Autotrophic picoplankton in tropical reservoirs: a hydrobiological approximation of their abundance, dynamics and diversity

By

FERNANDO PANTOJA AGREDA

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
in
MARINE SCIENCES
(Biological Oceanography)

UNIVERSITY OF PUERTO RICO
MAYAGÜEZ CAMPUS
2016

Approved by:

_____	_____
Ernesto Otero Morales, PhD Chairman, Graduate Committee	Date
_____	_____
Carlos J. Santos Flores, PhD Member, Graduate Committee	Date
_____	_____
Luis R. Pérez Alegría, PhD Member, Graduate Committee	Date
_____	_____
Juan G. González Lagoa, PhD Member, Graduate Committee	Date
_____	_____
Linda W. Beaver, PhD Representative of Graduate Studies	Date
_____	_____
Ernesto Otero Morales, PhD Director, Department of Marine Sciences	Date

Abstract

In the present study, the abundance, dynamics and diversity of photosynthetic picoplankton were studied, with emphasis on the picocyanobacteria community, in two tropical reservoirs of Puerto Rico (Cerrillos and Lucchetti). Evolutionary changes in autotropic picoplankton over the limnological cycle, as well as environmental factors that control its population were analyzed. Autotrophic picoplankton cells were identified and quantified using flow cytometry and epifluorescence microscopy. These techniques allowed the identification of two populations of picoplanktonic organisms in the Cerrillos Reservoir. The picocyanobacteria (phycoerythrin-rich *Synechococcus* type) were dominant throughout the study, with maximum abundance of 6.6×10^4 cells mL^{-1}. Picoeukaryotes were two orders of magnitude less abundant. The biomass of autotrophic picoplankton averaged of 0.56 mg L^{-1} and the contribution to the total chlorophyll-*a* was 11%. Chlorophyll-*a* concentration reflected the annual variation of autotrophic picoplankton. Its concentration was highest during the period of maximum stratification when solar radiation and nutrients increased. The picoplanktonic community was structured in accordance to thermal stratification, nutrient and light availability.

Limnological conditions and seasonal dynamics of the picocyanobacteria community in two tropical reservoirs of different trophic status were compared. Environmental parameters that control the trophic status, abundance and biomass of picocyanobacteria of the reservoirs were analyzed and compared. The application of Carlson trophic state index showed the Cerrillos Reservoir as oligomesotrophic and Lucchetti as eutrophic. Flow cytometry techniques allowed clear differentiation between the picocyanobacteria and other groups of picoplankton in both systems. The results suggest differences in productivity and abundance of picocyanobacteria between reservoirs. The picocyanobacteria were present throughout the year in the two reservoirs,

however their abundance and biomass were significantly higher in the oligo-mesotrophic reservoir than in the eutrophic system. The temporal and vertical dynamics of picocyanobacteria were compatible with the period of stratification and mixing, showing a unimodal pattern with a maximum peak of abundance during the summer, an important difference from the lakes in temperate latitudes which have two fluctuations in the picoplankton density during the annual cycle.

Finally, 16S DNA samples from the two reservoirs with different trophic status were compared to determine the composition and diversity of the picocyanobacteria community through the environmental metagenomics technique. Greater part of the sequences grouped at the phylum Cyanobacteria of Cerrillos reservoir were dominated by *Synechococcus*, representing 29% of the total genera found, and with a few sequences of *Cyanobium* (5%). In the Lucchetti reservoir, the picocyanobacteria group comprised two genera: *Synechococcus* representing 2%, and *Cyanobium* with 0.1% of all genera of the microbial community. The picocyanobacteria community in the ecosystems studied was diverse and variable, and the strains were dispersed in the tree of polyphyletic origin. Most lineages were unique to a single ecosystem, however certain strains were present in both environments and their closest relatives are in different geographic regions and with contrasting limnological characteristics. It appears that the trophic state of the reservoirs studied significantly influences the diversity, composition and abundance of picocyanbacteria community. Picocyanobacteria exhibited high plasticity and can easily adapt to eutrophic conditions, however their community was best developed in oligomesotrophic environments.

Resumen

Se estudió la abundancia, la dinámica y diversidad del picoplancton fotosintético con énfasis en la comunidad de picocianobacterias en dos embalses tropicales de Puerto Rico (Cerrillos y Luccheti. Se analizó, la evolución del picoplancton a lo largo del ciclo limnológico, así como los factores ambientales que controlan su población. Las células del picoplancton autotrófico se identificaron y cuantificaron usando citometría de flujo y microscopía de epifluorescencia. Estas técnicas permitieron la identificación de dos poblaciones de organismos picoplanctónicos en el embalse Cerrillos. Las picocianobacterias (con ficoeritrina tipo *Synechococcus*) fueron dominantes durante todo el estudio, con máxima abundancia de 6.6×10^4 células mL^{-1}. Los picoeucariotes fueron dos órdenes de magnitud menos abundantes. La biomasa del picoplancton autotrófico tuvo un promedio de 0.56 $\mu g\ L^{-1}$, y la contribución media de picoplancton autotrófico al total de clorofila-*a* fue de 11%. La concentración de clorofila-*a* reflejó la variación anual del picoplancton autotrófico. Su concentración fue más alta durante el periodo de máxima estratificación, cuando la radiación solar y los nutrientes aumentaron. La comunidad picoplanctónica se estructuró de acuerdo a la estratificación térmica, nutrientes y disponibilidad de luz.

En adición, este estudio comparó las condiciones limnológicas y la dinámica estacional de la comunidad de picocianobacterias en dos embalses tropicales de diferente estado trófico. Se analizaron los parámetros ambientales que controlan el estado trófico, la abundancia y biomasa de picocianobacterias y se comparó entre embalses. La aplicación del índice de estado trófico Carlson mostró al embalse Cerrillos como oligomesotrófico y a Lucchetti como eutrófico. La técnica de citometría de flujo permitió diferenciar claramente la población de picocianobacterias de otros grupos del picoplancton en ambos sistemas. Los resultados sugieren que existen diferencias en la productividad y la comunidad de picocianobacterias entre los dos embalses. Las

picocianobacterias estuvieron presentes durante todo el año en ambos embalses. Sin embargo, la abundancia y biomasa fue significativamente mayor en el embalse oligo-mesotrófico que en el sistema eutrófico. La dinámica temporal y vertical de picocianobacterias es compatible con el período de estratificación y mezcla, que muestra un patrón unimodal con un pico de máxima abundancia durante el verano, diferencia importante con relación a los lagos de latitudes templadas que presentan dos fluctuaciones en la densidad del picoplancton durante el ciclo anual.

Por ultimo, muestras de DNA 16S de dos embalses con diferente estado trófico se compararon para conocer la composición y diversidad de las picocianobacterias utilizando un análisis de metagenómica ambiental. La mayor parte de las secuencias agrupadas al phylum Cyanobacteria del embalse Cerrillos fueron clasificadas como *Synechococcus*, el cual representa el 29% del total de los géneros encontrados, y un menor número de secuencias fueron clasificadas como *Cyanobium* (5%). En contraste, el grupo de picocianobacterias del embalse Lucchetti presento una menor proporción respecto a la población microbiana, *Synechococcus* (2%) y *Cyanobium* (0,1%) . La comunidad de picocianobacterias en los ecosistemas estudiados es diversa y variable, sus cepas se dispersan en el árbol de origen polifilético. La mayoría de los linajes fueron exclusivos de un solo ecosistema, sin embargo ciertas cepas estuvieron presentes en ambos ambientes y sus parientes más cercanos se encuentran en diferentes regiones geográficas y con características limnológicas contrastantes. Parece ser que el estado trófico de los embalses estudiados, influye significativamente en la diversidad, la composición y la abundancia de la comunidad de picocianobacterias. Las picocianobacterias muestran una alta plasticidad y puede adaptarse fácilmente a las condiciones eutróficas, sin embargo se desarrollan mejor en ambientes oligotróficos.

Copyright

In presenting this dissertation in fulfillment of the requirements for the degree of Doctor in Philosophy at the University of Puerto Rico, I agree that the library shall make its copies freely available for inspection. Therefore, I authorize the Library of the University of Puerto Rico at Mayagüez to copy my dissertation completely or partially solely for educational purposes. Each copy must include the title page. I further agree that extensive copying of this dissertation is allowed solely for scholarly purposes. However, any copying or publication of this dissertation for commercial purposes or financial gain is prohibited without my written permission.

Dedicatoria

A mis padres:

Por su amor, dedicación y por enseñarme que los sueños se logran a base de esfuerzo y sacrificio. Ha sido un privilegio ser su hijo.

A mis adorados hijos:

Luciana y Mateo, por todos los instantes que necesitaron mi presencia y no pude estar a su lado; siempre me decían, no te vayas!, vuelve pronto!

A mi esposa:

Por su paciencia, tolerancia y comprensión; gracias por darme la oportunidad de vivir a tu lado.

A mis hermanos:

Con su ejemplo y apoyo me enseñaron a seguir adelante y a formar una gran familia.

A mis amigos:

Porque me han brindado su apoyo incondicional y por compartir conmigo buenos y malos momentos.

Acknowledgments

I sincerely thank members of the graduate committee for their suggestions and manuscript reviews. First, I want to thank my supervisor, Dr. Ernesto Otero, for giving me the opportunity to reach this goal and for placing his trust in me to complete successfully this dissertation. I want to thank Dr. Carlos Santos Flores for the trust, support, will and friendship. Also, I want to thank Dr. Luis Pérez Alegría for the equipment and material provided to work in the reservoirs and for his valuable motivation to keep me going. My admiration and gratitude to Dr. Juan González Lagoa for agreeing to be part of my committee and sharing his experience and knowledge. Thanks to Dr. John Kubaryk for his valuable comments and suggestions to improve significantly the manuscript. A special thanks to doctors Carlos Rodríguez Minguela and Carlos Ríos for their teachings in the techniques of molecular biology and bioinformatics. Thanks to Dr. Raúl Macchiavelli for the help in the application of statistical tests. I also wish to thank Dr. Ingrid Padilla for her motivation for research and for letting me be part of her lab group. My gratitude to the doctors, David Sotomayor and Gustavo Martínez for their support and confidence in my work during my graduate studies. In the same way, I thank Gaspar Pons and Darien López (DRNA), managers of the reservoirs, and all their staff for their valuable help in the arduous field days. To my friend José Almodóvar (UPRM), for the great job in electron microscopy, epifluorescence and Nomarski microscopy. Special thanks to Dr. Ernesto González (UCV) for the application of the program SURFER and suggestions in the experimental design. Thanks also to Dr. Luis Soler (USGS) for providing me the bathymetric studies of the reservoirs. To my fellows and friends of Environmental Engineering, Marine Sciences and Biology with whom I shared so many hours of work and good moments. I wish to thank the support and understanding of all administrative staff of the departments of Marine Sciences, Biology and Civil Engineering at Mayagüez, for making my stay an easier one during my doctorate in the Enchantment Island. It is difficult to name each and every one of my friends and colleagues who have made this work possible, but they all will always have my friendship and gratitude for all their contributions and support. I want to thank my family for their unconditional support despite the distance. Finally, thanks to my wife Andrea and my children: Luciana and Mateo, for their love and patience.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER III **Page**

CHAPTER IV

LIST OF FIGURES

CHAPTER II

CHAPTER III

CHAPTER IV

Chapter I GENERAL INTRODUCTION

The term picoplankton was first introduced in the size classification of plankton published by Sieburth et al. (1978). The picoplankton is the fraction of plankton composed of small organisms (0.2 to 2.0 microns) including prokaryotic and eukaryotic cells (Winder 2009). The picoplankton is represented by members of the three domains (Bacteria, Archaea and Eucarya) of the phylogenetic tree proposed by Woese et al. (1990). This community plays an important role in the ecology of marine and freshwater ecosystems, becoming an essential resource of energy to the food chain and playing an important task in recycling nutrients and biogeochemical cycles (Azam et al. 1983).

Archaea, were believed to be restricted to certain extreme environments such as anaerobic sediments, hot springs and environments with high salinity. However, their distribution have been found to be widespread based on studies using DNA molecular techniques in diverse marine and freshwater environments (Massana et al. 1997). The relative abundance of Archaea in the sea increases starting at depth of 250 m, becoming as abundant as the bacteria at depths greater than 1000 m (Karner et al. 2001). Apparently, these microorganisms are an important component in prokaryotic associations, but there is limited information about their physiology and ecology relative to other microbe groups.

Bacteria are the most diverse and abundant fraction within the picoplanktonic group, and also play an essential role in fundamental ecological processes of aquatic ecosystems. Given their abundance and metabolic versatility, these organisms are capable of inhabiting a variety of environments, from those that offer ideal conditions for growth to extreme environments such as hypersaline systems whose features prevent the development of any other life form (Horner Devine et al. 2003). In aquatic environments, bacteria that live suspended in the water column are called

bacterioplankton. This community includes autotrophic and heterotrophic prokaryotes, whose wide distribution and biomass production may constitute a high percentage of total primary production (Stockneret al. 2000). This fraction represents a fundamental component in the flow of energy through the microbial loop, connecting the carbon and nutrient cycles with the scheme of conventional food web. In addition, the bacterial community is responsible for a high percentage of aerobic respiration. Total anaerobic respiration in aquatic systems is one of the largest reservoirs of carbon and nutrients, representing a key component in aquatic food webs and biomass as well as a significant source of food for many organisms (Azam et al. 1983).

The eukaryotic picoplankton, including autotrophic and heterotrophic organisms with the presence of nucleus, have an abundance between 10^2 and 10^4 cells mL^{-1} in lakes (Johnson and Sieburth 1982). The picoeucaryotes (Peuk) are a very diverse group and have chlorophyll-*a* in all autotrophic representatives, and have accessory pigments such as chlorophyll b, C1, C2, C3, fucoxanthin, peridinin, zeaxanthin, and others. The Peuk are widely distributed in the ocean and freshwater systems and are found throughout the water column (Lopez-Garcia et al. 2001). They contribute a significant biomass and high primary productivity in ocean and coastal regions (Worden et al. 2004).

Autotrophic picoplankton in aquatic ecosystem

The autotrophic picoplankton (APP) is composed primarily of picocyanobacteria and to a lesser number by Picoeukaryotes. The picocyanobacteria, *Synechococcus* and *Prochlorococcus* are dominant in marine ecosystems (Chisholm et al. 1988). In freshwater, Pcy (phycoerythrin-rich) *Synechococcus* and *Cyanobium* are the most representative, dominating the surface and deep layers (Callieri 2008). In most freshwater systems, the photosynthetic bacterioplankton contributes up to 70% of total carbon production (Callieri 2008). In particular, in ultraoligotrophics lakes between

50 and 70% of the annual flow of carbon is attributed to cells of 1 to 2 μm (Caron et al. 1985). Given their abundance and metabolic and physiological versatility, these microorganisms are capable of inhabiting a variety of environments, becoming an essential resource of energy to the food chain and fulfilling an important task in recycling nutrients and biogeochemical cycles (Cotner and Biddanda 2002; Falkowski et al. 2008).

In freshwater ecosystems, picocyanobacteria dominate pelagic environments and may be present as single cells or microcolonies (Crosbie et al. 2003; Stockner et al. 2000) presenting a greater diversity of morphotypes than those observed in marine environments. From multiple ecological studies we know that picocyanobacteria, like many other groups of prokaryotes, have a great capacity to adapt and can acclimate easily to different environmental conditions such as different levels of light or nutrients (Callieri 2008; Stomp et al. 2007).

Study methods of picoplankton

Because of their small size and simple morphologies picoplankton commynity are difficult to observe by optical microscopy, in which they appear as small spheres of different colors, without appreciable morphological structures. Observations by electron microscopy can give more background on the ultrastructure of cells and their external morphology (Johnson and Sieburth 1982), but often the morphological characteristics are not enough to describe a species or classify it within a taxonomic group (Komárek et al. 2004).

Another major problem in the study of picoplankton are the protocols and methodology for the collection of samples. It is important to collect a representative sample of biomass fractions separated from the larger phytoplankton. Fractionation by class size through filters of different pores and materials allows recovery of the fraction of picoplankton between 0.2 and 2 microns; however, caution should be exercised with pressure filtration, the volume of filtrate water, and the

dissolved solids content in order to get successful results in the extraction of chlorophyll and their identification of cells under epifluorescence microscopy.

The use of epifluorescence microscopy allows the determination of autotrophic picoplankton abundance in the water column through the composition of pigments (Ting et al. 2002). Both picocyanobacteria and Picoeukaryotes, belonging to different algal groups, have greater abundance of certain accessory pigments in proportion to chlorophyll allowing identification and quantification through the use of high performance liquid chromatography (HPLC) (Sarmento et al. 2008). There are well identified marker pigments. However there are also certain limitations, to the use of there markers related to the complexity involved in interpreting HPLC data from environmental samples and the fact that the pigment ratio marker chlorophyll-*a*, established to identify a taxon, are based on culture data (Bel Hassen et al. 2008). Isolation in pure culture is one of the best methods for identifying species in a picoplanktonic community. However, many species, especially marine ones, are difficult to grow, and even more so the majority can not be grown in culture; thus isolation in pure culture provides limited information about the structure of a community.

Thanks to the incorporation of new techniques such as flow cytometry, great accomplishments have been made in the ecology and taxonomy of picoplankton, allowing more accuratel quantification of organisms less than < 2 μm in size whose importance until now has been underestimated. Flow cytometry provides simultaneous information on abundance, the relative cell size, granularity or internal structure and the relative fluorescence intensity. The flow system introduces and restricts the cells for individual analysis, the optical system excites the sample and collects the light signals coming from the same, and the electronic system converts the optical signal into an electronic signal and digitizes for computer analysis. A flow cytometer

measures aligned cells in very thin water flow to which light beams are focused; how this light is deflected provides information about the size and shape of the cell and the fluorescence is detected and converted into a signal processed by a computer. In this way, flow cytometry allows cell counts and categorize them. Cytometers exist that are able to recover cells for identification and/or culture (Marie et al. 2005). When analyzing a water sample by flow cytometry, groups can be clearly distinguished that make up the autotrophic picophytoplankton (picocyanobacteria and picoeukaryotes), but to distinguish organisms at the level of genera it is necessary to have calibrations based on size and fluorescence of each taxon. Many of these attributes (cell size, content of chlorophyll and DNA content) are observed in cultivable species (Marie et al. 1995).

The introduction of molecular biology for the study of aquatic organisms has allowed to an enormous advance in our knowledge of picoplanktonic communities. In particular, the field of microbial aquatic ecology has benefited from a great development of various techniques developed to study not only the identity, but also the activity and microbial genomics of picoplankton (Azam 2004). The use of these molecular techniques in microbial ecology has revolutionized the understanding of aquatic ecosystems. For the first time, it is possible to determine the composition of picoplankton without the need to look at it under a microscope or cultivating, because that composition was obtained from the sequence of a gene present in all living organisms and coding for the small subunit rRNA.

Comparative gene analysis of phylogenetically informative genetic material (such as rRNA or rDNA), has helped identify new phylogenetic lineages, expanding the map of biogeographical distribution of microorganisms and their functional relations (Laybourn-Parry and Pearce 2007). Phylogenetic analysis of the 16S rRNA genes for bacteria and picocyanobacteria, and 18S rRNA for picoeukaryote is a very useful tool in evaluating phylogenetic relationships (Callieri 2008).

Diversity of freshwater picoplankton

Smaller size phytoplankton fractions such as the populations of *Prochlorococcus, Synechococcus* and *Cyanobium* are the most conspicuous components of picophytoplankton in oceans and freshwater systems (Callieri 2008). The analysis of these fractions has recently been made using the analysis of environmental DNA and the design of specific probes. These studies indicate that picoplankton biodiversity in reservoirs is still unexplored. In recent years, several new taxa have been described from the genus to the class level, by combining conventional techniques with new molecular methodologies (Kawachi et al. 2002).

In freshwater systems, phenotypic picocyanobacteria diversity has been observed and used to study its relationship with phylogenetic composition of these communities. The simple morphology of picocyanobacteria (Pcy) requires cytomorphological, physiological and biochemical methods to determine genera and species (Potter et al. 1997). Cytomorphological, physiological and biochemical methods are used to determine genera and species since the simple morphology of picocyanobacteria does not provide enough information. (Potter et al. 1997). One example of this phenotypic classification of Pcy populations is that which relies on yellow and red autofluorescence to distinguish phycoerythrin (PE)-rich cells and phycocyanin (PC)-rich cells (Ernst, 1991).

Molecular studies comparing genetic sequences of a microbial community have shown great diversity of bacterioplankton in lakes and reservoirs. Molecular techniques such as fingerprinting as T-RFLP (Terminal-Restriction Fragment Length Polymorphism) and DGGE (Denaturing Gradient Gel Electrophoresis) are used to analyze the dynamics of the structure and biodiversity of communities in environmental samples from aquatic systems (Hewson and Fuhrman 2004).

Currently genetic and molecular studies show that Pcy are a polyphyletic group with a complex taxonomy (Robertson et al., 2001). According to Callieri (2008), *Synechococcus, Cyanobium* and *Cyanothece* dominate the prokaryote picophytoplankton of inland waters. *Synechococcus* is the most abundant, and physiologically and genetically diverse genus of Pcy (Callieri and Stockner, 2002). The freshwater *Synechococcus* and *Cyanobium* species together with the marine *Synecochococcus* and *Prochlorococcus,* form a bootstrap-supported "picocyanobacterial clade" separated from the rest of the cyanobacterial radiation, and these four groups dominate the APP of the freshwater and marine ecosystems (Ernst et al. 2003; Crosbie et al. 2003).

Picoeukaryotes are less abundant than other groups; however, they are the most diverse group of picoplankton with both phototrophic and heterotrophic representatives. This group is composed mostly of green algae (Chlorophyta) and diatoms (Bacillariophyta), and to a lesser degree cryptomonad (Cryptophyta), chrysomonids (Chrysophyta) and dinoflagellates (Dinophyta) (Callieri and Stockner, 2002). Eukaryotic organisms of the picoplanktonic fraction are widely distributed in lakes and reservoirs, and along the entire water column (López-García et al. 2001). These organisms contribute a significant biomass and high primary productivity in oceanic and coastal regions (Massana 2011; Worden et al., 2004). However, based on present information held about phylogenetic groups comprising the eukaryotic component of picoplankton in freshwater environments, their abundance lowis relatively. Phylogenetic analyses based on 18S rRNA sequences indicates that the most common freshwater picoeukaryotes are the unicellular genera *Choricystis* and *Myconastes*. Some of the unicellular forms may also be found as colonies, indicating the ability of strains to form microcolonies.

Limnological antecedents of reservoirs in Puerto Rico

The limnology of just a few of the more than 32 reservoirs in Puerto Rico, has been studied in in detail. The studies that have been conducted have not paid attention to the dynamics and structure of picophytoplankton communities or the relationship of these community characteristics with the physicochemical variables of these water bodies. The examination of picophytoplankton communities in Puerto Rico has the potential of showing or corroborating substantial community differences with similar systems in temperate zones.

Candelas and Candelas (1964), in the 50's, made the first limnological study in reservoirs of Puerto Rico. They selected large lakes (Guajataca, Dos Bocas, Caonillas, Matrullas, Guayabal, Patillas and Cidra) that best mimicked the conditions of natural lakes without extreme fluctuations in water level. The results of their study showed that water transparency, among these lakes had large variations but generally the Secchi disk visibility was low, one meter on average. The maximum temperature recorded was 31 °C and the minimum 22 ^{o}C without extensive thermal stratification even being noticed. All lakes were considered slightly alkaline, with pH range of 7 to 8. The dissolved oxygen ranges were 0.2 - 11.2 mg L^{-1}. In all the lakes studied, oxygen was present to a depth of 6 meters.

Valido (1975) included an overview of the Guajataca reservoir limnology and planktonic organisms, finding that this system depends physically and limnologically on two factors: the Guajataca river hydraulics and the weather conditions such as air temperature, rain and wind. Referring to plankton, *Perinidium* was the dominant taxon in number and volume in samples of phytoplankton, and species of *Keratella* were the most common in the zooplankton.

Carvajal (1979) conducted a chemical, physical and biological characterization of lakes La Plata, Cidra, Dos Bocas, Carite, Lucchetti and Loco. Within the parameters evaluated, the total

phosphorus was presented as orthophosphate by 18%, however correlations between the nitrogen and phosphorus were not performed. The organic load input in the drainage basins of the lakes was estimated at approximately 8%. The values of total coliforms, some metals and phosphorus compounds exceeded the threshold values estabished by the Environmental Quality Board (EQB).

Tilly and García (1983) described the annual path of the different forms of nitrogen and phosphorus in the lake La Plata, and discussed the most important factors controlling nutrient dynamics in 13 reservoirs on the island. Significant positive correlations between concentrations of nitrogen and phosphorus levels and primary productivity were found. This study concluded that the levels of nutrient loading are based on the area of the drainage basin, precipitation, the coefficient of runoff and the final concentration of nutrients.

The dissolved oxygen content in the lakes of Puerto Rico has been studied by Candelas and Candelas (1964), Martínez (1979), Quiñones (1980), Sotomayor et al. (2009) and Pantoja (2006, 2009), Martinez et al. (2014). These works, which together describe to some extent most of the reservoirs on the island, show a deficiency of dissolved oxygen below surface levels, a zone of rapid reduction of oxygen from 4 to 6 meters, and anaerobic conditions at the bottom of most lakes.

Martínez et al (2005) proposed the development of numerical nutrient criteria for lakes of Puerto Rico. Based on data from 14 stations in different reservoirs, values below the 25th percentile of the frequency distribution of these lakes were selected for each of the following parameters: total nitrogen, total phosphorus, chlorophyll-*a* and Secchi depth. Values of 19 $\mu g\ L^{-1}$ for total phosphorus, 0.48 $\mu g\ L^{-1}$ for total nitrogen, 7.3 $\mu g\ L^{-1}$ for chlorophyll-*a* represented an estimate of the numerical criteria for lakes of Puerto Rico.

Pantoja-Agreda et al (2009) studied the chemistry variables and phytoplankton dynamics of Guajataca reservoir and found low values of transparency due to the high amount of solids and

organic matter suspended in the water column to be classified. A stable thermal stratification was recorded during most of the year, which allowed to classify the reservoir as warm monomictic. Dissolved oxygen in the surface was relatively high, and showed anoxic conditions in the hypolimnion throughout the study period. Median concentrations of nutrients, especially available phosphorus, were detected by applying the rate of Carlson Index. The relation TN: PT was above 9, indicating that the potentially limiting nutrient in the reservoir was phosphorus. Chlorophyll-*a* reflected the annual variation of phytoplankton biomass. The concentration was higher in the summer when there was greater availability of nutrients and sunlight. The diversity and abundance of phytoplankton was low; a total of 22 taxa belonging to 20 genera and 6 classes were recorded. Euglenophyta was the dominant group throughout the year, contributing 43.9% to the total biomass. Dinoflagellates (*Peridiniumm* and *Peridiniopsis*) were the second largest group, with 34.9% of the algal biomass, and the third group was the Chlorophyta, with 10.71%. The structure of the phytoplankton community was established basically in relation to the availability of nutrients and light, and their behavior closely followed variations in climate, particularly the rainfall regime in the region (Pantoja 2006).

Sotomayor et al. (2008) described the limnological conditions in two reservoirs in Puerto Rico with low nutrient concentrations. Both systems remained stratified at least 9 months. Primary productivity was probably limited by light because there was no algal seston and phosphorus. Both reservoirs showed similar phytoplankton biomass, nutrient concentration and trophic status, but varied in the degree of hypolimnetic anoxia. Dissolved oxygen in the hypolimnetic zone of Guajataca reservoir was never greater than 2.1 mg L^{-1} and quickly decrease during stratification. In Cerrillos no complete mixing was observed and anoxia remained for most of the year.

Nutrient levels, related to ecological thresholds of deterioration in the reservoirs of Puerto Rico, were studied by Martínez et al. (2014). The numeric nutrient criteria were established by evaluating the relationship between TN and TP, and different ecological thresholds of deterioration. A Chlorophyll-*a* concentration of 24 μg L^{-1} was selected as the threshold for impairment where the degree of penetration of light is significantly reduced due to an excess of primary productivity, making it difficult for the reservoir to meet the criteria for aquatic life. Concentrations of 0.035 mg L^{-1} for TP and 0.43 mg L^{-1} for TN were associated with impairment, and can be considered as the thresholds for nutrients. Values of 0.026 mg L^{-1} for TP and 0.41 mg L^{-1} for TN were established as numerical criteria for nutritional standards of the reservoirs of Puerto Rico.

Rodríguez (2014) studied diatoms in 6 reservoirs in Puerto Rico with a broad spectrum of trophic conditions. A total of 32 taxa were recorded. The most abundant taxa in these lentic systems in Puerto Rico were: *Achnanthidium* sp., *Navicula* sp. and *Ulnaria* sp. In mesotrophic reservoirs, the dominant genera were *Navicula, Synedra* and *Ulnaria*. In eutrophic reservoirs, *Ulnaria* and *Synedra* were the most common species. Puerto Rican water reservoirs are singularly characterized by the presence of the genus *Ulnaria* which wasalways above 25% of the relative abundance regardless of trophic status.

The present research is structured in manuscript form and consists of five chapters. The first chapter is a general introduction on the picoplankton community, with special emphasis on the ecology of picocyanobacteria. The second chapter studies the temporal and vertical dynamics of the autotrophic picoplankton of both prokaryotes and eukaryotes in a subtropical reservoir in Puerto Rico and aims to determine the environmental factors that influence the patterns of distribution and abundance of the picoplankton community during an annual cycle. The third

chapter describes and compares the structure of picocyanobacteria in two tropical reservoirs of contrasting trophic state: seasonal and vertical variations in abundance and biomass are compared with the environmental variables that control its presence. The study determines if the seasonal and vertical variations of the community of picocyanobacteria is related to the environmental conditions and if the abundance and biomass of the dominant species present differences in reservoirs with different trophic states. In the fourth chapter, the genetic diversity of the picocyanobacteria community was analyzed in two reservoirs with contrasting limnological characteristics, aiming to provide the first data on the structure of the smallest fraction of the phytoplankton through the application of molecular techniques, in order to understand the role of the picocyanobacteria in the biogeochemical processes of lakes and reservoirs in the neotropic. Finally, in chapter five a section is presented summarizing the main conclusions along with the most important findings.

References

Azam F, Fenchel T, Field JG, Gray JS, Meyer-Riel RA, Thingstad F. 1983. The ecological role of water column microbes in the sea. Mar Ecol Prog Ser. 10: 257-263.

Azam F, Worden A. 2004. Microbes, molecules, and marine ecosystems. Science. 303:1622-1624

Bel Hassen M, Drira Z, Hamza A, Ayadi H, Akrout F, Issaoui, H. 2008. Summer phytoplankton pigments and community composition related to water mass properties in the Gulf of Gabes. Est Coast Shelf Sci. 77: 645-656.

Callieri C, Stockner JG. 2002. Freshwater autotrophic picoplankton: rewiew. J Limnol. 61:1-14.

Callieri C. 2008. Picophytoplankton in freshwater ecosystems: the importance of small-sized phototrophs. Freshw Rev. 1:1-28.

Candelas G, Candelas G. 1964. Plankton studies on Puerto Rico freshwater lakes. Physical and chemical nature. Carib J Sci. 4 (4): 451-458.

Carvajal JR.1979. Ecological survey of lakes. Final report. Project N. F-4. Puerto Rico. Departamento de Recursos Naturales PR. 52p.

Caron DA, Pick FR, Lean DRS. 1985. Chroococcoid cyanobacteria in Lake Ontario: seasonal and vertical distribution during 1982. J Phycol. 21:171-175.

Chisholm SW, Olson RJ, Zettler ER, Goericke R, Waterbury JB, Welschmeyer NA. 1988. A novel free living prochlorophyte abundant in the oceanic euphotic zone. Nature. 334: 340-343.

Cotner JB, Biddanda BA, 2002. Small players, large role, microbial influence on biogeochemical processes in pelagic aquatic ecosystems. Ecosystems. 5:105-121.

Crosbie ND, Teubner K, Weisse T. 2003. Flow-cytometric mapping provides novel insights into the seasonal and vertical distributions of freshwater autotrophic picoplankton. Aquat Microb Ecol. 33:53–66.

Ernst A.1991. Cyanobacterial picoplankton from Lake Constance I. solation by fluorescence characteristics. J Plankton Res. 13:1307–1312.

Ernst A, Becker S, Wollenzien UIA, Postius C. 2003. Ecosystem-dependent adaptive radiations of picocyanobacteria inferred from 16S rRNA and ITS-1 sequence analysis. Microbiology. 2003: 149: 217.

Falkowski PG, Fenchel T, Delong EF. 2008. The microbial engines that drive earth's biogeochemical cycles. Science. 320:1034-1039.

Hepperle D, Schlegel I. 2002. Molecular diversity of eukaryotic picoalgae from three lakes in Switzerland. Intern Review of Hydrobiol. 87:1-10.

Hewson I, Fuhrman JA. 2004. Richness and diversity of bacterioplankton species along an estuarine gradient in Moreton bay, Australia. Appl environ Microbiol. 70:3425-33.

Horner-Devine MC, Leibold MA, Smith VH, Bohannan BJ. 2003. Bacterial diversity patterns along a gradient of primary productivity. Ecology Letters. 2003; 6: 613-622

Johnson PW, Sieburth JMcN. 1982. In-situ morphology and occurrence of eukaryotic phototrophs of bacterial size in the picoplankton of estuarine and oceanic waters. J Phycol. 18: 318-327.

Karner, MB, DeLong EF, Karl DM. 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature. 409: 507-510.

Kawachi MI, Inouye D, Honda CJ, O´Kelly JC, Bailey R, Bidigare R, Andersen RA. 2002. The Pinguiophyceae *classis nova*, a new class of photosynthetic stramenopiles whose members produce large amounts of omega-3 fatty acids. Phycological Res. 50:31-47.

Komárek J Cepák V, Kaštovský J, Sulek J. 2004. What are the cyanobacterial genera *Cyanothece* and *Cyanobacterium*? Contribution to the combined molecular and phenotype taxonomic evaluation of cyanobacterial diversity. Algol Stud. 113:1–36.

Laybourn-Parry J, Pearce DA. 2007. The biodiversity and ecology of Antarctic lakes: models for evolution. Phil Trans Royal Soc. 362: 2273- 2289.

Li WKW. 1998. Annual average abundance of heterotrophicbacteria and *Synechococcus* in surface ocean waters. Limnol Oceanogra. 43:1746-1753.

Lee-Borges J. 2003. Contribution of picoplankton to phytoplankton dynamics and biooptics of the Eastern Caribbean Sea. Ph.D. Thesis. University of Puerto Rico. Mayagüez. P.R. 133 pp.

Lefranc M, Thénot A, Lepère C, Debroas D. 2005. Genetic diversity of small eukaryotes in lakes di ering by their trophic status. Appl Environ Microbiol. 71:5935-5942.

López-García, P, López-López A, Moreira, D, Rodríguez-Valera F. 2001. Diversity of free-living prokaryotes from a deep- sea site at the Antarctic Polar Front. FEMS Microbiol Ecol. 36: 193-202.

Massana R, Murray AE, Preston CM, DeLong EF. 1997. Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara Channel. Appl Environ Microbiol. 63: 50–56.

Massana R. 2011. Eukaryotic picoplankton in surface oceans. Annu Rev Microbiol. 65: 91–110.

Marie D, Simon N, Vaulot D. 2005. Phytoplankton cell counting by flow cytometry. In R. A. Anderson (ed.), Algal Culturing Techniques. Elsevier Academic Press. pp. 253–268.

Martínez R. 1979. Estudio comparativo de la limnología de los embalses mayores de Puerto Rico. M.S. Thesis. University of Puerto Rico. Río Piedras. 75p.

Martínez GA, D Sotomayor, L Pérez. 2005. Determination of numeric nutrient criteria in lakes and reservoirs of Puerto Rico. Final progress report. University of Puerto Rico. College Agricultural Sciences. Agronomy and Soils Department. 59 pp.

Martínez-Rodríguez GA, Sotomayor-Ramírez D, Macchiavelli R, Santos-Flores C, Pérez-Alegría L. 2014. Nutrient levels associated with ecological thresholds of impairment in reservoirs of Puerto Rico. Final Report Submitted to The Environmental Quality Board of Puerto Rico. October, 2014.

Pantoja-Agreda F.2006. Dinamica fisicoquimica y fitoplanctonica del embalse Guajataca, Puerto Rico. Master Thesis. Universidad de Puerto Rico. Mayaguez. 206 p.

Pantoja-Agreda F, Sotomayor D, Martínez G. 2009. Phytoplankton dynamics of the Guajataca Reservoir. Verth. Internat. Verein Limno. 30. 7:1096-1100.

Potter DT, Lajeunesse C, Saunders GW, Anderson A. 1997. Convergent evolution masks extensive biodiversity among marine coccoid picoplankters. Biodivers. Conserv. 6:99-107.

Quiñonez F. 1980. Limnology of Lake Loíza, Puerto Rico. U.S Geological Survey. Water Resources Investigations. 109 pp.

Robertson BR, Tezuka N, Watanabe MM. 2001. Phylogenetic analyses of *Synechococcus* strains (Cyanobacteria) using sequences of 16S rDNA and part of the phycocyanin operon reveal multiple evolutionary lines and reflect phycobilin content. J Syst Evol Microbiol. 51:861–871.

Rodriguez Lola. 2014. Net-Plankton Diatoms of Puerto Rican Water Reservoirs as Potential Bioindicators of Trophic Status. Master Thesis. Universidad de Puerto Rico. Mayaguez. 132 p.

Sarmento H, Unrein F, Isubmisho M, Stenuite S, Gasol J, Descy JP. 2008. Abundance and distribution of picoplankton in tropical oligotrophic Lake Kivu, eastern Africa. Freshwater Biol. 53:756-771.

Sieburth JM, Smetacek V, Lenz J. 1978. Pelagic ecosystem structure: heterotrophic compartments of the plankton and their relationship to plankton size fractions. Limnol Oceanogr. 23:1256-1263.

Simon N, Lebot N, Marie D, Partensky F, Vaulot D. 1995. Fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes to identify small phytoplankton by flow cytometry. Appl. Environ Microbiol. 61:2506-2513.

Sotomayor D, Martínez G, Pantoja F. 2008. Limnological assessment of two reservoirs in Puerto Rico. Verth Internat Verein Limnol. 30(3):521-527.

Stomp M, Huisman J, Vörös L, Pick, FR, Laamanen, M, Haverkamp T, Stal LJ. 2007. Colorful coexistence of red and green picocyanobacteria in lakes and seas. Ecology Letters. 10:290-298.

Stockner J, Callieri C, Cronberg G. 2000. Picoplankton and other non- bloom forming cyanobacteria in lakes. In: Whitton BA, Potts M (eds) The ecology of cyanobacteria: their diversity in time and space. Kluwer Academic Publishers, Dordrecht 195–238, 688 pp.

Tilly L, García J. 1983. Background for management of tropical reservoirs in Puerto Rico. International symposium of North American Lake Mangement Society (NALMS). 16 pp.

Ting CS, Rocap G, King J, Chisholm SW. 2002. Cyanobacterial photosynthesis in the oceans: the origins and significance of divergent light-harvesting strategies. Trends Microbiol. 10:134-42.

Valido A. 1975. Food habit studies of *Tilapia mossambica* and *Dorosoma petenense* in Guajataca Reservoir, Puerto Rico. Master Thesis. University of Puerto Rico. 65p.

Wehr JD. 1993. Efects of experimental manipulation of light phosphorus supply on competition among picoplankton and nanoplankton in a oligotrophic lake. Can J Fish Aquat Sci. 50:936-945.

Winder M. 2009. Photosynthetic picoplankton dynamics in Lake Tahoe: temporal and spatial niche partitioning among prokaryotic and eukaryotic cells. J Plankton Res. 31:1307-1320.

Worden AZ, Nolan JK, Palenik B. 2004. Assessing the dynamics and ecology of marine picophytoplankton: the importance of the eukaryotic component. Limnol Oceanogr. 49:168–179.

Woese CR, Kandler O, Wheelis ML. 1990. Towards a natural system of organisms: proposal of the domains Archaea, Bacteria and Eukarya. PNAS 87: 4576-4579.

Chapter II

Autotrophic picoplankton assemblages in a subtropical reservoir: temporal and vertical dynamics in abundance and biomass

Accepted to Journal of Freshwater Ecology

Fernando Pantoja Agreda[1*] and Ernesto Otero Morales[1]

[1]Department of Marine Sciences, University of Puerto Rico at Mayagüez
PO Box 9000, Mayagüez 00681 Puerto Rico

[*]Corresponding author: fernando.pantoja@upr.edu

Abstract

The composition, biomass and dynamics of autotrophic picoplankton (APP, size range of 0.2 to 2 μm) in a subtropical reservoir (Cerrillos, Puerto Rico) were examined from January to December, 2013. Samples were collected monthly in the limnetic zone. Because of their small size APP were identified and quantified using flow cytometry and epifluorescence microscopy. The warm monomictic nature of this reservoir resulted in thermal stratification during most of the year. Near surface dissolved oxygen was relatively high; however, hypoxic conditions were present in the hypolimnion during the whole period of study. Flow cytometry analyses allowed the identification of two populations of picoplanktonic organisms. Picocyanobacteria groups (phycoerythrin-rich *Synechococcus* type) were dominant throughout the study, with maximum abundances of 6.6×10^4 cells mL^{-1}, although picoeukaryotes were temporally important. Annual mean total chlorophyll *a* (Chl-*a*) in the euphotic zone was of 7.54 ± 3.41 μg L^{-1}. Fractionated Chl-*a* exhibited a relatively constant pattern: ranging from 0.24 to 1.99 μg L^{-1}. The average contribution of APP to total Chl-*a* concentration was 11 %. Chl-*a* concentration reflected the annual variation in picoplankton, its concentration being higher during summer when solar radiation was also higher. The picoplanktonic community was structured in accordance to thermal stratification, nutrients and light availability. Understanding the dynamics of picoplankton and their response to environmental change are important

because they are a good indicator of the trophic status of reservoirs, especially drinking water reservoirs, and can guide management decisions.

Keywords: Chlorophyll, flow cytometry, picocyanobacteria, *Synechococcus*, nutrients, oligotrophic.

Introduction

Autotrophic picoplankton (APP) are defined as the fraction of phytoplankton within the size range of 0.2 and 2 μm, including prokaryotic and eukaryotic cells (Callieri 2007). They are distributed worldwide and are ubiquitous in all types of lakes of varying trophic states (Stockner 1991). The APP community is composed predominantly of picocyanobacteria (Pcy), regardless of the trophic status, while Picoeukaryotes (Peuk) are numerically less abundant (Winder 2009). The genera *Procholorococcus* and *Synechococcus* dominate the picocyanobacteria community in marine waters (DuRand et al. 2001). In freshwater ecosystems, species of the phycoerythin-rich genus *Synechococcus* and phycocyanin-rich genus *Cyanobium* are the most commonly observed (Callieri 2007, Winder 2009).

The APP community plays an important role in the carbon production in ocean and freshwater ecosystems (Callieri et al. 2002). Picoplanktonic species are considered the main contributors to phytoplankton biomass and primary production (Worden et al. 2004). They can fix annually 50 to 70% of the carbon in oligotrophic freshwater ecosystems (Caron et al. 1985). The activity of picoplankton constitutes an important resource of energy in the aquatic microbial loop and plays a major role in biogeochemical processes (Cotner and Biddanda 2002).

Changes in environmental conditions in temperate lakes seem to support the coexistence of multiple picocyanobacteria genotypes (Callieri 2012). In temperate lakes, the typical seasonal cycle of Pcy shows a bimodal pattern with a spring or early summer peak, which corresponds to

the onset of stratification, and a second peak during autumn (Stockner et al. 2000). In contrast, peak abundances of Peuk are normally observed only during the spring isothermal mixing, early thermal stratification (Callieri and Stockner 2002), while low numbers occur during the rest of the year (Crosbie et al. 2003). In tropical freshwaters, Sarmento et al. (2008) reported a constantly high abundance of photosynthetic picoplankton (mainly *Synechococcus*) during all seasons; similar results were found by Stenuite et al. (2009) in Tanganyika Lake, where the abundance of this group remained high throughout the year. Tropical lakes and reservoirs generally have a constant temperature during the entire year, with a monomictic thermal regime and a long period of stratification in deep water bodies (Lewis 2000).

Transparency and light conditions in the water column also have a major impact in the composition of the photosynthetic picoplankton (Sarmento et al. 2008). According to Stockner et al. (2000) underwater light conditions are very important for the growth and production of photosynthetic picoplankton, and their abundance and distribution within the water column can change rapidly with different thermal and light regimes. Waterbury et al. (1986) observed in laboratory experiments that the optimum growth rate of *Synechococcus* occurs under low light conditions. However, during *in situ* studies picocyanobacteria and picoeukaryotes have been found at a variety of depths and light irradiances (Nagata 1994, Callieri and Pinolini 1995); thus, suggesting that the penetration of light is directly related to vertical distribution of Pcy (Pick 1991).

The availability of light and nutrients exert a strong effect on the seasonal dynamics of APP (Winder 2009). According to Schindler (2006), phosphorus (P) is considered the limiting nutrient in temperate lakes. In contrast, studies in tropical lakes and ultraoligotrophic Patagonian Lakes show nitrogen (N) as the limiting nutrient of phytoplankton growth (Lewis 2002, Diaz et al. 2007). The importance of co-limitation by N and P has commonly been observed in marine, lakes,

and reservoirs systems throughout the world (Dzialowski et al. 2005, Smith 2006). Similar results were observed in Caribbean warm tropical reservoirs (Pantoja-Agreda et al. 2009), where the N/P ratios suggested a general trend towards co-limitation of both nutrients.

There is relatively scarce information on the structure, composition and the population dynamics of the freshwater autotrophic picoplankton, and factors controlling phytoplankton growth and productivity (Callieri and Stockner 2002). The high contribution of picoplankton to primary productivity, biomass and trophic dynamics in tropical lakes and reservoirs have not received the same level of inquiry that has been given to non-picoplanktonic cyanobacteria and other phytoplankton groups (Nagata et al. 1994, Ivanikova 2006). The aim of the present study is to expand our knowledge on the abundance and composition of picoplankton community of warm subtropical reservoirs, while addressing the link of these communities with environmental variables throughout an annual cycle.

Methods

Study area description

The research was carried out in Cerrillos Reservoir, a subtropical reservoir located in the south of Puerto Rico (18˚04'N; 66˚40'W), at an elevation of 170 meters above sea level (m a.s.l.). Constructed in 1991, this reservoir provides flood protection, water supplies and recreation for the city of Ponce (Soler-López 2011). The reservoir has a surface area of 1.32 km², a drainage area of 45.3 km²; the mean depth is 25 m with a maximum depth of 65 m near the dam. The annual average annual rainfall is 236 cm. The typical dry season is from December to March, and the wet season from May to November. Sedimentation has reduced the storage capacity of Cerrillos Reservoir from 38.03 in 1991 to 37.26 million m^3 in 2008 (Soler-López 2011), and its mean water residence time is 0.87 times yr^{-1}. Sotomayor et al. (2008) defined this reservoir as warm monomictic and described its temperature and dissolved oxygen dynamics.

Sampling and environmental parameters

Water samples (24) were collected monthly in the central region of the reservoir (25 m maximum depth) from January 2013 to December 2013, using a 2L horizontal Van Dorn bottle at surface, 1 %, 0.1 % and 0.01 % photosynthetically active radiation (PAR). Vertical profiles of temperature, dissolved oxygen, pH, conductivity and oxidation-reduction potential (redox) were obtained using a CTD12 multiparameter probe (Applied Microsystems Inc., Canada). Photosynthetically active radiation (PAR) was measured at different depths using an underwater Licor LI-192 quantum sensor. Vertical attenuation coefficients were calculated according to the equation of Kirk (1994). The euphotic zone was defined as the depth where PAR reaches 1% of surface PAR ($Z_{1\%}$). Total Nitrogen (TN) and Total Phosphorus (TP) concentrations were

determined after acid digestion by the molybdate-ascorbic acid method (Valderrama 1981). Nitrate and orthophosphate were analyzed according to Standard Methods (APHA 1998) after sample filtration through GF/F (Whatman, 0.7 μm nominal pore size) filters.

Picoplankton abundance and biomass estimation

Autotrophic picoplankton cells were analyzed using flow cytometry, and estimated cell biovolume measurements by epifluorescence microscopy. For the flow cytometric counts of APP populations, 4 mL freshwater samples were collected from each depth and fixed immediately using cold 25%-glutaraldehyde (final concentration 2%), stored in darkness at 4 C, and processed within 2 weeks. Samples were analyzed in an Accuri C6 (Becton Dickinson) flow cytometer equipped with an air-cooled argon laser (488 nm blue and 620 nm red excitation). Each sample was run for 4 min at fast flow rate (66 μL min^{-1}). Filters for fluorescence emission were used forward scatter (FSC) and side scatter (SSC), indicative of cell size, shape and refractive index, green fluorescence (FL1= 530 nm) from phycobilin, orange fluorescence (FL2= 585 nm) from phycoerythrin, and red fluorescence (FL3= 670 nm) from Chl-*a*. Fluorescent beads (yellow-green microspheres, Polysciences Inc.) of 1 μm diameter were used to calibrate the side scatter signal and to control the internal volume standards. Picocyanobacteria and picoeukaryotes populations were identified and enumerated using three-dimensional gate base on red fluorescence (FL3) versus side scatter (SSC), and red fluorescence (FL3) versus orange fluorescence (FL2). Flow cytometry data were collected and analyzed using CFlow sofware 2012.

Chl-*a* concentration was determined fluorometrically for total phytoplankton and picophytoplankton. Water samples of 250 mL were filtered on Whatman GF/F glass fiber filters (0.7 μm pore size) for the total Chl-*a* content. To measure chlorophyll *a* content of the picophytoplankton fraction, a 250-500 mL water sample was separated by differential filtration,

through a 2 μm pore size polycarbonate filter and the filtrate was subsequently filtered through a 0.2 μm pore size polycarbonate filter (Whatman, Nucleopore). Pigments were extracted in darkness and ≤ 500 mm Hg partial vacuum. The filters were frozen at -20 C and analyzed within a month. Chlorophyll was extracted following sonication of the filters for 5 min in 10 mL of hot 90% ethanol (Nusch 1980) and kept over 24 hours in darkness at 4 °C. The extracts were analyzed in a Turner Design 10-AU Fluorometer, using an emission wavelength of 650 nm before and after acidification with 100 μL of HCL 0.12N. Calibration of the fluorometer was done using Chl-*a* pure standard (Sigma C-6144, 1 mg).

Data Analysis

Analysis of variance (ANOVA) one-way was used to determine seasonal and vertical differences ($P<0.05$) in abiotic and biotic variables; the data were normalized using log transformation. Pearson correlations of picophytoplancton abundances and limnological variables were calculated for stratification and circulation periods. All statistical analyses were completed using MINITAB version 16.1. The values of autotrophic picoplankton abundance, Chl-*a*, and selected environmental parameters were plotted by isolines as functions of depth and time with the Surfer (Golden, Sofware 2012) program, using Kriging interpolation method.

Results

Environmental variables

The average surface water temperature was 28.1 ± 1.46 °C and 26.04 ± 1.16 °C at 25 m. Minimum water column temperature was found during Feb (24.3 °C at 25 m deep), whereas maximum temperatures were observed in Jul (28.3 °C at 1 m deep) during the summer season. A stable thermal stratification was observed during most of the year (Apr to Oct) with one mixing period in the dry and cold season (Nov to Mar). The average thermocline depth was 8.3 m and reaching a maximum of 12 m in Sep. The seasonal change in the vertical profile was typical of warm monomictic lakes. Maximum differences between the surface and bottom temperatures > 4.8 °C were observed in Aug. The largest gradient of temperature in the water column (about 1.2 °C m^{-1}) occurred during late stratification.

According to PAR values, the euphotic zone average was 8 m during the mixing period and 4.6 m during the stratification period. The Z_{eup} reached its maximum in Mar (9.8 m) and its minimum in Jul (4.6 m). The depth of 0.1% of PAR was deeper than 12 m and that of 0.01% PAR was often around 22 m.

The dissolved oxygen (DO) concentration generally was higher in the upper water layer than in the lower and followed the dry and wet seasonality. The average O_2 in the water column was 3.26 ± 0.61 mg L^{-1}. The average concentration at the surface was 7.61 ± 0.72 mg L^{-1} while 2.26 ± 1.3 mg L^{-1} below the thermocline. Lake Cerrillos developed a clinograde DO profile during the months of Jul and Aug, with DO concentrations greater than 7 mg L^{-1} in the epilimnion, and anoxic conditions in the bottom with DO concentrations below 1 mg L^{-1}. The hypolimnion remained anoxic during a large fraction of the stratified period. In the cold and dry months (Dec

to Mar) a trend to mixture is observed, forming a more homogeneous water column, where the average DO difference between the surface and the bottom is 3.6 mg L^{-1}.

Nitrogen and phosphorus concentrations in the Cerrillos Reservoir fluctuated through the year. The mean photic zone TN concentration was 0.21 ± 0.06 mg L^{-1}, with minimum values (0.09 mg L^{-1}) during Dec and maximum values (0.33 mg L^{-1}) during May. A similar pattern was observed for TP, with mean photic zone concentration of 0.01 ± 0.0019 mg L^{-1}, minimum and maximum values of 0.006 mg L^{-1} and 0.011 mg L^{-1} in Nov and Jun, respectively. The patterns of seasonal change in the levels of TN and TP showed a similar tendency, higher concentrations during the summer months (May-Sep) and lower at the onset and throughout the mixing period of the cold and dry months (Nov-Feb). The mean concentration of nitrate in the photic zone was 0.025 ± mg L^{-1}; varied from 0.001 mg L^{-1} in Apr to 0.18 mg L^{-1} in Jul. The nitrate concentrations did not exhibit any large differences between the upper and lower water layers in the reservoir. Phosphate concentrations were always low, below the detection limits of the methods used (<0.005 mg L^{-1}).

Chlorophyll-a concentration of total and APP size fractions

The mean photic zone total Chl-*a* concentration in Cerrillos was 7.54 ± 3.41 μg L^{-1}, with minimum values in Apr (4.50 μg L^{-1}) during the mixing to 1% of surface PAR (6-8 m) Fig 3A. Higher concentrations were measured during the stratification in the upper layer (0-1 m) corresponding to 99 % of surface PAR with maximum values in Aug (24.4 μg L^{-1}) (Fig. 3A). Autotrophic picoplankton chlorophyll-*a* concentration (≤ 2 μm fraction) was generally low with a mean photic zone concentration of 0.72 ± 0.28 μg L^{-1}. The maximum value was observed in Jun (1.99 μg L^{-1}) during the thermal stratification in the surface and the minimum in Feb (0.24 μg L^{-1})

at a depth of 6-8 m ($Z_{1\%}$) during the circulation period (Fig. 2). The average contribution of APP to total Chl-*a* concentration in the surface, estimated by size fractionation, during the study was 10.9 % and varied from 0.99% (Sep) to 20.5% (Jun) (Fig. 3B). Chl-*a* concentration reflected the annual variation in picoplankton density, its concentration being higher during summer, when solar radiation was also higher. The average difference in Chl-*a* concentration between the surface and the limit of the photic zone was 0.55 μg L^{-1}. Concentrations reached similar values in the entire column during the mixed period. The highest peaks were obtained in the rainy season and lowest during the dry season. The Pearson correlations analysis showed that Chl-*a* concentrations had significant positive correlations ($P<0.001$) with nitrate and TN ($r=0.54$ and $r=0.62$, respectively) and negative correlations with pH and DO. Chl-*a* was significantly different between two depths: 1% and 0.1% surface PAR (ANOVA $P<0.05$).

Picoplankton abundances

According to the size determinations and the predominant pigments analyzed by flow cytometry and epifluorescence microscopy, different populations of APP were identified in the Cerrillos Reservoir. These were composed of small prokaryotic and eukaryotic cells: one population of phycoerythrin-rich picocyanobacteria (*Synechococcus*) solitary coccoid, ovoid cells and two picoeukaryotes populations.

Picocyanobacteria characterized by their high phycoerytrhrin (orange fluorescence) were dominant APP during most of the year. The mean concentration of total Pcy in the photic zone was of 2×10^4 cell mL^{-1}, with minimum values in Feb (0.6×10^4 cell mL^{-1}) at $Z_{1\%}$ during the mixing period (Fig. 4) Higher concentrations were measured during the stratification period with maximum values in Jun (6.6×10^4 cell mL^{-1}) at surface. The vertical profiles showed high

abundance in the photic zone and a considerable decrease below the thermocline (Fig. 6). At $Z_{0.01\%}$ (22 m) the Pcy abundance was the lowest (0.05-0.1 $\times$ 10^4 cell mL^{-1}). The Pearson correlations between Pcy and environmental variables showed that the abundance was positively correlated with nitrate ($r=0.75$, $P<0.001$), TN ($r=0.82$, $P<0.001$), light availability ($r=0.65$, $P<0.001$) and chlorophyll-a ($r=0.70$, $P<0.001$).

The annual pattern of picoeukaryotes, characterized by their high chlorophyll-*a* (red fluorescence), showed a less abundant and more uniformly distributed population than Pcy. The overall average abundance in the euphotic zone was of 1.2 $\times$ 10^4 cell mL^{-1}, with maximum values of 2.1 $\times$ 10^4 cell mL^{-1} in Oct, at the surface, while the abundance decreased to a minimum of 0.3 $\times$ 10^4 cell mL^{-1} in Jul to 1% of surface PAR (Fig. 5). No statistically significant differences were observed among depths within the euphotic zone (ANOVA $P<0.05$). In contrast to Pcy, the picoeukaryotes density was only positively related to light availability ($r=0.45$, $P<0.001$) and chlorophyll-*a* ($r= 0.46$, $P<0.001$).

Discussion

The Cerrillos reservoir behaves as a warm monomictic system, with a mixing period during the cool dry season (Nov to Feb) and a stable stratification during most of the year (Mar to Oct). Similar stratification and mixing patterns in reservoirs of Puerto Rico were found by Pantoja-Agreda et al. (2009) and Sotomayor et al. (2008), as well as in other latitudes (Macek et al. 2009, Hernandez-Aviles et al. 2010). The high surface temperatures (>28 °C) and a long period of thermal stratification are features of Cerrillos reservoir, allowing higher rates of decomposition in the hypolimnion, restricting the distribution of picoplankton and other aerobic organisms to the top 12 m surface layer. The depth (Zmax≈ 65 m) and hydraulic residence time (0.87 times yr^{-1}) (Soler-Lopez 2011) that characterize this reservoir sustain a long and stable stratification during the summer, allowing for a better adaptation and development of autotrophic picoplankton. The formation of a stable thermocline during most of the year decreases nutrient transport to the epilimnion, and results in low hypolimnetic oxygen concentration and picoplankton abundance. The high temperatures, approaching 26 °C in the hypolimnion, support high oxygen consumption creating anoxic conditions and subsequently high anaerobic microbial metabolism in deeper areas during most of the year.

Environmental and hydrological characteristics of the Cerrillos Reservoir are decisive for the composition and abundance of autotrophic picoplankton. The temporal and vertical dynamics of autotrophic picoplankton coincide with the period of circulation and stratification. Picocyanobacteria abundance showed a unimodal seasonal cycle throughout the year, increasing exponentially in late May after the onset of stratification, and reached the largest number of organisms in the summer during the full stratification. The high peaks of abundance were mainly

in Jun with higher densities than 6×10^4 cells mL^{-1}. This unimodal pattern is different to that which occurs in most lakes in temperate zones, where the peaks of Pcy follow a typical bimodal pattern with high concentrations in spring or early summer and a second peak in the summer or fall (Stockner et al. 2000).

The increase in picocyanobacterial abundance during the stratification was possibly linked to the thermal structure of the lake, which affects the dynamics of Pcy in response to density gradients caused by the thermocline, that prevents sedimentation of organisms to deeper layers (Callieri et al. 2012). According to Winder and Hunter (2008), changes in the thermal stratifications processes affect the temporal and vertical dynamics of phytoplankton assemblages. The positive correlation between the abundance of Pcy and temperature ($r = 0.40$ $P < 0.01$) indicates that the increase in water temperature in summer promotes the growth of autotrophic picoplankton in the photic zone, and corroborate the results of other studies showing that the higher water temperatures during the stratification has a positive relationship with high concentrations of autotrophic picoplankton (Callieri and Piscia 2002, Crosbie et al. 2003, Horn and Horn 2008). The increase in picoplankton abundance in the epilimnion and its relationship with higher temperatures have been observed in other lakes and reservoirs, including Alchichica (Macek et al. 2009), Kinneret (Yacobi 2006) and Saidenbach (Horn and Horn 2008). The abundance of the Pcy in the period of stratification decreases significantly at depths below 0.1% PAR, possibly by the light conditions and the long period of anoxia that prevails most of the year below metalimnion. In the colder months, when the water temperature decreases, changes in the abundance coincides with the mixing period, resulting a decrease in the density of Pcy from Dec to Mar. The densities of Pcy have greater variations in the dry season when mixing occurs in the water column. This breaking of the thermocline during the circulation period allows greater light penetration, as is evidenced

by microcolonies of Pcy observed at depths of $Z_{0.1\%}$. According to Moore (1995), light limitation severely restricts the development of *Synechococcus* to deeper layers due to the absence of accessory pigments (Chl-b_2).

The abundance of Peuk was two times lower than Pcy, and with a temporal pattern different to from Pcy. Sarmento et al. (2008) and Stenuite et al. (2009) have found to be two orders of magnitude lower in Peuk density relationship to Pcy in tropical lakes. In this study, the greatest abundances of Peuk were found in Oct and Nov at the end of stratification. In contrast, a study on Lake Tahoe showed that Peuk have opposite seasonal dynamics, the maximum abundance was during the mixed period in late winter and early spring (Winder 2009). In our study, during mixing the picoeukaryotes density decreases, and its distribution in the water column was similar. Increased density picoeukaryotes at the end of stratification and the beginning of the circulation period, can be related to resuspension and nutrient availability from hypolimnion toward outer layers, regardless of loss in light penetration.

Other important factors that may be limiting the composition and abundance of autotrophic picoplankton are water transparency and light penetration. In the Cerrillos Reservoir, the highest concentration of APP was present in the photic zone ($Z_{1\%}$ = 6.5 m), similar to other tropical marine ecosystems (Atlantic and Pacific) and freshwater bodies (Lake Tahoe); the picocyanobacteria of the genus *Synechococcus* dominates the surface layers and mostly above the thermocline (DuRand et al. 2001, Winder 2009). The vertical profiles indicate that growth of Peuk and Pcy is limited to the shallower region of the epilimnion. This was observed in Sep, with peak concentrations at 25% PAR (depth 2 meters), and APP abundances decreased significantly at depths below 1% PAR. According to Passoni et al. (1997; cited by Callieri and Stockner 2002), autotrophic picoplankton shows maximum abundance peaks between 25 and 50 % PAR in large and deep lakes. The great

adaptability of Pcy to different light regimes allows greater development in high light (Postius and Boger 1998). Pcy concentration during the summer in the superficial layers with higher solar radiation appears to be controlled by light regime, which promotes higher density of phycoerythrin-rich species (i.e. *Synechococcus* spp.) more resistant to high-irradiance than other picoplanktonic organisms such as *Prochlorococcus* spp. and picoeukaryotes (Sommaruga et al. 2005). The Pcy of tropical freshwater ecosystems are well adapted to high solar radiation on the surface (> 1500 μE $m^{-2}s^{-1}$). Stenuite et al. (2009) observed that the Pcy numbers were high in the photic zone, and that these organisms are well suited to high light conditions that prevail throughout the year in Tanganyika, a tropical lake where phytoplankton may be exposed to irradiance above 2000 μE $m^{-2}s^{-1}$ at the surface level.

The biomass obtained by fractionated filtrations showed temporal and vertical dynamics in accordance to the distribution of autotrophic picoplankton. Two peaks were observed during the study period: the first, in full stratification (June), corresponding to maximum picocyanobacteria; and the second, at the end of the stratification and start of mixing (Oct-Nov), which coincides with the highest peak of the picoeukaryotes. Chl-*a* concentration in the water column also coincides with the vertical variation of picoplankton with depth. The highest concentration was found at the top of the photic zone, between 0 and 2 meters deep. No Chl-*a* deep peaks were observed during the stratification period. Only one Chl-*a* peak was observed: 1.18 μg L^{-1} at 12 meters ($Z_{0.01\%}$). This observation coincided with the relaxation of the thermocline in the month of Nov during the onset of mixing. This deeper peak coincided with a pulse in picoeukaryotes density, which has been attributed to a decrease in solar irradiance and predation (Pilati and Wurtsbaugh 2003) and increased availability of nutrients from the bottom, which favors Peuk growth relative to that of Pcy (Callieri and Stockner 2002). In our study, APP contribution to the total phytoplankton

biomass, expressed in terms of Chl-*a*, was on average 11 %. In summer, APP became responsible for up to 21 % of photosynthetic activity, with values within the range from 0.2 to 43 % reported for fresh water ecosystems (Stockner 1988). APP biomass showed a contrasting behavior with the >2 μm fraction phytoplankton. In May, APP chlorophyll (< 2 μm fraction) increased exponentially and reached its maximum peak in Jun (1.99 μg L^{-1}), while Chl-*a* concentration in the larger fraction decreased. The opposite trend was observed in Aug and Sept, when a maximum concentration of Chl-*a* was observed for the fraction > 2 μm and minimum concentrations for the APP. The increase in total chlorophyll was related to a bloom of *Ulnaria ulna,* a large diatom species that dominated phytoplankton communities at Cerrillos Reservoir during summer.

According to Carlson trophic-state index, Cerrillos reservoir was classified as in transition from oligotrophic to mesotrophic (Pantoja et al 2016, Sotomayor et al. 2009). The high abundance of APP is associated with oligotrophic ecosystems (Stocker 1991) since higher surface-to-volume in Pcy and Peuk enable these species to compete advantageously for nutrients against larger phytoplankters, especially during low nutrient conditions (Vörös et al. 1998, Raven 1998, Drakare et al. 2003, Cellamare et al. 2010).

Pcy abundance and biomass were highest in summer and decreased gradually in winter, similar to the dynamics of TN, which reached its maximum concentration during full stratification. Both abundance and biomass of Pcy showed positive correlations with nitrate and TN concentrations. These correlations suggest that rain, runoff and river discharge patterns modulate changes in the development of autotrophic picoplankton populations. This “nitrate” trend was not observed for phosphate, as it was always undetected, suggesting that this form of phosphorus is precipitated rapidly and, thus may play a role in limiting phytoplankton growth in this system. Although TP was indeed detected, no covariation with APP was found suggesting that this form

of phosphorus may not be rapidly cycled or it has limited importance for the production of autotrophic picoplankton (Horn and Horn 2008). Similarly, Lavallée and Pick (2002) did not find correlative support for inorganic phosphorus stimulation of APP growth rates.

The present study suggests that the penetration of light, nutrient availability and trophic status of the reservoir are important factors that determine the abundance and biomass of autotrophic picoplankton. Understanding the ecology of picoplankton and their response to environmental change shows that it is a good indicator of the trophic state of reservoirs and may have important implications for the management of water quality, especially the potential use of reservoirs for drinking water.

Acknowledgments

We are grateful to the Department of Natural Resources group for access to the facilities and their help in field. Jose Almodovar for microscopy laboratory assistance. We thank Dr. Maribella Domenech for the introduction to and assistance with the flow cytometry. Thanks are due Dr. Ingrid Padilla and Perla Torres for their laboratory support and nutrients analysis, and to Dr. Carlos Santos for his constructive comments to previous versions of this manuscript.

References

American Public Health Association. 1998. Standard methods for the examination of water and wastewater. Am. Publ. Health Ass. Washington.

Callieri C. 2007. Picophytoplankton in freshwater ecosystems: the importance of small sized phototrophs. Freshwater Rev. 1:1-28.

Callieri C. 2010. Single cells and microcolonies of freshwater picocyanobacteria: a common ecology. J Limnol. 69:257-277.

Callieri C, Caravati E, Corno G, Bertoni R. 2012. Picocyanobacterial community structure and space-time dynamics in the subalpine Lake Maggiore (N.Italy). J Limnol. 71:95-103.

Callieri C, Karjalainen SM, Passoni S. 2002. Grazing by ciliates and heterotrophic nanoflagellates on picocyanobacteria in Lago Maggiore, Italy. J. Plankton Res. 24:785-796.

Callieri C, Pinolini ML. 1995. Picoplankton in Lake Maggiore, Italy. Int. Rev. ges. Hydrobiol. 80:491-501.

Callieri C, Piscia R. 2002. Photosynthetic efficiency and seasonality of autotrophic picoplankton in Lago Maggiore after its recovery. Freshwater Biol. 47:941-956.

Callieri C, Stockner JG. 2002. Freshwater autotrophic picoplankton: a review. J Limnol. 61:1-14.

Caron DA, Pick FR, Lean DRS. 1985. Chroococcoid cyanobacteria in Lake Ontario: seasonal and vertical distribution during 1982. J Phycol. 21:171-175.

Cellamare M, Rolland A, Jacquet S. 2010. Flow cytometry sorting of freshwater phytoplankton. J Appl Phycol. 22:87-100.

Cotner JB, Biddanda BA, 2002. Small players, large role, microbial influence on biogeochemical processes in pelagic aquatic ecosystems. Ecosystems. 5:105-121.

Crosbie ND, Teubner K, Weisse T. 2003. Flow cytometric mapping provides novel insights into the seasonal and vertical distributions of freshwater autotrophic picoplankton. Aquat Microb Ecol. 33:53-66.

Diaz M, F. Pedrozo, C. Reynolds P, Temporetti. 2007. Chemical composition and the nitrogen-regulated trophic state of Patagonian lakes. Limnologica. 37:17-27.

Dove A, Chapra SC. 2015. Long-term trends of nutrients and trophic response variables for the Great Lakes. Limnol Oceanogr. 60:696-721.

Drakare S, Blomqvist P, Bergström AK, Jansson M. 2003. Relationships between picophytoplankton and environmental variables in lakes along a gradient of water colour and nutrient content. Freshwater Biol. 48:729-740.

DuRand MD, Olson RJ, Chisholm SW. 2001. Phytoplankton population dynamics at the Bermuda Atlantic Time-series station in the Sargasso Sea. Deep-Sea Res. 48:1983-2003.

Dzialowski AR, Wang SH, Lim NC, Spotts WW, Huggins DG. 2005. Nutrient limitation of phytoplankton growth in central plains reservoirs, USA. J Plankton Res. 27:587-595.

Hernández-Avilés JS, Macek M, Alcocer J, López-Trejo B, Merino-Ibarra M. 2010. Prokaryotic picoplankton dynamics in a warm-monomictic saline lake: temporal and spatial variation in structure and composition. J Plankton Res. 32:1301-1314.

Horn H, Horn W. 2008. Bottom-up or top-down – How is the autotrophic picoplankton mainly controlled? Results of long term investigations from two drinking water reservoirs of different trophic state. Limnologica. 38:302-312.

Ivanikova 2006. Lake Superior phototrophic picoplankton: nitrate assimilation measured with a cyanobacterial nitrate-responsive bioreporter and genetic diversity of the natural community. Doctoral Thesis. College of Bowling Green State University.

Ivanikova NV, Popels LC, McKay RML, Bullerjahn GS. 2007. Lake Superior supports novel clusters of cyanobacterial picoplankton. Appl Environ Microbiol. 73: 4055-4065.

Kirk JTO. 1994. Light and Photosynthesis in Aquatic Ecosystems. Inited Kingdom. Cambridge University Press. 509p.

Lavallée BF, Pick FR. 2002. Picocyanobacteria abundance in relation to growth and loss rates in oligotrophic to mesotrophic lakes. Aquat Microb Ecol. 27:37-46.

Lewis WM Jr. 2000. Basis for the protection and management of tropical lakes. Lake Reserv. Manage. 5:35-48.

Lewis WM Jr. 2002. Causes for the high frequency of nitrogen limitation in tropical lakes. Verh. Internat. Verein. Limnol. 28:210-213.

Macek M, Alcocer J, Lugo-Vázquez A, Martínez-Pérez ME, Peralta-Soriano L, Vilaclara-Fatjó G. 2009. Long term picoplankton dynamics in a warm-monomictic, tropical high altitude lake. J Limnol. 68:183-192.

Moore L. R, Goericke R. Chisholm S. W. 1995. Comparative physiology of *Synechococcus* and *Prochlorococcus:* influence of light and temperature on growth, pigments, fluorescence and absorptive properties. Mar. Ecol. Prog. Ser. 116:259-275.

Nagata TK, Takai K, Kawanobe D, Kim R, Nakazato N, Guselnikova N, Bondarenko O,

Mologawaya T, Kostrnova V, Drucker Y, Satoh Y. Watanabe. 1994. Autotrophic picoplankton in southern Lake Baikal: abundance growth and grazing mortality during summer. J Plankton Res. 16: 945-959.

Nusch EA. 1980. Comparison of different methods for chlorophyll and phaeopigments determination. Arch. Hydrobiol. Beih. Ergebn. Limnol. 14: 14-36.

Pantoja-Agreda F, Sotomayor D, Martínez G. 2009. Phytoplankton dynamics of de Guajataca Reservoir. Verth. Internat. Verein Limnogoly. Vol 30. part 7:1096-1100.

Passoni S, Callieri C, Heinimaa S. 1997. Dinamiche di distribuzione del picoplancton autotrofo nel Lago Maggiore. In: Callieri and Stockner 2002.

Pick FR. 1991. The abundance and composition of freshwater picocyanobacteria in relation to light penetration. Limnol Oceanogr 36:1457-1462.

Pilati A, Wurtsbaugh W. A. 2003. Importance of zooplankton for the persistence of a deep chlorophyll layer: a limnocorral experiment. Limnol Oceanogr. 48: 249-260.

Postius C, Böger P. 1998. Different interactions of phycoerythrin-rich and phycocyanin-rich *Synechococcus* spp. with diazotrophic bacteria from the picoplankton of Lake Constance. Arch Hydrobiol. 141:181-194.

Raven JA. 1998. The twelfth Tansley Lecture. Small is beautiful: the picophytoplankton. Funct. Ecol. 12:503-513.

Sarmento H, Unrein F, Isubmisho M, Stenuite S, Gasol J, Descy JP. 2008. Abundance and distribution of picoplankton in tropical oligotrophic Lake Kivu, eastern Africa. Freshwater Biol. 53:756-771.

Schindler DW. 2006. Recent advances in the understanding and management of eutrophication. Limnol Oceanogr. 51:356-363.

Smith VH. 2006. Responses of estuarine and coastal marine phytoplankton to nitrogen and phosphorus enrichment. Limnol Oceanogr. 51:377-384.

Soler-Lopez L.R. 2011. Sedimentation survey of Lago Cerrillos, Ponce, Puerto Rico, April-May 2008: U.S. Geological Survey Scientific Investigations Report 2011-5057.

Sommaruga R, Hofer JS, Alonso-Saez L, Gasol JM. 2005. Differential sunlight sensitivity of picophytoplankton from surface Mediterranean coastal waters. Appl Environ Microbiol. 71: 2154-2157.

Sotomayor D, Martínez G, Pantoja F. 2008. Limnological Assessment Of Two Reservoirs In Puerto Rico. Verth Internat Verein Limnogoly. Vol 30. part 3:521-527

Stenuite S, Tarbe AL, Sarmento H, Unrein F, Pirlot S, et al. 2009. Photosynthetic picoplankton in Lake Tanganyika: biomass distribution patterns with depth, season and basin. J Plankton Res. 31:1531-1544.

Stockner JG. 1988. Phototrophic picoplankton: An overview from marine and freshwater ecosystems. Limnol Oceanogr. 33:765-775.

Stockner JG. 1991. Autotrophic picoplankton in freshwater ecosystems: the view from the summit. Int Rev ge. Hydrobiol. 76:483-492.

Stockner J, Callieri C, Cronberg G. 2000. Picoplankton and other non-bloom forming cyanobacteria in lakes. In: Whitton, B.A., and M. Potts (Eds), The Ecology of Cyanobacteria. Their Diversity in Time and Space. Kluwer Academic Publishers, Dordrecht, The Netherlands: 195-238.

Valderrama JC. 1981. The simultaneous analysis of total nitrogen and total phosphorus in natural waters. Mar Chem. 10:109-122.

Vörös L, Callieri C, Balogh KV, Bertoni R. 1998. Freshwater picocyanobacteria along a trophic gradient and light quality range. Hydrobiologia. 369/370:117–125.

Waterbury J, Watson W, Valois FW, Franks DG. 1986. Biological and ecological characterization of the marine unicellular cyanobacterium *Synechococcus*. Can Bul. Fish Aquat Sci. 214:71-120.

Winder M. 2009. Photosynthetic picoplankton dynamics in Lake Tahoe: temporal and spatial niche partitioning among prokaryotic and eukaryotic cells. J Plankton Res. 31:1307-1320.

Winder M, Hunter DA. 2008. Temporal organization of phytoplankton communities linked to chemical and physical forcing. Oecologia. 156: 179-192.

Worden A. 2004. Assessing the dynamics and ecology of marine picophytoplankton: the importance of the eukaryotic component. Limnol Oceanogr. 49:168-179.

Yacobi YZ. 2006. Temporal and vertical variation of chlorophyll a concentration, phytoplankton photosynthetic activity and light attenuation in Lake Kinneret: Possibilities and limitations for simulation by remote-sensing. J Plankton Res. 28:725-736.

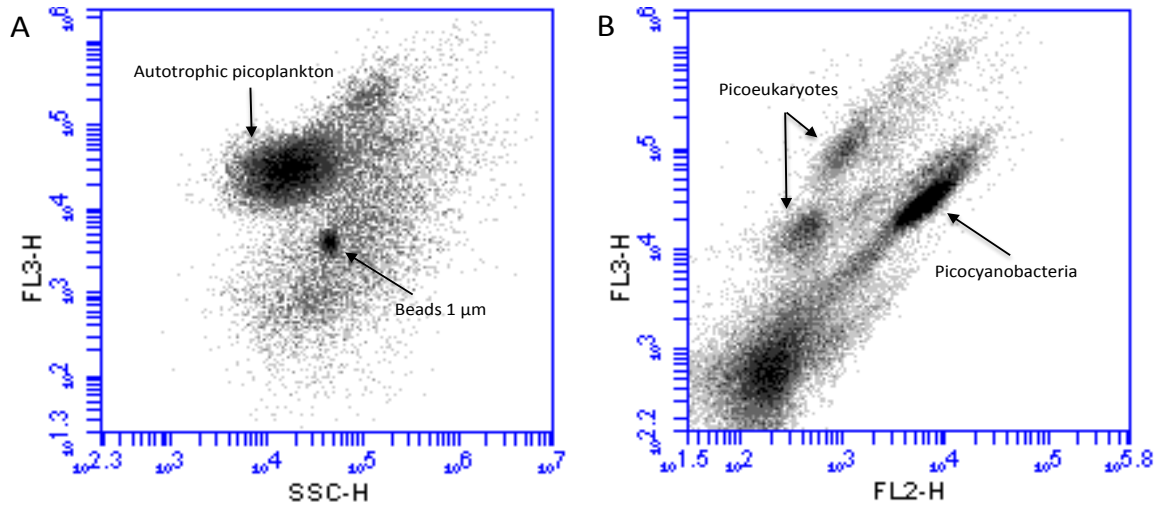

Figure 1. Cytograms by flow cytometry analysis samples from the surface water: A. Chlorophyll (FL3) vs. side scatter (SSC) and B. Chlorophyll (FL3) vs. phycoerythrin (FL2) fluorescence.

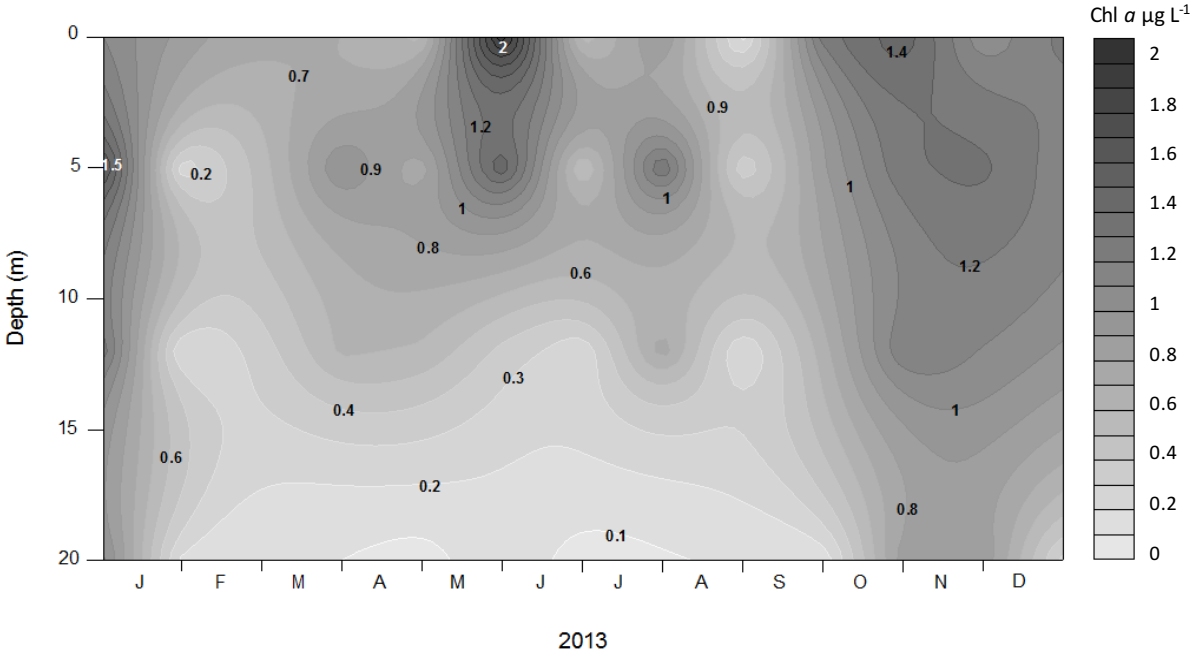

Figure 2. Seasonal and vertical distribution of chlorophyll *a* concentration (µg L^{-1}) for the picoplankton fraction in the Cerrillos Reservoir.

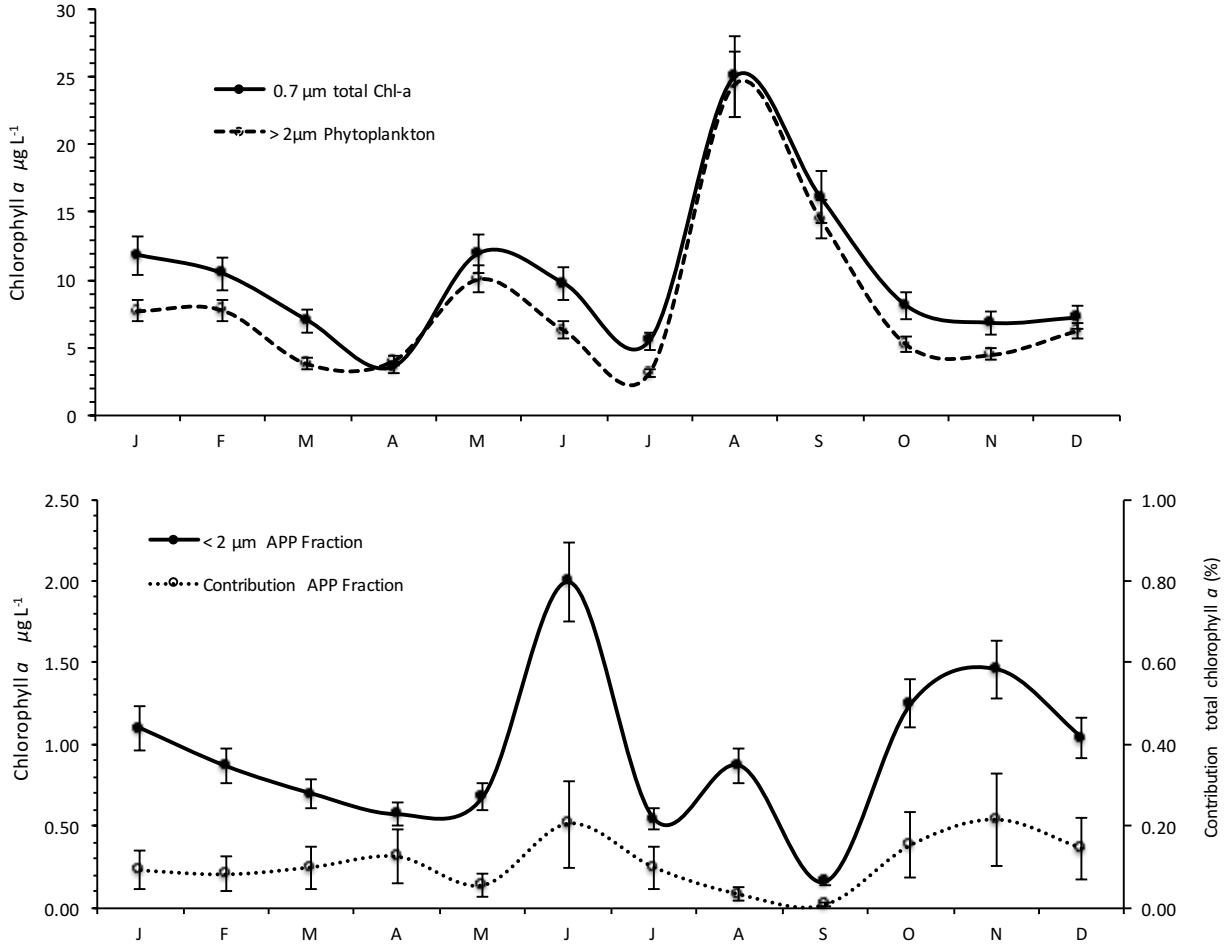

Figure 3. Total chlorophyll-*a* (0.7 µm fraction) and > 2 µm fraction of phytoplankton (A). Autotrophic picoplankton (APP) chlorophyll-*a* concentrations (< 2 µm fraction) and their relative contributions to total chlorophyll-*a* in the Cerrillos Reservoir, Puerto Rico from January to December 2013 (B). Bars represent ± standard error.

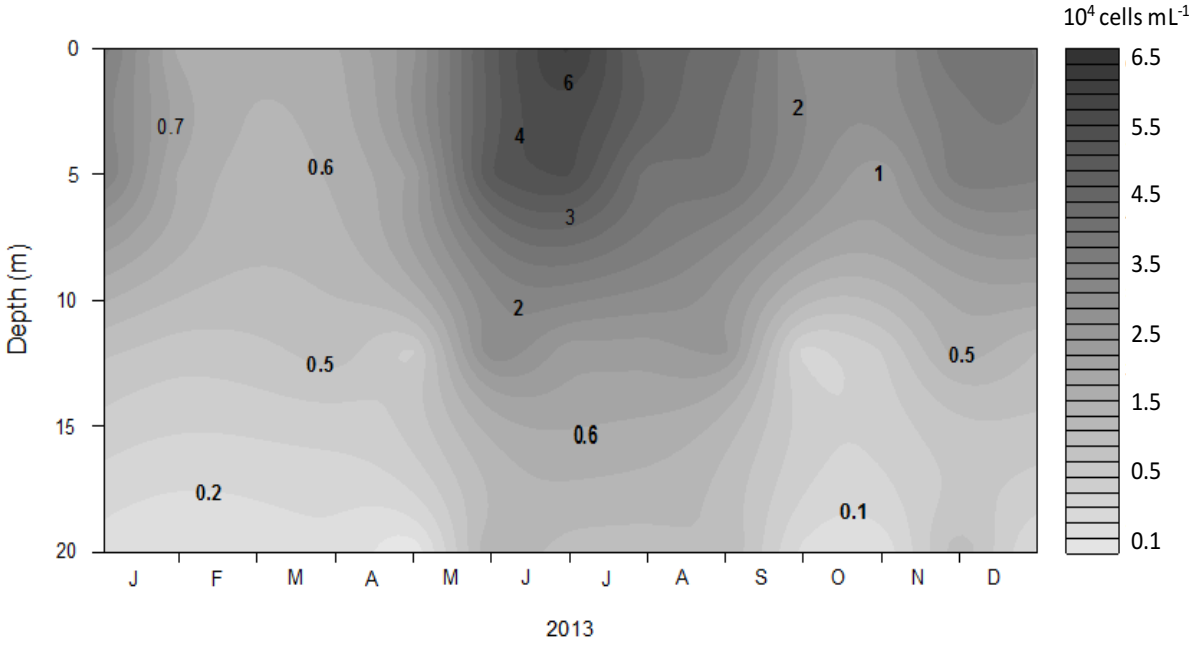

Figure 4. Seasonal and vertical distributions of picocyanobacteria (cells mL^{-1}) in the Cerrillos Reservoir.

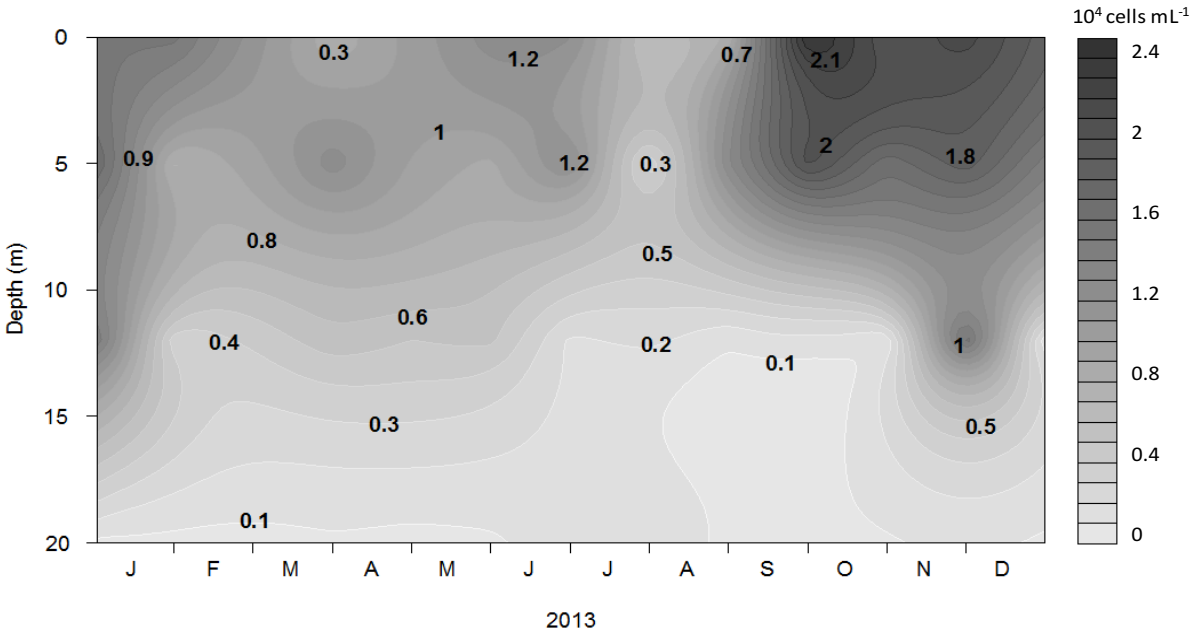

Figure 5. Seasonal and vertical distributions of picoeukaryotes (cells mL^{-1}) in the Cerrillos Reservoir.

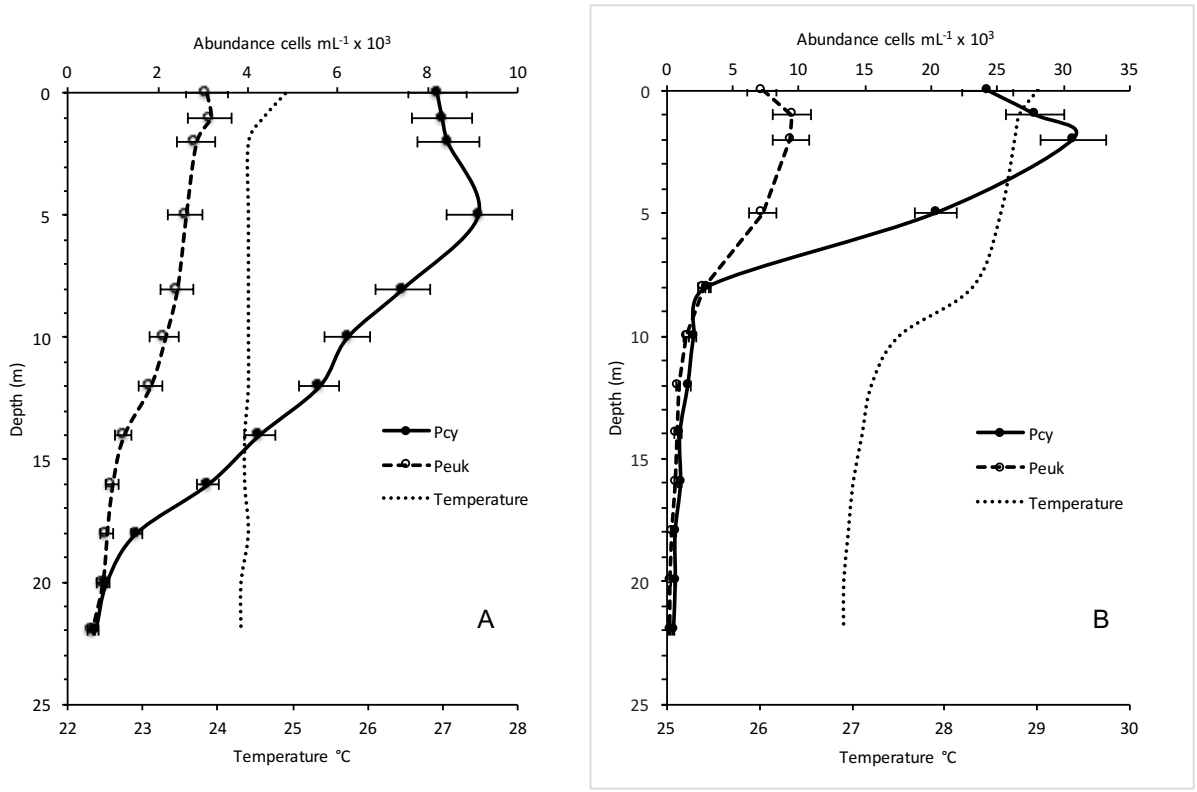

Figure 6. Vertical profiles of temperature (°C), picocyanobacteria (Pcy, cells mL^{-1}) and picoeukaryotes (Peuk, cells mL^{-1}) during: A. Mixis (Mar) and B. Stratification (Sep) in Cerrillos Reservoir from January to December 2013. Bars represent ± standard error.

Chapter III

Picocyanobacteria communities in two warm subtropical reservoirs of contrasting trophic state: environmental factors controlling their distribution and biomass

Fernando Pantoja-Agreda[1*], Carlos J. Santos-Flores[2], Ernesto Otero-Morales[1]

[1]Department of Marine Sciences, University of Puerto Rico at Mayagüez
[2]Department of Biology, University of Puerto Rico at Mayagüez
PO Box 9000, Mayagüez 00681 Puerto Rico

[*]Corresponding author: fernando.pantoja@upr.edu

Abstract

We compared the limnological conditions and seasonal dynamics of the picocyanobacteria community, in two tropical reservoirs of Puerto Rico with a different trophic state. Differences in environmental parameters, abundance and biomass of picoplankton between reservoirs and depths were analyzed. Both reservoirs are warm monomictic and they remain stratified during most of the year. The dissolved oxygen in the surface was high for both reservoirs; however, the low solubility of dissolved oxygen at high temperatures in the hypolimnion, and a high microbial metabolism in deep layers produces anoxic conditions during the stratification. The application of the Carlson's Trophic State Index showed the Cerrillos Reservoir as mesotrophic and Lucchetti as eutrophic. The flow cytometry technique allowed to clearly differentiate the picocyanobacteria from other picoplankton groups in both systems. The results suggest that there are differences in water quality and the picoplankton community between the two reservoirs. The picocyanobacteria were present throughout the year in both reservoirs, however their abundance and biomass was significantly greater in the mesotrophic reservoir than in the eutrophic one. The temporal and vertical dynamics of picocyanobacteria was consistent with the period of stratification and mixing, showing a unimodal pattern with maximum abundance peak during the summer. No significant correlation was found between picocyanobacteria abundance and total phosphorus. This could explain the structure and dynamics of picocyanobacteria which does not depend on the supply of phosphorus in the systems. Picocyanobacteria exhibit high plasticity and can easily adapt to eutrophic conditions; however,

they were best developed in an oligomesotrophic environment, where transparency of water is higher and nutrient concentrations are low, especially when phosphorus is limiting.

Keywords: Eutrophication, Picoplankton, Chlorophyll, Anoxia, Tropical, Flow cytometry.

Introduction

Eutrophication is a process associated with the nutrient enrichment of the aquatic systems. The increase in nutrient load experienced by these systems comes primarily from human activities. These increased loads affect the phytoplankton structure and dynamics, changing its diversity and species abundances, altering primary production and causing the deterioration of water quality (Shindler 2006). An increased primary production usually decreases light penetration and the lower levels within the aquatic systems experience periods of hypoxia or anoxia.

In Puerto Rico, man-made lakes or reservoirs are crucial as water supplies for human consumption and agriculture, flood control, and recreation. Understanding the relationships between trophic state and phytoplankton is essential for the management of these reservoirs. Phytoplankton composition has been considered worldwide as an excellent bioindicator of trophic state because it is determinated by the biological, chemical and physical properties of the systems (Reynolds 1984).

Picoplankton is the fraction of plankton comprised by organisms in the 0.2 -2 μm size range, and it encompasses autotrophic and heterotropic species. This community plays a major role in freshwater and marine ecosystems as a source of energy and nutrient cycling and biochemical cycles (Azam et al. 1983, Li and Harrison 2001). The autrotrophic picoplankton (APP) can contribute to over 70% of the total primary productivity in aquatic ecosystems (Stockner & Antia 1986, Worden et al. 2004, Callieri 2008). APP is primarily composed by picocyanobacteria (Pcy) and, to lower extent, Picoeukaryotes (Peuk). APP in marine environments

is dominated by Pcy in the genera *Synechococcus* and *Prochlorococcus* (Chisholm 1988), while phycoerythrin-rich cyanobacteria in the genus *Synechococcus* dominate in freshwaters (Stockner et al. 2000, Callieri et al. 2007).

In tropical areas, picoplankton is strongly influenced by rain and drought regimes (Horne & Goldman 1994). Picocyanobacteria, in particular, respond to changes in chemical and physical conditions. Differences in composition, productivity and biomass of Pcy have been measured in freshwater systems with contrasting trophic states (Stockner 1991). Winder (2009) observed dominance by Pcy in the water column of oligotrophic lakes, with maximum values of Pcy abundance under isothermal conditions and a uniform distribution during mixing periods. In an alpine oligo-mesotrophic lake light proved to be more limiting than nutrient concentrations for Pcy growth (Crosbie et al. 2003).

Horn & Horn (2008) found a lower relative contribution of APP in a mesotrophic lake than in an oligotrophic one. However, in some temperate eutrophic lakes APP had high contributions to annual fixed carbon (Vorös et al. 1991). Similar abundances of APP were observed in tropical oligrotrophic lakes from Africa as compared to temperate eutrophic lakes. understood that light availability, trophic web structure and competition among species were the main controlling factors for APP in these systems (Sarmento et al. 2008).

According to the model proposed by Stockner (1991), oligotrophic waters that have low phosphorus concentrations are characterized by a high abundance of APP and these microbes make a major contribution to photosynthetic biomass. The objective of the present study was to relate the temporal and vertical variations of Pcy with environmental parameters in two subtropical reservoirs of contrasting trophic states.

Materials and Methods

Study sites

Cerrillos and Lucchetti are warm subtropical reservoirs located in the south and southwestern of Puerto Rico, respectively. The morphometric characteristics, location and uses of these two reservoirs are summarized in Table 1.

Sampling

The freshwater samples (24) were taken monthly in the course of an annual study in both reservoirs. A single sampling station was located in the deepest area. Biological and chemical samples were obtained using a 2 Liters Van Dorn bottle at different depths between 0 and 25 meters: surface 99% Photosynthetically active radiation (PAR), limited photic zone (1% PAR), metalimnion or depth mixing (0.1% PAR) and hypolimnion (0.01% PAR). Vertical profiles of temperature, pH, dissolved oxygen, conductivity, irradiance (PAR), total dissolved solids (TDS) and oxidation-reduction potential were measured using a multiparameter data sonde (Hydrolab DS4a, Loveland, CO, USA). Water transparency was determined with a Secchi Disk and euphotic zone (Zeu) was defined as the depth where PAR was 1% of surface light.

Chemical analysis

Total Nitrogen (TN) and Total Phosphorus (TP) were analyzed by acidic and buffered persulphate oxidation method (Valderrama 1981). Nitrate, orthophosphate and other ions were determined by the US Environmental Protection Agency (EPA) method 300.0 using ion chromatography, after sample filtration through GF/F (Whatman, 0.7 µm pore size) filters. The average annual values of water transparency and surface concentrations of total phosphorous and chlorophyll-*a* were used to calculate the trophic status index of Carlson [=TSI] (1977).

Chlorophyll-*a* concentration of Pcy were determined fluorometrically. Volumes (250 ml) of the water samples were filtered through Whatman GF/D glass fiber filters (47 mm diameter, 2.7 µm pore size). The filtrate was subsequently filtered through a 0.2 µm pore size polycarbonate filter (Whatman, Nucleopore). Chlorophyll-*a* was extracted in 10 mL with 90% acetone in darkness for 24 h at 4 °C. The concentration was measured with a Turner Designs 700 fluorometer using an emission wavelength of 675 nm. Calibration of fluorometer was done using Chl *a* pure standard (Sigma Chemical-6144, 1 mg).

Picoplankton analysis

Samples for picoplankton counts were processed for flow cytometry and epifluorescence microscopy. We used flow cytometry to measure Pcy abundances. A 4 mL subsample from each depth sample was fixed immediately using cold glutaraldehyde 25% (final concentration 2%) and stored in darkness at 4˚C. Counting was performed using an Accuri C6 (Becton Dickinson) flow cytometer equipped with an air-cooled Argon laser (488 nm blue and 620 nm red excitation). Analyses were run for 4 min at fast flow rate (90 µL min^{-1}). Fluorescent beads (1 µm diameter, yellow-green microspheres, Polysciences Inc.) were used to calibrate the side scatter signal and to control the internal volume standards. Pcy populations were identified and enumerated using three-dimensional gates based on side scatter (SSC) for cell size versus red fluorescence (FL3) and orange fluorescence (FL2) versus red fluorescence (FL3) (Fig 1). Data were collected and analyzed using CFlow sofware 2012. More details concerning flow cytometry analysis are provided in (Pantoja-Agreda et al. 2016).

Pcy populations were confirmed by epifluorescence microscopy. A volume of 10 ml from each water sample was fixed in formalin (2%), filtered through black polycarbonate membrane filter (0.2 µm pore size; 25 mm diameter), stored in darkness at -20 °C and processed within 4 weeks. The filters were mounted on microscope slides with a drop of lowest auto-fluorescence immersion oil (refractive index, n=1.518). Pcy were enumerated using epiflurescence microscopy (Olympus IX71, equipped with HOB 100W lamp, at 1000X magnification). A minimum of 400 cells was counted distributed over at least 20 microscope fields. Pcy were differentiated based upon their fluorescence characteristic. The autofluorescence of Pcy (phycoerytrhrin containing) was visible under green-light excitation (Olympus set: excitation filter 480-590 nm; emission filter 590 nm; dichroic mirror DM 570), producing yellow-orange fluorescence. Chlorophyll-*a* fluorescence under blue light excitation (Olympus set: excitation filter 420-480 nm; emission filter 520 nm; dichroic mirror DM 500) produced a red emission.

Data Analysis

Analysis of Variance (ANOVA) were used to compare the average values of Pcy abundance and environmental parameters in both reservoirs. Regression analysis and Pearson correlation coefficients were used to determine the relation between Pcy density and environmental variables. The program Minitab 16.1 for Windows was used for statistical analyses. Principal component analysis (PCA) of physical, chemical and biological parameters was used to determine the environmental factors controlling abundance and biomass of Pcy.

Results

The mean water temperature at Cerrillos was 27.2 °C, varying from 26.1 °C in Feb to 28.9 °C in Sep (Table 2). Due to its depth (65 m), the variations in temperature within the column allowed a very stable thermal stratification from Apr to Oct, with a mixing period from Nov to Mar (Fig 2). The thermocline reached down to 7 - 9 m (Fig 2). The thermal difference between the surface and the bottom was around 3.2 °C. In contrast, Lucchetti reservoir was only partially stratified from Apr to Oct, with a mixing period also from Nov to Mar (Fig. 2). Mean temperature at surface was 27.8 °C, varying from 26.3 °C in Feb to 29.4 °C in Jul (Table 2). The difference in temperature between the epilimnion and the hypolimnion was less than 2.8 °C. More details about the thermal behavior of Cerrillos and some reservoirs in Puerto Rico are given by Sotomayor et al. (2008) and Pantoja et al. (2009).

In Cerrillos water transparency as expressed by Secchi depth, in Cerrillos varied from 1.8 m in Jul to 3.5 m in Jan, with a mean value of 2.6 m (Fig 3). Secchi depth in Lucchetti was lower, with a mean value of 1.4 m, and varying from 0.9 m en August to 2.5 m in Feb (Fig 3). The values for Secchi disk depth were significantly higher in Cerrillos than in Lucchetti ($F=23.4$; $p<0.05$). In both reservoirs, the maximum transparency was measured during the mixing period while the lowest values occurred during stratification (Fig. 3). The euphotic zone in Cerrillos surpassed 7 m deep, while Zeu in Lucchetti it was shallower and had a mean value of 4.8 m (Table 2).

Dissolved oxygen (DO) concentration in the surface was high for both reservoirs (Fig. 2). DO had a mean value of 6.5 mg L^{-1} in Cerrillos, with a minimum of 5.06 mg L^{-1} in Nov and a maximum of 10.2 mg L^{-1} in Sep (Table 2). Lucchetti had a mean DO value of 8.0 mg L^{-1}, with a minimum of 6.8 mg L^{-1} in Jan and a maximum of 12.1 mg L^{-1} in Jun (Table 2). Mean values for oxygen saturation were 84.8% for Cerrillos and 94% for Lucchetti. Lucchetti presented oxygen

supersaturation in the epilimnion during summer months. During the stratification periods, DO decreased drastically with depth in both reservoirs, thus generating clinegrade profiles. Anoxia (DO < 1 mg L^{-1}) occurred below 6 m in Lucchetti and 10 m in Cerrillos (Fig. 2). Hypolimnion became completely anoxic from May to Oct in Cerrillos and from Apr to Nov in Lucchetti.

Both reservoirs had relatively high pH near the surface (alkaline waters). Mean pH was 7.8 in Cerrillos and 8.3 in Lucchetti. Conductivity values suggested that both reservoirs have poorly mineralized waters. Mean conductivity near the surface was 246.8 μS cm^{-1} in Cerrillos and 268 μS cm^{-1} in Lucchetti, while mean values near the bottom were 278.9 μS cm^{-1} in Cerrillos and 354.7 μS cm^{-1} in Lucchetti (Table 2)

Mean TN near the surface was 0.91 mg L^{-1} for Lucchetti and reached a maximum in Oct (1.57 mg L^{-1}) and a minimum in Apr (0.21 mg L^{-1}) (Table 2). In contrast, TN in Cerrillos was 3 to 4 times lower, with a mean of 0.21 mg L^{-1}, a minimum of 0.07 mg L^{-1} in Feb, and a maximum of 0.54 mg L^{-1} in May. Annual mean values for nitrate were 0.025 mg L^{-1} for Cerrillos and 0.32 mg L^{-1} for Lucchetti. In Cerrillos, during stratification the nitrate increased with depth from 0.012 mg L^{-1} near the surface to 0.15 mg L^{-1} at 25 m deep, without the formation of a nitrocline. Nitrate concentration did not differ with depth during mixing, as evidenced in Feb (Fig. 2, top). During the stratification in Lucchetti, nitrate increased with depth, from 0.04 mg L^{-1} near the surface to 0.25 mg L^{-1} at 25 m, and a nitrocline became evident at 12 m. Over mixing, nitrate concentrations were not homogeneous and also increased with depth (Fig. 2, bottom).

Cerrillos presented TP concentrations lower than in Lucchetti, with a mean of 0.01 mg L^{-1}, a minimum in Nov (0.005 mg L^{-1}) and a maximum in Jul (0.02 mg L^{-1}) (Table 2). TP concentrations in Lucchetti were less variable and had a mean of 0.04 mg L^{-1}, with a minimum in Feb (0.009 mg L^{-1}) and a maximum in Oct (0.08 mg L^{-1}) (Table 2). TP was significatively higher

in Lucchetti than in Cerrillos ($F=7.56$; $p<0.05$) (Fig. 3). Orthophosphate was also much higher in Lucchetti, with mean values near surface of 0.015 mg L^{-1}. In Cerrillos, orthophosphate was below 0.005 mg L^{-1} (Table 2).

Phytoplankton biomass, expressed as Chl *a,* was relatively high at Lucchetti, with mean values at the surface of 19.2 μg L^{-1}, a minimum in Feb (7.2 μg L^{-1}) and a maximum in Sep (43.8 μg L^{-1}) (Table 2). Chl *a* was considerably lower in Cerrillos, with an annual mean of 8.56 μg L^{-1}, a minimum in Apr (3.5 μg L^{-1}) and maximum in Sept (16.1 μg L^{-1}) (Table 2). Chl *a* concentrations were significatively different between reservoirs ($F=11.23$; $p<0.05$). Deep Chl *a* maxima (DCM) were observed in both reservoirs during stratification; these "pulses" at the limit of the photic zones were detected in Sept at 6 m in Lucchetti (24.1 μg L^{-1}) and in Aug at 10 m in Cerrillos (6.29 μg L^{-1}).

Carlson's Index values, as a function of water transparency, indicate that Cerrillos is a mesotrophic reservoir (TSI= 46.2) and Lucchetti is a eutrophic system (TSI=54.2). Carlson's Index based on mean TP concentration suggest Cerrillos as oligotrophic (TSI=37.3) and Lucchetti as eutrophic (TSI=57.3). Ch-*a* concentration points towards a oligo-mesotrophic state for Cerrillos (TSI=50.5) and an eutrophic state for Lucchetti (TSI=59.5).

Cytometric analyses allowed the rapid detection of Pcy populations and their differentiation from Peuk populations based on cell size and fluorescence emitted by the photosynthetic pigments (Fig. 1). Fluorescence emission by *Synechococcus* was confirmed by epifluorescence microscopy. Cytograms indicated high Pcy densities in the photic zone of Cerrillos, with mean value of 2.2 x 10^4 cells m L^{-1}, maximum during stratification in Jun (6 x 10^4 cells m L^{-1}) and minimum during mixing in Feb (8.2 x 10^3 cells mL^{-1}) (Fig. 4). Densities were around an order of magnitude and significantly lower in Lucchetti ($F=8.79$; $p<0.05$), with a mean

value near the surface of 9.2 x 10^3 cells mL^{-1}, a maximum in Jul (1.5 x 10^4 cells mL^{-1}) and minimum in Nov (5.2 x 10^3 cells mL^{-1}) (Fig. 5). Pcy density profiles showed a marked decrease with depth below the lower limit of the photic zone (1% PAR), with very low densities yearlong in the hypolimnion.

The fraction of the picoplanktonic biomass (P-Chl *a*) represented by *Synechococcus* in Cerrillos had a mean of 0.88 μg L^{-1}, with a minimum in Sept (0.36 μg L^{-1}) and maximum in Jun (1.99 μg L^{-1}) (Fig. 6). In Lucchetti these Chl *a* values had a mean of 0.56 μg L^{-1}, with a minimum during mixing in Feb (0.22 μg L^{-1}) and maximum during stratification in May (0.88 μg L^{-1}) (Fig. 6). Picoplankton contribution to total chlorophyll (T- Chl *a*) varied from 2.2% in September to 20.5% in Jun (mean = 12.3%) at Cerrillos, and from 1.2% in Oct to 5.1 % in May (mean = 3.02%) at Lucchetti (Fig. 6). In Cerrillos, this picoplankton contribution to Chl *a* increased during full stratification (Summer) and the beginning of the mixing period, when solar radiation was higher. In Lucchetti, the contribution of the < 2 μm fraction was observed during the warm stratified period, when total Chl *a* was lower. Significant differences were obtained in the picoplankton Chl *a* ($F= 11.23$, $p< 0.05$) and in the Pcy contribution to T-Chl *a* ($F=4.4$, $p< 0.05$) between both reservoirs.

Pearson's correlations and Principal Component Analysis (PCA) for Pcy densities and environmental variables indicated that axes 1 and 2 explained 62.6% of the variation in Cerrillos, and the environmental parameters were significantly correlated with axis 1 (Fig 7). Axis 1 clearly separates Jun from the rest of months, and it was strongly associated with Pcy abundance and the P-Chl *a* concentration. High, positive correlations were obtained among variables Pcy, P-Chl *a*, TN, Sd, pH and temperature during stratification. The right angle between Pcy and TP indicates a lack of correlation between these two variables. The parameters DO and T-Chl *a* were negatively

correlated with Pcy, however the DO was associated to the cold months, such as Jan and Feb. Axis 2 separated the sampling of Aug from those of Mar and Apr; it also showed a positive correlation between DO and T-Chl *a*, and a negative correlation between T-Ch *a* and conductivity.

PCA of environmental variables and Pcy concentrations in Lucchetti indicated that axes 1 and 2 represent 71% of total variation (Fig. 7). As in Cerrillos, in the PCA for Lucchetti axis 1 showed high correlations among most environmental variables. Pcy abundance was positively correlated to TN, T-Chl *a*, P-Chl *a* and the highest values for oxygen and temperature. The coldest months, during mixing, were associated to pH values and water transparency (Sd). Pcy abundance showed a negative correlation with the last two variables. Variables more strongly correlated with axis 2 were conductivity and TP; however, these two were not correlated to Pcy.

Discussion

Both reservoirs had similar surface temperatures and showed thermal maxima during stratification and minima during mixing. Both can be considered warm monomictic with a short mixing period in February and a long stratification from Apr to Oct. However, Lucchetti had higher thermal homogeneity and less stable stratification due to its shallower depth, higher wind regime and shorter water residence. Mixing and stratification patterns were similar to those determined for other comparable tropical freshwater bodies (Tavera & Martínez 2005, Macek et al. 2009, Pantoja-Agreda et al. 2009, Sotomayor et al. 2008). Temperatures above 25 °C in the hypolimnion of both reservoirs sustain an anaerobic metabolism in the lower layers, creating anoxic conditions over most of the year, especially in the summer months.

DO dynamics were closely related to temperature in both reservoirs; however, this relation was more evident in the epilimnion of Cerrillos during stratification. DO and Chl *a* concentrations were strongly correlated in both reservoirs. Epilimnetic DO values above 8 mg L^{-1} in the summer represented saturation conditions and evidenced the effects of phytoplanktonic activity. Phytoplankton contributed significantly to the oxygen budget of both reservoirs, but physical processes such as oxygen diffusion from the atmosphere seems important in Cerrillos during the coldest months.

Water transparency in both reservoirs was lower in the warmer months (stratification period) and greater in the cooler and drier months. Lower transparency could also be related to higher concentrations of solids brought in by rivers during rain events and to higher phytoplankton biomass during stratification. Similar results were observed in two reservoirs of Puerto Rico (Sotomayor et al. 2008); lower transparency in the summer and a deeper euphotic zone during the winter (mixing period).

The correlation between light attenuation and Pcy abundance was significantly high. Thus, low values for total phytoplankton density and a consequent higher transparency seem to play major roles in allowing the establishment of Pcy. In Lucchetti, phytoplankton growth also diminishes light penetration into the system but allochthonous turbidity (i.e. sediments carried by the river flow) better explains light extinction in the water column. The relative importance of allochthonous turbidity upon light penetration has been pointed out in subtropical and tropical reservoirs and lakes (Guarino et al. 2005, Ribeiro et al. 2005).

In Cerrillos, TP concentrations were relatively low and constant during the study and there was just a slight increase in the summer stratification. During mixing there was a decrease in TP concentration at surface level and a more even distribution along the water column. In contrast, TP concentrations were significantly higher at Lucchetti, and values showed a wider range of fluctuations in time. Higher concentrations were measured between the end of the stratification period and the onset of mixing, with a marked reduction during the winter months. Precisely, higher TP concentrations occurred in the summer, when nutrient input is related to rainfall. TP concentrations above 20 μg L^{-1} usually imply eutrophication (Correll 1998), as does occur in Lucchetti, where TP exceeds 50 μg L^{-1}. Sediment load at Lucchetti (8.29 ton km^2 $year^{-1}$) is much higher than in Cerrillos (1.069 ton km^2 $year^{-1}$) (Soler-López et al. 2011); thus, nutrients seem to be trapped in the sediments, and they become highly available for the phytoplankton during subsequent mixing events.

In both reservoirs, Chl *a* concentrations were higher at the end of the stratification period and eventually decreased during mixing. In Cerrillos, high Chl *a* concentration in the epilimnion was measured in Sep, at the end of stratification, possibly caused by summer blooms of diatoms and dinoflagellates (genera *Ulnaria* and *Peridinium*, respectively).

The diatom *Ulnaria ulna* was the most abundant species in a net plankton study done by Rodriguez (2014) in Cerrillos. According to Rosen (1981), dinoflagellates are common in oligotrophic waters and are tolerant to a wide variation in pH; these microorganisms are also tolerant to drastic changes in light availability (Pollingher 1988). The previous two genera thrive mainly in alkaline waters and are bio-indicators of conditions characterized by organic matter enrichment combined with low phosphorus availability, particularly in stratified oligo-mesotrophic systems (Reynolds et al. 2002, Pantoja-Agreda et al. 2009). The high Chl *a* concentration in Lucchetti in October coincided with TP maxima, stressing the importance of this nutrient for phytoplankton > 2 μm. In both reservoirs, Chl *a* and DO concentrations were positively correlated, which suggests that photosynthesis is more important than atmospheric diffusion at the water-air interfase.

According to the classification by Carlson (1977), and taking into account total Chl *a*, TP concentration and water transparency, Cerrillos is in the transition from an oligotrophic to a mesotrophic reservoir, so it is considered herein as oligo-mesotrophic. On the other hand, Lucchetti reservoir had high TP and Chl *a* concentration, together with a reduced transparency, typical of a eutrophic system.

Hydrologic and environmental parameters were correlated to trophic state and picoplankton structure and dynamics. Temporal and vertical distributions of Pcy were related to stratification and mixing events in both reservoirs. Pcy showed a unimodal distribution with maximum abundance in the summer. Maximum Pcy abundance in Cerrillos was determined in Jun, during full stratification. Maximum Pcy abundance in Lucchetti was obtained between Jun and Aug, at the end of the stratification. The unimodal behavior of Pcy abundance has been reported for tropical (Hernández-Avilés 2010) and subalpine lakes (Winder 2009), as well as from temperate systems like Lake Maggiore (Callieri et al. 2012), Lake Constanza (Gaedke & Weisse

1998), Marne reservoir (Celllamare et al, 2010) and Eagle Mountain reservoir (Grover & Chrzanowski 2006). However, many temperate lakes show a bimodal pattern in picoplankton abundance with a peak in spring or early summer and a second one in late summer or autumn (Stockner et al. 2000).

Flow cytometry allowed the differentiation of the Pcy fraction from the rest of the phytoplankton community in both reservoirs. Cytograms with low Chl *a* and high phycoerythrin fluorescence signaled the dominance by species of *Synechococcus*. Pcy were detected yearlong in Cerrillos, but became more abundant in the summer by nutrient availability and stability in the water column.

In Cerrillos, Pcy abundance and TP concentration were not correlated. Thus, phosphorus might not be a major limiting factor for Pcy in oligo-mesotrophic systems (Horn & Horn 2008). The same lack of correlation between phosphorus and Pcy productivity was also found by Callieri et al. (2010) and Lavelle & Pick (2002). Wehr (1990) also observed a higher Pcy abundance in an eutrophic lake during the summer, exactly when phosphorus concentration was low. The small size and high surface/volume ratio in Pcy should provide advantages over larger size phytoplankters in the incorporation of phosphorus during periods of nutrient-limitation (Jannson et al. 1996, Drakare et al. 2003, Cellamare et al. 2010). In mesotrophic waters, other members of the phytoplankton have shown high correlations with phosphorus concentrations (Naselli-Flores & Barone 1998, Blomqvist et al. 1994).

It is a given fact that light availability is a factor that affects all the members of the phytoplankton community. However, light penetration seems to play a more important role determining Pcy abundance than nutrients do in a eutrophic reservoir. In Lucchetti, the most abundant algal groups were cyanobacteria and euglenophytes. These algae prevail when there are

high concentrations of nutrients and decaying organic matter (Rahman et al. 2007). Also, common species in the reservoir, like members of *Chroococcus* and *Euglena* are indicative of high nutrient and organic matter concentrations (Komárek & Anagnostidis, 2001, John et al. 2003).

Total nitrogen (TN) concentrations were significantly different between both reservoirs and much higher in Lucchetti than Cerrillos. However, the temporal fluctuations of this nutrient produced similar patterns in both reservoirs. As with Pcy abundance, TN reached maxima in the summer, during full stratification, and both diminished significantly during mixing. Indeed, Pcy abundance and biomass were positively correlated with TN in both reservoirs. Thus, nitrogen dynamics seem important for the Pcy in both systems, regardless of their differing trophic states. Evidence that nitrogen is a major limiting factor for Pcy communities was provided by Buskey et al. (2003), who enriched mesocosms with nitrogen and obtained a substantial increase in the density of *Synechococcus* sp. Similarly, Cai and Kong (2013) obtained a positive relation between dissolved inorganic nitrogen (DIN) and Pcy abundance in a large, shallow, eutrophic lake (Lake Chaohu).

Pcy abundance maxima were detected at the epilimnion in both reservoirs; however, the profiles indicated high densities in Cerrillos at depths below 0.1% PAR, especially when water transparency was high due to the lack of recent heavy-rain. Hernández-Avilés et al. (2010) and Camacho et al. (2003) found that Pcy were relatively abundant at the interface between the metalimnion and the hypolimnion in deep lakes that were limited by light and nutrients. Pcy have well-known capacity to thrive below the photic zone (Nagata et al. 1994; Callieri & Piscia 2002). In Lucchetti water transparency was relatively low, restricting Pcy mainly to the layers near the surface and allowing very little growth below 1% PAR during stratification.

In the present study, it was observed that Pcy prevailed in the more transparent, oligomesotrophic waters of Cerrillos reservoir; meanwhile Pcy were scarcer and limited to the euphotic zone in the eutrophic Lucchetti reservoir. In both reservoirs, the fraction of the total Chl *a* concetration corresponding to picoplankton was higher in the summer, during stratification. In Cerrillos, the highest values for algal biomass, was coincident with maximum Pcy abundance; but there was a second peak near the beginning of the mixing period in Nov, which possibly corresponds to an increase of Peuk (Pantoja et al. 2016). In Lucchetti, maximal Chl *a* concentrations were measured between May and Jun, which coincided with highest Pcy abundance and TN values. According to Stockner (1991), autotrophic picoplankton reaches maximal abundance at the end of the summer in marine freshwater environments.

Chl-*a* concetrations were significantly different between both reservoirs, with much higher values recorded in Cerrillos. However, the Chl *a* values were lower than those recorded in other systems with similar trophic states (Sarmento et al. 2008, Lavellé & Pick 2002, Wakabayashi & Ichise 2004). The comparatively lower algal biomass values determined for Lucchetti were correlated with reduced light penetration. However, foraging by zooplankton was not considered in this study, but it is an important controller of Pcy biomass (Vidal et al. 2007).

The relative contribution of APP to total biomass is very variable among lakes of different trophic states: 70% in lake Tanganyka (Stenuite et al. 2009), 40% in lake Baikal (Nagata et al. 1994), 28% in Andean ultraoligotrophic lakes (Callieri et al. 2007), 27.9 % in Alchichica lake (Adame et al. 2008) and 21% in lake Kivu (Sarmento et al. 2008). We found that Pcy were an important year-round fraction of the subtropical freshwater phytoplankton. The Pcy in the oligomesotrophic Cerrillos presented a comparatively higher contribution to total phytoplankton (12.3%). The rest of the Chl *a* in the samples could be attributed to the diatoms and dinoflagellates

that comprised the most of the phytoplankton with size above 3 μm. Picoplankton can comprise over 50% of total phytoplankton biomass in oligotrophic waters (Vörös et al. 1998, Bell & Kalf 2001).

Pcy contribution to the total algal biomass (as Chl *a*) in Lucchetti reservoir was below 3.2%. Low Pcy contributions to the system Chl *a* budget can be related to the low water transparency and presumably to the selective pressure of small-sized filtering zooplankters (rotifers are known to dominate the zooplankton in Lucchetti; unpublished data from the UPR Agriculture Experiment Station, Project Z-297). Sommaruga and Roberts (1997) concluded that the contribution of APP to total biomass relatively low in hypereutrophic waters.

Results favor the concept that lakes and reservoirs with lower trophic states, such as Cerrillos, exhibit higher abundances and biomass of Pcy (Stocker 1991) and further support the use of this microbial community as indicators of water quality and trophic state. The members of the Pcy community have high plasticity and can easily adapt to eutrophic conditions; however, they flourish more under high water transparency and low concentrations of nutrients, especially under phosphorus limitation.

The present study is an exploration of the relative Pcy contribution to the photosynthetic biomass in only two subtropical reservoirs with different trophic states. Future studies should replicate trophic states by incorporating more reservoirs into them. Most of the reservoirs in Puerto Rico are already in the eutrophic category, so there are many candidates to compare with Lucchetti reservoir.

References

Adame MF, Alcocer J, Escobar E. 2008. Size-fractionated phytoplankton biomass and its implications for the dynamics of an oligotrophic tropical lake. Freshwater Biol. 53:22-31.

Azam F, Fenchel T, Field JG, Gray JS, Meyer LA, Thingstad F.1983. The ecological role of water column microbes in the sea. Mar Ecol Prog Ser. 10:257-263.

Bell T, Kalff J.2001. The contribution of picophytoplankton in marine and freshwater systems of different trophic status and depth. Limnol Oceanogr. 46:1243-1248.

Blomqvist P, Pettersson A, Hyenstrand P.1994. Ammonium- nitrogen: A key regulatory factor causing dominance of non- nitrogen-fixing cyanobacteria in aquatic systems. Arch Hydrobiol. 132:141-164.

Buskey EJ, Deyoe H, Jochem FJ, Villareal TA. 2003. Effects of mesozooplankton removal and ammonium addition on plank- tonic trophic structure during a bloom of the Texas 'brown tide': a mesocosm study. J Plankton Res. 25:215-228.

Cai Y, Kong F. 2013. Diversity and dynamics of picocyanobacteria and the bloom-forming cyanobacteria in a large shallow eutrophic lake (lake Chaohu, China). J Limnol. 72:473-484.

Callieri C, Piscia R. 2002. Photosynthetic efficiency and seasonality of autotrophic picoplankton in Lago Maggiore after its recovery. Freshwater Biol. 47:941-956.

Callieri C, Modenutti B, Queimaliños C, Bertoni R, Balseiro E. 2007. Production and biomass of picophytoplankton and larger autotrophs in Andean ultraoligotrophic lakes: differences in light harvesting efficiency in deep layers. Aquat Ecol. 80:345-362.

Callieri C. 2008. Picophytoplankton in freshwater ecosystems: the importance of small-sized phototrophs. Freshwater Rev.1:1-28.

Callieri C. 2010. Single cells and microcolonies of freshwater picocyanobacteria: a common ecology. J Limnol. 69:257-277.

Callieri C, Cronberg G, Stockner JG. 2012. Freshwater picocyanobacteria: single cells, microcolonies and colonial forms. The Ecology of Cyanobacteria II: Their Diversity in Time and Space, 2nd edn. Springer, New York, NY.

Camacho A, Miracle MR, Vicente E. 2003. Which factors determine the abundance of picocyanobacteria in inland waters? A comparison among different types of lakes and ponds. Arch Hydrobiol. 3:321-338.

Carlson RE. 1977. A trophic state index for lakes. Limnol Oceanogr. 22:361-369.

Cellamare M, Rolland A, Jacquet S. 2010. Flow cytometry sorting of freshwater phytoplankton. J Applied Phycol. 2:87-100.

Chisholm SW. 1988. Rapid diversification of marine pico- phytoplankton with dissimilar light-harvesting structures in- ferred from sequences of *Prochlorococcus* and *Synechococcus*. J Mol Evol. 16:188-201.

Correll DL. 1998. The role of phosphorus in the eutrophication of receiving waters: A review. J Environ Qual. 27:261-266.

Crosbie ND, Teubner K, Weisse T. 2003. Flow-cytometric mapping provides novel insights into the seasonal and vertical distributions of freshwater autotrophic picoplankton. Aquat Microb Ecol. 33:53-66.

Drakare S, Blomqvist P, Bergström AK, Jansson M. 2003. Relationships between picophytoplankton and environmental variables in lakes along a gradient of water colour and nutrient content. Freshwater Biol. 48:729-740.

Gaedke U, Weisse T. 1998. Seasonal and interannual variability of picocyanobacteria in Lake Constance. Arch Hydrobiol Special Issue Adv Limnol. 53:143-158.

Grover JP, Chrzanowski TH. 2006. Seasonal dynamics of phytoplankton in two warm temperate reservoirs: association of taxonomic composition with temperature. J Plankton Res. R 28:1-17.

Guarino AWS, Branco CWC, Diniz GP, Rocha R. 2005. Limnological characteristics of an old tropical reservoir (Ribeirão das Lages Reservoir, RJ, Brazil). A Limnol Bras. 17:129-141.

Hernández-Avilés JS, Macek M, Alcocer J, López-Trejo B, Merino-Ibarra M. 2010. Prokaryotic picoplankton dynamics in a warm-monomictic saline lake: temporal and spatial variation in structure and composition. J Plankton Res. 32:1301-1314.

Horn H, Horn W. 2008. Bottom-up or top-down – How is the autotrophic picoplankton mainly controlled? Results of long term investigations from two drinking water reservoirs of different trophic state. Limnologica. 38:302-312.

Horne AJ, Goldman CR. 1994. Limnology. McGraw-Hill. New York, New York, USA.

Jansson M, Blomqvist P, Jonsson A, Bergström AK. 1996. Nutrient limitation of bacterioplankton, autotrophic and mixo- trophic phytoplankton, and heterotrophic nanoflagellates in Lake Örtrasket. Limnol Oceanogr. 41:1552-1559.

John DM, Whitton BA, Brook AJ. 2003. The Freshwater algal flora of the British Isle. An Identification guide to Freshwater and Terrestrial algae. The Natural History Museum. Cambridge University Press.

Knoll LB, Vanni MJ, Renwic, WH. 2003. Phytoplankton primary production and photosynthetic parameters in reservoirs along a gradient of watershed land use. Limnol Oceanogr. 48:608-617.

Komarek J, Anagnostidis K. 2001. Cyanoprokaryota. Oscillatoriales. Susswasserflora von Mitteleuropa Vol 19. Gustav Fisher Jena. Part 2.

Lavallée BF, Pick FR. 2002. Picocyanobacteria abundance in relation to growth and loss rates in oligotrophic to mesotrophic lakes. Aqua Microb Ecol. 27:37-46.

Li WKW, Harrison WG. 2001. Chlorophyll, bacteria and picophytoplankton in ecological provinces of the North Atlantic. Deep-Sea Res. II 48.10:2271-2293.

Macek M, Alcocer J, Lugo-Vázquez A, Martínez-Pérez MaE, Peralta-Soriano L, Vilaclara-Fatjó G. 2009. Long term picoplankton dynamics in a warm-monomictic, tropical high altitude lake. J Limnol. 68:183-192.

Nagata T, Takai K, Kawanobe K, Kim D, Nakazato R, Guselnikova N, Bondarenko N, Mologawaya O, Kostrnova T, Drucker V, Satoh Y, Watanabe Y. 1994. Autotrophic picoplankton in southern Lake Baikal: abundance growth and grazing mortality during summer. J Plankton Res. 16:945-959.

Naselli-Flores L, Barone R. 1998. Phytoplankton dynamics in two reservoirs with different trophic state (Lake Rosamarina and Lake Arancio, Italy). Hydrobiologia. 369/370: 163-178.

Pantoja-Agreda F, Sotomayor D, Martínez G. 2009. Phytoplankton dynamics of de Guajataca Reservoir. Verh Internat Verein Limnol. 30(7):1096-1100.

Pantoja-Agreda F, Otero-Morales E. 2016. Autotrophic picoplankton assemblages in subtropical reservoir: temporal and vertical dynamics in abundance and biomass. J Fresh Ecol. Vol. 0. Iss. 0.0

Pollingher U.1988. Freshwater armored dinoflagellates: growth, reproduction, strategies, and population dynamics. In: Sandgren, CG., ed. Growth and reproductive strategies of freshwater phytoplankton. Cambridge: Cambridge University Press.

Rahman MM, Jewel MAS, Khan S, Haque MM. 2007. Study of euglenophytes bloom and its impact on fish growth in Bangladesh. Algae. 22(3):185-192.

Reynolds CS. 1984. Phytoplankton periodicity: the interactions of form, function and environmental variability. Freshw Biol. 14:111-142.

Reynolds CS, Huszar V, Kruk C, Naselli-Flores L, Melo S. 2002. Towards functional classification of the freshwater phytoplankton. J Plankton Res. 24:417-428.

Ribeiro LHL, Brandimarte AL, Ksishi RT. 2005. Formation of the Salto Caxias reservoir (PR): an approach on the eutrophication process. A Limnol Bras. 17(2):155-165.

Rodríguez VL. 2014. Net-plankton diatoms of Puerto Rican water reservoirs as potential bioindicators of trophic status. Master thesis. University of Puerto Rico, Mayaguez, PR.

Rosen G. 1981. Phytoplankton indicators and their relations to certain chemical and physical factors. Limnologica. 13:236-296.

Sarmento H, Unrein F, Isubmisho M, Stenuite S, Gasol J, Descy JP. 2008. Abundance and distribution of picoplankton in tropical oligotrophic Lake Kivu, eastern Africa. Freshw Biol 53: 756-771.

Schindler DW. 2006. Recent advances in the understanding and management of eutrophication. Limnol Oceanogr. 51:356-363.

Soler-Lopez LR. 2011. Sedimentation survey of Lago Cerrillos, Ponce, Puerto Rico, April-May 2008: U.S. Geological Survey Scientific Investigations Report. 2011-5057.

Sommaruga R, Robarts RD. 1997. The significance of autotrophic and heterotrophic picoplankton in hypertrophic ecosystems. FEMS Microb Ecol. 24:187-200.

Sotomayor D, Martínez G, Pantoja F. 2008. Limnological assessment of two reservoirs in Puerto Rico. Verh Internat Verein Limnol. 30(3):521-527.

Stenuite S, Tarbe AL, Sarmento H, Unrein F, Pirlot S, Thill M, Lecomte B, Leporcq, Gasol JM, Descy JP. 2009. Photosynthetic picoplankton in Lake Tanganyika: biomass distribution patterns with depth, season and basin. J Plankton Res. 31:1531-1544.

Stockner JG, Antia NJ. 1986. Algal picoplankton from marine and freshwater: a multidisciplinary perspective. Can J Fish Aquat Sci. 43:2472-2503.

Stockner J, Callieri C, Cronberg G. 2000. Picoplankton and other non-bloom forming cyanobacteria in lakes. In: Whitton, B.A., Potts, M. (Ed) The Ecology of Cyanobacteria. Their Diversity in Time and Space. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Stockner JG. 1991. Autotrophic picoplankton in freshwater environments: the view from the summit. Int Rev Ges Hydrobiol. 76:483-492.

Tavera R, Martinez-Almeida V. 2005. Atelomixis as a possible driving force in the phytoplankton composition of Zirahuén, a warm-monomictic tropical lake. Hydrobiologia 533: 199-208.

Valderrama GC. 1981. The simultaneous analysis of total nitrogen and total phosphorus in natural waters. Mar Chem. 10:109-122.

Vidal L, Rodríguez-Gallego L, Conde D, Martínez-López W, Bonilla S. 2007. Biomass of autotrophic picoplankton in subtropical coastal lagoons: Is it relevant? Limnetica. 26:441-452.

Vörös L, Gulyás P, Németh J. 1991. Occurrence, dynamics and production of picoplankton in Hungarian shallow lakes. Int Revue Ges Hydrobiol. 76:617-629.

Vörös L, Callieri C, Balogh KV, Bertoni R. 1998. Freshwater picocyanobacteria along trophic gradient and light quality range. Hydrobiologia. 369(370):117-125.

Wakabayashi T, Ichise S. 2004. Seasonal variation of phototrophic picoplankton in Lake Biwa (1994–1998). Hydrobiologia. 528:1-16.

Winder M. 2009. Photosynthetic picoplankton dynamics in Lake Tahoe: temporal and spatial niche partitioning among prokaryotic and eukaryotic cells. J Plankton Res. 31:1307-1320.

Wehr JD. 1990. Predominance of picoplankton and nanoplankton in eutrophic Calder Lake. Hydrobiologia. 203:35-44.

Worden AZ, Nolan JK, Palenik B. 2004. Assessing the dynamics and ecology of marine picophytoplankton: The importance of the eukaryotic component. Limnol Oceanogr. 49:168-179.

Table 1. Location and morphometric characteristics of Cerrillos and Lucchetti Reservoir in Puerto Rico.

Feature	Cerrillos	Lucchetti
Location	18°04' N - 66°24' W	18°05' N - 66°5' W
Area (km^2)	2.1	2.8
Drainage area (km^2)	45.3	44.8
Volume (10^6 m^3)	58.2	11.1
Mean depth (m)	25	11
Maximum depth (m)	65	25
Altitude (m a.s.l)	170	175
Water Retention time (yr^{-1})	0.87	2.9
Uses	Water supplies/Flood protection	Water supplies/Irrigation

Table 2. Mean (minimum and maximum) values of physical and chemical variables in the Cerrillos and Lucchetti Reservoirs.

Parameter	Cerrillos			Lucchetti		
	Mean	**Min**	**Max**	**Mean**	**Min**	**Max**
Secchi Disk (m)	2.6	1.8	3.5	1.	0.9	2.5
Photic Zone (m)	7	4.8	9.5	4.8	2.4	6.7
Water Temperature (°C)	27.2	26.1	28.9	27.8	26.3	29.4
Dissolved Oxygen ($mg\ L^{-1}$)	6.5	5.06	10.2	8	6.8	12.1
pH	7.8	7.4	8.6	8.3	7.3	9.1
Conductivity ($\mu S\ cm^{-1}$)	246.8	237	262	268	244.5	287.2
Salinity (ppt)	0.11	0.09	0.13	0.12	0.11	0.14
Total Dissolved Solid ($mg\ L^{-1}$)	157.1	151	162	1740	1532	1838
Total Nitrogen ($mg\ L^{-1}$)	0.21	0.07	0.54	0.91	0.21	1.57
Total Phosphorus ($mg\ L^{-1}$)	0.01	0.005	0.02	0.04	0.009	0.08
Nitrates ($mg\ L^{-1}$)	0.025	0.053	0.016	0.32	0.08	1.02
Orthophosphate ($mg\ L^{-1}$)	0.008	<0.005	0.011	0.015	0.009	0.07
Iron ($mg\ L^{-1}$)	0.06	0.02	0.07	0.05	0.03	0.08
Chlorides ($mg\ L^{-1}$)	13.3	7.35	20.2	20.8	10.1	29.4
Sulphates ($mg\ L^{-1}$)	2.8	0.9	5.6	8.05	6.2	9.3
Chlorophyll *a* ($\mu g\ L^{-1}$)	8.56	3.5	16.1	19.2	7.2	43.8
Primary production ($mg\ C\ m^{3}\ h^{-1}$)	65.23	39.84	87.11	91.5	80.12	170.4
Turbidity (NTU)	2.2	0.9	7.2	3.8	1.2	9.9
Trophic status	Oligomesotrophic			Eutrophic		

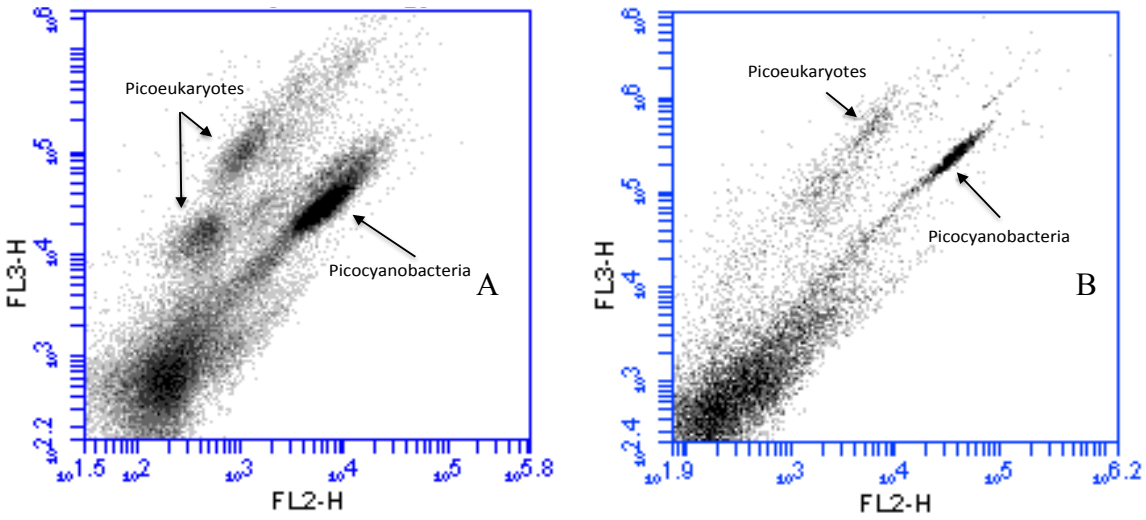

Fig 1. Cytograms by flow cytometry analysis of samples from the surface water: Chlorophyll (FL3) vs. phycoerythrin (FL2) fluorescence in the Cerrillos (A) and Lucchetti (B) reservoirs in Puerto Rico. Picocyanobacteria populations differ from picoeukaryotes in size and fluorescence emission.

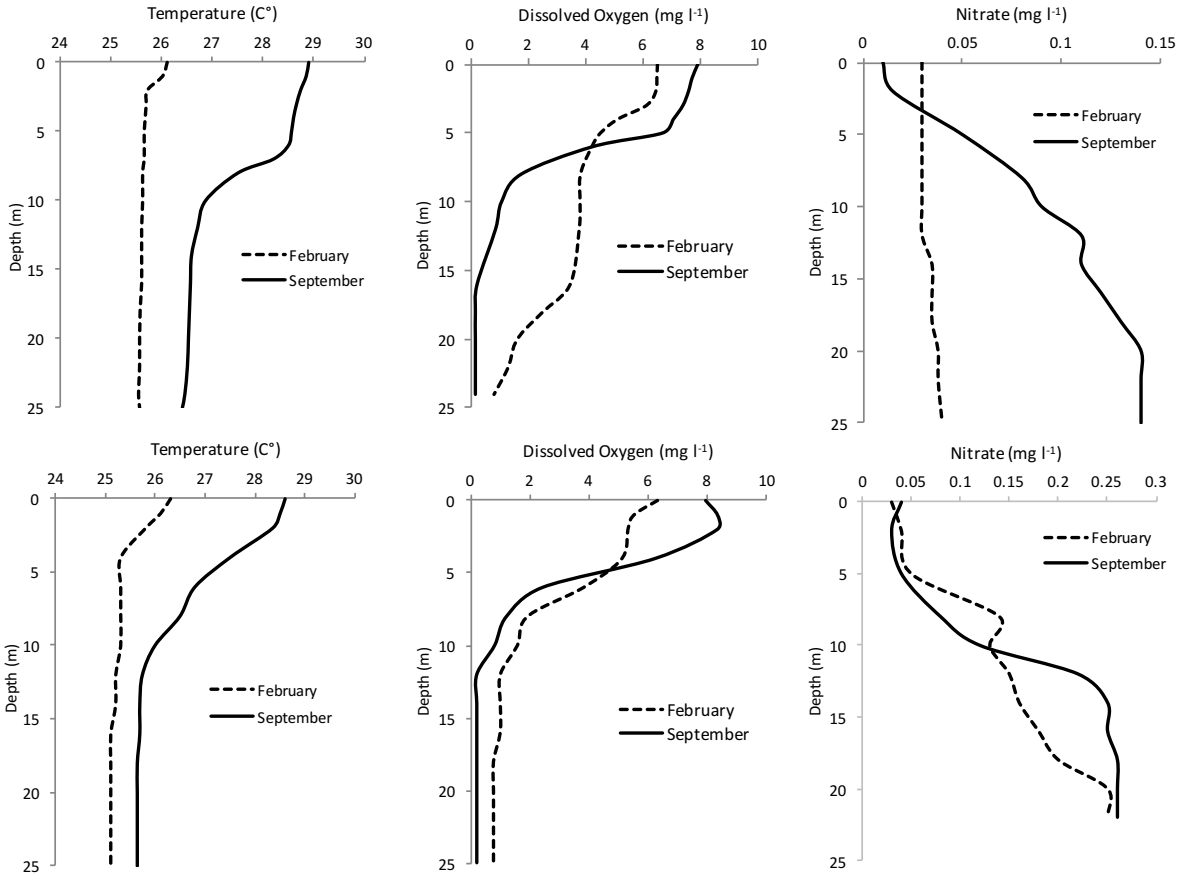

Fig 2. Vertical profiles of temperature (°C), dissolved oxygen (mg L^{-1}) and nitrate (mg L^{-1}) during the wet (September) and dry (February) seasons in the Cerrillos (top) and Lucchetti (bottom) reservoirs in Puerto Rico, during 2013.

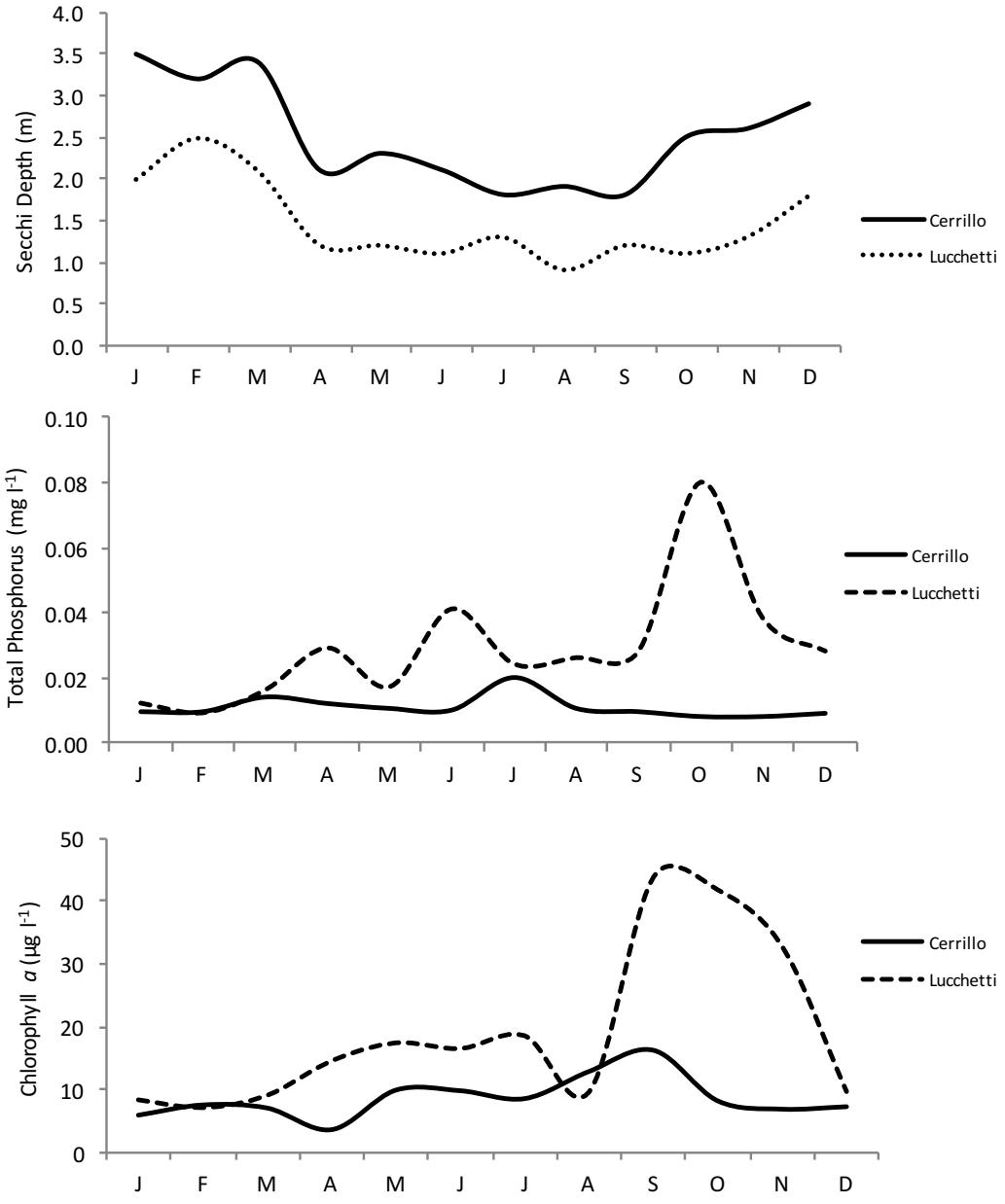

Fig 3. Seasonal fluctuations in the trophic state parameters: Secchi disk depth (top), total phosphorus (middle) and chlorophyll *a* (bottom) in Cerrillos and Lucchetti Reservoirs in Puerto Rico, during 2013.

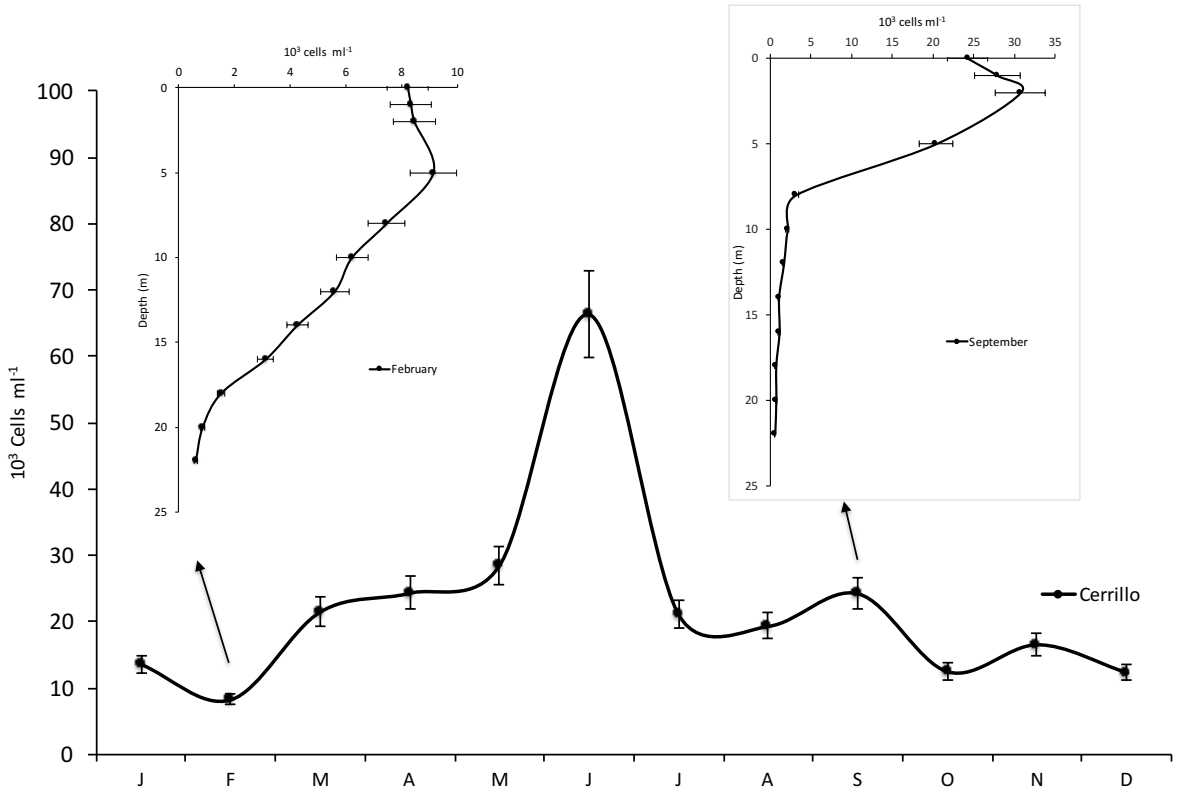

Fig 4. Seasonal variation of picocyanobacteria abundance in the Cerrillos Reservoir. Arrows indicate vertical profiles during the stratified period (Sep) and mixing period (Feb) of 2013. Bars represent ± standard errors.

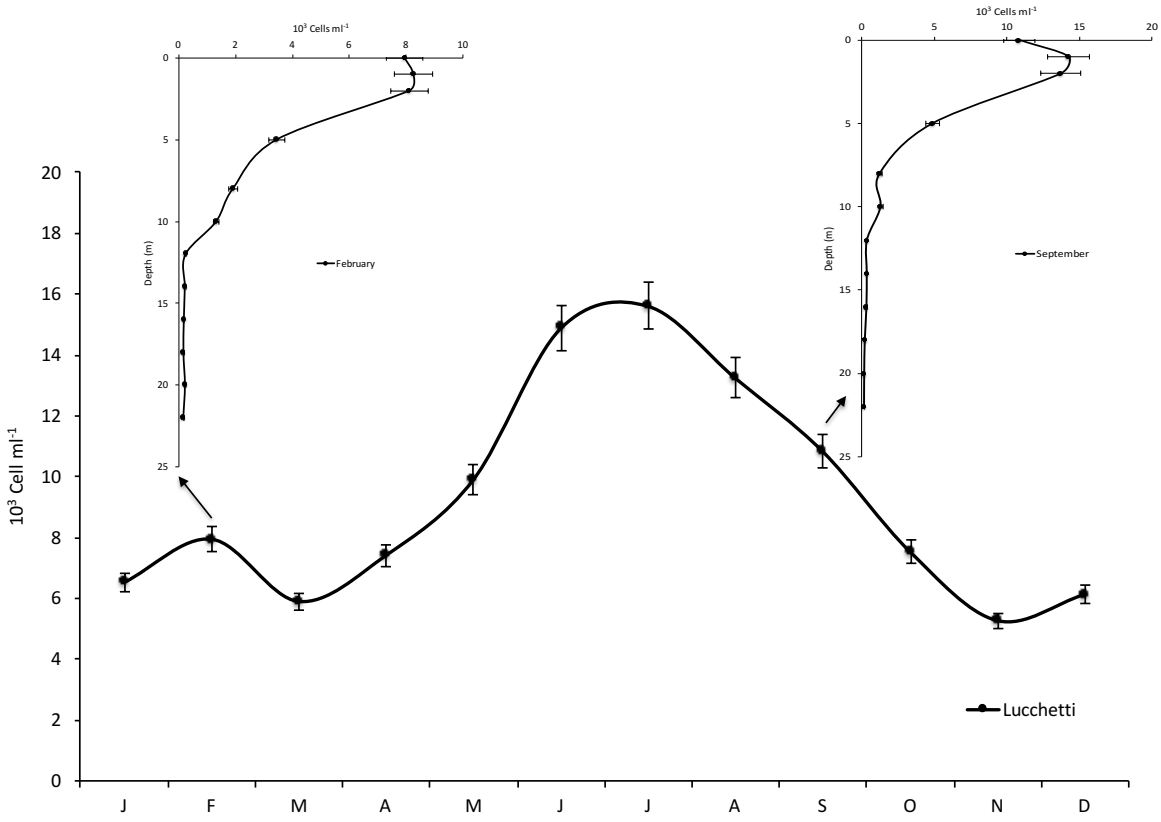

Fig 5. Seasonal variation of picocyanobacteria abundance in the Lucchetti Reservoir. Arrows indicate vertical profiles during the stratified period (Sep) and mixing period (Feb) of 2013. Bars represent ± standard errors.

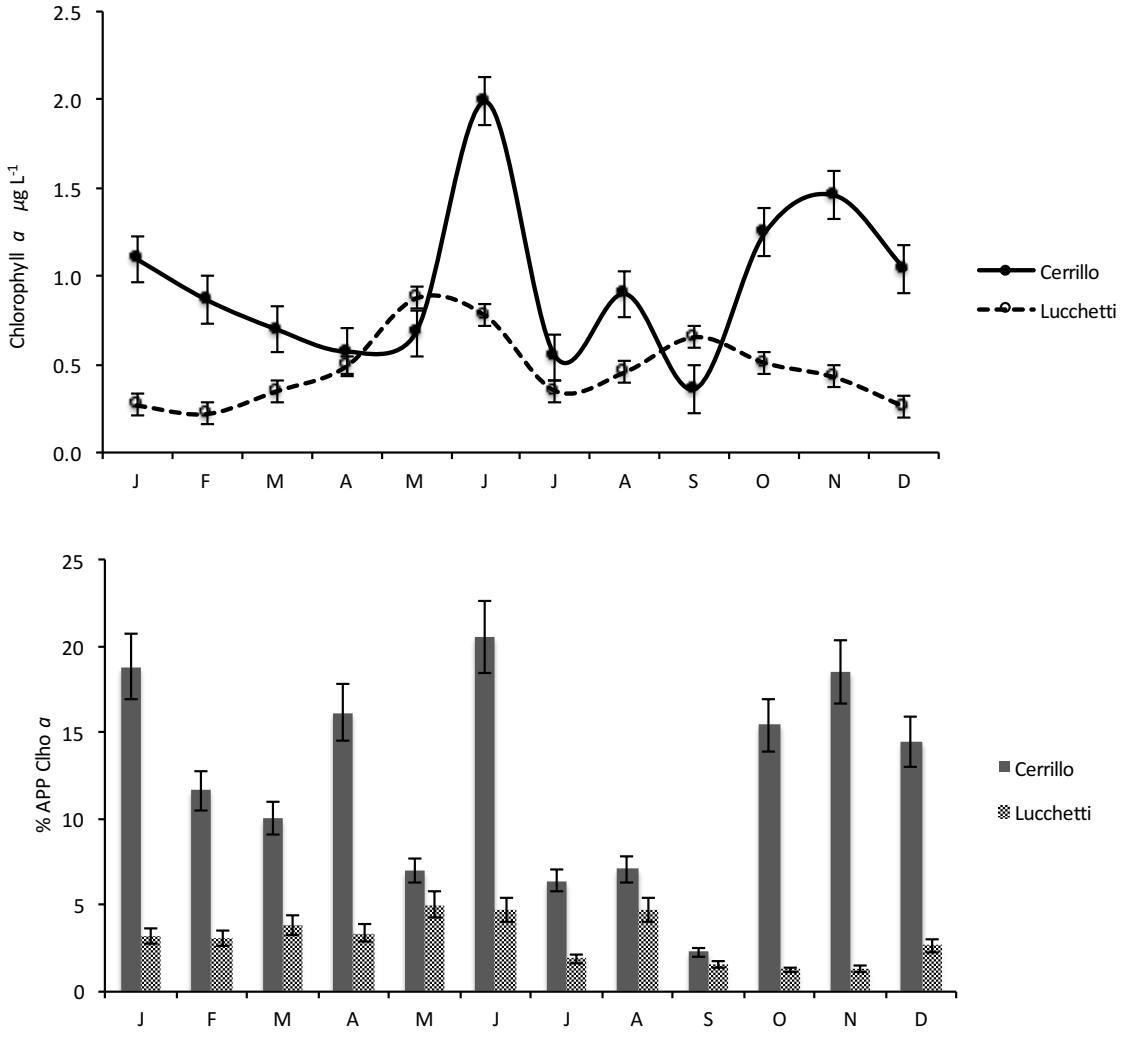

Fig 6. Seasonal variations of autotropic picoplankton (APP) chlorophyll *a* concentration in Cerrillos and Lucchetti Reservoirs (top). Relative contribution of APP to total chlorophyll *a* in the Cerrillos and Lucchetti Reservoirs (bottom) during 2013. Bars represent ± standard error.

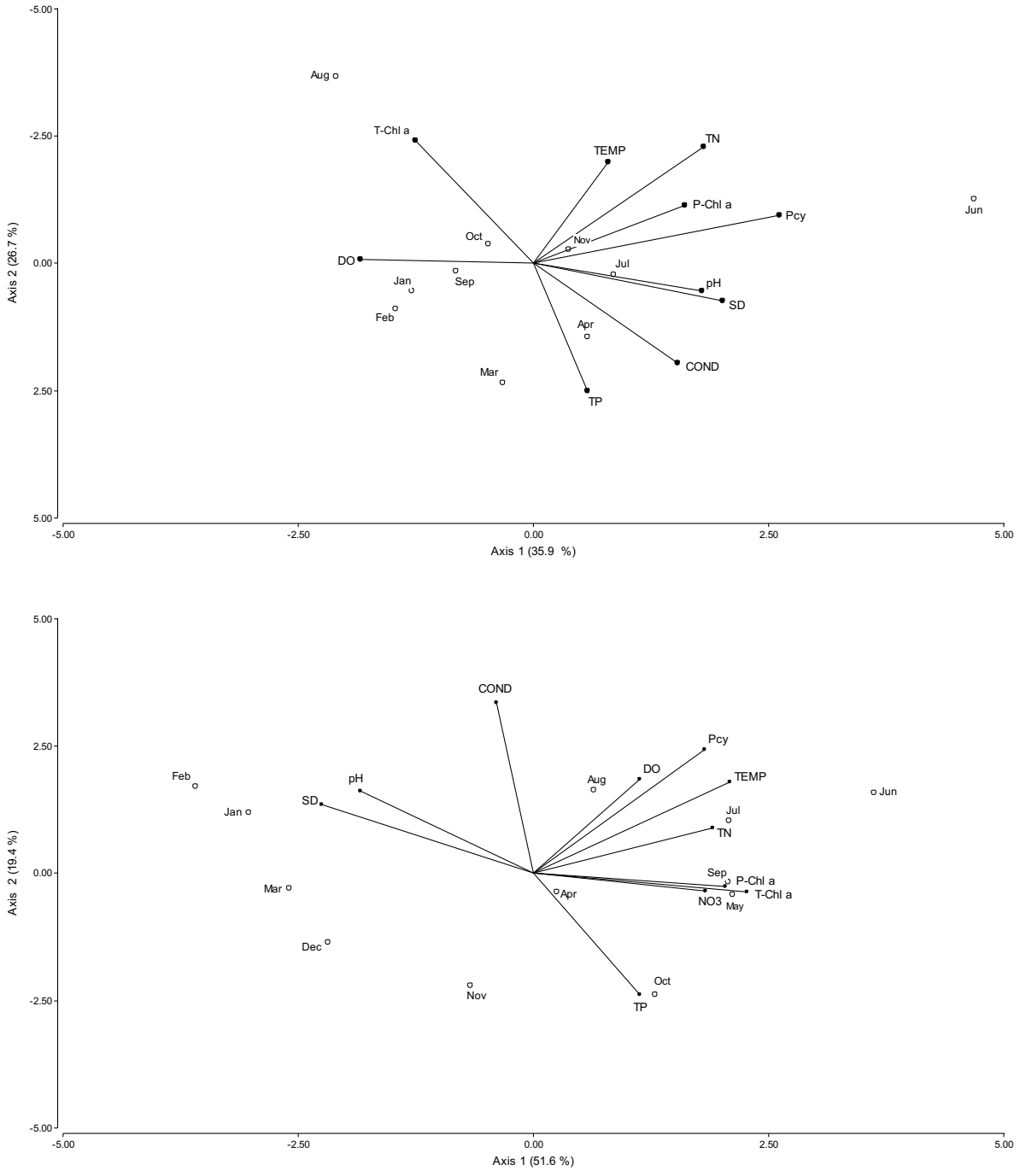

Fig 7. Biplot of PCA analysis. Vectors represents environmental variables, point symbols represent sampling dates for Cerrillos Reservoir (top) and Lucchetti Reservoir (bottom).

Chapter IV

Picocyanobacteria diversity in two warm tropical reservoirs of contrasting trophic state

Fernando Pantoja-Agreda[1*], Carlos Rodriguez-Minguela[2], Ernesto Otero-Morales[1]

[1]Department of Marine Sciences, University of Puerto Rico at Mayagüez
[2]Department of Biology, University of Puerto Rico at Mayagüez
PO Box 9000, Mayagüez 00681 Puerto Rico

[*]Corresponding author: fernando.pantoja@upr.edu

Abstract

Water samples from two reservoirs with different trophic states were compared by using environmental metagenomics to determine the composition and diversity of picocyanobacteria. Water samples from a eutrophic reservoir (Lucchetti) had significantly higher relative abundance, and bacterial community was more diverse than the one in an oligotrophic reservoir (Cerrillos). A greater number of Operational Taxonomic Units (OTUS) sequences were observed for the Lucchetti reservoir, indicating greater genetic diversity by sample size. The phylogenetic analyses of 29 representative sequences from the Luchetti and the Cerrillos reservoirs revealed that the structure of the picocyanobacteria community at these sites is polyphyletic and dominated by lineages associated with the order Chroococcales of the Cyanobacteria. The picocyanobacteria community in the ecosystems studied is diverse and variable; strains are dispersed in the 16S rRNA tree polyphyletic origin. Results preliminarily suggest that the trophic state of the reservoirs studied significantly influences the diversity, composition and abundance of the picocyanbacteria community.

Keywords: Diversity, Trophic state, 16S rRNA, metagenomics, *Cyanobium, Synechococcus*

Introduction

Autotrophic picoplankton (APP) are unicellular, photosynthetic prokaryotes and eukaryotes present in oceans and freshwater ecosystems, which have a cell size in the range of 0.2 to 2 μm. Among these two groups, representatives of the picocyanbacteria are known to dominate the composition of APP communities (Winder 2009). The genus *Synechococcus* has been reported to be wide spread across APP communities from marine and freshwater environments. In contrast, other picocyanobacteria affiliated to the the genera *Prochlorococcus* and *Cyanobium* tend to be associated with marine and freshwater settings, respectively (Stockner et al. 2000, Callieri et al. 2007). Autotrophic picoplankton are known to generate as much as 70% of the total carbon production in aquatic ecosystems, serving as an essential energy resource in the food chain and as a critical catalyst for biogeochemical cycling (Cotner and Biddanda 2002, Li et al. 2001, Callieri 2008, Greisberger et al. 2007).

Knowledge of the diversity in and abundance of the bacterioplankton community and its relationship with environmental factors is of great importance for understanding the role of picocyanobacteria in biogeochemical cycles and the food web, as this would allow more effective management of lakes and reservoirs, especially in relation to potential use for drinking water. Research in tropical fresh water systems has focused on ecology and composition of phytoplankton, and in studies of water quality and limnological aspects.

The picoplankton in marine and freshwater environments traditionally have been studied by epifluorescence microscopy and flow cytometry. These techniques allow the identification of picocyanobacteria by the fluorescence of their pigments unlike other bacteria. The bacterioplankton morphological plasticity, in combination with molecular techniques, offer a more favorable approach to understand the structure and diversity of bacterioplankton. Phylogenetic

analysis of 16S rRNA gene sequences for bacteria and picocyanobacteria, and 18S rRNA for Pico eukaryotes, have proved to be useful tools in evaluating phylogenetic relationships for the APP (Callieri 2008).

Morphologically similar picocyanobacteria populations can show spatial and temporal variations in their genetic structure that respond differently to environmental changes and biological pressures such as: nutrient availability, light intensity and predation (Postius and Ernst 1999). Due to their high plasticity Different *Synechococcus* strains and most of the prokaryotes can be adapted to different environmental conditions, levels of light intensity and trophic state, as well as variations in seasonal and vertical dynamics (Vörös et al. 1998, Wakabayashi and Ichise 2004, Callieri et al. 2007).

Freshwater picocyanobacteria contribute substantially to primary production and biomass in oligotrophic lakes in temperate and tropical zones. Despite their ecological importance, little is known about biodiversity and physiology of picocyanobacteria in tropical freshwater systems. The majority of research is based on cyanobacteria forming algal blooms such as: *Anabaena, Nostoc,* and *Microcystis*, common in tropical and warm, nutrient rich environments (Ivanikova 2006).

Morphological characteristics and differences based on pigmentation are insufficient to provide a robust taxonomic classification of picocyanobacteria (Ernst et al. 2003). Small size, simple morphological structure and difficulty in being cultured in the laboratory are factors that limit the estimation of their biodiversity. Therefore, knowledge of the phylogenetic relationships and understanding lineage is crucial for the study of structure and diversity of APP (Sánchez-Baracaldo 2008). DNA sequences of ribosomal operon and molecular phylogeny have proved very useful tools to differentiate between morphologically similar strains of *Synechococcus* and other picocyanobacteria (Crosbie et al. 2003, Ernst et al. 2003, Ivanikova et al. 2007). Molecular tools

based on PCR and phylogenetic analysis of 16S rRNA genes have been used to study the community picocyanobacteria in natural environments. The gene sequences from samples of water can provide a considerable amount of information on the species composition, role and diversity of aquatic microbial communities in aquatic continental systems (Dorigo et al. 2005).

The aim of this study was to know the diversity of photosynthetic prokaryotes from rRNA gene sequencing using molecular techniques in tropical reservoirs of different trophic status; and if the composition of the community picocyanobacteria with the trophic status of the systems. This study provides the first data on the diversity of photosynthetic picoplankton community in Caribbean tropical reservoirs using molecular techniques.

Materials and Methods

Study sites

The study was conducted in two reservoirs with different trophic state: Cerrillos Reservoir (Oligomesotrophic) and Lucchetti reservoir (eutrophic). Both are warm subtropical systems located at the south and southwestern regions of Puerto Rico, respectively. The morphometric characteristics, location and uses of these two reservoirs are summarized in Table 1.

The reservoirs were sampled during the stratification (June) and circulation period (February). Temperature, pH, dissolved oxygen, electrical conductivity and dissolved solids were measured using a multiparameter probe (Hydrolab DS4a, Loveland, CO, USA). Light penetration was measured using a radiometric LI-COR probe with underwater spherical sensor (PAR), whereas water transparency was assessed using a Secchi disk. The total biomass of phytoplankton was estimated by measuring chlorophyll-*a* concentration in fiberglass filters (Whatman GF/D of 47 mm diameter and 0.7 µm pore size). Similarly, picocyanobacterial biomass was estimated through chlorophyll *a* measurements using a fractional filtration procedure with filters having pore sizes ranging from of 2.7 µm to 0.2 µm. Chlorophyll *a* was extracted with 90% acetone for 24 hours in the dark. Measurements were carried out with a Turner Designs fluorometer 700, using excitation/emission wavelengths of 675 nm.

For molecular diversity analysis, five water samples were collected at both reservoirs from different depth within the photic zone at a single sampling station. Samples were obtained using a 2L Van Dorn bottle. A total of 250 mL of water was prefiltered through Whatman GF/D fiberglass filters of 47 mm diameter and 2.7 µm pore size to remove larger plankton. Subsequently, the picoplankton fraction was concentrated in polycarbonate filters (Whatman Nucleopore) with pore size 0.2 µm and frozen at -80 °C until DNA extraction.

Filtered biomass was sent to Research and Testing Laboratory (Lubbock, Texas) for total DNA extraction, sequencing and downstream bioinformatics analyses of PCR- amplified 16S rRNA genes (V1-V3 region) using universal (28F 5'-GAGTTTGATCNTGGCTCAG-3'; 519R 5'-GTNTTACNGCGGCKGCTG-3) and picocyanobacteria-specific primers (CYA359F 5'-GGGGAATYTTCCGCAATGGG-3'; CYA 781R 5'-ACTACTGGGGTATCTAATCCCATT-3') fused to sequencing adapters for the Illumina MiSeq platform according to the method described by Kozich et al. (2013). Rarefaction analysis (Pommier et al. 2007) was conducted to evaluate changes in diversity among sampling sites (Cerrillos and Lucchetti).

Phylogenetic analysis.

Five sequences (approximately $\leq$ 99.7 identical) of picocyanobacterial 16S rRNA genes were selected from a subset of 100 reads (377-380bp long) representative of the total sequencing data generated from samples retrieved at the surface level (0m) and at the deepest level of photic zone (5m) of the Cerrillos and lucchetti reservoirs. Based on the $\leq$ 99.7% identity criterion, only four sequences were detected (and further analyzed) for the subset corresponding to the sample collected at the Cerrillos reservoir from a depth of 5 m (02/2016). All representative sequences (n=29) were independently searched against 16S rRNA genes from type, cultured and uncultured strains using the RDP II, EZ-taxon, SILVA and NCBI's RefSeq databases (Cole et al. 2014, Chun et al. 2007, Quast et al. 2013, O'Leary et al. 2016). The experimental sequences were aligned against that of their closest type strain or database match using the MUSCLE alignment tool from the MEGA6 software package (Tamura et al. 2013). An identity matrix was constructed for the aligned sequences using the BioEdit software (Hall 1999) to further assess levels of relatedness and to complement information derived from phylogenetic analyses. The alignment was used to evaluate phylogenetic relationships through the construction of a Neighbor-Joining dendrogram

using MEGA6. Evolutionary distances were estimated using the p-distance method (number of base differences per site). Nodal support was tested with 2000 bootstrap replications. Gaps in the alignment were treated as pair wise deletions. The final data set included a total of 381 positions.

Results

The picocyanobacteria diversity observed between reservoirs was compared through the rarefaction method, which shows a comparison between clones of sites with different sample sizes. The rarefaction curve represents sequences with similarity of 97% among them, which corresponds to the taxonomic genus level, and showed the change in the expected value of species richness according to the size of the sample. No asymptote was reached during rarefaction analysis for Bacteria at the Genus level (Fig. 1) suggesting under sampling. The values of the tag on the slopes of both reservoirs did not become constant. However, greater diversity of bacteria was observed in the Lucchetti reservoir. The rarefaction analysis for picocyanobacteria of both reservoirs showed an asymptotic trend, reaching a plateau. The slopes tended to be constant, reaching a saturation point by saturation sequence diversity. A greater number of OTUS sequences were observed for the Lucchetti reservoir, indicating greater picocyanobacteria diversity by sample size (Fig. 2).

Phylogenetic analyses revealed that 27 of the 29, 16SrRNA sequences (99-90% identical with respect to themselves) were affiliated to the order *Chroococales* of the picocyanobacteria. Eight of them were 97-98% identical to the type strain of *Cyanobium gracile* (Fig. 3 Cluster C) and comprised lineages detected in both reservoirs that were mostly retrieved from a depth of 0 m. Within this group, sequences of uncultured strains from fresh water reservoirs (China and Brazil) that were 98-98.6% identical to *Cyanobium gracile* were the closest matches to OTU's L0PC9518

and L5PC1115, whereas OTU C0PC5931 was the closest match (99% identical) for a *Synechococcus* strain isolated from Lake Taihu in China.

OTU's affliated to Cluster E were 99-100% identical among themselves and comprised the second largest group of the dendrogram. Most members of this cluster were recovered from Cerrillos at a depth of 5 m (n=5) with the exceptions of C0PC24533 which was recovered from 0 m and that of phylotype L5PC1112 which was the only OTU recovered from the Lucchetti reservoir (5 m). L5PC1112 and C5PC22494 were 100% identical with respect to each other and to an uncultured bacterium detected in a dam reservoir from Burkina Faso, North Africa. In contrast, Cluster D consisted of three phylotypes from Lucchetti (0 m) and a single representative of Cerrillos (5 m). Among these, OTU L0PC16911 was the closest match (99.7% identical) to a *Synechococcus* sp. isolated from Lake Biwa, Japan, whereas OTU's C5PC6686 and L0PC2109 were the closest relatives (97.8 and 99.4% identical, respectively) of an uncultured cyanobacteria detected in an unidentified lake in China. Additionally, phylotype L0PC1119 was 95.7% to an uncultured cyanobacteria described in a water purification plant in Japan.

Phylotype (PC0PC24981) was the only lineage related to the type strain of *Synechocccus rubescens* (97.3% identical; Cluster B). However, its closest match (99.7% identical) was an uncultured bacterium from Lake Havis, Hungary. In contrast, the type strain of *Synechocccus elongatus* appeared in an isolated branch, deep within the *Chroococales* assemblage. Likewise, its closest relative L5PC2104 (87.3% identical) came forth as a discrete lineage (black arrow) Fig 3. next to Cluster F, which contained two phylotypes (L0PC19802 and L0PC2105) 94.7 to 96% identical to an uncultured bacterium described in a lagoon from La Reunion Island, Indian Ocean.

Two OTU's retrieved from Luchetti at a depth of 5m (L5PC1109 and L5PC2107) were 99% identical to uncultured bacteria from coastal environments in Uruguay and India,

respectively. These were also the only phylotypes that grouped with the type strain of *Prochlorococcus marinus* (96 and 95% identical, respectively, Cluster A).

As observed for OTU L5PC2104, four additional phylotypes (L0PC2108, C5PC25157, C5PC7949 and C0PC22501; black arrows) emerged as separate lineages that were 90-96.8% identical among themselves and more distantly related (85-96.7% identical) from canonical species of picocyanobacteria.

Discussion

The analysis of 16S rRNA gene secuences from environmental samples allowed us to analyze the diversity and relative abundance of picocyanobacteria in two tropical reservoirs with different trophic state. There is scarce information about the structure of the picocyanobacteria in freshwater tropical ecosystems and with not much published research on the subject. Our research provides the first information on biodiversity and abundance of autotrophic picoplankton, particularly of picocyanobacteria, in Caribbean tropical reservoirs, through technical environmental metagenomics.

Bacterioplankton rarefaction curves revealed that the eutrophic reservoir showed higher diversity than the oligomesotrophic reservoir. The higher diversity of bacteria in Lucchetti, might be associated with different physical and environmental conditions in the water column such as: higher productivity, shallower depth, unstable thermal stratification, high turbidity, hypoxia and anoxia conditions at low depths, these factors could provide a variety of niches in the water column (Humayoun et al. 2003) giving the opportunity to colonize new environments for both aerobic and anaerobic bacteria.

Both rarefaction curves showed a logarithmic trend without reaching a plateau, suggesting that microbial diversity for both reservoirs was subestimated, confirming the great complexity of

the microbial diversity in tropical reservoirs. Rarefaction analysis of libraries built from the DNA of bacterioplankton indicated a complex microbial community with a relatively high species richness. The phyla Proteobacteria and Actinobacteria dominant in the Lucchetti reservoir. Their metabolic versatility and genetics allows them to easily adapt to adverse environmental conditions (Pinhasi et al. 2004, Zwisler et al. 2003, Newton et al. 2007) and thus explains how they dominate aquatic bacterioplankton and are the most cosmopolitan groups in lakes. The prevalence of Actinobacteria in the photic zone of both reservoirs, especially their greater relative abundance in Cerrillos, could be related to the resistance of these organisms to the prolonged UV radiation in tropical environments (Warnecke et al. 2005). However, the presence of Actinobacteria as a one of the dominant groups was also expected since this groups is associated with terrestrial systems and the decomposition of complex organic molecules (Thomson et al. 2011; Bai et al 2016).

The rarefaction curve for cyanobacteria reached an asymptote and quickly become saturated forming a stable plateau, suggesting that the number of sequences obtained represent a significant fraction of the samples of picocyanobacteria in both reservoirs. Rarefaction analysis for specific primers of cyanobacteria also suggest that most of the genera of picocyanobacteria present in Cerrillos and Lucchetti reservoirs have been captured, allowing to generate a reliable description of the composition and structure of the community picocyanobacteria in the samples of these two environments.

In agreement with the taxonomic assignments obtained by deep sequencing analyses of partial rRNA genes, the phylogenetic analyses of 29 representative sequences from the Lucchetti and the Cerrillos reservoirs revealed that the structure of the picocyanobacteria community at these sites is polyphyletic and dominated by lineages associated with the order Chroococcales of the Cyanobacteria (n=27). Only two of the analyzed OTU's were affiliated to the Procholorales and

these were detected at Lucchetti. Previous ecological, physiological and molecular (16S rRNA-based) surveys have documented complex, polyphyletic community structures of picocyanobacteria in fresh water environments (Robertson et al. 2001, Callieri 2007; Sánchez-Baracaldo et al. 2008 and Cai et al. 2010). Among all the representative sequences, 8 phylotypes recovered from the photic zone were 97-98% identical to the type strain of *Cyanobium gracile* (Fig. 3 Cluster C). However, Cluster C appeared to be dominated by phylotypes detected at the surface level (n=6). Sequences of uncultured strains identified in fresh water reservoirs from China (Tingxi-mesotrophic) and Brazil (Tucurui-oligomesotrophic) clustered within Group C and emerged as the closest matches (>99identical) to OTU's L0PC9518 and L5PC1115, whereas OTU C0PC5931 was the closest match (99% identical) for a *Synechococcus* strain isolated from Lake Taihu-eutrophic in China. The similarity with phylotypes originally detected in lakes and reservoirs from distinct geographical regions and different trophic states, suggest that there is no relationship between the trophic state of reservoirs and phylogenetic location of the recovered genotypes belonging to Cluster C. Representatives from the genus *Cyanobium* are known to be highly diverse and widely distributed across settings with different limnological characteristics (Crosbie et. al 2003, Sanchez-Baracaldo et al. 2008, Ivanikova 2006).

Three phylotypes detected at the surface level at the Lucchetti reservoir, L0PC16911, L0PC1119 and L0PC2109, and a single OTU retrieved from a depth of 5m at Cerrillos (C5PC6686) grouped in Cluster D with freshwater isolates from the genera C*yanobacterium* and *Synechococcus*. OTU L0PC16911 was the closest match to a *Synechococcus* sp. isolated from Lake Biwa, Japan (99.7% identical), whereas OTU's C5PC6686 and L0PC2109 were the closest relatives of an uncultured cyanobacterium detected in an unidentified lake in China (99.4 and 97.8 % identical, respectively). Lake Biwa sequences appear to be restricted to certain particular

freshwater environments according to their geographical location (Crosbie et al. 2003; Ivanikova 2007). Ecological studies at Lake Biwa have demonstrated that maximum abundance of picocyanobacteria at the surface level occurs during the summer or fall (Nagata et al. 1996). Likewise, the maximum abundance of picocyanobacteria in surface waters at the Lucchetti reservoir has been observed during the summer (Pantoja-Agreda et al. 2016).

Cluster E was dominated by 6 phylotypes (99-100% identical among themselves) which were detected near the limit of the photic zone (5m deep). Five of these were recovered from Cerrillos and 1 from Lucchetti. OTU C0PC24533 was the only member of this group recovered from the surface level, suggesting a prevalence of populations endemic to the Cerrillos reservoir. The structure of picocyanobacteria communities may vary depending on the trophic state of the environment and certain phylotypes may be enriched under specific limnological conditions. L5PC1112 and C5PC22494 were 100% identical with respect to each other and to an uncultured bacterium detected in a dam reservoir from Burkina Faso, North Africa. The presence of these identical phylotypes across local reservoirs and systems from distant latitudes demonstrates that novel, understudied lineages are widespread in nature.

Cluster F was the closest assemblage with respect to the type strain of *Synechocccus elongatus* which appeared as an isolated branch, deep within the *Chroococales* group. This cluster contained two phylotypes (L0PC19802 and L0PC2105) detected at the surface level of the eutrophic reservoir (Lucchetti) which were 94.7 to 96% identical, respectively to an uncultured bacterium described in a lagoon from La Reunion Island, Indian Ocean. OTU L5PC2104 appeared as a discrete lineage (black arrow) next to Cluster F and was the closest relative (87.3% identical) the type strain of *Synechocccus elongatus*. Representatives from the genus *Synechocccus* are found mainly and in great abundance in nutrient-rich environments (Melo and Huszar 2000). In

contrast, a single OTU (PC0PC24981) detected at the surface level of the oligomesotrophic reservoir (Cerrillos) was the only lineage related to the type strain of *Synechocccus rubescens* (97.3% identical; Cluster B). However, its closest match (99.7% identical) was an uncultured bacterium from Lake Havis, Hungary. Notably, the *Synechocccus rubescens* (GenBank accession no. AF317076) and *Synechocccus elongatus* (GenBank accession no. NR_074309) type strains clustered virtually at opposite ends within the Chroococcales clade, suggesting a distant relationship among them. Moreover, their full length 16S rRNA genes were 91.1% identical, a value which is below the proposed threshold sequence identity level of ≥ 94.5% for the identification of bacteria at the genus rank (Garza et al. 2014).

Cluster A was comprised of the type strain of *Prochlorococcus marinus* and 2 OTU's retrieved from Lucchetti (5m) (L5PC1109 and L5PC2107) that were 99% identical to an uncultured bacterium detected at coastal environments in Uruguay and India, respectively. Phylotypes L5PC1109 and L5PC2107 were 96 and 95% identical, respectively to the type strain of *Prochlorococcus marinus.* Freshwater environments are known to sustain diverse communities of picocyanobacteria across relatively small spatial scales which can include populations from marine ecosystems as well (Sánchez-Baracaldo 2005). Although over one fourth of the analyzed phylotypes were associated with the *C. gracile* clade (cluster C), and most of these were detected near the surface level, the closest relative for various environmental phylotypes were taxa identified at different geographical regions around the globe that also had distinct limnological characteristics.

As observed for OTU L5PC2104, four additional phylotypes (L0PC2108, C5PC25157, C5PC7949 and C0PC22501; black arrows) Fig 3, emerged as separate lineages that were 90-96.8% identical among themselves and more distantly related (85-96.7% identical) from canonical

species of picocyanobacteria, suggesting the presence of potentially novel species and genera. In general, the constructed phylogeny showed that the picocyanobacteria community associated with the sampled ecosystems is diverse and could be significantly different from known species as judged by identity levels ranging from 87.6 to 98.4% between partial 16S rRNA genes from environmental phylotypes and type strains.

References

Bai Y, Eijsink VG, Kielak AM, van Veen J A, de Boer W. 2016. Genomic comparison of chitinolytic enzyme systems from terrestrial and aquatic bacteria. Environ Microbiol. 18:38-49.

Cai H, Wang K, Huang S, Jiao N, Chen F. 2010. Distinct patterns of picocyanobacterial communities in winter and summer in the Chesapeake Bay. Appl Environ Microbiol 76:2955-2960.

Callieri C. 2007. Picophytoplankton in freshwater ecosystems: the importance of small-sized phototrophs. Freshw Rev.1:1-28, DOI: 10.1608/FRJ/1.1.1.

Callieri C, Corno G, Caravati E, Galafassi S, Bottinelibi M, Bertoni R. 2007. Photosynthetic characteristics and diversity of freshwater *Synechococcus* at two depths during different mixing conditions in a deep oligotrophic lake. J Limnol. 66:81-89.

Callieri C. 2008. Picophytoplankton in freshwater ecosystems: the importance of small-sized phototrophs. Freshw Rev. 1:1-28.

Cole JR, Q Wang, JA Fish, B Chai, DM McGarrell, Y Sun, CT Brown, A Porras-Alfaro, CR. Kuske, JM Tiedje. 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucl. Acids Res. 42.

Chun J, Lee JH, Jung Y, Kim M, Kim S, Kim BK, Lim YW. 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int J Syst Evol Microbiol. 57:2259-61.

Cotner, JB and Biddanda BA. 2002. Small players, large role: microbial influence on biogeochemical processes in pelagic aquatic ecosystems. Ecosystems. 5:105-121.

Crosbie ND, Pöckl M, Weisse T. 2003. Dispersal and phylogenetic diversity of nonmarine picocyanobacteria, inferred from 16S rRNA gene and cpcBA-intergenic spacer sequence analyses. Appl Environ Microbiol. 69:5716-5721.

Dorigo U, Volatier L, Humbert J. 2005. Molecular approaches to the assessment of biodiversity in aquatic microbial communities. Water Res. 39: 2207-2218.

Ernst A, Becker S, Wollenzien UI, Postius C. 2003. Ecosystem dependent adaptive radiations of Picocyanobacteria inferred from 16S rRNA and ITS-1 sequence analysis. Microbiology. 149: 217-228.

Greisberger S, Dokullil MT, Teubner K. 2007. A comparison of phytoplankton size-fractions in Mondsee, an alpine lake in Austria: distribution, pigment composition and primary production rates. Aquat Ecol. 42:379-389.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.Nucleic Acids Symposium Series. Vol. 41:95-98.

Humayoun, SB, Bano N, Hollibaugh JT. 2003. Depth distribution of microbial diversity in Mono Lake, a meromictic soda lake in California. Appl Environ Microbiol. 69:1030-1042.

Ivanikova 2006. Lake Superior phototrophic picoplankton: nitrate assimilation measured with a cyanobacterial nitrate-responsive bioreporter and genetic diversity of the natural community. Doctoral Thesis. College of Bowling Green State University.

Ivanikova NV, Popels LC, McKay RML, Bullerjahn GS. 2007. Lake Superior supports novel clusters of cyanobacterial picoplankton. Appl Environ Microbiol. 73: 4055-4065.

Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol. 79(17):5112-20.

Li WKW, Harrison WG. 2001. Chlorophyll, bacteria and picophytoplankton in ecological provinces of the North Atlantic. Deep-Sea Res II. 48.10:2271-2293.

Melo, S. & Huszar, V.L.M. 2000. Phytoplankton in an Amazonian flood-plain lake (Lago Batata, Brasil): diel variation and species strategies. J Plankton Res. 22(1):63-76.

Nagata T, K. Takai, K Kawabata, M Nakanishi, J Urabe. 1996. The trophic transfer via a picoplankton-flagellate- copepod food chain during a picocyanobacterial bloom in Lake Biwa. Arch Hydrobiol. 137:145-160.

Newton RJ, Jones SE, Helmus MR, McMahon KD. 2007. Phylogenetic ecology of the freshwater Actinobacteria acl lineage. Appl Environ Microbiol. 73:7169-7176.

O'Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, Rajput B, Robbertse B, Smith-White B, Ako-Adjei D, Astashyn A, Badretdin A, Bao Y, Blinkova O, Brover V, Chetvernin V, Choi J, Cox E, Ermolaeva O, Farrell CM, Goldfarb T, Gupta T, Haft D, Hatcher E, Hlavina W, Joardar VS, Kodali VK, Li W, Maglott D, Masterson P, McGarvey KM, Murphy MR, O'Neill K, Pujar S, Rangwala SH, Rausch D, Riddick LD, Schoch C, Shkeda A, Storz SS, Sun H, Thibaud-Nissen F, Tolstoy I, Tully RE, Vatsan AR, Wallin C, Webb D, Wu W, Landrum MJ, Kimchi A, Tatusova T, DiCuccio M, Kitts P, Murphy TD, Pruitt KD. 2016. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res. 4:44(D1):D733-45.

Pantoja-Agreda F, Otero-Morales E. 2016. Autotrophic picoplankton assemblages in subtropical reservoir: temporal and vertical dynamics in abundance and biomass. J Fresh Ecol. Vol. 0. Iss. 0.0

Pinhassi J, Sala MM, Havskum H, Peters F, Guadayol Ò, Malits A, Marrasé C. 2004. Changes in bacterioplankton composition under different phytoplankton regimens. Appl Environ Microbiol. 70:6753-6766.

Pommier T, Canbäck B, Riemann L, boström KH, Simu K, lundberg P, *et al*. 2007. Global patterns of diversity and community structure in marine bacterioplankton. Mol ecol. 16: 867-80.

Postius C, Ernst A. 1999. Mechanisms of dominance: coexistence of picocyanobacterial genotypes in a freshwater ecosystem. Arch Microbiol. 172:69-75.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013 Jan:41(Database issue):D590-6.

Robertson BR, Tezuka N, Watanabe MM. 2001. Phylogenetic analyses of Synechococcus strains (cyanobacteria) using sequences of 16S rDNA and part of the phycocyanin operon reveal multiple evolutionary lines and reflect phycobilin content. Int J Syst Evol Microbiol. 51:861-871.

Sánchez-Baracaldo P, Hayes PK, Blank CE. 2005. Morphological and habitat evolution in the cyanobacteria using a compartmentalization approach. Geobiology. 3:145-165.

Sánchez-Baracaldo P, Handley BA, Hayes PK. 2008. Picocyanobacterial community structure of freshwater lakes and the Baltic Sea revealed by phylogenetic analyses and clade-specific quantitative PCR. Microbiology. 154:3347-3357.

Soler-Lopez LR. 2011. Sedimentation survey of Lago Cerrillos, Ponce, Puerto Rico, April-May 2008: U.S. Geological Survey Scientific Investigations Report. 2011-5057.

Stockner J, Callieri C, Cronberg G. 2000. Picoplankton and other non-bloom forming cyanobacteria in lakes. In: Whitton BA, and M. Potts (Eds). The Ecology of Cyanobacteria. Their Diversity in Time and Space. Kluwer Academic Publishers, Dordrecht, The Netherlands: 195-238.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 30:2725-2729.

Thompson FL, Bruce T, Gonzalez A, Cardoso A, Clementino M, et al. 2011. Coastal bacterioplankton community diversity along a latitudinal gradient in Latin America by means of V6 tag pyrosequencing. Arch microbial. 193:105-114.

Vörös L, Callieri C, Balogh KV, Bertoni R. 1998. Freshwater picocyanobacteria along a trophic gradient and light quality range. Hydrobiologia. 369/370:117-125.

Wakabayashi T, Ichise S. 2004. Seasonal variation of phototrophic picoplankton in Lake Biwa. Hydrobiologia. 528:1-16.

Warnecke F, Sommaruga R, Sekar R, Hofer JS, Pernthaler J. 2005. Abundances, identity, and growth state of actinobacteria in mountain lakes of different UV transparency. Appl Environ Microbiol. 71:5551-5559.

Winder M. 2009. Photosynthetic picoplankton dynamics in Lake Tahoe: temporal and spatial niche partitioning among prokaryotic and eukaryotic cells. J Plankton Res. 31:1307-1320.

Zwisler W, Selje N, Simon M. 2003. Seasonal patterns of the bacterioplankton community composition in a large mesotrophic lake. Aquat Microb Ecol. 3:211-225.

Table 1. Location and morphometric characteristics of Cerrillos Reservoir and Lucchetti Reservoir

Feature	**Cerrillos**	**Lucchetti**
Location	18°04' N- 66°24' W	18°05' N- 66°5' W
Area (km^2)	2.1	2.8
Drainage area (km^2)	45.3	44.8
Volume (10^6 m^3)	58.2	11.1
Mean depth (m)	25	11
Maximum depth (m)	65	25
Altitude (m a.s.l)	170	175
Water Retention time (yr^{-1})	0.87	2.9
Trophic State	Oligomesotrophic	Eutrophic
Uses	Water supplies/flood protection	Irrigation/water supplies

Table 2. Environmental and biological parameters of Cerrillos and Lucchetti Reservoirs in Puerto Rico.

Reservoir	Trophic Status	Secchi disk	Temperature	Oxygen	pH	Conductivity	Total Chlorophyll	Picoplankton Chlorophyll	Pcy Abundance
		m	°C	mg L^{-1}		µS cm^{-1}	µg L^{-1}	µg L^{-1}	Cells mL^{-1}
Cerrillos	Oligomesotrophic	2.4	25.4	6.2	7.8	236	1.7	0.13	$21x10^3$
Lucchetti	Eutrophic	1.8	24.7	6.56	8.05	290	3.2	0.08	$9x10^3$

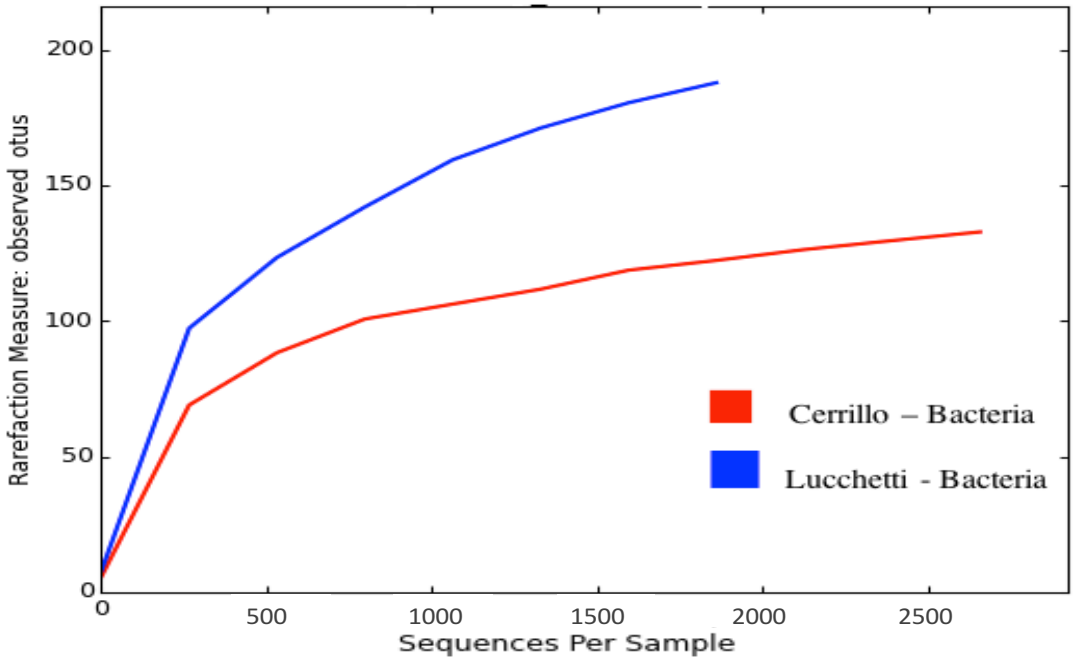

Figure 1. Rarefaction curves for bacteria from Cerrillos and Lucchetti Reservoir.

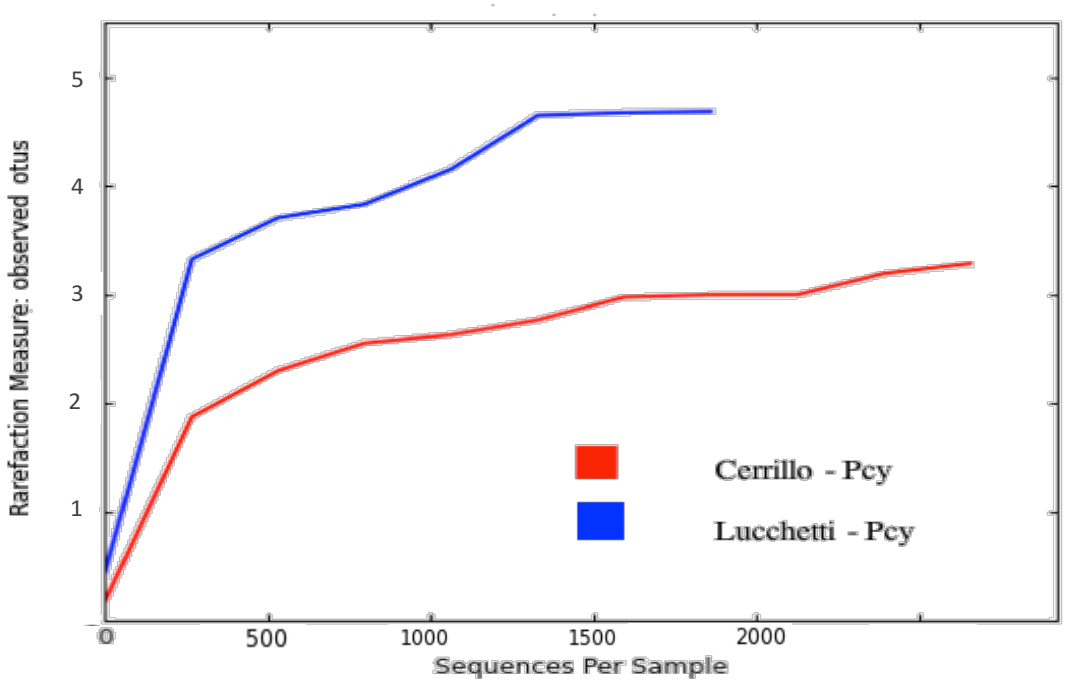

Figure 2. Rarefaction curves for Picocyanobacteria from Cerrillos and Lucchetti Reservoir.

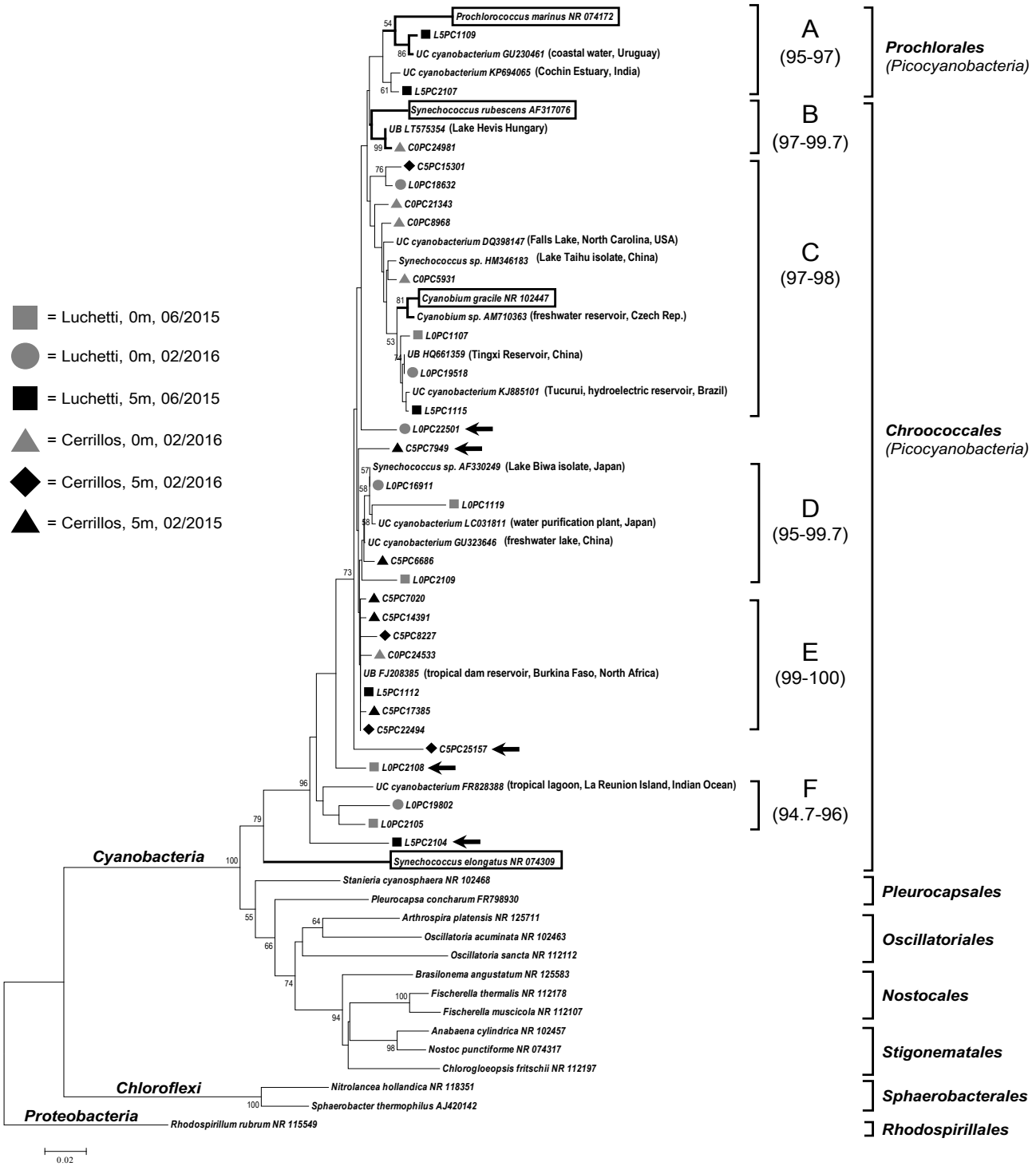

Figure 3. Neighbor-Joining dendrogram illustrating the relationships of representative 16S rRNA phylotypes recovered from Lucchetti and Cerrillos reservoirs (0-5m) with respect to representatives of the picocyanobacteria. Type strains representative of the phylum *Chloroflexi* were included as an outgroup. Letter (A-F) represent the main clusters revealed by the topology of the dendrogram. The sequence identity percent range across members of each is shown in parentheses. The following codes were used for the designation of OTU's: UB = uncultured bacterium; UC = uncultured; C0PC = Cerrillos reservoir, 0 m, picocyanobacterium; C5PC, = Cerrillos reservoir, 5 m, picocyanobacterium; L0PC = Lucchetti reservoir, 0 m, picocyanobacterium; L5PC = Lucchetti reservoir, 5 m, picocyanobacteria.

Chapter V

OVERALL CONCLUSIONS

- This doctoral dissertation is the first to of study of the ecology and diversity of the picoplankton community in the Caribbean freshwater environments. The results are an important contribution to the understanding of ecology, structure and diversity of the components of bacterioplankton in the region, providing ecological information about picoplanktonic autotrophic communities that develop in these systems.

- The contrasting trophic status of systems could influence the structure and dynamics of picoplanktonic communities inhabiting in these systems. A lower concentration of nutrients, and therefore the trophic state, could be a determining factor in the structure of the local picoplanktonic community in the reservoirs studied.

- Environmental factors such as temperature, light, and nutrients might be responsible for the abundance and dynamics of the autotrophic picoplankton. However, it is suggested that the control by biotic factors such as grazing, competition, and parasitism which are not considered in this study could control the abundance and lead to temporal succession of the autotrophic picoplankton.

- In both reservoirs, the highest values of chlorophyll-*a* did not occur in the surface layer of the photic zone, perhaps it is due to photoinhibition by prolonged exposure to high

irradiance of the tropical zones and larger phytoplankton that causes a decrease in the growth of picoplankton and photosynthetic rates.

- The picoplankton density obtained by epifluorescence microscopy was consistent with the results obtained by flow cytometry. These techniques demonstrated efficiency and sensitivity in the detection and quantification of both prokaryotic and eukaryotic cells, allowing greater discrimination by fluorescence emission between different cell types.

- Autotrophic picoplankton abundance was different between periods and increased during the summer especially in Cerrillos reservoir, where it reached maximum concentrations in full stratification. Autotrophic picoplankton, did not show marked seasonality as in temperate zones where bimodal patterns are registered.

- In both reservoirs studied, different genera of picocyanobacteria were identified. The dominant type along the entire limnologic cycle is the genera *Synechococcus sp*. The rest of the morphotypes of autotrophic picoplankton like *Cyanobium sp*, had highly variable distributions, with significant fluctuations on the type of dominant species.

- The picocyanobacteria community in the ecosystems studied is diverse and dynamic; the strains are scattered in the 16S rRNA tree of polyphyletic origin. Many of the lineages were restricted to a single ecosystem, however certain strains were present in both environments and their closest relatives have been recorded from different geographic regions with contrasting limnological characteristics.