x, R2 = 0.911, p=0.0001 for March, y = 23.6-0.304x, R2 = 0.7106, p=0.0002 for May, and y = 19-0.0897x, R2 = 0.33, p=0.03 for December, Figure 2) and was negatively correlated with rainfall and discharge. The strongest linear relationship with salinity and time was observed in March and weakened with each successive sampling period. Patterns in nitrate for the March and May samplings was a gradual rise starting at day one, a peak between day 5-7, and a decline over the remainder of the sampling event ($y=2.19+0.0027x-0.0307[x-7.5]^2$, $R^2=0.86$, p=0.0001 for March, and $y=2.97+0.059x-0.0519[x-7.5]^2$, $R^2=0.75$, p=0.0005, Figure 2). In December, nitrate concentrations were much lower and diminished over the duration of the sampling (y = -0.0758x + 3.0219, R2 = 0.6611, p=0.02, Figure 2). Temperature showed no consistent trend during the March or December sampling, however, temperatures decreased over the first half of the May sampling and increased over the last seven days ($y=21.37+0.097x-0.11[x-7.5]^2$, $R^2=0.66$, p=0.0026, Figure 2). Temperature ranges did not overlap between sampling periods and were highest in May and lowest in December, consistent with seasonality. Light extinction coefficients were on average very high and variable during both May and December (Figure 2). Patterns

for all physical variables were nearly identical for both high and low tide, therefore, only low tide data are presented.

Phytoplankton Community

Three rapid increases in chlorophyll *a* occurred during the March and May samplings (Figure 3). While chlorophyll *a* increased throughout the March sampling ($y = 0.40367x + 7.297$, $R2 = 0.8106$, $p=0.01$), there was no clear trend in chlorophyll *a* in either the May or December sampling. Total Chlorophyll *a* concentration was highest in March and lowest in December. In March, changes in chlorophyll *a* were most strongly influenced by salinity, and this factor explained 56.5% of the variation (Table 1, Figure 4). Differences in chlorophyll *a* in May were strongly related to temperature and light attenuation and these variables accounted for 34.9% of chlorophyll *a* change (Table 2, Figure 5). The smallest amount of chlorophyll *a* variation explained by abiotic parameters was observed in December where 25.1% of the variation was attributable to salinity alone (Table 3, Figure 6).

I observed 28 diatoms, 5 chlorophytes, and 3 dinoflagellate species over all sampling periods, however, the chlorophyte and dinoflagellate species were relatively rare (see appendix A for a complete list of species and species abbreviations). Overall, diatom species composition was very similar among sampling periods. There was an 89% community similarity of all diatoms between the March and May sampling, 84% similarity between March and December, and 92% similarity between the May and December sampling events. The four most common species (occurring in greater than 95% of samples) encountered in the March sampling were *Chaetoceros socialis, Coscinodiscus sp., Nitzshia longissima,* and *Skeletonema costatum*. Dominant species in

May included *Coscinodiscus sp., Nitzschia longissima,* a *Pinnularia sp*., and *Thalassionema sp.* and December was dominated by a *Ceratulina sp., Coscinodiscus sp.,* and *Skeletonema costatum.* The *Ceratulina sp.* was only observed during December. Transitions in the relative abundance of each dominant species were observed within each sampling period and were most pronounced in March and May. During both of these samplings large, non-chain forming species including *Coscinodiscus sp.* and *Nitzschia longissima* constituted a larger proportion of the community early and there was a transition towards more chain-forming species including *Melosira sp.*, and *Skeletonema costatum* at the end of the sampling period. These chain-forming species, especially *Skeletonema costatum,* also occurred in much higher abundances in March compared to any other month. In December, non-chaining forming species (mainly *Coscinodiscus sp. 2*, and *Ceratulina sp.*) made up the largest proportion of the community in the middle and beginning of the sampling period while *Skeletonema costatum* was most abundant at the end of the period (Table 4, Figure 7). Overall densities of all species were much lower in December.

Relationship Between Environmental Variables and Dominant Species

The strongest relationship between community structure and water quality was observed in March where the abiotic conditions measured cumulatively accounted for 42.9% of the variance in species composition and salinity was the most important factor (RDA, $p=0.001$, Figure 4). Most species were negatively associated with salinity. In addition to salinity, nitrate, temperature, and rainfall also explained a significant proportion of the variation in species abundances (RDA, $p<0.05$, Figure 5) and rainfall and nitrate exhibited the highest variability (Table 5, Figure 4). There were positive

relationships between most taxa and rainfall, nitrate, discharge, and temperature (Figure 4). Multiple regression analysis of the common diatom species in March further emphasized the importance of salinity. Salinity alone explained a significant amount of the variation in 7 of the 10 most common species (Table 1).

The environmental variables explained the least amount of variation (22.9%) in dominant species in May and temperature was the most important variable (RDA, $p=0.001$, Figure 5). Most species detected increased with increasing temperatures. Along with temperature, salinity, nitrate, light attenuation, rainfall, and discharge also explained a significant proportion of changes in species abundance (RDA, $p<0.05$, Figure 5) and rainfall and light attenuation demonstrated the highest variability over this period (Table 5). As in March, the majority of species appeared to be more common under lower salinities and exhibited positive relationships with nitrate, light attenuation, rainfall, and discharge (Figure 5). Multiple regression analysis showed that salinity and nitrate were important in explaining changes in the abundance of *N. longissima*, salinity alone exhibited a significant relationship with *Coscinodiscus sp. #2* density, and salinity and light attenuation had a significant effect on *Pinnularia sp. #3*.

The abiotic factors together accounted for 28 % of the variation in species composition during the December sampling (RDA, $p=0.001$, Figure 6) and temperature was the most important component. Most species identified exhibited a positive relationship with temperature. Along with temperature, salinity, light attenuation, rainfall, and discharge all explained significant amounts of the variation in species abundances ($p<0.05$, Table 5) and rainfall and discharge were most variable over this period. There was a negative relationship between the abundance of most species and

salinity, and a positive relationship with rainfall, light attenuation, and discharge (Figure 6). Multiple regression analysis showed a significant pattern for 6 of the 10 common species with temperature, light attenuation, salinity, and nitrate all contributing to the models (Table 3).

Discussion

Consistent with previous studies of estuaries, large diatoms constituted the major proportion (over 90%) of the phytoplankton assemblage within and among all sampling periods and *Coscinodiscus sp.*, *Melosira sp.*, *Nitzschia longissima*, *Skeletonema costatum*, *Pinnularia spp.*, and *Thalassionema sp.* were detected in nearly all samples. However, there were short-term shifts in the abundance of individual species within sampling events for all seasons. In March and May these shifts were consistent and pronounced. Non-chain forming species including *Coscinodiscus* and *Nitzschia longissima* made up a larger percentage of the community in the beginning of the sampling period when salinity was high and nutrients were low. In the middle of the sampling period when nutrients were highest, intermediate densities of both solitary species (*Coscinodiscus* and *Nitzschia longissima*) and chain-forming species were observed. At the end of the sampling period when salinity was low and nutrients were decreasing, Chain-forming species including *Skeletonema costatum* and *Melosira sp.* comprised a higher portion of the assemblage. Lower nitrate concentration at the end of the sampling period may be due to utilization by these species as there is some lag time (2-3 days) associated with increased nutrients and changes in density (Mallin et al. 1991, Cloern, 1987). In December, *Skeletonema costatum* dominated late in the sampling and solitary species like *Coscinodiscus* and *Ceratulina* were more abundant towards the

beginning and middle of the period. Both chain-forming and solitary species of diatoms have been shown to exhibit high rates of productivity (Mallin et al. 1991, Cloern and Dufford, 2005), however, they may have different effects on energy transfer through food chains. For example, most copepods generally select large, solitary diatoms like *Coscinodiscus* or *Nitzschia* species as prey (Frost, 1972). These diatoms contain high concentrations of PUFAs and are beneficial to larval fish which feed on the copepods (Sommer et al. 2002, Wichard et al. 2007). On the other hand, small, chain-forming species may promote a less efficient grazer pathway as they are consumed by small flagellates and contribute more to the microbial loop (Sommer et al. 2002). There is also a lot of evidence which suggests many chain-forming species including *Skeletonema costatum* and *Melosira* species synthesize aldehydes that may have negative effects on copepod reproduction (Miralto et al. 1999, Bochdansky and Bollens, 2004).

My analyses indicate that the transitions in species abundance within all sampling periods could be explained by the environmental variables measured. Salinity had a strong effect on community structure during all samplings, and it was most important during March when there was a consistent decrease in salinity over time. This finding is consistent with previous research suggesting diatoms are sensitive to changes in salinity (Muylaert et al. 2009). In March and May chlorophyll *a* and the abundance of individual species generally increased with decreasing salinity. However, overall abundance was greatest during high salinity conditions in December and this may be due to the positive association with salinity and a *Ceratulina sp.* which was only observed during this period. *Ceratulina sp.* are generally slow-growing and are thus associated with lower rates of primary production (Fahnenstiel et al. 1995). The relationship between salinity

and phytoplankton community structure has traditionally been examined by looking at distinctly different salinity zones or relating seasonal changes to phytoplankton communities. This has revealed a division in typically "freshwater" diatom species including *Navicula* and *Melosira sp.* and "marine" diatoms including *Fragillaria* and *Nitzschia* species (McIntire, 1978, Seppala et al. 2005, Madhu et al. 2007). This study has further demonstrated both positive and negative relationships between individual species and salinity over short time periods following changes in discharge at a fixed location within the SKE. However, seasonal differences in phytoplankton species make-up were small and could not be attributed to salinity. It is also important to note that changes in salinity were correlated with differences in rainfall and discharge. While there is some evidence that increased rainfall and instantaneous discharge may dislodge diatoms and other phytoplankton species from the benthos, the direct effects of these variables in this study were small (Karentz and McIntire, 1977). More importantly, these variables may have driven the observed changes in salinity.

Although nitrate can be a key determinant of estuarine phytoplankton community structure in long-term studies of estuaries (D'Costa and Anil, 2010), its effect on species abundance within and among sampling events in this study was relatively small compared to some of the other abiotic factors. In estuaries where nitrate has been shown to have large effects on phytoplankton community structure, concentrations are much higher (20-30µM) than we observed in the SKE, suggesting nitrate availability may not have been high enough to drive a large shift in the community (Piehler et al. 2004, Dominguez et al. 2011). However, previous research in the SKE has indicated nutrient concentrations are increasing and may reach much higher (up to 10x) concentrations

(Verity, 2002a). When comparing among seasons, nitrate had its largest effect in March when the lowest initial values were observed suggesting nutrient limitation of abundant taxa. Many of the common species including *Coscinodiscus sp., Cocconeis sp.,* and *Nitzschia longissima* exhibited positive relationships with nitrate within all sampling events. These species are large, productive, non-chain forming diatoms which should promote direct, efficient energy transfer to higher trophic levels in the SKE (Sommer et al 2002). Seasonal evaluations of phytoplankton communities have shown peak abundance of these taxa during spring bloom periods which often coincide with higher nutrient availability (D'Costa and Anil, 2010). This study has also shown that overall densities of diatoms are highest in spring, however, there are also frequent short-term changes which can be explained partly by variability in nitrate concentration.

Temperature had the greatest influence on species composition in December when the coolest temperatures were observed and in May when the warmest temperatures were recorded. Short-term increases in temperature during these periods promoted growth of many common species including *Nitzschia longissima,* multiple *Pinnularia sp.*, and a *Raphoneis sp.* suggesting the temperature optimum for these taxa is higher than the ranges observed in both May and December (Resende et al. 2005, Wang et al. 2008). These species are all productive and of suitable size for direct grazing by consumers like calanoid copepods which are abundant in temperature estuaries (Frost 1972). While temperature has been shown to be a key factor promoting seasonal abundance of non-diatom species in estuaries (Buric et al. 2007), my findings suggest that temperature may also be important in explaining variation in many common diatom species over short time scales.

Higher light extinction coefficients (increased turbidity) were associated with increases in diatom abundance during both May and December likely because these species are best adapted to low light levels (Bledsoe and Phlips, 2000). Extinction coefficients for the SKE were consistent with studies of estuaries where diatom abundance is strongly influenced by turbidity (Cloern, 2000). Also, the high attenuation observed in the SKE may help explain the low abundance of species from non-diatom phytoplankton classes (Bledsoe and Phlips, 2000). Increases in densities of *Thalassiosira decipiens* and *Pleurosigma elongatum* in particular coincided with high light attenuation. Both of these species are known to synthesize aldehydes which may negatively affect the growth and reproduction of primary consumers like calanoid copepods (Wichard et al. 2005). While seasonal differences in phytoplankton species make-up have been attributed to changes in light availability, the short-term patterns between turbidity and individual species in this study have not been previously been demonstrated in the field (Mallin et al. 1991).

Changes in the phytoplankton community occur quickly and have the potential to affect estuarine production and trophic transfer. For example, the direct short-term relationship between the abundance of individual diatom species and decreasing salinity suggests that increases in river discharge due to rain events promotes pulses of primary production in the SKE. These pulses can lead to long term increases in consumer populations including calanoid copepods and juvenile fishes. However, effects on higher trophic levels may differ depending on species composition. Larger diatoms like *Coscinodiscus* and *Thalassiosira* species are directly grazed by abundant copepods and promote an efficient energy pathway while smaller species like *Chaetoceros socialis* may

route primary production through the microbial loop. Harmful bloom-forming diatoms like *Skeletonema costatum* and *Nitzschia sp.* may also contribute a large proportion of their biomass to the microbial loop. Therefore, characterizing short-term relationships between individual phytoplankton species and how these relationships differ between seasons are important for understanding long-term consumer health in estuaries.

Conclusion

Although the taxa present did not change within or among sampling events, I have shown that changes in the abundance of each species in the phytoplankton community can occur not only among seasons, but over very short periods of time. Changes in the abundance of dominant taxa were observed over each 14-day sampling event and significant relationships between the environmental variables and individual species were established. Since differences in species composition can alter estuarine production and energy transfer to higher trophic levels, evaluations of phytoplankton species make-up should also include changes over small temporal scales, particularly following short-term environmental perturbations such as storm events that are likely to increase in severity and frequency with changing climatic patterns.

Table 1. Multiple regression models of the relationship between significant environmental variables, chlorophyll *a*, and the dominant taxa in the March 30-April 12 sampling. N represents nitrate, T is temperature, Sal is salinity, and K is light attenuation. P-values for all equations included are <0.05.

Targets	Possible factors (N, N^2, T, T^2, Sal)	R^2
Chl *a*	5.65 - 0.163 Sal	56.5%
Nitzschia longissima	13.0 - 0.534 Sal	48.6%
Thalassionema sp.	4.05 - 0.211 Sal	14.9%
Pinnularia sp. #1	8.04 - 0.391 Sal	31.2%
Pinnularia sp. #2	10.7 - 0.478 Sal	43.0%
Pinnularia sp. #3	8.23 - 0.376 Sal	17.5%
Skeletonema costatum	13.4 - 0.582 Sal	42.9%
Chaetoceros socialis	12.5 - 0.520 Sal	38.2%

Table 2. Multiple regression models of the relationship between significant environmental variables, chlorophyll *a*, and the dominant taxa in the May 14-May 28 sampling. P-values for all equations included are <0.05.

Targets	Possible factors (N, N^2, T, T^2, K, K^2 , Sal)	R^2
Chl *a*	- 0.728 + 0.0939 T + 0.273 K	34.9%
Nitzschis longissima	11.017-0.426 Sal-0.165N^2	64.3%
Coscinodiscus sp. #2	4.97 - 0.158 Sal	15.4%
Pinnularia sp. #3	8.84 - 0.355 Sal - 0.377 K^2	35.2%

Table 3. Multiple regression models of the relationship between significant environmental variables, chlorophyll *a*, and the dominant taxa in the December 4-December 17 sampling. P-values for all equations included are <0.05.

Targets	Possible factors (N, N^2, T, T^2, K, K^2, Sal)	R^2
Chl *a*	- 0.218 + 0.0666 Sal	25.1%
Coscinodiscus sp. #2	- 20.6 + 0.0326 T2	43.9%
Pinnularia sp. #1	- 4.30 + 2.54 K	32.4%
Pinnularia sp. #2	- 0.09 - 0.204 Sal + 0.0158 T^2	55.3%
Skeletonema costatum	1.39 - 0.189 Sal + 0.0105 T^2	42.4%
Ceratulina sp.	- 23.5 + 0.320 N2 + 0.316 Sal	60.1%
Cocconeis sp.	- 8.05 + 0.0343 T^2	40.7%

Table 4. Changes in the abundance of dominant species over each sampling period. Density given is in number of cells per milliliter (±1SEM) for high and low tide combined.

	March			May			December		
Species	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Coscinodiscus sp 2.	2.8±0.4	2.4±1.1	3.2±1.0	3.8±0.8	1.9±0.6	5.4±1.2	0.4±0.2	2.4±0.3	0.9±0.1
Ceratulina sp.	--	--	--	--	--	--	0.8±0.1	1.0±0.2	0.3±0.04
C. socialis	0.9±0.2	7.0±1.5	12.5±3.8	1.4±0.1	0.4±0.1	1.8±0.5	0.6±0.04	0.2±0.01	0.2±0.04
Melosira sp. 1	0.9±0.1	--	7.5±1.8	--	--	--	--	0.6±0.1	0.2±0.08
N. longissima	0.5±0.1	3.5±1.8	6.3±1.0	1.9±0.4	0.5±0.1	3.7±0.6	0.1±0.03	0.7±0.2	0.2±0.04
Pinnularia sp. 1	0.6±0.1	02±0.05	2.2±0.5	0.4±0.1	1.1±0.2	0.5±0.1	0.2±0.1	0.2±0.01	0.1±0.04
S. costatum	0.7±0.2	1.1±0.2	14.0±2.0	0.3±0.01	0.3±0.05	1.4±0.2	1.1±0.4	1.7±0.5	1.5±0.5
Thalassionema sp.	0.7±0.2	0.4±0.1	4.7±1.1	2.5±0.6	1.3±0.8	2.1±0.5	0.3±0.1	0.8±0.1	0.4±0.1

Table 5. Percent of variance explained and coefficient of variation for environmental factors in the redundancy analysis for each season. Only significant factors ($p<0.05$) are included.

Sampling Period	Factor	Percent of Variance Explained	Coefficient of Variation
March	Salinity	33%	8.87
	Nitrate	3.9%	35.90
	Temperature	3.1%	5.28
	Rainfall	3.2%	173.48
May	Salinity	2.8%	7.09
	Nitrate	1.2%	29.88
	Temperature	6.8%	8.26
	Rainfall	1.9%	96.81
	Light Attenuation	4.7%	38.37
	Discharge	4.4%	8.33
December	Salinity	5.2%	8.88
	Temperature	12.3%	5.27
	Rainfall	2.2%	150.06
	Light Attenuation	3.5%	37.60
	Discharge	4.4%	25.64

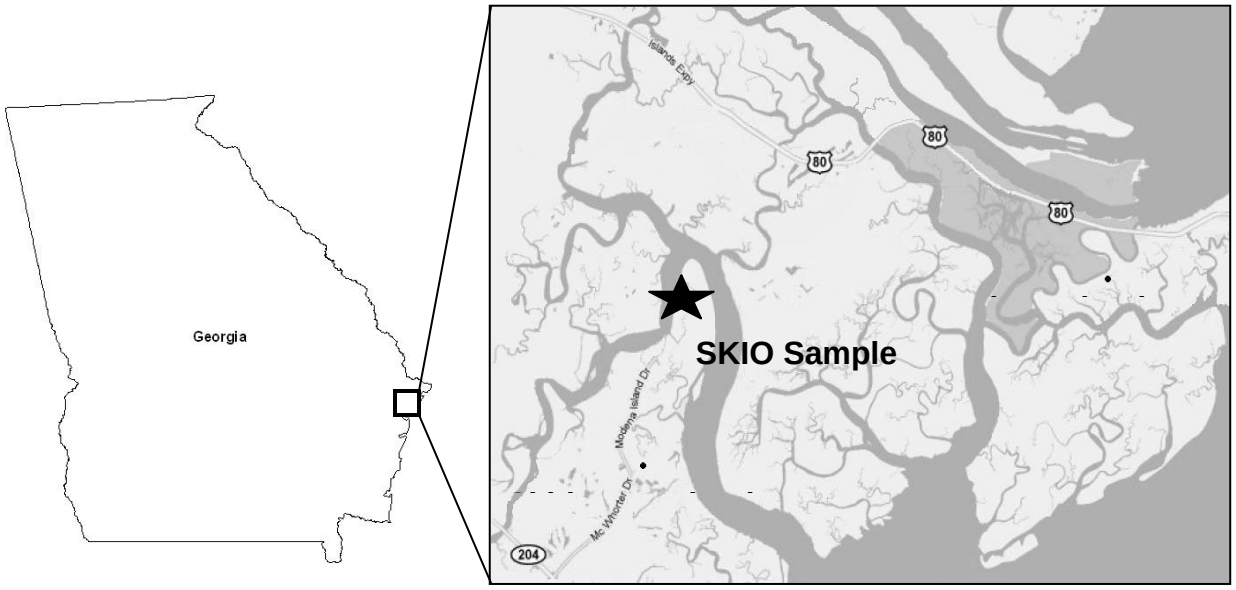

Figure 1. Map of the Skidaway River estuary and the sample site at the Skidaway Institute of Oceanography (SKIO).

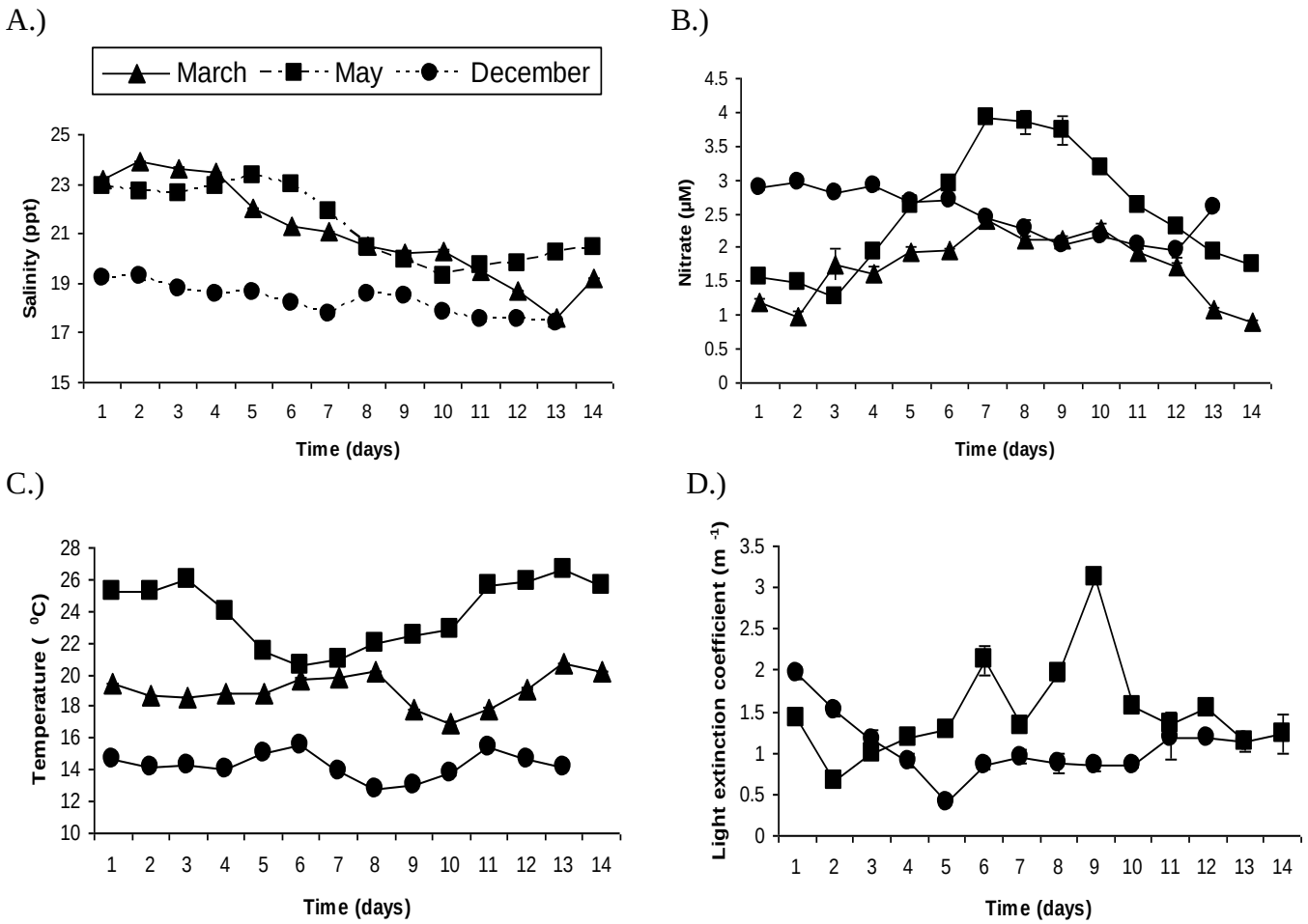

Figure 2. Water quality data at low tide for all sampling events including: A.) salinity B.) nitrate C.) temperature D.) light extinction coefficient. Error bars are ± 1 standard error (n=14).

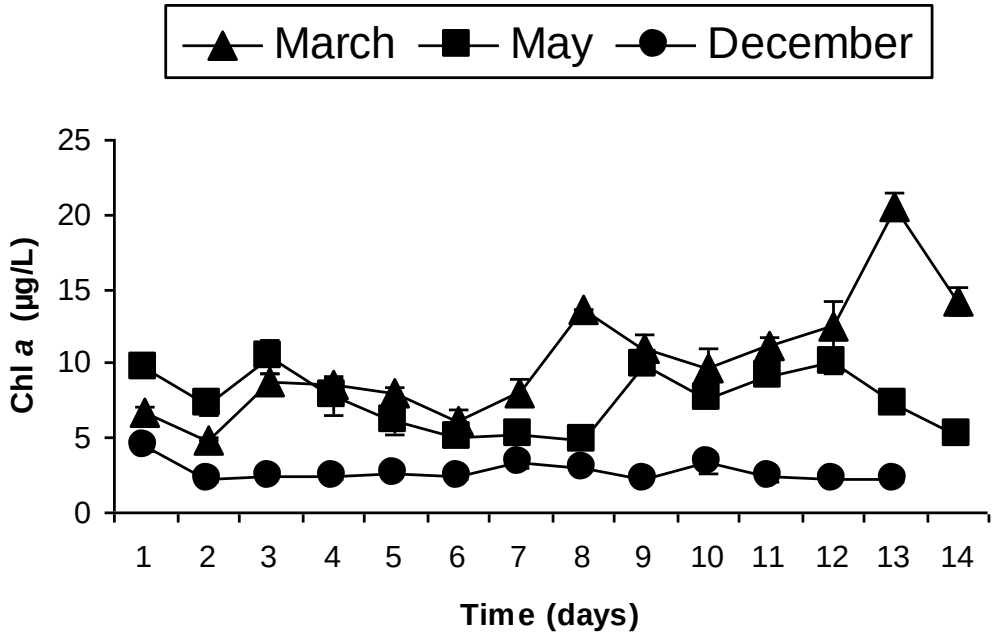

Figure 3. Total phytoplankton abundance as indicated by chlorophyll *a* at low tide for all sampling periods. Error bars are ± 1 standard error (n=14).

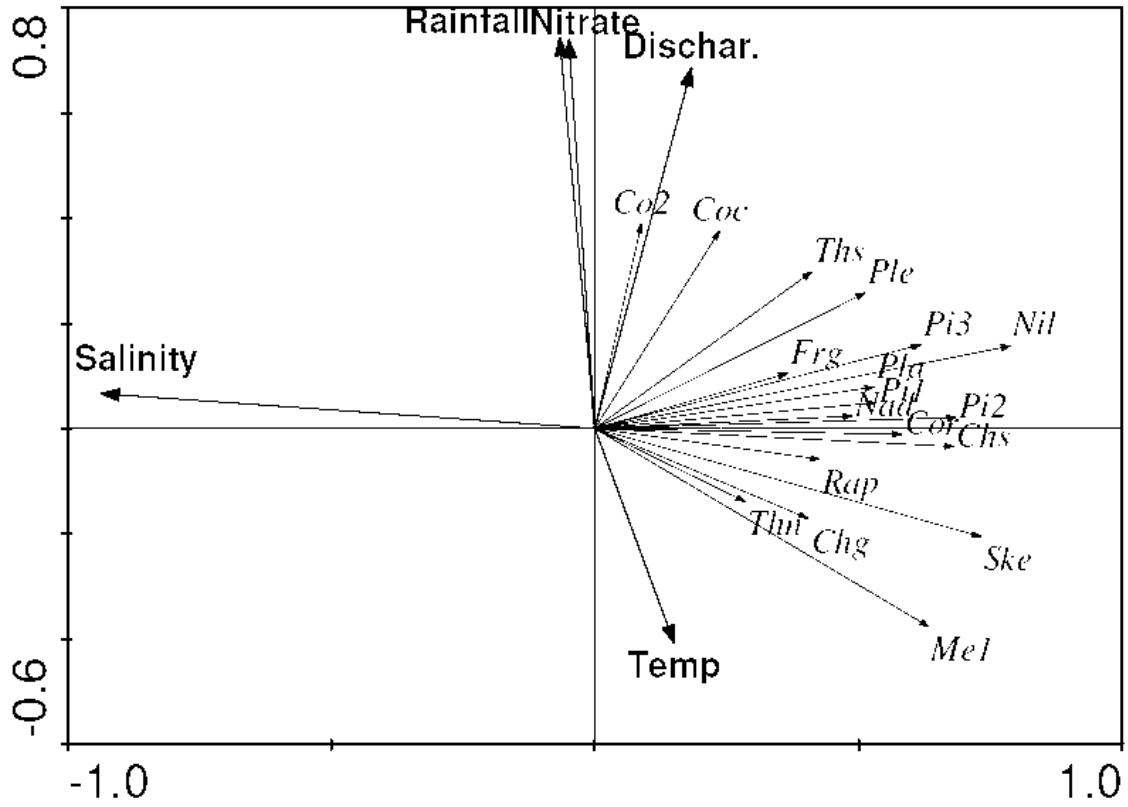

Figure 4. Biplot of species and environmental variables in Redundancy Analysis from the March 30-April 12 sampling. (Sum of all canonical eigenvalues = 0.429, p= 0.001)

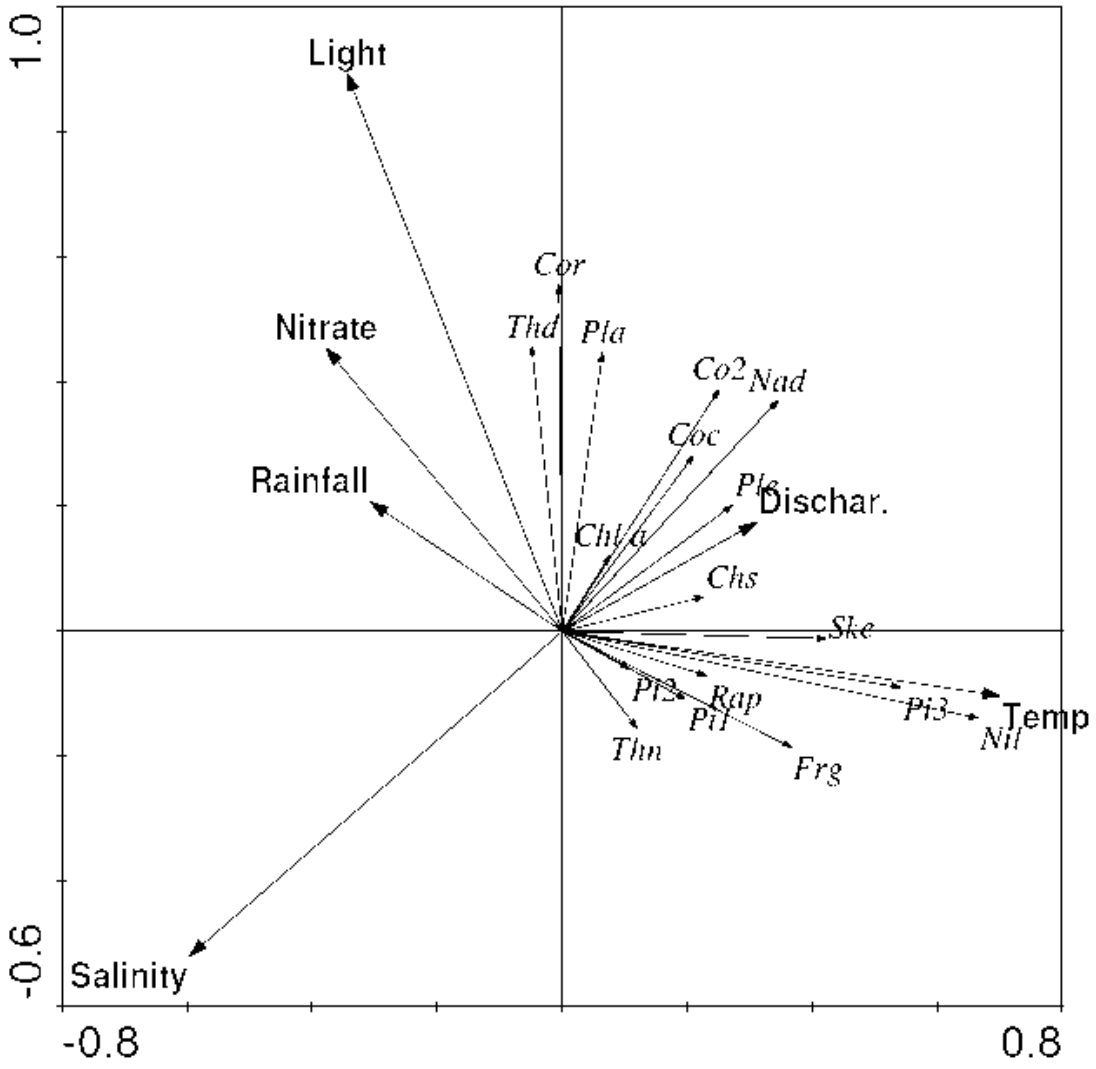

Figure 5. Biplot of species and environmental variables in Redundancy Analysis from the May 14-May 28 sampling. (Sum of all canonical eigenvalues = 0.229, p= 0.001).

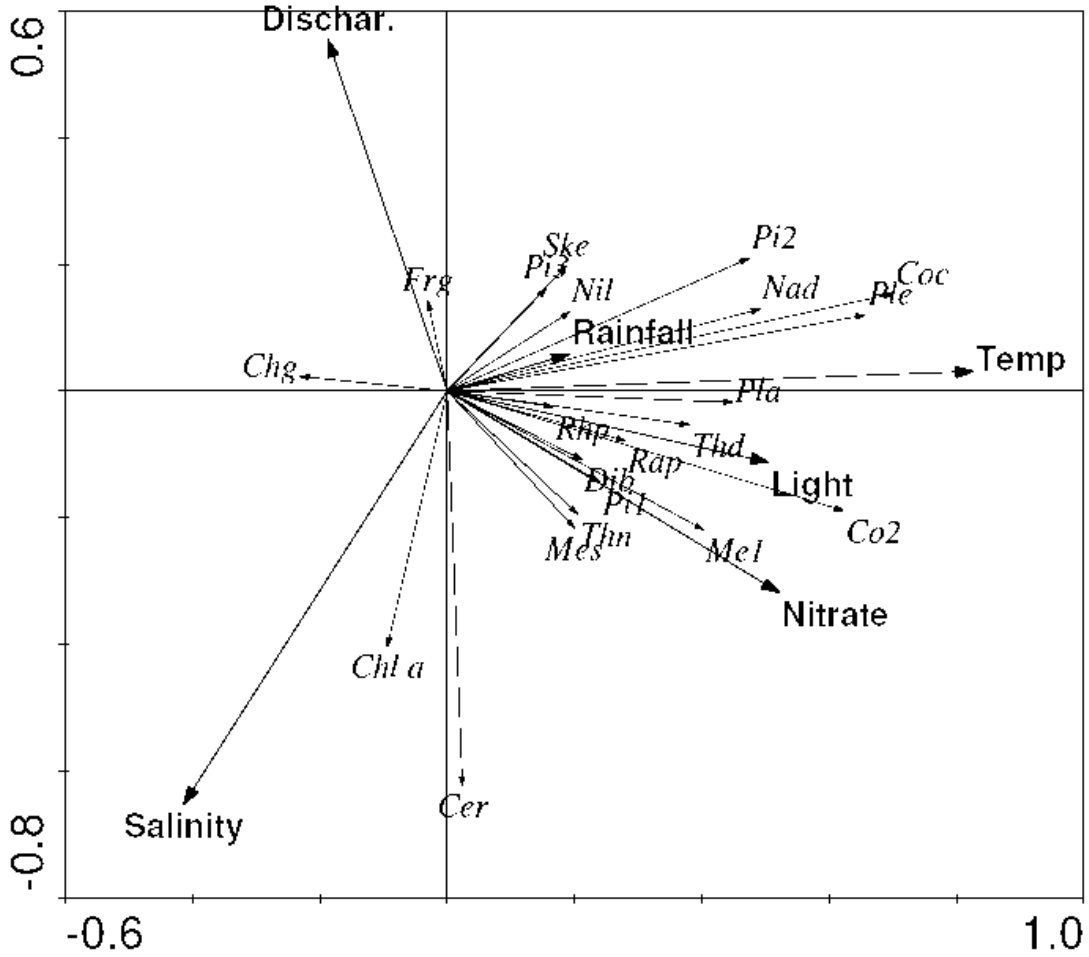

Figure 6. Biplot of species and environmental variables in Redundancy Analysis from the December 4-December 17 sampling. (Sum of all canonical eigenvalues = 0.279, p=0.001).

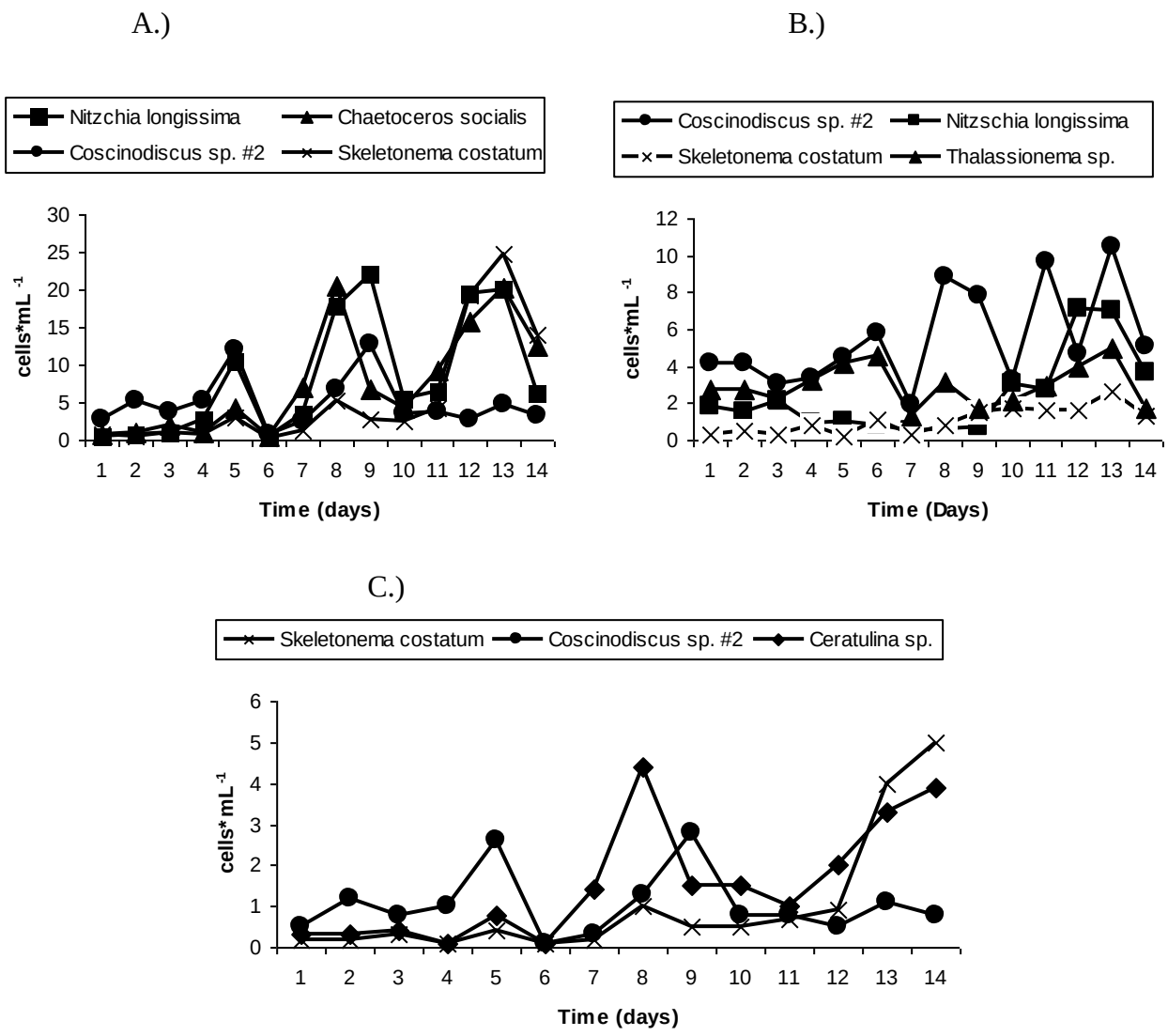

Figure 7. Transition in the abundance of dominant species in A.) March B.) May and C.) December.

CHAPTER 2

PCR AND MORPHOLOGICAL ANALYSIS OF SEASONAL DIFFERENCES IN PHYTOPLANKTON SPECIES COMPOSITION OF THE SKIDAWAY RIVER ESTUARY

Introduction

Estuarine phytoplankton exhibit seasonal variation in species composition and abundance due to bottom-up control by abiotic factors including nutrient availability, temperature, and salinity (Acuna et al. 2008, Thompson et al. 2008). Shifts in dominant phytoplankton species can affect estuarine productivity and food webs as some species may have little nutritional value to consumers (Cloern and Dufford, 2005) or be toxic (Tang et al., 2003). Also, changes in the size of phytoplankton species available are important since very small (less than 8µm) taxa are not grazed directly by most consumers and may contribute to the microbial loop, a less efficient energy pathway (Sommer et al. 2002, Cloern and Dufford, 2005). Therefore, an accurate and complete assessment of phytoplankton diversity and species make-up is essential to understanding the ecology of estuarine ecosystems.

Most studies which seek to understand phytoplankton ecology at the species level have traditionally employed visual analysis to identify and enumerate phytoplankton taxa and relate species composition to differences in abiotic conditions. However, the validity of these methods has been questioned as many species (like dinoflagellates in the *Alexandrium* genus) cannot be differentiated based on morphology alone (Anderson et al. 1999, Savin et al. 2004). The Utermöhl method, where preserved phytoplankton are settled and counted using an inverted microscope (at 100x-400x

magnification), is the most commonly employed visual protocol used to determine phytoplankton composition and abundance (Bledsoe and Phlips, 2000). This method is very useful in quantifying total density of large phytoplankton, however, many species require much greater resolution to detect subtle morphological differences as nanoplankton typically range from 2 to 20 micrometers (Hewes and Holm-Hansen, 1983, Cloern, 2005, Ellison and Burton, 2005). Identification with higher resolution electron microscopy can aid in sorting out the phenotypic differences between smaller species, but does not allow for density determination (Ellison and Burton, 2005). Most studies employing the Uterhmohl method suggest that estuaries are dominated by large diatoms (>20µm) like *Coscinodiscus, Nitzschia, Ditylum,* and, *Pleurosigma* species, and these genera are generally associated with high primary productivity and direct, efficient transfer of energy to higher trophic levels as they are selected for by higher order primary consumers like calanoid copepods (Sommer et al. 2002, Cloern and Dufford, 2005). However, techniques including chlorophyll analysis, HPLC, and flow cytometry (which are used for determining phytoplankton class and size composition) have indicated that the smaller size fraction of plankton may constitute a much larger proportion of estuarine biomass than indicated from visual counts (Remsen et al., 2004, Verity 2002b).

Recently PCR-based molecular methods have been used to describe unprecedented diversity in phytoplankton communities (Moon van der Staay et al. 2004). In these methods, phytoplankton are filtered from water samples, DNA is extracted, specific genes are amplified, and gene sequences analyzed to determine species composition (Fawley et al., 2004). Sequences are compared with each other to determine the number of similar sequences and many are identified by comparison to known

sequences in DNA databases (Fawley et al., 2004, Countway, 2005). A shift towards the use of PCR methods has risen from the idea that limitations with visual identification have underrepresented nanoplankton diversity, and most studies employing these techniques have been aimed at assessing diversity (Moon van der Staay, 2004). In contrast to visual analysis, molecular studies usually identify taxa less than 20µm in diatmeter including chlorophytes like *Nannochloropsis* and *Ostreococcus* and flagellates like *Ceratium, Protaspis,* and *Rhodomonas* (Zeidner et al. 2002). These taxa are generally associated with higher incidence of harmful algal blooms and less efficient energy transfer as they are not grazed directly by large consumers (Sommer et al. 2002, Cloern and Dufford, 2005). It is possible that the abundance of nanoplankton in molecular analyses may be due to preferential DNA extraction of smaller species or other biases in the PCR process (Potvin and Lovejoy, 2009). Very few studies have compared differences in the same phytoplankton assemblage using both PCR and morphological identification to determine how the potential biases of each may affect our view of community structure (Savin et al. 2004).

The purpose of this study was to compare PCR-based methods and visual analysis of phytoplankton species composition and diversity in the Skidaway River Estuary (SKE) during the spring and winter of 2009. I hypothesized that visual analysis would show a diatom-dominated community consisting mainly of *Coscinodiscus, Skeletonema, Nizschia,* and *Pinnularia* species while molecular analysis would yield a higher proportion of chlorophytes including *Nannochloropsis* and *Ostreococcus* species, and dinoflagellates like *Gonyaulux* and *Ceratium* species. I also expected overall species diversity to be higher using molecular methods.

Methods

Sampling

The Skidaway River Estuary (SKE, 32^{o} 37' 05.64'' N, 81^{o} 52' 43.71'' W, Figure 1) is well-mixed, tidally-dominated and receives low freshwater input from the Savannah and Ogeechee rivers except following major rain events (Verity 2002). To compare the use of PCR analysis and visual techniques in characterizing seasonal differences in phytoplankton species composition, 5 replicate 500 mL surface water samples were collected at both high and low tide on April 4th, 12th, December 5th, and 17th, 2009 in the Skidaway River Estuary. Phytoplankton species composition and biomass were evaluated using visual analysis, PCR methods, and chlorophyll *a* quantification. Measurements of dissolved oxygen, salinity, temperature, and pH were also taken to characterize environmental conditions during each season with temperature and nutrients exhibiting significant differences between seasons (Table 6). Visual samples used for comparison in this study were part of a larger study on phytoplankton ecology in the Skidaway River examining short-term variation in species compsotion and dates were chosen as days of high (April 12th and December 17th) and low (April 4th and December 5th) biomass (chlorophyll *a*) within respective seasons.

Chlorophyll *a* Concentration

Total phytoplankton biomass was measured using chlorophyll *a* concentrations (Lehman, 2000, Verity 2002). Phytoplankton were concentrated from a 100 mL volume onto a Whatman GF/F glass fiber filter. Following extraction of chlorophyll from cells

on each filter in 90% acetone at -20^{o}C in the dark for 24 hr., chlorophyll *a* was measured using a Turner Designs-700 flurometer (EPA Method 404, Arar and Collins, 1997).

Visual Identification of Phytoplankton

Samples for visual analysis of phytoplankton species composition were preserved by adding approximately 0.5 mL of Lugol's solution to 50mL water samples. Each sample was concentrated using centrifugation and resuspended in 1mL. Phytoplankton identification and counts were performed using a Sedgewick-Rafter counting chamber and an inverted microscope at 200x magnification (Bledsoe and Phlips, 2000, Cloern and Dufford, 2005). Counts for each sample were completed when 100 individuals of the three most numerous taxa had been tallied and only species that could be identified at least to the genus level were included in further analysis. (Cloern and Dufford, 2005).

PCR Analysis

100 mL of each water sample was filtered onto a 25mm Whatman GF/F glass fiber filter for DNA analysis. A small portion was removed from the center of each filter for DNA extraction using standard techniques (Qiagen kit). Silica beads were used to rupture cells in the extraction process to maximized lysis of cell walls (Savin et al. 2004). An approximately 1500 base pair portion of the 18S rDNA gene was amplified by PCR using universally conserved primers EukR and 528f (Viprey and Guillou, 2008). This gene was chosen to eliminate the possibility of recovering bacterial sequences and it is the most commonly used gene in phytoplankton barcoding studies maximizing the possibility for identifying sequences. Next, PCR products were TA-cloned into a plasmid vector (TOPO pCR 4), transformed into competent *E. coli* cells, and plated on agar plates (Invitrogen). Ninety six random clones (1 plate) were isolated for sequencing from each

date. Each clone was sequenced using an Applied Biosystems 3500 Analyzer and manually edited in Sequencher to yield a 300-700 base pair fragment and aligned with each other to determine the number of identical sequences. Greater than a 98% similarity was used to identify unique taxonomic units and assign them to known species in Genbank. A distance-based phylogenetic tree was constructed for each season using the PAUP 8.0 program for the purpose of clustering unknown sequences with known ones.

Data Analysis

Species richness and diversity were compared for each method and between seasons using Shannon's index for both visual and molecular data. Shannon's index ranges from about 1 to 3.5 with lower values indicating less diversity. Differences in the presence/absences of taxa were also examined using Sorensen's similarity index. This index ranges from 0 to 1 with 1 representing communities that are identical in terms of the species present. Total phytoplankton biomass and the abundance of dominant taxa (five most common species) from visual analysis were tested for normality using the Shapiro-Wilkes test and homogeneity of variance using Levenes' tests. This data was log transformed to meet normality assumptions and compared between seasons using T-tests in JMP 8. Data from both dates within each season were pooled prior to analyses as taxonomic composition was very similar (Sorensen's index >0.90) within seasons.

Results

Spring

Visual analysis of species composition in spring revealed 22 different diatoms, 4 chlorophytes, and 1 dinoflagellate species. Large, centric diatoms *Chaetoceros socialis*, *Melosira sp.*, *Skeletonema costatum*, and *Nitzschia longissima* were the most abundant

taxa (Table 7). Diversity was high during the spring sampling yielding a Shannon diversity value of 2.87. Molecular analysis in spring yielded a very different view of the community. We observed 8 different species of which 4 were dinoflagellates and 4 were chlorophytes (Figure 8). The DNA library was dominated by dinoflagellates, mainly *Gymonodinium sanguineum* (Table 8). *Gymonodinium sanguineum* was observed 62 times and made up over half of the observed sequences. Due to the abundance of this one species, sequence diversity was low over this period yielding a Shannon value of 1.01. Three of the dinoflagellate species were unknown but shared high sequence similarity (>95%) and grouped closely with *Gymnodinium sanguineum* in phylogenetic analysis (Figure 8).

Species composition in winter based on visual analysis was also dominated by centric diatoms including *Chaetoceros socialis, Melosira sp., Skeletonema costatum,* and *Nitzschia longissima* and diversity was high yielding a Shannon index of 2.83. I observed 23 diatoms, two chlorophytes, and two dinoflagellates (Table 7). Molecular characterization of species make-up in winter showed that the community was comprised of 6 diatoms, 7 dinoflagellates, 2 chlorophytes, 7 cryptophytes, and 1 Prasinophyte. Sequence diversity was also high with a Shannon index of 2.85. Diatoms including *Minutocellus polymorphus, Guinardia delicatula, Coscinodiscus granii,* and a *Nitzschia* species appeared in the DNA library. Although present, *Gymnodinium sanguineum* was much less common in winter. There were also many sequences recovered during winter that did not match any known species in the Genbank database with greater than 98% similarity, but were assigned to class based on cluster analysis (Figure 11).

Species composition in both seasons appeared to be very similar based on visual observation and the similarity index between seasons was 0.78. In contrast, there was low similarity (Sorensen's index = 0.12) between seasons based on molecular data and *Gymondinium* and *Ostreococcus* species were the only taxa shared between seasons (Table 8). If we combine taxa observed using both methods there is also low similarity between seasons with a Sorensen's index of 0.59. Another key difference between spring and winter based on visual analysis was that abundance of the most common taxa was 4 times higher in spring (Figure 9, T-test, $p<0.05$) and this trend was also evident based on chlorophyll *a* analysis (Figure 10, T-test, $p<0.001$).

Discussion

As predicted, visual identification showed a phytoplankton community dominated by the centric diatoms *Chaetoceros socialis, Melosira sp., Skeletonema costatum, Coscinodiscus sp., and Nitzschia longissima* during both spring and winter and overall species diversity was high. Abundance of these taxa has been documented for other temperature estuaries and they are generally associated with high rates of primary productivity and direct, efficient energy transfer to upper level consumers (Cloern, 1987, Bledsoe and Phlips, 2000). Although some morphological studies have shown higher diversity of dinoflagellates like *Gymonidinium* and *Gonyaulux* species and cyanobacteria like *Anabaena* species in spring, I was able to detect little difference between seasons (Ramus et al. 2003). The main difference observed visually was that there was a much greater density of all species in spring compared to winter consistent with a spring bloom (Cloern and Dufford, 2005).

In contrast, PCR-based analysis revealed a community dominated by smaller species and there were large differences in taxonomic composition between seasons. In spring, no diatoms were observed and a very large proportion of the sequences were identified as the dinoflagellate, *Gymnodinium sanguineum*. This species is common in coastal waters and is associated with harmful algal blooms worldwide (Horner et al. 1997, Smayda, 1997, Smayda 2002). The abundance of *G. sanguineum* resulted in much lower species diversity compared to visual characterization. Other species identified from DNA sequences in spring included the dinoflagellate, *Protaspis oblique* and chlorophyte species in the *Tetraselmis, Nannochloropsis,* and *Ostreococcus* genuses. These species have been commonly documented as members of the nanoplankton community in open ocean systems but have been documented less frequently in estuaries (Zeidner et al. 2002, Buric et al. 2007).

Sequence analysis in winter yielded a much higher diversity than sequence analysis in spring and included species of diatoms, chlorophytes, dinoflagellates, and cryptophytes (including a few genera that were detected visually). Diversity during this period was similar and high based on both the morphological and molecular methods, and it is important to point out that only 55 sequences were included in the molecular data compared to thousands of cells counted visually. Greater molecular diversity in winter contrasts with visual studies in estuaries that suggest the community is dominated by a few, large diatom species during this season, but may help explain higher recovery of diatom sequences as they are expected to comprise a larger proportion of the community (Ramus et al 2003, Tilman et al. 1986).

Discrepancies between morphological and PCR analysis may be attributable to biases or inherent limitations of each technique. When using light microscopy, detecting differences or even the presence of very small cells (<20µm) like *Nannochloropsis* and *Ostreococcus* species can be difficult, especially in turbid, estuarine samples which include lots of detritus. However, visual methods allow efficient and accurate determination of the density of larger species (Savin et al. 2004). In contrast, molecular studies have shown a clear bias towards smaller cells and these species tend to be recovered more commonly in sequence libraries, even in artificial communities where species composition is manipulated to include an equal number of small and large-celled species (Savin et al. 2004, Potvin and Lovejoy). Diatoms in particular are recovered in low frequency and it is possible that lysing the cell walls of bigger species (especially those with silica cell walls) may be difficult. This leads to lower efficient of DNA extraction from these individuals even though they are expected to contain more genomic DNA (Savin et al. 2004, Cavilier-Smith, 2005). Several studies have documented that diatom species are often only recovered when they comprise a very large proportion of the population (Lovejoy et al. 2007). Another possible explanation for preferential recovery of small species is that some classes of phytoplankton (dinoflagellates in particular) may contain multiple gene copies which can skew their contribution to the PCR product (Farrely et al. 1995). There are many other steps in the PCR process that may be susceptible to PCR bias (See appendix B for a full discussion) and because of this, extracting densities or species abundance from sequence data is difficult. However, it is clear that to attain a complete view of phytoplankton species make-up, both

morphological and molecular identification are needed. Identifying species in both large and small size classes is important as individuals from both groups may form harmful algal blooms. Also, many large (*Coscinodiscus* sp.) and small species (*Nannochloropsis* and *Ostreococcus* sp.) are not consumed by selective grazers like calanoid copepods and route most of their production through the microbial loop, a less efficient energy pathway (Sommer et al. 2002, Frost, 1972). Therefore, a complete characterization of species composition is needed to understand estuarine health and function.

Table 6
Mean dissolved oxygen, pH, salinity, temperature, and nitrate between seasons. Means include ± 1 SEM and significant differences between seasons are indicated with * (T-test, $p<0.05$)

Season	**DO (mg/L)**	**pH**	**Sal. (ppt)**	**Temp. (^{o}C)**	**Nitrate (µM)**
Spring	7.17±0.08	7.78±0.009	21.62±0.37	*19.47±0.12	*1.43±0.15
Winter	7.61±0.06	7.62±0.03	20.03±0.58	*14.36±0.18	*3.21±0.11

Table 7
Phytoplankton species observed during both spring and winter using visual analysis.

Season	Species	Density (cells mL^{-1})	Taxonomic group
Spring	Asterionella sp.	1.4	Diatom
	Chaetoceros gracile	1	Diatom
	Chaetoceros socialis	7.7	Diatom
	Chaetoceros sp. #3	1.2	Diatom
	Closterium gracile	0.8	Chlorophyte
	Cocconeis sp.	0.09	Diatom
	Coscinodiscus radiatus	1.2	Diatom
	Coscinodiscus sp. #2	1.7	Diatom
	Gonyaulux verior	0.8	Dinoflagellate
	Mamiella sp.	0.4	Chlorophyte
	Melosira sp. #1	7.5	Diatom
	Melosira sp. #2	1.3	Diatom
	Melosira sulcata	4.3	Diatom
	Microactinum sp.	1.5	Chlorophyte
	Navicula didyma	0.5	Diatom
	Nitzschia longissima	3.3	Diatom
	Pinnularia sp. #1	1.4	Diatom
	Pinnularia sp. #2	1.8	Diatom
	Pinnularia sp. #3	1.7	Diatom
	Pleurosigma angulatum	0.4	Diatom
	Pleurosigma elongatum	0.6	Diatom
	Raphoneis sp.	1.5	Diatom
	Rhizoselenia pungens	0.4	Diatom
	Scenedesmus ellipticus	3.1	Chlorophyte
	Skeletonema costatum	7.9	Diatom
	Thalassionema sp.	1.0	Diatom
	Thalassiosira decipiens	2.1	Diatom
Winter	Biddulphia longicuris	0.2	Diatom
	Ceratium sp.	0.1	Dinoflagellate
	Ceratulina sp.	0.5	Diatom
	Chaetoceros gracile	0.0	Diatom
	Chaetoceros socialis	0.2	Diatom
	Chaetoceros sp.	0.1	Diatom
	Cocconeis sp.	0.7	Diatom
	Coscinodiscus sp.	1.9	Diatom

Ditylum brightwellii	0.2	Diatom
Fragillaria sp.	0.1	Diatom
Gonyaulux verior	0.1	Dinoflagellate
Mamiella sp.	0.1	Chlorophyte
Melosira sp.	1.3	Diatom
Melosira sulcata	0.3	Diatom
Microactinum sp.	0.1	Chlorophyte
Navicula didyma	0.4	Diatom
Nitzschia longissima	0.2	Diatom
Pinnularia sp. #1	0.1	Diatom
Pinnularia sp. #2	0.5	Diatom
Pinnularia sp. #3	0.3	Diatom
Pleurosigma angulatum	0.3	Diatom
Pleurosigma elongatum	0.8	Diatom
Raphoneis sp.	0.2	Diatom
Rhizoselenia pungens	0.2	Diatom
Skeletonema costatum	1.3	Diatom
Thalassionema sp.	0.4	Diatom
Thalassiosira decipiens	0.3	Diatom

Table 8
Phytoplankton species observed during both spring and winter using molecular analysis. UK denotes a taxa that did not match any known sequence in the GenBank database but was assigned to class based on similarity to known sequences and phylogenetic analysis.

Season	**Species**	**# Observed**	**Taxonomic group**
Spring	Gymnodinium sanguineum	62	Dinoflagellate
	UKdino	1	Dinoflagellate
	UK1	1	Dinoflagellate
	UK5	1	Dinoflagellate
	Tetraselmis sp.	1	Chlorophyte
	Nannochloris sp.	9	Chlorophyte
	Ostreococcus tauri	2	Chlorophyte
	UK4	1	Chlorophyte
Winter	UKstram2	3	Diatom
	Rhodomonas sp.	2	Cryptophyte
	Ostreococcus sp.	6	Chlorophyte
	Minutocellus polymorphus	4	Diatom
	Guinardia delicatula	5	Diatom
	Gymnodium sanguineum	8	Dinoflagellate
	UK1	5	Dinoflagellate
	UK2	4	Cryptophyte
	Dinoflagellate #1	2	Dinoflagellate
	UKdin2	2	Dinoflagellate
	UKstram1	2	Diatom
	UKdin1	1	Dinoflagellate
	Coscinodiscus granii	1	Diatom
	Pedinella sp.	1	Dinoflagellate
	Pseudopirsonia mucosa	1	Cryptophyte
	Nitzschia sp.	1	Diatom
	Chrysochromulina sp.	1	Prasinophyte
	Mymecia sp.	1	Chlorophyte
	Protoperidinium sp.	1	Dinoflagellate
	Hemiselmis sp.	1	Cryptophyte
	UK4	1	Cryptophyte
	UKc1	1	Cryptophyte
	UKc2	1	Cryptophyte

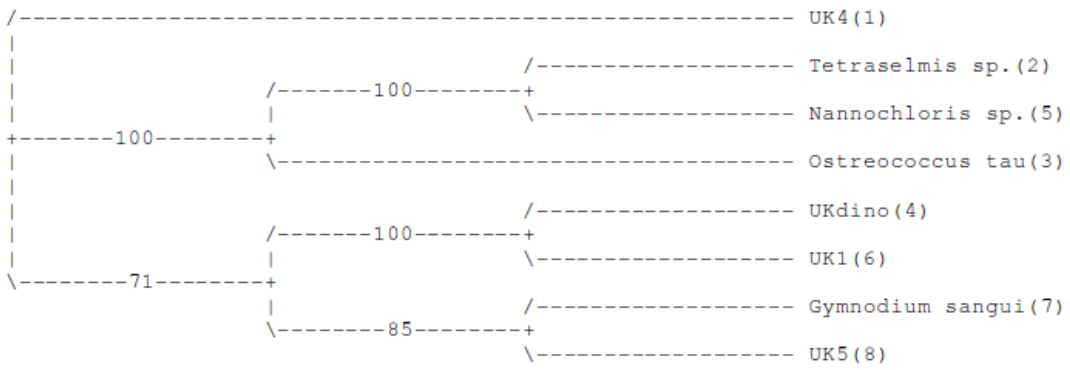

Figure 8. Phylogenetic tree of phytoplankton species observed during spring sampling. Tree is based on distance and was created in PAUP 8.0. UK denotes a sequence which did not match any known sequence in the GenBank database.

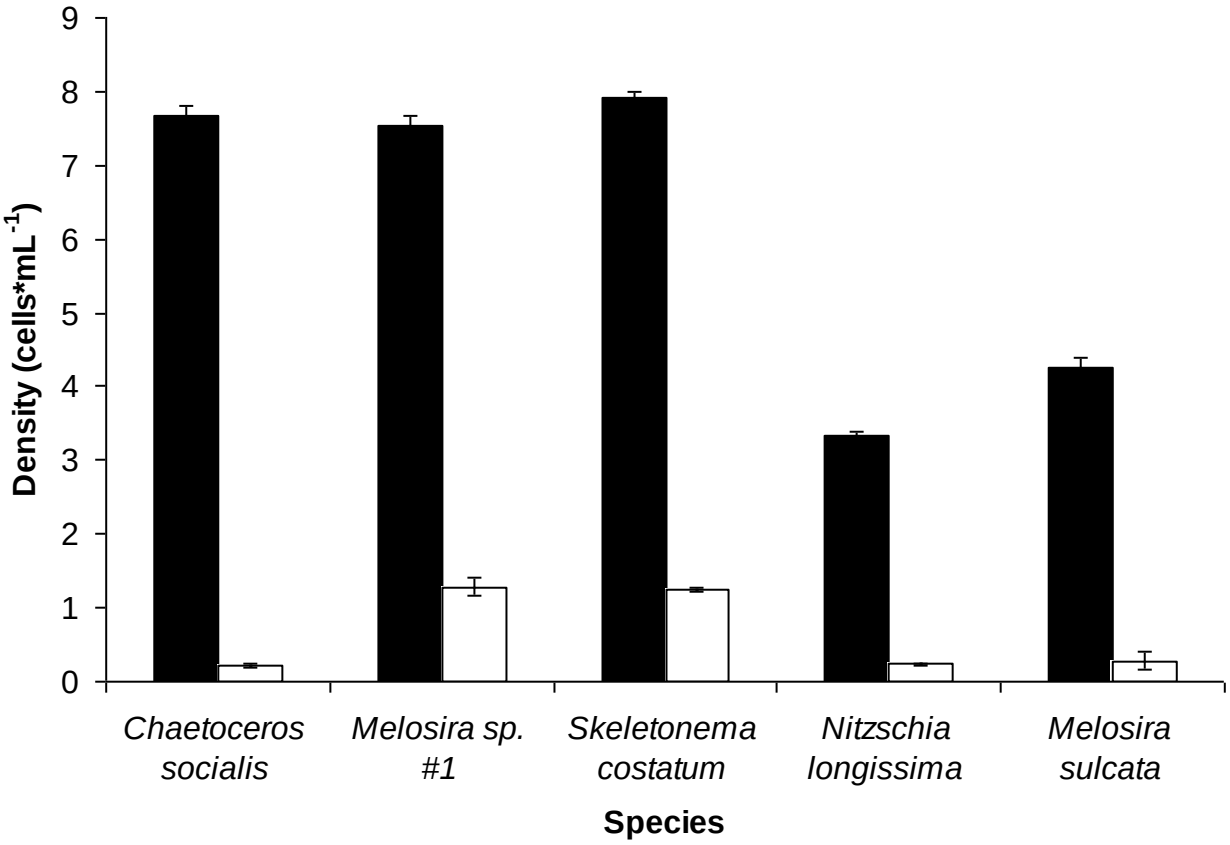

Figure 9. Dominant species based on visual analysis for spring (■) and winter (□). Error bars are +/- 1 SEM (n=10). All shaded and open bars for each species are significantly different from one another (T-test, $p<0.001$).

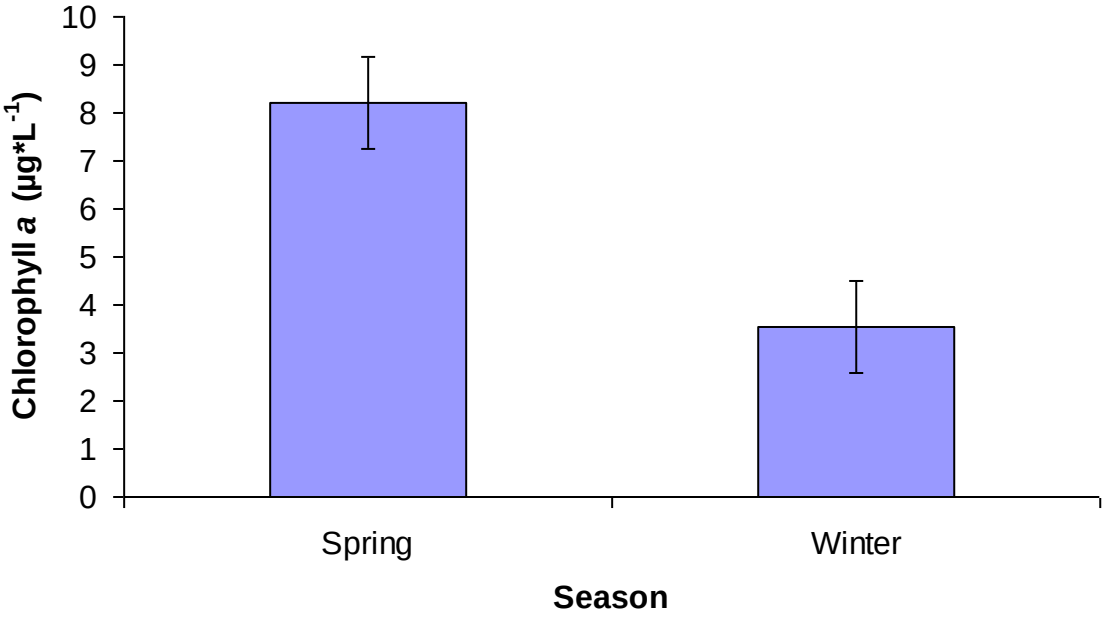

Figure 10. Total phytoplankton biomass in spring and winter based on chlorophyll *a* analysis. Error bars are ±1SEM (n=10). Bars are significantly different (T-test, $p<0.001$).

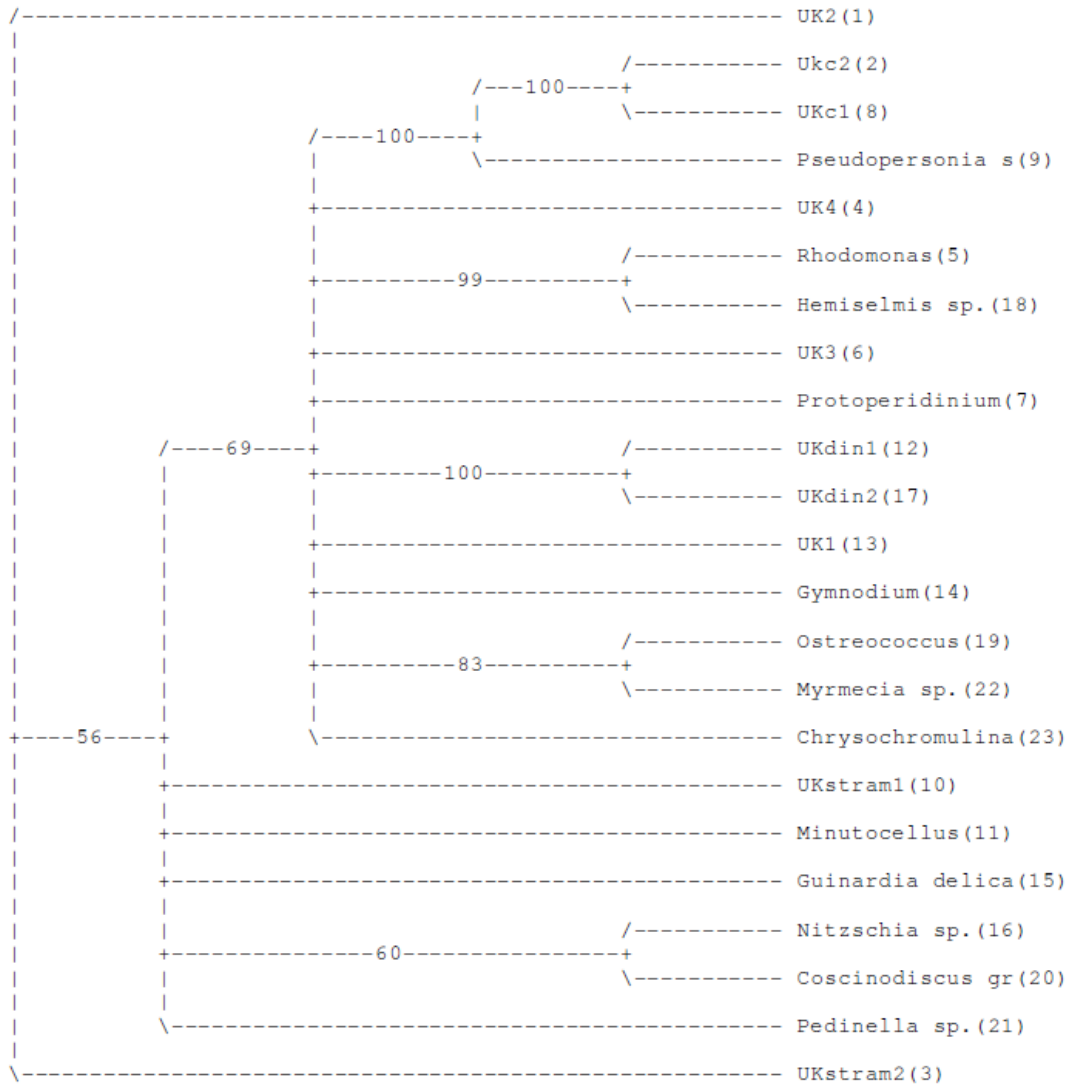

Figure 11. Phylogenetic tree of phytoplankton species observed during winter sampling.

CHAPTER 3

NUTRIENT ADDITIONS INFLUENCE PHYTOPLANKTON SPECIES COMPOSITION IN THE SKIDAWAY RIVER ESTUARY

Introduction

Nutrients, in particular nitrogen and phosphorus, have been identified as a limiting factor of phytoplankton abundance in many estuarine ecosystems (Pederson and Borum, 1996, Piehler et al. 2004, Domingues et al. 2011). While nitrogen is considered the primary limiting nutrient in most temperate estuaries, there is some evidence of seasonal shifts between nitrogen and phosphorus limitation (Domingues et al. 2005, Fisher et al. 2006). For example, phosphorus may become limiting during periods of high biomass production including spring blooms (Pedersen and Borum, 1996, Fisher et al. 2006). Blooms that result from higher nutrient availability can often stimulate productivity, however, if the species that comprise these blooms are toxic or exhibit low primary productivity, they may be harmful to grazers and the system as a whole.

Increases in nitrogen and phosphorus have been associated with the growth of specific taxonomic classes or individual species of phytoplankton (Ramus et al., 2003, Piehler et al. 2004, Cloern and Dufford, 2005, Domingues et al. 2011). Pulses of nutrients can increase the abundance of dinoflagellate species like *Kryptoperidinium foliaceum* and *Gymnodium* species which are generally less productive, toxic to consumers, and have a greater potential to form harmful algal blooms (Ramus et al. 2003, Tang et al. 2003, Springer et al. 2005, Domingues et al. 2011). However, higher nutrient

concentrations may also increase the abundance of benign dinoflagellates including *Ceratium* and *Protaspis* species and large productive diatoms like *Coscinodiscus* and *Nitzschia* species (Cloern and Dufford, 2005, Pedersen and Borum, 1996). Since not all species within broad taxonomic classes are necessarily bad for the system and each species is associated with its own benefits, characterizing changes in species composition due to nutrient enrichment is important for understanding changes in primary productivity and higher trophic levels.

Determining changes in the taxonomic make-up of estuarine phytoplankton attributable to nutrient concentration alone is difficult to measure in the field as there is concurrent variability in other abiotic conditions (this variability is particularly high in estuaries). Higher freshwater discharge coupled with increased nutrient concentrations have been shown to promote the growth of many large, productive diatoms including *Skeletonema costatum, Melosira sp.,* and *Coscinodiscus sp.*, however, these changes also coincide with decreasing salinity and higher turbidity in estuaries (Cloern and Dufford, 2005, Domingues et al. 2005). A microcosm approach where only nutrient concentrations are manipulated is valuable for evaluating nutrient effects on species composition because the influence of other environmental variables is eliminated (Bishop et al. 1984, Piehler et al. 2004). However, extrapolating results of microcosm or mesocosm experiments to the natural environment may not be realistic: enclosing phytoplankton in small volumes reduces or removes natural processes including mixing and advection, grazing, and nutrient uptake (Dugdale and Wilkerson, 1989, Oviatt et al. 1989, Kudela and Dugdale, 2000). Despite limitations, experiments still provide valuable

insights into the relationship between nutrient availability and the abundance of individual phytoplankton species (Piehler et al. 2004, Domingues et al. 2011).

Long term field study the Skidaway River Estuary (SKE) showed a significant correlation between phytoplankton abundance, size class composition, and nutrients both seasonally and over a ten year period between 1986 and 1996. Overall biomass and the abundance of small phytoplankton classes (<8µM) were highest in late spring/summer and were strongly correlated with temperature and nutrient concentration. There was also a long-term trend of higher biomass and greater abundance of smaller size class phytoplankton each year that appeared to be associated with increasing nutrient concentration over the study period (Verity, 2002b). While the small size fraction of phytoplankton can sometimes be associated with high productivity and doubling time, these species are not efficient at transferring energy to higher trophic levels as they are only grazed by ciliates and other small heterotrophs (Sommer et al. 2002). Nutrient concentrations are expected to increase with higher development in coastal areas, and we know little about the potential impact of increased nutrient availability on individual phytoplankton species. The purpose of our study was to investigate potential influence of inorganic nitrogen and phosphorus additions on phytoplankton species composition in the Skidaway River estuary using a controlled, microcosm experiment. We hypothesized that the addition of nitrate and phosphate decreases the proportion of diatom species like *Skeletonema costatum* and *Coscinodiscus radiatus* while promoting the diversity of dinoflagellates, chlorophytes, and cyanobacterial species, like *Gymnodinium*, *Gonyaulux*, *Nannochloropsis*, and *Anabaena*. Because the Skidaway River is thought to be nitrogen

limited, we expected increased nitrate concentration to have a stronger impact on species abundance and composition than phosphate concentration.

Methods

The Skidaway River Estuary (SKE, 32^{o} 37' 05.64'' N, 81^{o} 52' 43.71'' W) is a well-mixed, tidally dominated estuary to which freshwater input from the Savannah and Ogeechee rivers is low except following major rain events (Verity 2002). To examine estuarine phytoplankton community response to increased nutrients under manipulated conditions, phytoplankton from the SKE were exposed to either nutrient enrichment or control treatments. Translucent, plastic, 10L containers were filled with surface water from the SKE on May 23, 2009 and incubated for 96 hours in flow through tanks utilizing water from the estuary to maintain ambient temperature. One layer of neutral density fiberglass window screening was used to reduce ambient irradiance by 50%, thus providing saturating light intensity but preventing photoinhibition (Piehler et al. 2004). Treatments were created by adding sodium nitrate to a final concentration 15 µM nitrate and potassium phosphate to a final concentration of 5µM phosphate in a fully-crossed factorial design with 5-fold replication for a total of 20 microcosms which were numbered and randomly assigned to treatments. This created the following addition treatments: A.) Control, No addition B) Nitrate only C)Phosphate only and D) Nitrate and Phosphate. The selected nutrient concentrations were ecologically relevant based on historical highs in the Skidaway River Estuary (Verity, 2002a). Initial nutrient concentrations and biomass are reported in Table 9. There was higher biomass in the control compared to the other treatments at the start of the experiment (Oneway ANOVA, df=3, F=3.8, p=0.031). The experiment lasted for 4 days as phytoplankton have the

potential to respond 2-3 days following nutrient inputs (Kudela and Dugdale, 2000, Piehler et al. 2004, Carter et al. 2005). Sampling occurred at 0, 48, and 96 hours post-addition.

Nutrient depletion over the course of the experiment was measured from 125 ml water samples from each replicate which were filtered through 48mm Whatman GF/F glass fiber filters, and frozen until analysis. Dissolved oxygen, pH, salinity, and conductivity were also measured at each time point to make sure there were no differences between treatments. Lastly, samples for total biomass and analysis of phytoplankton species composition were taken. Species identification and enumeration were carried out visually for all replicates at all time points and molecular analysis was conducted for one replicate of each treatment at the midpoint of the experiment where the largest visual difference in community composition was observed. Both visual analysis of phytoplankton species composition and molecular identification methods were used because visual methods may underestimate the diversity and abundance of small phytoplankton species and molecular methods may underestimate the presence of large species (e.g. chlorophytes and dinoflagellates) in the community (Ellison and Burton, 2000).

Sample Analysis

Nutrient Concentrations:

To analyze nitrate and phosphate concentrations, 125 mL surface water samples were vacuum filtered through 47mm Whatman GF/F glass fiber filters. The water samples were frozen and analyzed at JBL Analytical Laboratory at the University of Georgia in Athens, GA. Briefly, analysis of nitrate involved the reduction of nitrate to

nitrite and treatment with acid to produce a colored soluaiton (U.S. EPA Method 353.2). For phosphate, ammonium molybdate and antimony potassium tartrate reacted with phosphoru to form an acid complex that was reduced to an intensely blue-colored complex by ascorbic acid (U.S. EPA Method 365.5). The amount of color was then measured using an autoanalyzer (LACHAT) and related to concentration. Limits of detection were >1µM for nitrate and >0.1µM for phosphate.

Chlorophyll *a* Concentration

Total phytoplankton biomass was measured using chlorophyll *a* concentrations (Lehman, 2000, Verity 2002). Phytoplankton were concentrated from a 100 mL volume onto a Whatman GF/F glass fiber filter. Following extraction of chlorophyll from cells on each filter in 90% acetone at -20^{o}C in the dark for 24 hr., chlorophyll *a* was measured using a Turner Designs-700 flurometer (EPA Method 404, Arar and Collins, 1997).

Visual Identification of Phytoplankton

Samples for visual analysis of phytoplankton species composition were preserved by adding approximately 0.5 mL of Lugol's solution to 50mL water samples. Each sample was concentrated using centrifugation and resuspended in 1mL. Phytoplankton identification and counts were performed using a Sedgewick-Rafter counting chamber and an inverted microscope at 200x magnification (Bledsoe and Phlips, 2000, Cloern and Dufford, 2005). Counts for each sample were completed when 100 individuals of the three most numerous taxa had been tallied and only species that could be identified at least to the genus level were included in further analysis. (Cloern and Dufford, 2005).

PCR Analysis

100 mL of each water sample was filtered onto a 25mm Whatman GF/F glass fiber filter for DNA analysis. A small portion was removed from the center of each filter for DNA extraction using standard techniques (Qiagen kit). Silica beads were used to rupture cells in the extraction process to maximized lysis of cell walls (Savin et al. 2004). An approximately 1500 base pair portion of the 18S rDNA gene was amplified by PCR using universally conserved primers EukR and 528f (Viprey and Guillou, 2008). This gene was chosen to eliminate the possibility of recovering bacterial sequences and it is the most commonly used gene in phytoplankton barcoding studies maximizing the possibility for identifying sequences. Next, PCR products were TA-cloned into a plasmid vector (TOPO pCR 4), transformed into competent *E. coli* cells, and plated on agar plates (Invitrogen). Ninety six random clones (1 plate) were isolated for sequencing from each date. Each clone was sequenced using an Applied Biosystems 3500 Analyzer and manually edited in Sequencher to yield a 300-700 base pair fragment and aligned with each other to determine the number of identical sequences. Greater than a 98% similarity was used to identify unique taxonomic units and assign them to known species in Genbank.

Data Analysis

Data was tested for normality using Shapiro-Wilkes W test and equality of variances using Levene's test. Differences in nutrient concentrations and total phytoplankton biomass between treatments at all time points were log transformed and analyzed using ANOVA and Tukey-HSD post-hoc comparisons in JMP 8. Redundancy analysis was performed using CANOCO 4.5 to determine significant relationships between overall species composition determined by visual analysis and treatments at all

time points. Data included in redundancy analysis were log +1 transformed prior to analysis and only species observed in greater than 50% of all samples were included (Suikkanen et al. 2007). Densities of individual species that exhibited strong relationships with treatments in the redundancy analysis were further compared using ANOVA. Species diversity was compared between treatments using Shannon's diversity index for both visual and molecular data at 48 hours after initiation of treatment.

Results

By 48 hours after initiation of incubation all nitrate had been consumed in the treatments without added N. Nitrate concentration was significantly higher in the N than in the NP addition (Oneway ANOVA, Tukey HSD, df=3, $F=20.8$, $p<0.0001$). At 96 hours nitrate was reduced below detection limits indicating rapid utilization (Figure 12A). Phosphate concentration exhibited a similar pattern with treatment. At 48 and 96 hours, phosphate concentration was highest in the P treatment and significantly higher in the NP treatment than in the control and N treatment (Oneway ANOVA, Tukey HSD, df=3, $F=39.8$, $p<0.0001$, Figure 12B).

By 48 hours after start of incubation significant differences in total phytoplankton biomass emerged. Biomass was highest in the NP treatment and significantly higher in the N treatment than in the control or P treatment (Oneway ANOVA, Tukey HSD, df=3, $F=38.7$, $p<0.0001$). At 96 hours, the biomass was similar in the N and NP treatments but both of these treatments were significantly different from the control and P only treatment (Oneway ANOVA, Tukey HSD, df=3, $F=27.4$, $p<0.001$, Figure 12).

Redundancy analysis of visual data revealed that treatment had a significant effect on species composition only at 48 hours after start of incubation, therefore, species

comparisons were focused on this time point. At 48 hours, treatment explained 27% of the variation in overall species composition (Redundancy analysis, sum of all canonical eigenvalues = 0.272, F=1.7, p=0.049, Figure 13). Twenty-six different species were observed across all treatments during this period. Diatoms were the dominant taxa, and only 2 dinoflagellates (*Ceratium* and *Gonyaulux* species) and 2 chlorophytes (*Staurastrum* and *Microactinum* species) were observed (Table 10). Diversity was highest in the N only treatment and was very similar across the other treatments and control (H = 1.91-2.06, Table 10). Some diatom species in the redundancy analysis including *Nitzschia longissima*, a *Thalassionema* species, and a *Pinnularia* species were most abundant in the N only treatment (Table 10, Figure 13, Oneway ANOVA, Tukey HSD, df=3, $p<0.05$). In contrast, the two *Melosira* species observed, *Coscinodiscus sp. #2*, *Cocconeis sp.*, and *Raphoneis* species were more common in the P only treatment (Table 10, Figure 13, Oneway ANOVA, Tukey HSD, df=3, $p<0.05$). Species composition among treatments based on molecular identification was differed from visual analysis. DNA libraries were comprised mainly of chlorophytes including a *Nannochloris* and *Chlamydomonas* species and dinoflagellates including a *Protaspis* species. Only 3 sequences belonging to a *Chlamydomonas sp.* and a *Syndiniales* dinoflagellate species were observed in the control, and diversity was lowest in this treatment. Diversity was highest in the P treatment (Table 11).

Discussion

Relationships between phytoplankton species composition and treatment emerged after 48 h indicating rapid nutrient utilization (Springer et al. 2005, Wetz et al. 2006). Similar to other nutrient enrichment experiments in temperate estuaries, diatom species

including *Skeletonema costatum, Melosira sulcata*, and a *Coscinodiscus* species made up the largest proportion of the community based on visual analysis across all treatments (Piehler et al. 2004, Domingues et al. 2011). However, we observed no relationship between treatment and increases in dinoflagellates, chlorophytes, and other smaller size class of phytoplankton taxa as previous studies have suggested (Piehler et al. 2004, Domingues et al. 2011).

Addition of only nitrate stimulated increases in the diatoms *Nitzschia longissima*, a *Thalassionema sp.,* a *Pinnularia sp.,* and *Skeletonema costatum* relative to the control. Of these species, *Nitzschia longissima, Skeletonema costatum,* and *Thalassionema sp.* are all bloom forming suggesting nitrate limitation of bloom forming species (Pratt et al. 1966, Vila and Maso, 2005, D'Costa and Anil, 2010). N addition also stimulated greater increases in overall biomass further supporting nitrogen limitation in the SKE (Piehler, 2004, Fisher et al. 2006). Higher availability of large, solitary phytoplankton taxa like *Thalassionema, Pinnularia,* and *Nitzschia longissima* may be beneficial for selective grazers like calanoid copepods as they generally choose large, individual food particles (Frost, 1972). However, greater abundance of small diameter, chain-forming species including *Skeletonema costatum* may stimulate feeding by ciliates and other lower level heterotrophs or contribute a large amount of primary production to the microbial loop (Sommer et al. 2002). Since ciliates and other diminutive heterotrophs are not grazed directly by higher order consumers like larval fishes, energy is transferred less efficiently.

Although phosphate did not appear to stimulate overall phytoplankton biomass or become limiting based on nutrient concentrations, there were increases in a number of individual species including *Melosira, Coscinodiscus, Cocconeis,* and *Raphoneis* species

in the P treatment. All of these taxa are large, productive diatoms, generally associated with high rates of primary productivity, and are not known to have toxic effects on consumers (Patten and Chabot, 1966). These species are also selected for by large consumers like copepods which are abundant in estuaries (Frost, 19722). Phosphorous limitation of *Melosira sp.* and *Coscinodisucs sp.* have been observed in freshwater systems (Henry et al. 2007), and our findings suggest its availability is also important in the SKE. Individual taxa have been observed to alter uptake rates of this nutrient in the presence of increased concentrations (Klausmeier et al. 2003).

The NP treatment appeared to induce the greatest change in overall phytoplankton biomass and lead to more rapid nutrient depletion than both the N and P treatments alone. Yet interestingly, there was only 1 species (a *Pinnularia sp.)* that appeared to be most common in the NP treatment. There was, however, a trend of higher densities of many species in the NP treatment compared to the control. It is possible that by dosing species with nitrogen and phosphorus together in an enclosed environment we reduced competition for both nutrients, allowing species to utilize them at an optimum N:P ratio different from the Redfield ratio of 16:1. There is some evidence for alteration of N:P uptake ratio based on nutrient availability in the environment which could explain intermediate increases in species which were most abundant in the N only or P only treatments (Klausmeier et al. 2003).

Molecular analysis of community structure indicated that all nutrient addition treatments were dominated by chlorophytes, including a *Nanochloropsis* and *Chlamydomonas* species and dinoflagellates including a *Protaspis* species. Increases in the contribution of these taxa to the community have been shown following nutrient

additions (Piehler et al. 2004). There was also a higher diversity of species recovered in all nutrient additions relative to the control and only two species, *Chlamydomonas* and *Syndiniales* were recovered in the control. This was due to a high incidence of sequence data from non-phytoplankton taxa in the control including both ciliates and copepod grazers. This finding may indicate lower abundance of small size classes of phytoplankton (which appear to be more common from molecular analysis) in the control. Differences in species observed using visual and molecular methods may be due to certain biases associated with each technique (See Chapter 2 for discussion).

Conclusion

Nutrients alone stimulated species-level changes in this experiment. Although extrapolating results from microcosms to the natural environment is difficult, the short incubation times used in the experiment should minimize bottle effects due to enclosing phytoplankton species. Also, there were relationships between individual taxa and both nitrate and phosphate concentration emphasizing the importance of examining nutrient effects at the species level. If we examined only biomass or class composition, it would appear that nitrogen alone stimulated changes in phytoplankton as P addition did not stimulate higher overall growth or differences in class composition. However, both N and P may be important determinants of species composition in the SKE. As development and population increases in coastal areas, there is a greater potential for nutrient increases in the SKE and long-term analysis has already shown this trend. I have demonstrated that increasing N and P may alter the composition of natural phytoplankton assemblages. Changes in species make-up may lead to variation in primary productivity as individual species possess unique growth rates and fluctuation in the efficiency of

energy transfer as some species are grazed directly by higher order consumers while many route their energy through the microbial loop.

Table 9. Summary of nutrient and biomass conditions at start of microcosm experiment. Biomass was significantly higher in the Control treatment compared to others (Oneway ANOVA, df=3, F=3.8, p=0.031).

Treatment	**Nitrate (µM)**	**Phosphate (µM)**	**Biomass (µg*L^{-1})**
C	3.77±0.46	0.78±0.02	8.30±0.32
N	14.61±2.06	0.81±0.03	6.45±0.13
NP	14.68± 2.05	4.58±0.13	7.16±0.11
P	3.72±0.46	4.72±0.05	5.75±0.20

Table 10. Abundance (cells dL^{-1}) of individual phytoplankton species by treatment after 48 hours of incubation (C-Control, P-Phosphate addition, NP-addition of nitrate and phosphate, and N-addition of nitrate only). -- indicates species that were not observed in a particular treatment.

Class	**Species**	**C**	**N**	**NP**	**P**
Diatom	*Asterionella sp.*	--	21	--	--
	Ceratulina sp.	--	--	9	--
	Chaetoceros socialis	--	101	--	28
	Cocconeis sp.	200	101	157	317
	Coscinodiscus sp.	550	789	965	1231
	Coscinodiscus radiatus	5	5	9	--
	Ditylum brightwellii	--	12	--	--
	Fragillaria sp.	4	28	--	8
	Melosira sp.	6	40	49	118
	Melosira sulcata	35	37	8	144
	Navicula didyma	24	63	57	84
	Nitzschia longissima	9	189	9	24
	Pinnularia sp. #1	7	75	--	23
	Pinnularia sp. #2	129	175	142	230
	Pinnularia sp. #3	34	92	147	65
	Pleurosigma angulatum	25	47	17	54
	Pleurosigma elongatum	183	198	188	199
	Raphoneis sp.	2	17	11	42
	Rhizoselenia pungens	--	14	14	--
	Skeletonema costatum	--	100	27	--
	Thalassionema sp.	173	375	93	225
	Thalassiosira decipiens	99	171	154	131
Dinoflagellate	*Ceratium sp.*	6	--	--	--
	Gonyaulux verior	3	--	--	--
Chlorophyte	*Staurastrum sp.*	2	--	--	--
	Microactinum sp.	--	48	--	--
Shannon's index	C=1.97±0.12	N=2.45±0.19	NP=1.87±0.14	P=1.99±0.19	

Table 11. Species composition based on molecular analysis. Unknown species were grouped to class based on similarity to known sequences in the Genbank database and phylogenetic analysis.

Treatment	Class	Species	# Observed
N	Chlorophyte	*Nannochloris sp.*	11
		Chlamydomonas sp.	7
		Tetraselmis sp.	7
	Dinoflagellate	*Gyrodinium dominans*	1
		Unknown sp.	1
	Chromerid	*Chromeria velia*	1
	Cryptophyte	*Cryothecomonas sp.*	1
NP	Chlorophyte	*Nannochloris sp.*	8
		Chlamydomonas sp.	5
		Tetraselmis sp.	3
	Dinoflagellate	*Protaspis sp.*	1
		Unknown sp.	4
		Unknown sp.	6
		Unknown sp.	3
		Unknown sp.	2
		Unkown sp.	1
	Diatom	*Minutocellus polymorphus*	2
P	Chlorophyte	*Nannochloris sp.*	3
		Chlamydomonas sp.	1
		Unknown sp.	1
	Dinoflagellate	*Protaspis sp.*	1
		Syndiniales sp.	1
		Unknown sp.	2
		Unknown sp.	1
		Unknown sp.	1
		Unknown sp.	1
		Unknown sp.	1
	Diatom	*Pinguiochrysis pyriformis*	1
		Cyclotella atomus	1
C	Chlorophyte	*Chlamydomonas sp.*	2
	Dinoflagellate	*Syndiniales sp.*	1
Shannon's index:	N=1.57	NP= 2.12 P=2.39	C=0.63

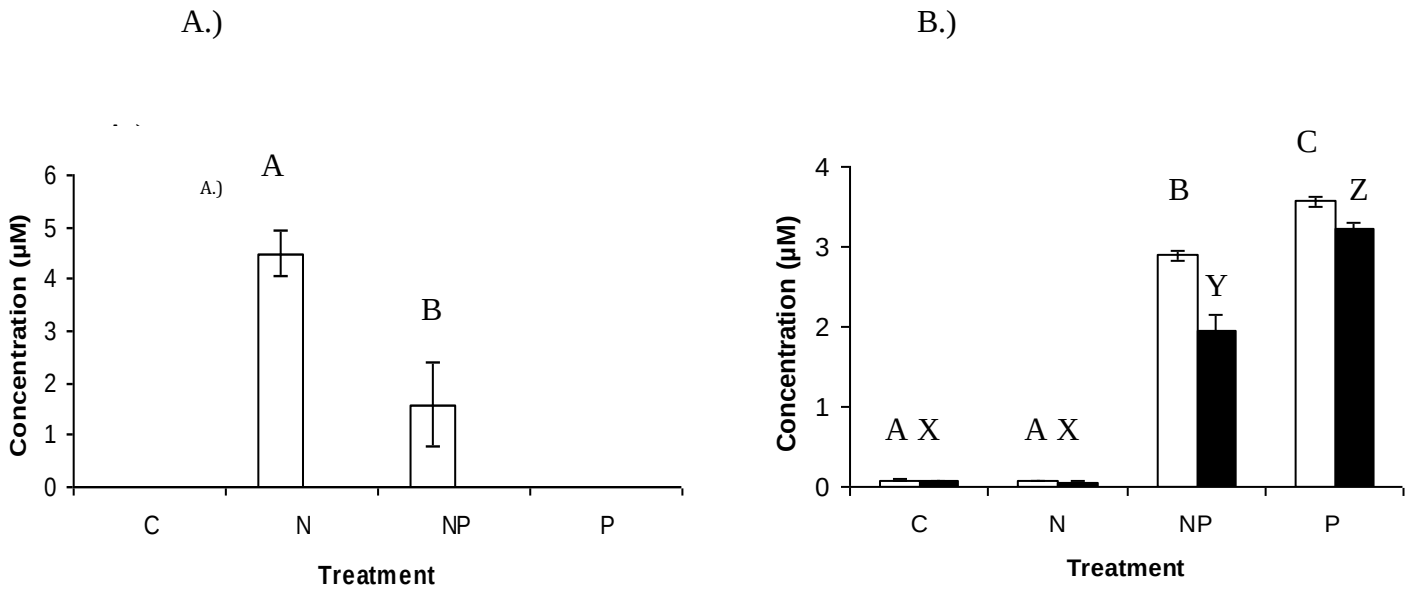

Figure 12. A.) Nitrate and B.) phosphate concentration by treatment at 48 □ and 96 ■ hours. Error bars are ±1SEM (n=5). Treatments that are significantly different at 48 hours are indicated by the letters A, B, or C. Treatments that are significantly different at 96 hours are indicated by the letters X or Y, or Z. For nitrate, only N and NP treatments at 48 hours were compared as concentrations were at 0 for the other treatments.

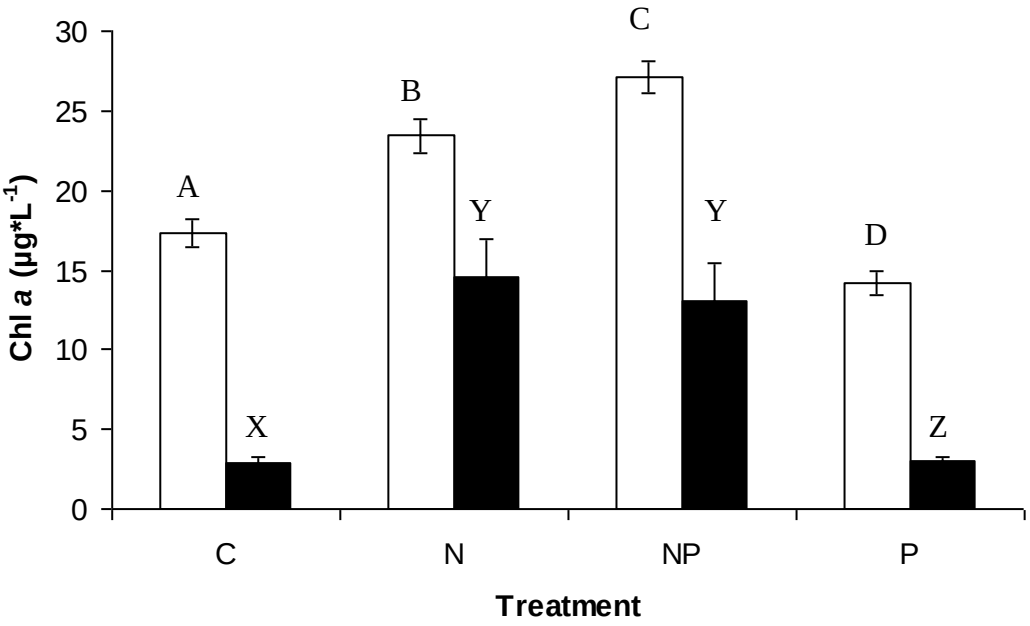

Figure 13. Total phytoplankton biomass at 48□ and 96■ hours following treatment. Error bars are ±1SEM (n=5). Treatments that are significantly different at 48 hours are indicated by the letters A, B, or C. Treatments that are significantly different at 96 hours are indicated by the letters X or Y, or Z.

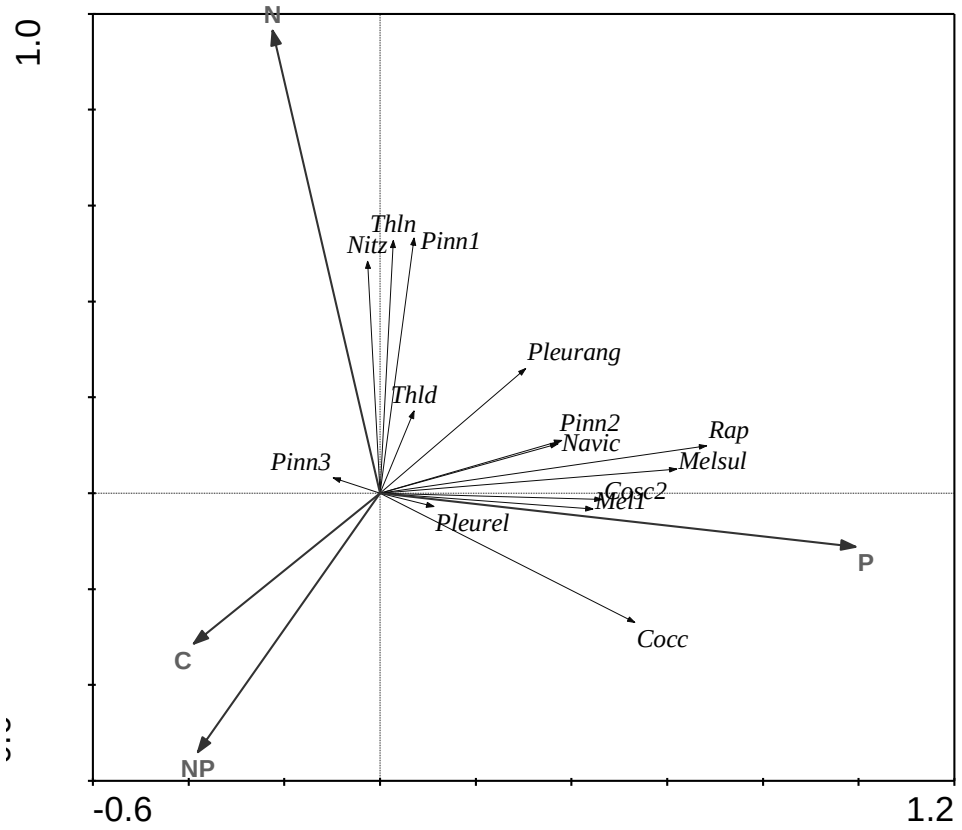

Figure 14. RDA biplot of species and treatments at 48 hours (C-Control, P-Phosphate addition only, NP-addition of nitrate and phosphate, and N-addition of nitrate only). Large arrows indicate treatments and small arrows indicate species.

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APPENDIX A

REDUNDANCY ANALYSIS SPECIES ABBREVIATIONS

Species	Abbreviation	Major Class
Asterionella sp.	Ast	Chlorophyte
Biddulphia longicuris	Bid	Diatom
Ceratium sp.	Cet	Dinoflagellate
Ceratulina sp.	Cer	Diatom
Chaetoceros gracile	Chg	Diatom
Chaetoceros socialis	Chs	Diatom
Chaetoceros sp. #3	Ch3	Diatom
Closterium gracile	Clg	Chlorophyte
Cocconeis sp.	Coc	Diatom
Coscinodiscus radiatus	Cor	Diatom
Coscinodiscus sp. #2	Co2	Diatom
Ditylum brightwellii	Dib	Diatom
Fragillaria sp.	Frg	Diatom
Gonyaulux spinifera	Gos	Dinoflagellate
Gonyaulux verior	Gov	Dinoflagellate
Leptocylindrius sp.	Lep	Diatom
Mamiella sp.	Mam	Diatom
Melosira sp. #1	Me1	Diatom
Melosira sp. #2	Me2	Diatom
Melosira sulcata	Mes	Diatom
Microactinum sp.	Mic	Chlorophyte
Navicula didyma	Nad	Diatom
Nitzschia longissima	Nil	Diatom
Pinnularia sp. #1	Pi1	Diatom
Pinnularia sp. #2	Pi2	Diatom
Pinnularia sp. #3	Pi3	Diatom
Pinnularia sp. #4	Pi4	Diatom
Pleurosigma angulatum	Pla	Diatom
Pleurosigma elongatum	Ple	Diatom
Raphoneis sp.	Rap	Diatom
Rhizoselenia pungens	Rhp	Diatom
Scenedesmus ellipticus	Sce	Chlorophyte
Skeletonema costatum	Ske	Diatom
Thalassionema sp.	Thn	Diatom
Thalassiosira decipiens	Ths	Diatom
Volvox	Vo	Chlorophyte

APPPENDIX B

USING MOLECULAR METHODS TO EXAMINE THE SPECIES COMPOSITION OF MICROBIAL COMMUNITIES

Abstract

Microorganisms account for a large portion of Earth's biodiversity, and they occupy essential niches in a wide variety of habitats. Evaluating the species composition of microbial communities is vital to understanding the ecology of any environment as each species can contribute differently to ecosystem processes. Traditionally, studies examining microbial communities have used phenotypic differences to differentiate and quantify species. While useful, visual analysis of microbial communities faces many difficulties and is often very time consuming. Increasingly, PCR-based molecular techniques are being used to determine microbial species composition, and these methods have revealed unprecedented diversity in bacterial, planktonic, and other microbial assemblages. Although the use of PCR offers many advantages over traditional methods, there are still difficulties associated with these techniques. This review will discuss the recent use of molecular techniques to identify microbial species, advantages over traditional analysis, and limitations.

Introduction

Microbes, those organisms too small to be seen with the naked eye, constitute a major proportion of Earth's biodiversity (Pham et al. 2008). These communities also serve essential functions such as primary production and decomposition in many unique environments (Niemi et al. 2004, Pontes et al. 2007). Since certain species often contribute differently to these processes, a detailed characterization of species

composition is necessary (Cloern and Dufford, 2005). However, our understanding of these communities has been limited in the past due to our inability to differentiate morphologically similar or cryptic species as traditional techniques have employed visual analysis of these assemblages (Cloern and Dufford, 2005, Pontes et al. 2007). Identification and enumeration of microscopic organisms using visual analysis is difficult because only subtle morphological differences exist between many species of bacteria and plankton and there may also be phenotypic variation within a species (Ellison and Burton, 2005, Pontes et al. 2007). Significant time and taxanomic expertise are needed to distinguish these minute differences and even then misidentification can occur. Also, since much of microbial diversity is thought to be undiscovered, more standardized methods with the ability to accurately describe new species are needed (Pham et al. 2008).

Recently, PCR-based molecular techniques have been used to look at the species make-up of microbial assemblages (Guillou et al. 2004, Pham et al. 2008). In these methods DNA is extracted, specific genes are amplified, and gene sequences analyzed to determine species composition (Fawley et al., 2004). The use of PCR-based analysis has begun to reveal unprecedented diversity in these communities as phenotypically similar species can more easily be differentiated using sequence data (Moon van der Staay, 2000). Previously undescribed species can also be delineated, described, and archived utilizing sequence data. The other major advantage to PCR methods over traditional techniques is that the lab techniques used to process samples follow cookbook procedures that are easy to perform. This reduces the amount of time and expertise needed to classify and enumerate different species (Ellison and Burton, 2005). While these

techniques offer great potential for a more efficient means of looking at microbial species composition, an understanding of some of the limitations these techniques is still needed.

Traditional Analysis

Historical analysis of species composition in microbial communities has relied solely on phenotypic differences to distinguish species (Pontes et al. 2007). This usually involves isolating and culturing individual species, or labor intensive identification using light microscopes. Culture techniques typically require dozens of dilutions to isolate a single species from mixed communties. Once a pure culture is obtained, substrate and chemical tests are used to characterize phenotypes. Many of these tests show overlap between species requiring hundreds of tests to adequately differentiate phenotypes (Hacene et al. 2004). Light microscopy can be used to identify species directly from environmental samples, but these techniques demand vast expertise and prior knowledge of microbial morphology (Cloern and Dufford, 2005). Both of these techniques are labor intensive, and due to phenotypic similarities between many species much of the community diversity is overlooked (Ellison and Burton, 2005). More efficient techniques are needed in studies of microbial communities.

DNA-DNA Hybridization

The first application of molecular techniques to help differentiate microbial species was with the use of DNA-DNA hybridization (Pontes et al. 2004). These techniques involve hybridization of genomic DNA extracted from a single pure culture with the DNA of another (Crosa et al. 1973). Generally, greater than 70% similarity between genomes is considered the same species (Hanage et al. 2004). While this

technique helps directly quantify differences between species, it still does not allow for identification from environmental samples.

PCR Analysis

More recently, PCR-based molecular methods have been used to identify species in microbial communities (Countway et al. 2005). These techniques typically employ amplification of ribosomal DNA from mixed environmental samples, cloning of individuals, and DNA sequencing. Species identity is determined by comparing sequences to known sequences in DNA databases (Betournay et al 2007). The major advantage of these techniques over traditional methods is that they allow identification directly from environmental samples and the protocols involved are relatively quick and easy to learn. The use of PCR techniques to identify microscopic organisms has revealed an unprecedented amount of diversity and holds great promise for future studies of microbial ecology (Moon-van der staay et. al 2005). However, while it is generally assumed that PCR techniques can give a more accurate estimation of the diversity of microbial communities when compared to visual analysis, the ability of PCR to quantify the actual proportion of species in mixed communities has been questioned (Farrelly et al. 1995). The main cause of this concern is that studies have documented "PCR bias" during some steps of the gene amplification process (Liesack and Stackebrandt, 1991). This bias is where one species can be favored during PCR amplification leading to an unreliable estimate of the actual proportion of that species in the community (Pham et al. 2008). A thorough understanding of the effects and causes of this bias is needed if these methods are to be commonly used in studies of microscopic communities. The remainder

of this paper will discuss some of the potential causes of this bias and other limitations associated with PCR techniques.

Primer Selection and Design

The first step in the PCR process is to design primers to amplify the gene of interest from your sample and primers can often be biased (Betournay et al. 2007). Primers are used in PCR analysis to selectively amplify DNA from a target group of organisms (Stiller and McClanahan, 2005). Typically, they are chosen by aligning a number of known gene sequences of the type of species targeted and selecting a 16-20 base pair region at the beginning and end of the sequence which is present across all the aligned species (Stiller and McClanahan, 2005). These 16-20 base pair regions are used as forward and reverse primers to bind to template DNA and amplify the selected genes (Ghosh et al. 2007). For example, studies have designed primers to amplify the 16S gene of phytoplankton from mixed water samples by aligning several species of phytoplankton and constructing primers from conserved regions in the aligned species that were not conserved in bacterial species (Stiller and McClanahan, 2005). Unfortunately, non-target species are often still amplified by many primer sets as a few undesirable species may have sequences complimentary to the primers (Betournay et al. 2007). Also, since every species of interest cannot be included in the alignment when selecting primers, primer design can sometimes fail to amplify target species that are not conserved (Stiller and McClanahan, 2005). This can bias quantification of actual species proportions (Betournay et al. 2007). As an increasing number of studies have documented amplification of non-target species (Stiller and McClanahan, 2005, Betournay et al.

2007), continuous experimentation with primer design is needed to determine the primer set which is most efficient at amplifying certain groups of organisms.

DNA Extraction and Purification

Another step in the PCR process that can be open to bias is DNA extraction (Farrelly et al. 1995). Template DNA of microbial communities can be extracted from a number of different mediums (i.e., water, soil, sediment) using a variety of kits and extraction procedures (Picard et al. 1992). The extraction procedure that is chosen is dependent on the extraction medium and target species (Carrigg et al. 2007). In many cases, the efficiency of extraction protocol to retrieve DNA is different between species as some species can be lysed more easily and DNA purified more efficiently due to the composition of cell walls or chemical conditions within the cell (Carrigg et al. 2007, Mumy and Finlay, 2004). This can lead to bias toward species whose DNA is more readily extracted. Studies which document this type of bias are typically aimed at optimizing extraction procedures for a specific microbial community by using a variety of extraction procedures to obtain template DNA from the same medium (Picard et al. 1992). Furthermore, extracted DNA must be purified or additional bias can occur (Carigg et al. 2007). This is because humic acids and other types of contamination interfere with the amplification process (Picard et al. 1992). The same studies which seek to optimize extraction protocol typically try to optimize purification procedures as well. It seems clear from these types of studies that optimization of these parameters can minimize any possible bias due to this step of the PCR process.

PCR Conditions and Cloning

The actual physical and chemical conditions during DNA amplification has also been shown to affect the amount of PCR product attained from different species (Reysenbach et al. 1992). PCR reactions are performed under different temperature cycles and chemical conditions, and these conditions are often optimized and set by the experimenter (Pham et al. 2008). In some cases, changes in PCR conditions can affect the amount of PCR product obtained. For example, Reysenbach et al. (1992) demonstrated that the addition of 5% acetamide to PCR reactions with archaeabacteria and yeast eliminated the inherent preferential amplification of yeast. The acetamide addition appeared to prevent selective priming of yeast rDNAs. Furthermore, many of the reactions with archaeabacteria alone, only worked when acetamide was added. This suggests that PCR conditions can play a major role in the amount of PCR product attained from individuals. While other studies have documented the effect of PCR conditions on the total yield of PCR product (Stiller and McClanahan, 2005) very few have documented species-specific differences in PCR product due to reaction conditions. Additional studies documenting species-specific bias due to PCR conditions are needed to determine its prevalence.

Once PCR amplification is attained, differential cloning efficiency can lead to further bias (Rainey et al. 1994, Pham et al. 2008). For example, Rainey et al. (1994) demonstrated that the cloning strategy selected (shotgun cloning, blunt end cloning, sticky end cloning) can lead to differential cloning of species as many of the techniques used are dependent on sequence composition. This problem can be reduced through optimized methodology and cloning strategies have been significantly improved in recent years (Pham et al. 2008).

Genome Effects

While most PCR bias can be reduced or eliminated through improved conditions and methodology (Picard et al. 1992, Liesack and Stackebrandt 1991), inherent differences in the genome of species may lead to a more consistent bias (Farrelly et al. 1995). For example, the number of gene copies within a species has been shown to bias proportions of bacterial species in known mixtures following PCR amplification (Farrelly et al. 1995). This bias is to be expected as all of the genes within an organism are available for amplification (Farrelly et al 1995). If one species has twice as many gene 16S gene copies as another, in an equal mixture of the two species, the one with more gene copies would constitute a larger proportion of the PCR product. This bias can be more difficult to eliminate than problems with the PCR process, but it can be reduced by selecting single copy genes (Pontes et al. 2007). Some known single copy genes include *rpoB, gyrB, recA, and dnaK* , however, fewer studies have used these genes in PCR studies of microbial communities, and sequence data may be more limited for single copy genes (Pontes et al. 2007).

Sequence Analysis

Another major obstacle in using PCR techniques to examine microbial communities is that sequence data can often be hard to interpret and analyze statistically. One of the first problems encountered when evaluating sequence data is defining what should be considered a unique species or taxonomic unit (Rivas et al. 2004). Species identity is typically obtained by comparing experimental sequences with known species sequences in databases. Many researchers have used a 97% similarity to known sequences to seperate species (Grattard et al. 2006, Heijs et al. 2007, Lu et al. 2008,

Marshall et al. 2008), however, some studies have shown only 1% differences between similar species (Ghosh et al. 2007). This makes the definition of a species or taxonomic unit in molecular studies variable. This problem could be considered analogous to the difficulty of sorting out morphological variation in visual analysis, however, by selecting genes with more variation between species this problem can be reduced.

Once a taxonomic unit is defined, the best methods for describing and comparing community sequence data can be difficult to decide on. Nearly all studies employing PCR methods to examine community composition incorporate a qualitative description of community composition where the recovered species and the number of clones of each species are listed (Marshall et al. 2008, Vaulot et al. 2008). More quantitative methods have used diversity indeces, species richness, and other compositional parameters to describe sequence data (Marshall et al. 2008). The most common and seemingly useful method for presenting sequence data is a phylogenetic tree or library approach and the vast majority of studies which use PCR to look at community structure typically employ this technique. The use of phylogeny can help determine the relatedness of unknown sequences to known species. Also, statistical methods for comparing phylogenetic libraries have been recently been developed (Pham et al. 2008). In general, these methods calculate a distance matrix between two libraries for comparison (Singleton et al. 2001). Advancement in these methods has allowed for the comparison of multiple libraries simultaneously and allowed researchers to look at the contribution of individual taxa to differences in libraries (Lozupone et al. 2006, Cole et al. 2007). As more studies employ PCR to evaluate and compare community structure, methods for presenting and statistically analyzing sequence data will continue to improve.

Conclusions and Future Research

The application of molecular, PCR-based techniques to characterize microbial communities holds great promise for the future as their use has already revealed a large amount of unknown diversity (Countway et al 2005, Pham et al. 2008). These techniques eliminate the problems associated with using phenotypic differences to distinguish species. Many of the drawbacks related to PCR which were discussed have been minimized by improved methodology over the past 15 years. The problems that remain, such as bias due to multi-copy genes can be eliminated through greater knowledge of species' genomes. Given the present pace of genome sequencing, this information will soon be readily available for most species. I believe the benefits of these techniques over traditional analysis greatly outweigh the drawbacks. Fewer of the recent studies using PCR to characterize microbial communities comment on the presence of PCR bias and most of the studies that were directly aimed at addressing PCR bias are outdated. Since techniques have been improved, more research on PCR bias is needed to evaluate the present state of the methodology. PCR is a highly efficient means of examining microorganisms and it will continue to advance our knowledge of microbial communities.