BENTHIC ALGAE AS BIOLOGICAL INDICATORS OF TROPHIC STATUS IN TROPICAL STREAMS

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ABSTRACT

This study describes the stressor-response relationships between water quality parameters and periphyton biomass on artificial and natural substrates of three tropical streams with contrasting trophic status. At the nutrient enriched study site, Río Piedras, a 2-day antecedent discharged volume exceeding 3.0×10^5 m^3 negatively impacted the periphyton biomass growth on natural substrates due to physical scouring. No significant negative effects of antecedent cumulative hydrologic discharge were observed at the two other sites, Río Mameyes and Río Guanajibo. To minimize the effect of physical scouring, concrete blocks were used as artificial growth media for periphyton biomass. A seven-day accrual time resulted in positive correlations between periphyton biomass with total phosphorus (TP) (R^2=0.74, $p<0.01$) and total nitrogen (TN) (R^2=0.58, $p<0.01$) suggesting that artificial substrates for periphyton growth could be a potential tool for setting the degree of biological impairments as related to nutrient concentrations in stream waters. The benthic chlorophyll-a limit of 70 mg/m^2 in natural substrates suggested by Dodds et al. (1998) was used to predict a 7-day periphyton biomass value indicative of impairment of 19.2 mg/m^2 in artificial substrates. The nutrient concentrations associated with said threshold (19.2 mg/m^2) were 0.169 mg TP/L and 1.64 mg TN/L. Numeric nutrient criteria suggested by Sotomayor-Ramírez et al. (2011) for Puerto Rico based on a frequency distribution approach are 0.160 mg TP/L and 1.70 mg TN/L which are similar to the predicted values obtained in this study. The periphyton community structure on the moderately nutrient enriched site experienced a shift from diatoms to cyanobacteria during ten days of incubation. Further studies should be conducted in order to validate the predictions made in this study as well as investigate the population dynamics of periphyton community on streams with moderate enriched nutrient conditions.

RESUMEN

Este estudio describe las relaciones estrés-respuesta entre los parámetros de calidad del agua y la biomasa del perifiton en sustratos artificiales y naturales de tres ríos tropicales con contraste en el estado trófico. En el sitio enriquecido de nutrientes, Río Piedras, un volumen de descarga hidrológica antecedente de 2 días al muestreo que excede 3.0 x 10^5 m^3 redujo la biomasa del perifiton colonizado en sustratos naturales. No se observaron efectos negativos significantes de descarga hidrológica antecedente cumulativa en los otros dos sitios, Río Mameyes y el Río Guanajibo. Para minimizar el efecto de abrasión, bloques de concreto se utilizaron como medio de crecimiento artificial para la biomasa de perifiton. Un tiempo de acumulación de siete días resultó en correlaciones positivas entre la biomasa del perifiton con fósforo total (TP) (R^2=0.74; p<0.01) y el nitrógeno total (TN) (R^2=0.58; p<0.01) sugiriendo que los sustratos artificiales para el crecimiento de perifiton podría ser una herramienta potencial para establecer el grado de alteraciones biológicas en relación con las concentraciones de nutrientes en las aguas de los ríos. El límite de clorofila-a béntica de 70 mg/m^2 en sustrato natural sugerido por Dodds et al. (1998) fue utilizado para predecir una biomasa de perifiton de 7 días en sustratos artificiales de 19.2 mg/m^2. Las concentraciones de nutrientes asociados con dicho umbral (19.2 mg/m^2) fueron 0.169 mg TP/L y 1.64 mg TN/L. Los criterios numéricos de nutrientes sugeridos por Sotomayor-Ramírez et al. (2011) para Puerto Rico, basados en un enfoque de distribución de frecuencias, son 0.160 mg TP/L y 1,70 mg TN/L, que son similares a los valores predichos obtenidos en este estudio. La estructura de la comunidad de perifiton en el sitio moderadamente enriquecido de nutrientes experimentó un cambio de diatomeas a cianobacterias durante los diez días de incubación. Nuevos estudios deben llevarse a cabo con el fin de validar las predicciones realizadas en este estudio, así como investigar dinámicas de

población de la estructura de la comunidad de perifiton en los ríos con condiciones enriquecidas moderadamente de nutrientes.

DEDICATION

To my parents: Norma and Jorge, and to my brother José María
To my friends
To the community

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INTRODUCTION

Puerto Rico has 96 basins with 115 rivers and 44 streams covering a total length of 8,120 km (PREQB, 2008). Currently, 22 basins with 51 rivers and one stream having a length of 4,618 km are monitored by the United States Geological Survey (USGS) under a cooperative agreement with the government of Puerto Rico (PREQB, 2010a). The USGS water quality station network is sampled on a quarterly basis for the analysis of selected physical and chemical parameters (PREQB, 2012). The data is used to prepare the List of Impaired Waters 303(d). As of 2010, the major causes of impairment were arsenic, cyanide, dissolved oxygen, pathogens, and turbidity with 41% of all river and stream miles having some cause for impairment (PREQB, 2010a).

Water quality standards are established to protect public welfare, enhance the quality of the water, and assure that designated uses are attained (USEPA, 2000). In Puerto Rico, surface waters such as rivers and lakes are referred as class SD[1], and their designated uses are: water source for drinking water supply, propagation and preservation of desirable species (including threatened and endangered species) and primary and secondary contact recreation (PREQB, 2010a). When specified uses are assigned to a body of water, water quality criteria are adopted to protect and maintain those uses (this is known as the anti-degradation policy) (USEPA, 2000).

Criteria are one of the elements of water quality standards specifying the amount of pollutant that can be present without impairing designated uses. Numeric criteria are measurable values, levels, or concentrations of water constituents that include physical, chemical and biological conditions. The U.S. Environmental Protection Agency (USEPA) has developed an

[1] Class SD refers to the surface waters designated as to raw source of public water supply, propagation and preservation of desirable species, and for primary and secondary contact recreation (PREQB, 2008)

official report with technical guidance to assist States, Tribes and Territories to develop numeric nutrient criteria (USEPA, 1998).

Although USEPA had stated that the second leading cause of water pollution in rivers and streams are nutrients (USEPA, 2013), in Puerto Rico nutrients are not officially considered a major contaminant, which may be due to inadequate standards currently been used for the identification of impaired waters (PREQB, 2010b). The existing numeric nutrient standards established for Class SD surface waters are 1 mg/L for total phosphorus (TP) and 10 mg/L for nitrate-N (NO_3-N) (PREQB, 2010c). The total P numeric criterion corresponds to the maximum discharge concentration allowed by USEPA for point sources as part of NPDES (National Pollutant Discharge Elimination Systems) permits. The nitrogen criterion is the maximum nitrate concentration permitted in drinking water (PREQB, 2010b). These nutrient standards were not developed by objective to protect ecological integrity of lotic systems. The PREQB has determined that in order to develop scientifically supported standards for ecosystem preservation, numeric nutrient criteria for rivers and streams are necessary. The biological characterization of organisms or bioprocesses can be used as ecological indicators of impairment. In this sense, the quantitative relationships between nutrients and ecological thresholds of algal biomass may prove useful for setting regulatory guidelines.

Periphyton communities in streams are the base of the food web that is mostly dominated by benthic algae (Barinova, 2011). Heterokontophyta or diatoms include a broad number of species that can inhabit a wide array of habitats including planktonic, benthic, standing and flowing waters. Many diatom species have specific water requirements so they have been used to monitor ecological characteristics of stream waters (Wehr and Sheath, 2003). Due to their rapid response to stressors, identification of specific species with a narrow range of tolerance to contaminants, diatoms have been used as biological indicators (Zheng and Paul, 2008; Potapova et al., 2004). Thus, many studies have examined the periphyton community response to a range of environmental variables such as pH, salinity, saprobity, and nutrients, and biotic indices founded primarily on diatom metrics such as relative abundances of tolerant and sensitive diatom species, richness and dominance species and trophic diatom indices (Hill et al., 2002; Smucker et al., 2013; Dodds et al., 1998; Bellinger et al., 2006). Likewise, many other studies have used non-taxonomical biological indicators to determine the water quality conditions based on approaches of benthic and planktonic algal biomass measured as chlorophyll-a and ash free dry mass, and periphyton metabolic activity (Biggs, 2000b; Boisson and Perrodin, 2006; Lavoie et a., 2004)

This thesis is divided in three parts. In the first chapter, I described the natural biological, physical and chemical conditions of three streams from different basins. In the second chapter, I examined benthic algae response to the physical and chemical parameters in the water column using artificial substrates. Finally in the third chapter, I assessed the taxonomical characterization of the periphyton community established on artificial substrates.

CHAPTER 1

ASSESSMENT OF PERIPHYTON BIOMASS ON NATURAL SUBSTRATES OF THREE TROPICAL STREAMS WITH CONTRASTING NUTRIENT STATUS

INTRODUCTION

Elevated nutrient concentrations in stream-waters affect aquatic life, human health and result in overall aesthetic impairment. Eutrophication from nitrogen (N) and phosphorous (P) causes excessive algal growth of species that can produce toxic compounds such as the dinoflagellate *Ostreopsis ovata* and several cyanobacteria genera (i.e. *Anabaena* and *Microcystis*) (Davidson et al., 2012; Granéli et al., 2011). Also, algal blooms cause nuisance problems resulting in changes in odor and taste (Metcalf and Codd, 2014). Excessive primary production will build up organic biomass-carbon in the aquatic system that upon decay decreases dissolved oxygen, and changes water pH, leading to fish and invertebrates die-off (Quinn and Gilliland, 1989; Welch, 1992). Ingestion of water by babies and infants with NO_3-N concentrations exceeding 10 mg/L can potentially cause methemoglobinemia, among other health concerns in infants and adults (USEPA, 1995). In terms of economics, nutrient enrichment increases the costs to clean the municipal water due to algal mats clogging the drinking water treatment plant filters (Nordin, 1985; Dodds and Welch, 2000).

When nutrients enter rivers, they can be taken by primary producers and transformed to and from organic or inorganic fractions in the water or in sediments. Nutrient cycling encompasses many transformations due to a range of abiotic and biotic processes operating between the water column and the sediments (Withers and Jarvie, 2008). Dissolved P may associate strongly with soil particles (i.e. clays, metal oxides) accumulating in the sediments, remaining as potential nutrient source for algae. Molecular nitrogen (N_2), found in the air, can be fixed by algae (i.e. cyanobacteria) and thus enter the aquatic system. Algae can use inorganic nitrogen in the form of ammonia and nitrate.

Nutrients contaminate waters from point and non-point sources. Point sources are confined and discrete conveyances from which pollutants are discharged, such as industries waste pipes and sewage treatment plants, and are thus controlled and regulated by environmental agencies (Withers and Jarvie, 2008; Mesner and Paige, 2011). However, nonpoint sources are more challenging to measure and control. The most common nonpoint nutrient sources originate from agricultural activities, urban areas, and construction sites with runoff being the primarily mode of transport into rivers (Carpenter et al., 1998). These sources are intermittent causing nutrient loading to rivers to significantly vary due to temporal (i.e. storm events) and spatial factors (i.e. land slopes). Therefore, the land use and land cover proportions could indicate the potential surface water quality conditions within a watershed.

Nutrient enrichment is one of the major causes of water impairment in continental U.S. surfaces waters (USEPA, 2013). Hence, efforts have been made to establish nutrient thresholds in order to reduce and improve water quality and ecological conditions (Chambers et al., 2012). In lotic waters, Dodds et al. (1997) suggested that a mean benthic algal chlorophyll-a biomass higher than 100 mg/m^2 is undesirable from a water quality perspective. The authors concluded that in order to maintain streams with benthic algae biomass below nuisance levels the mean TN and TP concentrations should be below 0.350 mg/L and 0.030 mg/L, respectively. Later, Dodds et al., (1998) used the cumulative frequency distribution approach to determine the boundaries of trophic state categories for TP, TN and mean benthic chlorophyll-a. The authors used the 1/3 percentile (Q_{33}) to set the boundary of oligo-mesotrophic and the upper percentile (Q_{66}) of meso-eutrophic state; for the latter category the limit proposed was 0.075 mg P/L, 1.50 mg N/L resulting in a mean chlorophyll-a biomass of 70 mg/m^2. The latter was proposed as an ecological threshold of impairment.

In Puerto Rico, the most recent study by Sotomayor-Ramírez and Martínez (2013) said approach to set the boundaries for three trophic state categories namely: non-enriched, enriched, and impaired. The authors used a historical water quality database from 1990 to 2003 in order to determine the limits of TP, TN and NO_3-N concentrations. The proposed lower limits for the impaired category were 0.160 mg P/L, 1.70 mg N/L, and 0.97 mg NO_3-N/L.

The present study will assess benthic algae response to nutrients in three streams having contrasting trophic status. The streams are within watersheds differing in several characteristics such as: flow regime, life-zone ecoregion, land cover, and geomorphology. The study will evaluate physical and chemical characteristics that affect the periphyton community structure that will serve to establish quantitative relationships between periphyton biomass and nutrient status.

OBJECTIVES

- Describe the temporal variations of algal biomass of three lotic systems of contrasting nutrient status.
- Establish quantitative relationships between algal biomass and abiotic factors.

MATERIALS AND METHODS

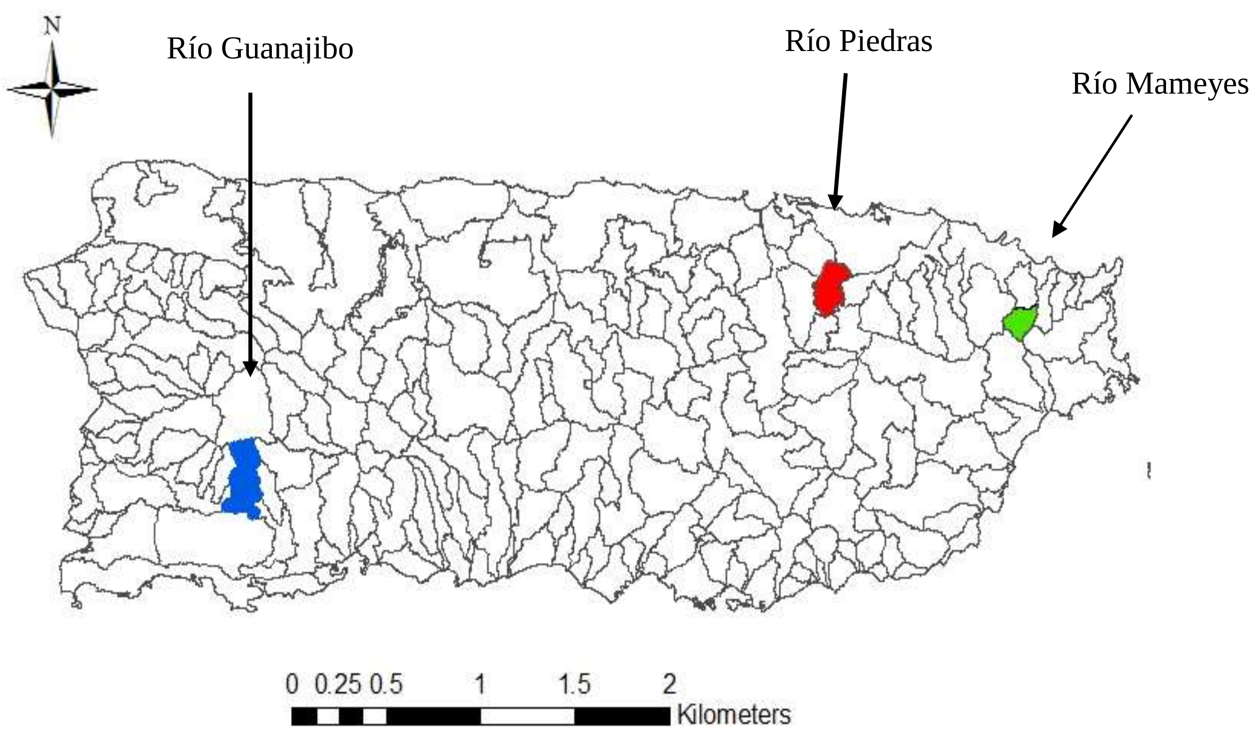
Study sites

The Mameyes watershed is located near the northeastern region of Puerto Rico and the principal river, Río Mameyes, is one of the five main rivers that originate at El Yunque National Rainforest. The watershed has a basin area of 40.3 km^2 and the river originates at approximately 1000 meters above sea level (m.a.s.l) extending 15 km from headwaters down to Palmer District (80 m.a.s.l) to its river mouth at the Atlantic Ocean (Ortiz-Zayas et al., 2005). The watershed is covered primarily with 93% of forest and pastures, and 5% of urban sectors located in the lowlands of Palmer and Río Mar. The mean annual precipitation ranges from 2,000 to 4,000 mm at higher elevations and rainfall events are frequent, with the largest rainfall events occurring in May and from September to November; February to April are usually the driest months (Ortiz-Zayas et al., 2005; DRNA, 2004b).

The sub watershed of Río Mameyes (RM) (Figure 1) has a catchment area of 17.2 km^2 from the headwaters to the sampling site (18°19’34” N, 65°44’55” W), with a length of 5.5 km (Appendix 1A). The sub-watershed comprises more than 99% of forests and shrublands and less than 0.05% of wetlands and urban zones (Table 1). The geology consists of sedimentary and plutonic (i.e. quartz and granodiorite) formations from the Eocene, Paleocene and Cretaceous period. In the northern parts of the watershed, quartz-dionite formations predominate, while in the southern part, closest to the sampling site, basaltic volcanic sandstone and siltstones predominate.

The Guanajibo watershed is located at the southwestern region of Puerto Rico with a basin area of 331 km^2 and originates at the boundary between Maricao and Sábana Grande municipalities. The headwaters are at an elevation of 750 m extending 36 km from north to southwest covering the municipalities of Sábana Grande, San Germán, and Hormigueros to end in the boundary between Mayagüez and Cabo Rojo coast. The watershed comprises ten sub-watersheds defined by United States Geological Survey (USGS); these include: Río Guanajibo, Río Grande, Río Cruces, Río Cupeyes, Río Caín, Río Duey, Río Rosario, Río Hondo, Río Viejo and a coastal watershed north and south of Río Guanajibo mouth. Due to the multiple tributaries that feed Río Guanajibo, this river is classified as a third order stream (DRNA, 2004a). The watershed has an heterogeneous land cover including approximately 50% forests, 26% shrublands and pastures, 13% agricultural lands, 9.5% urban zones and <2% of wetlands. The climate in this region is the driest in Puerto Rico with mean annual precipitation ranging from 1,100 to 1,650 mm; however, at higher elevations annual precipitation can reach 2000 mm.

Figure 1: Spatial location of the three sub watersheds selected this study.



The Río Guanajibo sampling site (RG) (18° 04’08” N, 66° 58’09” W) is located at the Sábana Grande municipality with a catchment area of 39 km^2 and at an elevation of 77 m.a.s.l (Figure 1). The catchment area includes mixed land uses of forest area (67%), shrublands and pastures (14.1%), urban zones (5.8%), agriculture (13.1%) and wetlands (0.12%) (Table 1). Two water plants, one for potable and the other for waste treatment purposes, managed by the Water and Sewer Authority (AAA, Spanish acronym) are located within the sub-watershed (Appendix 1B). The geology consists mainly of serpentine rock formations from the Miocene and Oligocene periods, as well as plutonic and sedimentary formations from the Cretaceous period. Quaternary alluvium deposits predominate in the lower part of the sub watershed and closest to the sampling site.

Table 1: Proportion in land cover distribution in the three sub-watersheds studied.

Study site[1]	Forests	Urban	Shrublands	Wetlands	Agriculture	Total area
	-------------------------------------	-------	---%---	-------	-------------------------------------	km^2
RM	99.65	0.02	0.29	0.03	--	17.2
RG	66.9	5.8	14.1	0.12	13.1	39.0
RP	30.1	49.0	20.3	0.58	--	30.0

[1]RM is Río Mameyes; RG is Río Guanajibo; RP is Río Piedras

The Río Piedras watershed originates in the slopes of the mountains of Trujillo Alto municipality at an approximate elevation of 300 m.a.s.l and runs from south to north ending at the Atlantic Ocean (DRNA, 2004c). The catchment area of the watershed (67 km^2) covers the northern part of the capital city of San Juan. The urban land covers 65.4% of the total catchment area followed by forests and pastures (31.6%), wetlands (3%) and no significant agricultural land use is registered. The north-northeast winds induce frequent evening orographic rains with mean annual precipitation range from 1,700 to 1,900 mm.

The Río Piedras sampling site (RP) (18°23’30” N, 66°03’28” W) is located at the Río Piedras district from San Juan municipality (Figure 1). The sub-watershed covers a catchment area of 30 km^2 extending 10.4 km (Appendix 1C). The land uses are primarily urban zones (49%), followed by 30.1% of forests and 20.3% of pastures; the remaining area is covered with water bodies (Table 1). The basin geology is from the Eocene, Paleocene and Cretaceous periods with plutonic rock formations of quartz and granodiorite composition; also, sedimentary formations and alluvium deposits are found in the lower part of the watershed.

Field Measurements

The study sites were visited from January to October 2013 at least once a month except in May in which none of the sites were visited due to recurrent storm events. Dissolved oxygen (DO), pH, specific conductance and temperature were gathered *in situ* using YSI Professional Pro (YSI, Yellow Springs, OH) calibrated according to manufactured instructions prior to each sampling. The stream water velocity was measured using a flow meter probe (Global Water Instrumentation, College Station, TX). The total width for the study site was divided in equal segments. The stream flow (m^3/s) was calculated as the sum of the product of the width, depth and water velocity measured in each segment.

Surface water samples were collected from the middle of the stream with a 1L polyethylene clear bottle for chemical analysis. The samples were preserved with 36N sulfuric acid to attain a sample water pH< 2.0, stored in a cooler with ice, and transported to the Soil and Water Quality Laboratory located at the Río Piedras Agricultural Experimental Station in Río Piedras, Puerto Rico. The chemical analysis included total phosphorus (TP; EPA method 365.4), nitrate as nitrogen (NO_3-N; EPA method 353.1) and total Kjeldahl nitrogen (TKN; EPA method

351.2). Prior to NO_3-N analysis, the sample was filtered using a 0.45µm GF/F filter(Glass Fiber type F, Whatman). Total nitrogen (TN) is the sum of TKN and NO_3-N concentrations.

One sample of periphyton biomass was collected at each sampling date, during the sampling period. The sample consisted of randomly selecting ten rocks from a 30 to 50 meter reach along the stream length. The surface of each rock was scrubbed by delineating a 6 cm diameter PVC ring to wash all the material of the outer area of the ring. The material within the ring was quantitatively transferred by gently scrubbing the material into a 500 mL amber bottle using a wash bottle containing streamwater. The composite sample consisted of water with the periphyton biomass from ten rocks which was diluted to a final volume of 500 mL (Gualtero-Leal, 2007). The sample was stored in a cooler with ice and transported to the Chemistry Laboratory of the Agronomy and Soils Department in University of Puerto Rico, Mayagüez.

Biological Characterization

For chlorophyll-a analysis, two subsamples were filtered through a Whatman GF/F filter membrane followed by the fluorometric analysis for chlorophyll-a in the presence of other pigments (Welschmeyer, 1994). After filtration, the filter was stored in a sealed 50 mL amber centrifuge tube below 0°C and was analyzed within 1 and 21 days. To extract the chlorophyll-a, 5 mL of 90% acetone solution was added to the 50 mL centrifuge tube. The filter was mashed using a glass stirring rod and 5 mL of 90% acetone was added to rinse the stirring rod and soak the mashed filter completely. The final volume on the centrifuge tube was 10 mL, and was stored below 0°C for an additional 22 to 24 hours. The tubes were centrifuged three times at a speed of 5000 rpm for five minutes. At the end of the first two centrifuges, the samples were decanted into a 15 mL amber tube and stored on a cooler. An additional 5 mL of 90% of acetone was added to the 50 mL tubes containing the filter and the sample was centrifuged again. After the third centrifuge, the final decanted (extracted) volume was 15 mL. The extracted volume was analyzed in a Turner Designs TD-700 fluorometer following the manufacturer specifications.

The final periphyton biomass was calculated as:

$$Chl - a_{corrected} = \frac{Chl - a_{uncorrected} \times V_{sample} \times V_{extracted} \times F.D.}{V_{filtered} \times n \times Area} \quad [1.1]$$

where Chl-$a_{corrected}$ is chlorophyll-a corrected after conversion to mg/m^2; Chl-$a_{uncorrected}$ is chlorophyll-a concentration obtained from the instrument in µg/L; V_{sample} is the sample volume (0.500 L); $V_{extracted}$ is the final volume after acetone extraction (0.015L); F.D is the dilution factor; $V_{filtered}$ is the filtered volume of the subsample; n is the number of rocks scrubbed; and Area is the marked area of the PVC ring (3.52565 x 10^{-3} m^2).

For ash-free-dry-mass (AFDM) analysis, two subsamples were filtered through a Whatman GF/F filter, previously cleaned of any organic impurity in a muffle furnace at 500°C for 1 hour. The procedure consists of gravimetrically quantifying the filtered material before and after a 2-hour incineration at 500°C. Prior to incineration, the filtrate was dried in the oven at 105°C for 24 hours. The AFDM was calculated as:

$$AFDM \left(\frac{g}{m^2}\right) = \frac{(W_1 - W_2) \times V_{sample}}{V_{filtered} \times n \times Area} \quad [1.2]$$

where W_1 is the weight of crucible + filter + filtered material after dried; W_2 is the weight of crucible + filter + filtered material after incineration; V_{sample} is the volume sample (0.500 L); $V_{filtered}$ is the volume of subsample filtered; n is the number of rocks scrubbed and poured into the bottle; and Area is the marked area of the PVC cylinder (3.52565 x 10^{-3} m^2).

The filtered volume for each sub-sample varied depending on the amount of turbidity associated from the biological material present in the sample. Frequently, samples from RG and RP were very concentrated; therefore the volume varied from 15 to 30 mL. On other occasions, the standard volume of the water filtered was 50 mL.

The periphyton community structure is composed of consumers (heterotrophs), primary producers and organic detritus. AFDM measures all the organic material present in the substrate without distinction between organisms. To understand the relative influence of autotrophs on the entire periphyton community, the autotrophic index (AI) was calculated based on the ratio of all periphyton biomass (AFDM, in mg/m^2) to algal biomass (chlorophyll-a, in mg/m^2). A higher numerical value indicates that heterotrophs dominate the community, and a lower numerical value indicates that autotrophs, meaning algae, predominates.

Biological oxygen demand (BOD)

Water samples were collected from the surface flow of the river in a 4L clean container and transported immediately to the laboratory. The sample was transferred into a 3L beaker and stirred in a stirring plate for 10 to 15 minutes to homogenize the dissolved oxygen saturation to between 90 and 100%. Ten 300 mL BOD clear bottles, previously sterilized in an autoclave, were wrapped in aluminum foil to prevent light penetration. Five bottles were filled and the dissolved oxygen concentration (BOD_0) was measured using YSI 5010/5100 DO probe meter. The other five bottles, filled with water sample and a magnet, were placed on a stirrer plate for 5 days under controlled temperature of 20°C. After 5 days, the DO was measured and recorded as BOD_5. The respiration was calculated as the difference in DO concentration between BOD_0 and BOD_5 to obtain the 5-day oxygen consumption rate.

Discharge evaluation

The mean daily discharge was gathered from the USGS monitoring network station in order to determine the effect of flow disturbance on periphyton biomass. The study sites were selected with the maximum possible proximity to USGS stations. The discharge database from the Río Piedras at El Señorial, PR (USGS 50048770) station is located 2.7 km upstream from the RP site. The USGS monitoring station at highway 119 in San Germán, PR (USGS 50131990) was 7 km downstream from the RG sampling site. Finally, Río Mameyes near Sabana, PR station (USGS 50065500) was the closest with 0.2 km downstream from the RM site.

Data analysis

In order to homogenize variance, all parameters were log transformed and normality was verified using Shapiro-Wilks test. Comparison of all the parameters among rivers was conducted with one way ANOVA and Tukey statistical test. Pearson correlations were generated to find correlations between environmental and biological parameters. Simple and stepwise regression analyses were performed to determine the best fit model for quantitative relationships between abiotic factors and periphyton biomass (Infostat Software, 2013 version)

The instantaneous daily discharge (m^3/s) was converted into mean daily volume (m^3/day). The sum of the volume discharged several days before sampling for periphyton biomass was calculated by the following equation:

$$V_n = \sum_{i=1}^{n} (Q_1 + \ Q_2 \ldots . + Q_n) \qquad [1.3]$$

where V_n is the volume accumulated for *n* days prior sampling in cubic meters (m^3) and Q_n is the sum of the mean daily discharge of n day in cubic meters per day (m^3/day). In order to determine the best fit of the flow data and periphyton biomass, the cumulative volume for 1, 2, 3, 5, 7, and

10 days prior sampling date was evaluated to generate regression curves between the cumulative volume and periphyton biomass for each study site.

The difference in catchment area between the sampling site and the USGS monitoring station will result in an overestimation or underestimation of the mean daily discharge. In order to correct for this difference, a conversion factor was calculated for two of the study sites (RG and RP) that had the major difference in basin area. The conversion factor was calculated as:

$$V_{site} = \frac{A_{site}}{A_{USGS}} \times V_{USGS} \qquad [1.4]$$

where V_{site} is the estimated mean daily volume for the sampling site in m^3, A_{site} is the sub watershed catchment area of the study site, A_{USGS} is the monitoring station sub watershed catchment area and V_{site} is the mean daily discharge (m^3) gathered from the USGS monitoring network station database.

RESULTS

Physical and chemical characteristics

Río Mameyes site is the widest stream studied having a mean width of 15.4 m and the instantaneous hydrologic flow ranged from 0.33 to 3.37 m^3/s (Table 2). Precipitation and discharge events rarely occurred during the study period in Río Guanajibo; hence, this site had the smallest stream mean width of 6.1 m and its measured flow values were the lowest ranging from 0.04 to 0.72 m^3/s. Río Piedras site had a mean width of 10.1 m with a stream flow range of 0.31 to 1.41 m^3/s.

Table 2: Descriptive statistics of the physical and chemical parameters measured on site for each study site during the period of January to October 2013.

Study site[1]	Flow	Stream width	Temperature	pH	Dissolved oxygen	Specific conductance
	(m^3/s)	(m)	(°C)		(mg/L)	(µS/cm)
RM	1.45 b[2]	15.40 c	22.67 a	7.83 a	8.22 b	91.45 a
RG	0.21 a	6.12 a	26.24 b	8.56 b	8.11 b	459.18 b
RP	0.61 a	10.10 b	25.99 b	7.85 a	6.95 a	411.32 b

[1]RM is Río Mameyes; RG is Río Guanajibo; RP is Río Piedras
[2]Common letters within a column indicates no statistical difference between study sites ($\alpha<0.05$).

Río Mameyes site showed the lowest mean temperature throughout the sampling period while no statistical differences ($p>0.05$) between RP and RG were observed. Specific conductance did not exhibit statistical differences between RG and RP which was higher than RM site. Río Mameyes and RP site did not show significant differences in pH with values nearly neutral. In RG, however, pH was higher with mean of 8.11 (range: 8.27 to 8.97) during the sampling period. The dissolved oxygen observed in RM and RG did not exhibit statistical differences with values above 8.0 mg/L most of the cases. In RP, however, the DO values observed were lowest (Appendix 2).

Nutrient concentrations (i.e. TN, TP, NO_3-N) differed significantly among study sites except for TKN-N concentrations in which no statistical differences were observed between RG and RM (Table 3). For RG and RP, NO_3-N contributed 70% of the total N in more than 40% of the cases. In contrast, for RM site most of the TN was attributed to TKN-N. The N:P ratio in the water column varied between 10.6 and 16.4 in RM and RP, respectively, while RG had intermediate ratio of 11.9. Nutrient ratios in periphyton biomass were not assessed.

Table 3: Median nutrient concentrations for the three study sites

Study site[1]	TKN-N	NO_3-N	TN	TP	N:P
	---------------------------mg/L----------------------				
RM	0.096 a[2]	0.062 a	0.180 a	0.010 a	16.4 b
RG	0.128 a	0.287 b	0.456 b	0.038 b	11.9 a
RP	0.376 b	0.821 c	1.22 c	0.121 c	10.6 a

[1]RM is Río Mameyes; RG is Río Guanajibo; RP is Río Piedras
[2]Common letters within a column indicates no statistical difference between study sites (α<0.05).

Pearson correlations were generated in order to relate physical and chemical characteristics. Dissolved oxygen was negatively correlated to TN (r= -0.68, p<0.01), NO_3-N (r=-0.68, p<0.01), and TP (r=-0.71, p<0.01) suggesting that oxygen consumption is associated with nutrients loads. Specific conductance was positively correlated to TP (r=0.78, p<0.01), TN (r=0.70, p<0.01) and NO_3-N (r=0.83, p<0.01). Also, specific conductance was associated with other physical parameters such as: temperature (r=0.60, p<0.01), pH (r=0.54, p<0.01) and DO (r=-0.40, p<0.01) (Appendix 3).

Periphyton biomass on natural substrates

The periphyton biomass was characterized based on chlorophyll-a concentration (chl-a), ash free dry mass (AFDM), and autotrophic index (AI). Río Guanajibo and RP exhibited statistically similar values of chl-a and AFDM with significant greater values than RM (Table 4). The AI values showed no significant differences among sites.

At RP site, two samples (sampled on July 24, 2013 and August 2, 2013) were considered as outlier observations given that both cases corresponded to samplings conducted after high flow events (Appendix 4). Therefore, these observations were eliminated for the descriptive statistics of biotic parameters and for the regression analyses between biotic and abiotic factors. Excluding these outliers, the chlorophyll-a concentration ranged from 17.3 to 108 mg/m^2 (CV=36%) whereas RG ranged from 8.4 to 48 mg/m^2 (CV=30%) and RM showed the least variability (CV=8%) with values ranging from 1.55 to 2.85 mg/m^2.

Table 4: Mean values for periphyton biomass based on non-taxonomical parameters

Study site[1]	Chlorophyll-a	AFDM	Autotrophic Index
	-------------mg/m^2-----------		
RM	2.15 a[2]	831 a	380 a
RG	21.70 b	9,343 b	426 a
RP	35.78 b	7,670 b	282 a

[1]RM is Río Mameyes; RG is Río Guanajibo; RP is Río Piedras
[2]Common letters within a column indicates no statistical difference between study sites (α<0.05).

Biological oxygen demand (BOD)

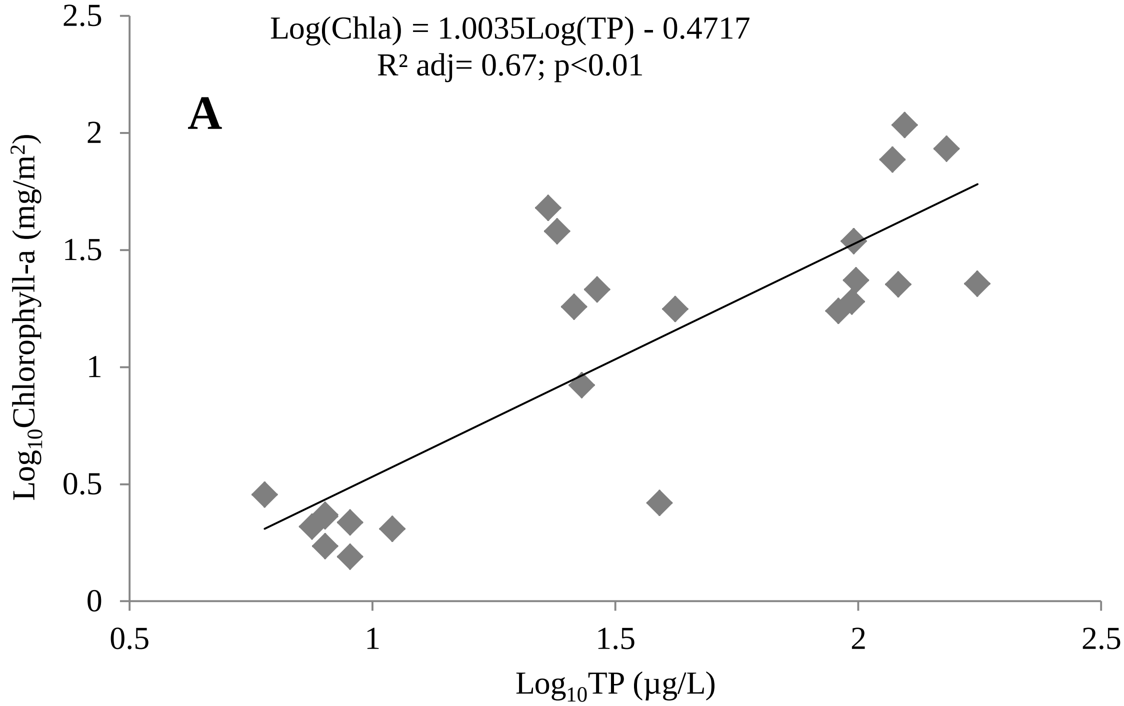
The water samples were gathered between July 2013 and March 2014. The dissolved oxygen concentration lost within 5 days (BOD_5) did not show statistical differences (F=1.52; p=0.2553) between study sites. However, the respiration the temporal fluctuations varied within study sites. The average DO consumption was 0.28 mg/L (CV=78%) in RM site, 0.82 mg/L (CV=90%) in RG site and 0.82 mg/L (CV=41%) in RP site.

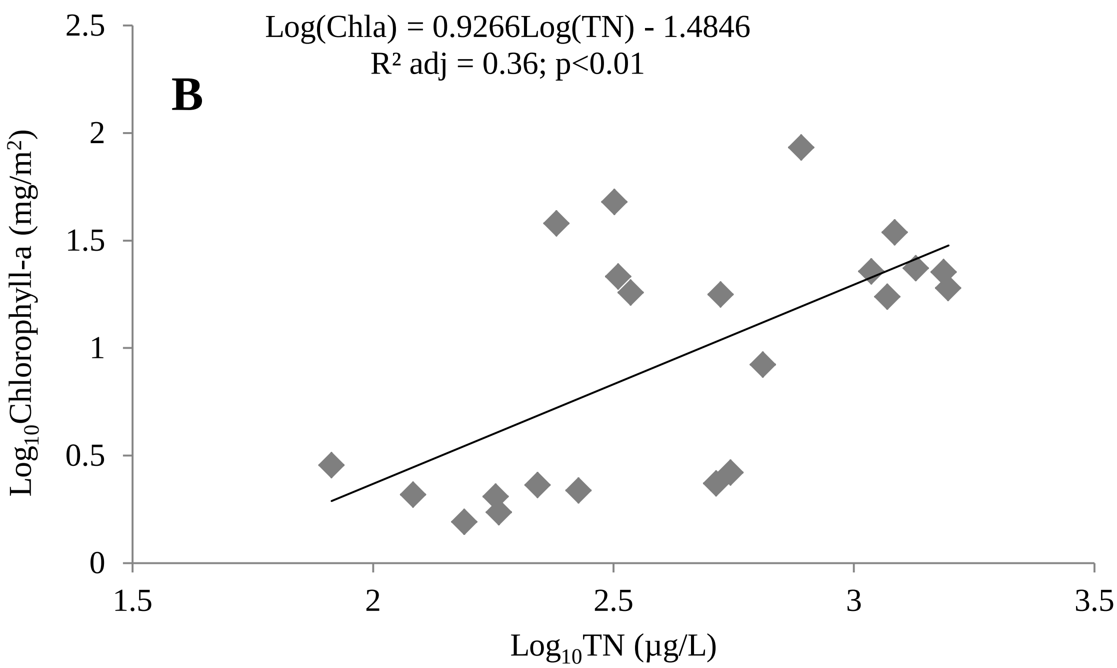
Biotic and abiotic relationships

The biological parameters exhibited significant correlations with nutrients: NO_3-N (r=0.70, p<0.01), TN (r=0.59, p<0.01), TP (r= 0.79, p<0.01); and physical water characteristics such as temperature (r=0.68, $p<0.01$) and specific conductance (r=0.92, $p<0.01$). In addition, the AFDM was positively correlated with chl-a (r=0.94, $p<0.01$).

Simple linear regressions were generated to describe the relationship between abiotic factors and periphyton biomass. The best fitted models (all variables log transformed) were periphyton biomass as a function of TP (R^2=0.67, $p<0.01$), NO_3-N (R^2=0.53, $p<0.01$), TN (R^2=0.36, $p<0.01$) and specific conductance (R^2=0.85, $p<0.01$). Simple models of TP and TN were used for predictive analyses since these are the most influential factors in algal biomass development (Figure 2).

Figure 2: Relationship between A) Total P and B) Total N concentrations and periphyton biomass on natural substrates.





Flow discharge evaluation

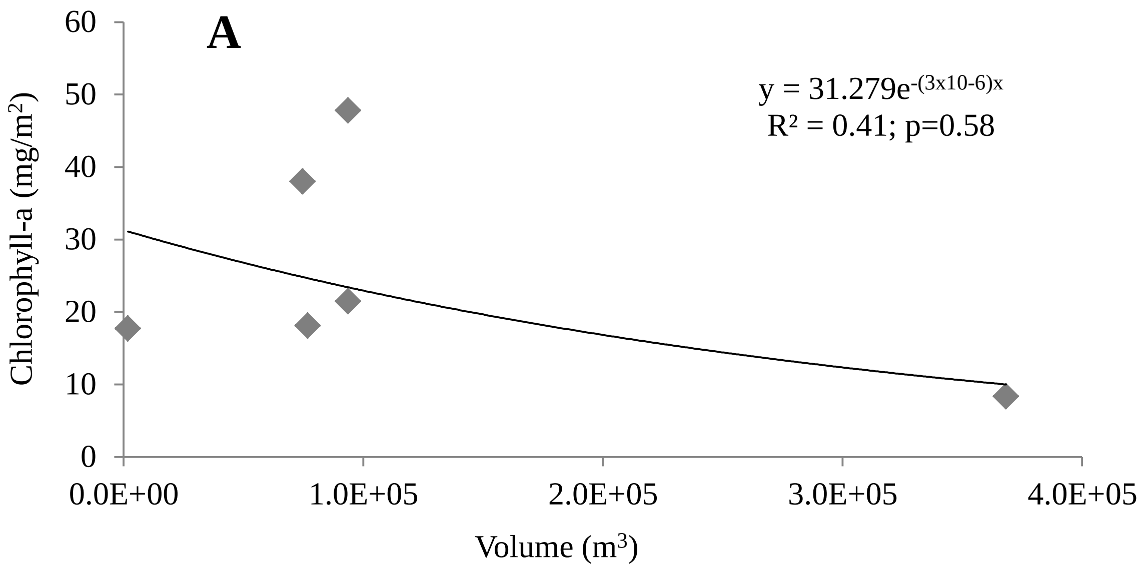
The watershed for the RP sampling site covered a larger catchment area (30 km^2) than the catchment area covered by the Río Piedras USGS gauging station (19.5 km^2); while the RG site had a smaller catchment area (39 km^2) than the catchment area covered by the Río Guanajibo USGS monitoring station (92 km^2). The conversion factor obtained from equation [1.4] for RP and RG was 1.564 and 0.43, respectively.

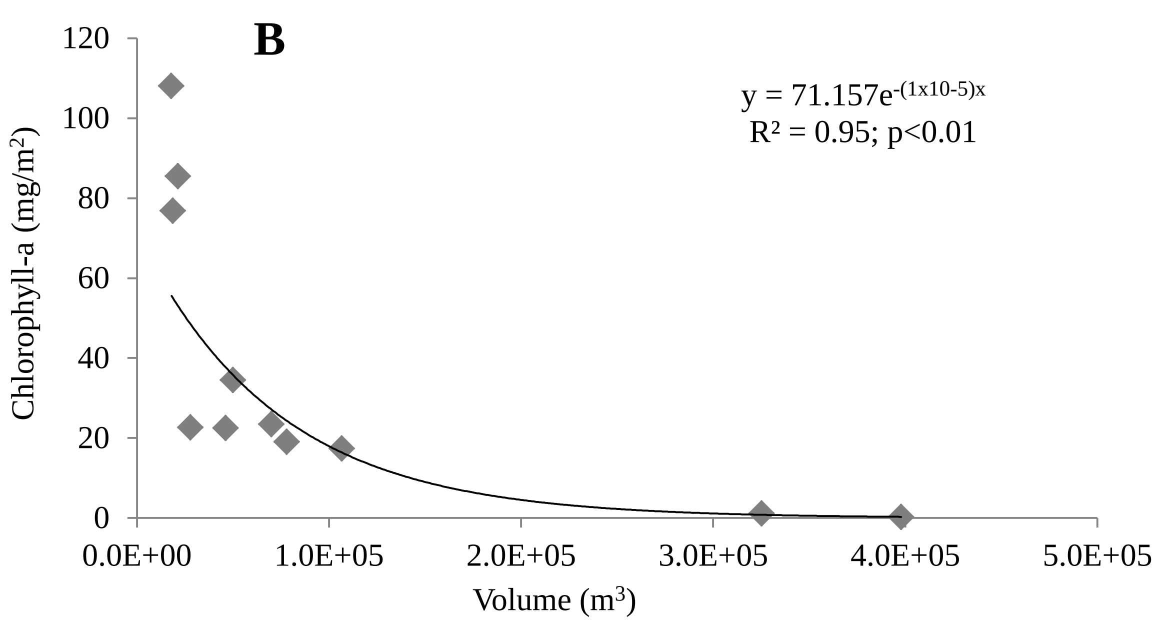
The effect of water discharge on periphyton biomass was evaluated by linear and exponential regression analyses for the discharged volume accumulated for 1, 2, 3, 5, 7 and 10 days prior sampling periphyton biomass (Appendix 5). Log transformed values were used to generate a linear model, though it was difficult to identify the inflection point at which the volume discharged is sufficient to drastically affect the periphyton community. Therefore, an exponential model was generated with the purpose of visually identifying the critical point where the volume destabilizes algal biomass accumulation

The cumulative volume for all the days evaluated could not explain the periphyton biomass variations observed in RM resulting in weak correlation coefficient ($R^2 < 0.10$) in both regression models (linear and exponential). For RG site, the linear regression generated a poor correlation ($R^2=0.01$) between periphyton biomass as a function of volume accumulated for 2 days. The exponential regression yielded a better fit but with no statistical significance ($R^2=0.41$; $p=0.58$) (Figure 3A). However, it is worth mentioning that from the dataset gathered, a single event with a 2-day cumulative volume of 3.7×10^5 m^3 resulted in a notable decline in periphyton biomass of 8.36 mg/m^2 compared to the mean chlorophyll-a value (21.70 mg/m^2).

In the case of RP site, cumulative volume explained most of the variability exhibited on the algal biomass (Figure 3B). The total volume accumulated 2 days prior the sampling date showed the best-fitted regression (R^2=0.95, p<0.01). In this model the two outlier cases (Appendix 4) were included. The model shows a gap in x-axis from $1x10^5$ to $3x10^5$ m^3 were no data was reported, thus it cannot be predicted how the periphyton assemblage will be affected within that range of volume. But it appears that antecedent cumulative volume greater than $3.0x10^5$ m^3 can impact negatively the periphyton biomass.

Figure 3: The effect of 2-day prior sampling cumulative volume on natural substrate in A) RG and B) RP.





DISCUSSION

The three streams studied contrasted significantly in their watershed characteristics such as land cover, geology, flow regime and climate ecological regions which reflect their differences in physical and chemical characteristics. The forested stream, RM, exhibited the lowest mean temperature, conductivity, and nutrient concentrations. This site is located at a higher altitude than the other two sites resulting in lower water temperature and the conductivity values are associated with the nutrient levels (Biggs and Close, 1989; Chetelat et al., 1999). The RG site showed mixed results as conductivity values were similar to RP site, despite the fact that the latter exhibited significantly higher nutrient concentrations. The elevated conductivity and pH levels in RG are associated to the significant presence of serpentine in large part of the basin which contributes minerals, especially magnesium, to the stream water due to the easily weathering (Dixon, 1989). Finally, RP site exhibited the highest nutrient concentrations that could be linked to the high degree urban land cover and of impervious surfaces that alter the precipitation infiltration capacity and increases the frequency and amplitude of floods adding more nonpoint nutrient sources. Blair et al. (2010) showed that larger population density increased runoff volume, peak flow rate and decrease runoff duration concluding that urbanization runoff is the leading cause of nonpoint source pollution.

Río Guanajibo watershed has heterogeneous land cover consisting of secondary forests, agricultural lands, and some urban areas. Uriarte et al. (2011) found that stream watersheds dominated by young forests and agriculture had greater DO than those draining from watersheds in which old forest as land cover predominates, as is the case for RM watershed. However, the levels of DO observed in RM and RG were statistically similar in this study. Diurnal DO concentrations near saturation are generally attributed to the metabolism dynamics. During the

10-month period, both sites showed minimal effects of soil erosion which would decrease water clarity due to suspended particles (Wetzel, 2001). The lower DO levels observed in RP site may be indicative of the potential effect of chemical reactions with organic and sediment loadings and nitrification processes (Wang et al., 2003; Odum, 1956). Nonetheless, all study sites met the dissolved oxygen quality standard for aquatic life (DO> 5mg/L) (PREQB, 2010a).

Stream-flow and nutrients were the most important factors explaining the variations observed in periphyton biomass among study sites. Nutrient availability influence periphyton growth biomass while frequency and severity of flood events modify the amount of time available for periphyton accumulation and assemblage strength (Murdock et al., 2004).

The un-enriched site showed positive correlations between nutrients and periphyton biomass suggesting that in RM site algal growth is controlled by nutrient availability. During the ten-month period, this site maintained similar levels of algal biomass and nutrients as that reported in Gualtero-Leal (2007) suggesting that the stream water and primary production characteristics have not changed with time.

The significant temporal variations in algal biomass at the nutrient enriched RP site was linked to the physical disturbances of high discharge events, which caused abrasion due to the water current force. After a high discharge event, substrates can be scoured, rolled over and moved affecting the periphyton community attachment (Lake, 2000). The volume accumulated two days before sampling showed a significant effect on the periphyton assemblages. The streambed in RP site was composed of soil sediments and rocks clearly not heavy enough to resist flood disturbances causing drastic changes in streambed structure after storm events. Evidently a flood event two days before sampling date that accumulates a volume higher than $3x10^5$ m^3 can potentially cause a substantial reduction in periphytic biomass due to the unstable stream bed. Biggs and Close (1989) showed that flood events greater than six times the antecedent base flow was sufficient to scour the periphyton biomass of the substrate. The effect of discharge volumes between $1x10^5$ to $3x10^5$ m^3 on periphyton biomass could not be defined with the dataset gathered in this study (Figure 3B). Based on these results, I suggest not sampling if a significant event occurs two days prior sampling date scheduled until a quasi-steady state is reached.

In the RG site, a flood disturbance that could have affected the periphyton biomass settlement was only recorded once (sampled on: October 17, 2013) in which the 2-day cumulative volume reached over 3.0 x 10^5 m^3 (Figure 3A). However, the reduction in biomass was not as significant as in RP site. These differences in flow regime between RG and RP may explain the statistical similarity in chlorophyll-a values despite their contrasting nutrient status. Among the multiple factors regulating benthic algae biomass, grazing and succession patterns might have been more influential on the temporal variability observed in RG. The lack of positive correlations between algal biomass and nutrients indicate that the range of nutrient concentration did not fluctuated significantly throughout the sampling period (Appendix 2E, 2G). Taxonomic analysis could have explained the changes in the periphyton community structure observed during the sampling period. One of the disadvantages of using periphyton biomass colonized on natural substrates as biological indicator for water quality is that the temporal variability associated with the successional patterns are inevitable to control given that the community undergoes changes in composition even when external factors, such as grazing, floods, and water quality, do not influence the community structure.

In the enriched site of RP, on three occasions the algal biomass was higher than the eutrophic chlorophyll-a limit of 70 mg/m^2 suggested by Dodds et al. (1998). It was observed that the time needed for periphyton to reach that limit was less than two weeks after a discharge event. Studies have shown that after four weeks the periphyton community reaches its peak biomass levels (Anping et al., 2011; Tien et al., 2009; Biggs, 1988).

According to the nutrient criteria proposed by Dodds et al. (1998), RM site with median TP of 0.010 mg/L would be considered as oligotrophic, RG site with median value of 0.038 mg/L can be considered mesotrophic (0.025-0.075 mg P/L) and RP with median value of 0.121 mg P/L would be classified as eutrophic. On the other hand, Sotomayor-Ramírez and Martínez (2013) related nutrient concentrations to trophic state indices of: non-enriched, enriched, and impaired. Based on TP and TN concentrations in the study sites, the suggested classification would be non-enriched for RM, and enriched for RG and RP.

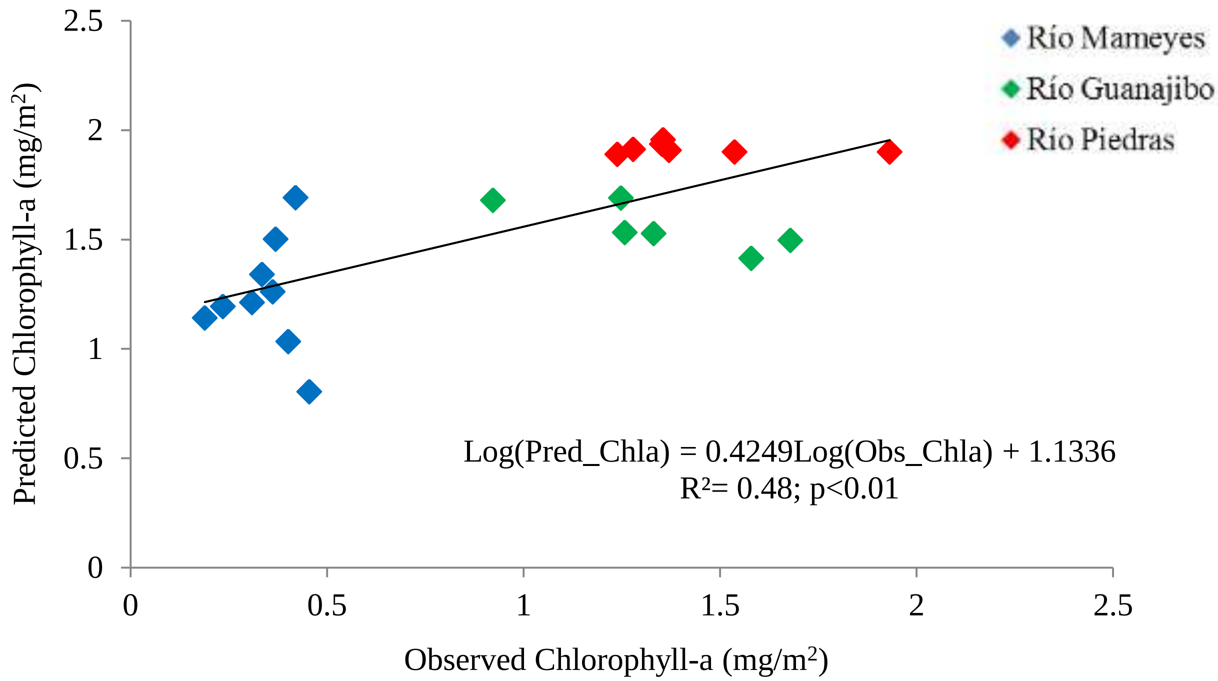
When considering all sites together, TP, TN, and specific conductance explained most of the chlorophyll-a variability in streams. Only the simple linear models with TN and TP were used as I presume that geology will influence specific conductivity in RG site. In order to predict the chlorophyll-a concentration corresponding to the limit for impaired threshold (0.160 mg P/L) suggested by Sotomayor-Ramírez et al., (2011), the linear equation between benthic chlorophyll a and TP was used (Figure 2A). The predicted value was 55 mg Chla/m^2, which is greater than the mean chlorophyll-a concentration for RP (35.8 mg/m^2) and slightly lower than the impairment threshold value (70 mg/m^2) suggested by Dodds et al. (1998). Of the nine sampling events where periphyton biomass and water TP concentration was collected in the nutrient enriched site, only on one case the observed TP concentrations was over 0.160 mg/L, and on three of the nine events observed the chlorophyll-a concentration reached higher values than 50 mg/m^2 (Appendix 6). To enhance the predictive relationship between TP and benthic chlorophyll a, it would be necessary to explore the periphyton biomass of streams that frequently exhibit nutrient levels close to 0.160 mg P/L.

The model determined by Dodds et al., (1997)[2]:

Log(mean chl-a) = -3.2236 + 2.826Log(TN) -0.431[Log(TN)]^2+ 0.255Log(TP); r^2=0.43 [1.5]

was used to predict chlorophyll-a concentrations with the reported values of TP and TN from dates that natural substrate and nutrients were sampled. A linear regression model (Figure 4) was generated relating the observed and predictor values (R^2=0.48; p<0.01). This result could potentially validate the regression model developed by Dodds et al. (1997) and use as an additional reference to estimate the chlorophyll-a values in streams of Puerto Rico.

Figure 4: Relationship between observed chlorophyll-a and that predicted from the equation [1.5] by Dodds et al., (1997) using measured nutrient concentrations in this study.



[2] Dodds et. al, (1998) Table 2, equation 7, page 1741

CONCLUSIONS

In this study, the selected streams contrasted in land use within each watershed, nutrients, and stream flow dynamics. The results show that watersheds with increasing percentage of urban land cover had greater nutrient concentrations. A linear regression model was developed between benthic algal biomass and nutrient concentrations. The results highlight the role of urban watersheds in influencing water quality conditions in Puerto Rico.

In some streams, increasing hydrologic flows caused a drastic reduction in periphyton biomass accumulation, even when nutrients increase periphyton biomass. The vulnerability of the periphyton community in a stream to be affected by high discharge volumes is influenced by the streambed composition, and the flow energy causing the biomass both shear stress and sloughing. Therefore, I would suggest that the streambed geomorphology and stream high flow recurrence must be taken into account prior to periphyton sampling in streams and rivers. Excluding high flood events, the use of benthic algae biomass as biological indicator of trophic status was effectively demonstrated. This work builds upon the work of Gualtero-Leal et al. (2010), by evaluating periphyton response to a wider range of nutrient concentrations and in streams differencing in geomorphologic characteristics.

Quantitative relationships were established between nutrients (i.e. TP and TN) and benthic chlorophyll a on natural substrates confirming that periphyton biomass can be a practical indicator of stream trophic status. Additionally, the regression model between the observed and the corresponding predicted periphyton biomass from the equations generated by Dodds et al. (1997) showed that the empirical model I have developed can be a useful tool to assess periphyton biomass levels in streams and to assess stream water trophic status.

Chapter 2

EVALUATION OF THE SEASONAL VARIATION IN PERIPHYTON GROWTH RATE USING ARTIFICIAL SUBSTRATES IN TROPICAL STREAMS

INTRODUCTION

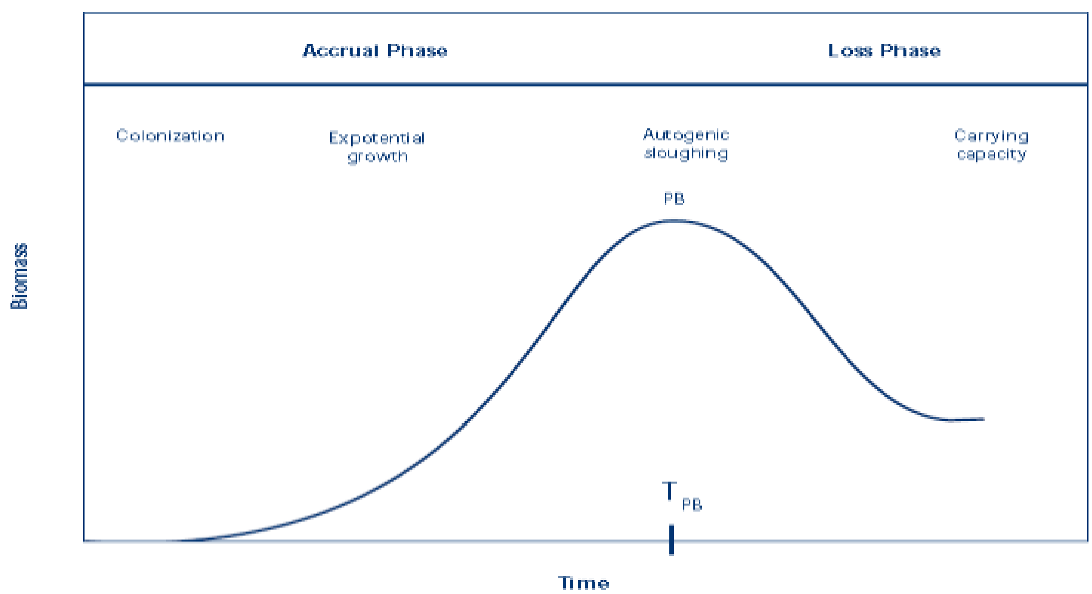
Benthic environments in streams are zones of relatively high biological activity in which processes such as nutrient cycling, primary production, and organic matter degradation are intensified (Hauer and Lamberti, 2006). Periphyton is the aquatic microbial community composed of algae, bacteria, fungi and protozoa which are attached to benthic surfaces. Algae dominate the community in areas where light and nutrients are highly available such as the stream benthos. In turn, periphytic algae are the food source of many invertebrates, fish, and crustaceous. Also, periphyton can modify stream water nutrient concentrations and can contribute to the dissolved oxygen fluctuations by the metabolic processes of photosynthesis and respiration (Wetzel, 2001).

The periphyton community usually exhibits many advantages over other biological indicators to be used as a tool for monitoring and assessing water quality. Benefits for using benthic algae as indicators of water quality impairment are their rapid growth rate, as so they can respond to changes in nutrient supply (Cushing and Allan, 2001). Also, since periphyton has a fixed habitat they cannot avoid contamination. There are multiple methods to study the periphyton community. One is abundance and diversity using a taxonomical approach and another is to use non-taxonomical parameters such as biomass and autotrophic indices (Biggs 1985; Chetelat et al., 1999; Wan-Omar, 2010; Black et al., 2011).

Periphyton colonization and growth likely exhibits a three-stage pattern of growth, represented by a sigmoid curve (Kevern et al., 1966; Biggs, 1988). First, initial colonization begins in which the new species adapt to their environment (substrata) and their growth increases slowly. After this lag-phase, a period of rapid growth occurs in response to favorable conditions, there is little or no competition because of available space in the substratum, and there is no physical disturbance. Third, a negative acceleration rate phase begins due to the primary production being balanced either by grazing, sloughing, nutrient limitation or other abiotic and biotic factors (Rastogi, 2004; Gotelli, 2008).

Biggs (1996) conceptualized a periphyton growth model describing the community as it undergoes a major disturbance (Figure 5). The peak biomass can be described as the net production stage before a major event happens. The time needed3 to reach the peak biomass varies depending on the availability of substrates and the effects of other biotic or abiotic factors. In humid tropical streams, variations on the accrual time to reach peak biomass are highly influenced by flood frequencies.

Figure 5: Periphyton community growth stage model describing the short-term periphyton accrual cycle before and after a major disturbance. Peak biomass (PB) is the maximum accrual cycle biomass; T_{PB} is the time to PB from initiation of colonization. The diagram is reproduced from Biggs (1996).



Artificial substrates have valuable advantages when used as the growth medium for periphyton community assessment in freshwater systems. Different materials have been used such as petri dish, ceramic tiles, glass slides, plastic and nutrient diffusion systems (Bothwell, 1985; Lavoie et al., 2004; Danilov and Ekelund, 2001; Fairchild et al., 1985; Tien et al., 2009). Important advantages of artificial substrates are that it is possible to know the age of the community being formed and that the factor causing the changes in the periphyton growth within the period of exposure can be identified by monitoring specific environmental and biotic parameters (Barinova, 2011). The use of artificial substrates increases reproducibility of experiments, reduces the effect of substrate-to-substrate variability, and reduces the variation that exists when studying algal biomass assemblages from natural substrates. In contrast, several studies have found that introduced substrates represented the natural community poorly and that the nature of the artificial substrate affected the patterns of colonization of periphytic algae (Tuchman and Stevenson, 1980; Danilov and Ekelund, 2001). However, Cattaneo and Amireault (1992) stated in their review on the use of artificial substrates for benthic algal studies that the choice of material is not crucial and that any variations are less important than those introduced by trophy, flood disturbances and the time available for colonization.

Several studies have coincided that after four weeks of exposure the periphyton community in the artificial substrate closely resembles that in natural substrate (Biggs, 1988; Tien et al., 2009). Anping et al., (2011) observed an increase in early successional stages (two weeks) and most of the species reached maximum levels between two to four weeks of exposure when glass slides as substrate media are used. Sekar et al., (2004) studied early stages of biofilm development in a lentic freshwater environment and found that the biofilm density increased within the first five to seven days and then decreased.

This chapter consists of two consecutive parts. First, an experiment was conducted to identify the optimum periphyton accrual time using artificial substrates. Once the optimum accrual time was selected, the seasonal variability in periphyton growth rate was assessed during the dry and wet seasons over a ten-month period.

OBJECTIVES

- Identify the optimum periphyton accrual time using artificial substrates as growth media
- Evaluate the seasonal variability in periphyton growth rate in three tropical streams
- Establish quantitative relationships between periphyton biomass accrued for a fixed time period and nutrient concentrations

MATERIALS AND METHODS

Study Sites

Three study sites Río Mameyes (RM) (18°19’34” N, 65°44’55” W), Río Guanajibo (RG) (18° 04’08” N, 66° 58’09” W) and Río Piedras (RP) (18°23’30” N, 66°03’28” W) were selected (Appendix 7). A detailed description of the study site basins was reported in Chapter 1. The three study sites have contrasting nutrient concentrations. Similar characteristics were considered such as: open canopy, gravel-bed substrates and continuous hydrologic flow. To maintain record of daily discharge events, sampling sites were selected near United States Geographical Survey (USGS) monitoring stations. The distance from the USGS monitoring station was approximately 0.2 km from Río Mameyes site, 2.7 km from Río Piedras site and 7 km for Río Guanajibo site.

The RM site the streambed substrates are dominated by gravel and rocks some large enough to apparently resist high discharge events. The substrates originate from sedimentary and plutonic formations including quartz and granodiorite. The study site where water and biological samples were collected had a total mean width of 15 m, but the artificial substrates were installed in a stream-section having mean width of 7 m since the other extent of the stream width was mostly shaded by riparian vegetation and the average water depth was approximately 1 m.

The geology of RG watershed is primarily serpentine, but has fewer parts as plutonic, sedimentary and alluvium deposits. The RG study site streambed is covered with rocks and in some sections with sediment. In order to anchor the artificial substrates, the river sections where large boulders predominate were considered. The total stream width had full open canopy with mean value of 6.12 m. On multiple visits, horses were seen walking through the river shore and drinking water from the stream and thus animal manure was visible in the stream’s surroundings.

The RP sampling site consists of small rocks and sediments primarily from land clearance for construction sites. The geology is mostly sedimentary and plutonic rocks. The streambed is constantly changing due to flood discharges. The sampling site had an open canopy with a total mean stream width of 10.10 m. The sampling site had areas of significant trash and debris accumulation that diverted the water current. The artificial substrates were installed in areas where rocks predominate.

Determination of the optimum periphyton accrual time

This experiment was conducted from January to October 2013 during dry and wet seasons. A dry season with minimal precipitation occurs from January to April. A wet season with frequent precipitation occurs from June to October. The assessment was conducted once in each season. In the dry season the experiment was completed during the first attempt at all sites, while in the wet season the experiment had to be repeated twice at each site due to loss of substrates (i.e. concrete blocks) caused by high flood disturbances.

The artificial substrate used as periphyton growth media were concrete blocks that measured 10 cm long, 8 cm wide and 8 cm height (640 cm^3). The surface area of the block used was 80 cm^2 and they were anchored to the surface level of the streambed rocks; the top of the concrete block was at least 10 cm below the water level. The substrates were obtained from Bloques Universales in Mayagüez, Puerto Rico[3].

Approximately 30 to 35 substrates were placed along the stream in an orderly way to facilitate the sample collection. Periods of accrual times of 3, 7 and 10 days were evaluated. At each accrual day, three replicates were randomly collected with each replicate being composed

[3] Bloques Universales, Inc. Carr 114 Km 0.4, Juanjebo St., Mayagüez, P.R.
The surface characteristics have not been defined. But, a sample can be obtained contacting Dr. Sotomayor-Ramírez at (787)-832-4040 ext. 5819; david.sotomayor@upr.edu

of three concrete blocks (i.e. 9 sampling units/sampling event). Additional concrete blocks were placed in the river in case that a high flow event would cause loss or moved down the blocks.

Periphyton colonization within the substrates at each accrual time was assessed. First, the surface area of the concrete blocks was treated by scrubbing the outer area of a 6 cm diameter PVC ring to eliminate all the material outside the ring. Then, all the material within the PVC ring was scrubbed gently with a soft toothbrush and washed into a 500 mL amber bottle with stream-water for a final volume of 500 mL. On occasions where the water was turbid, distilled water was used to fill the bottles containing benthic algae. The bottles were placed in a cooler with ice and taken to the laboratory where each replicate was divided into two sub-replicates for the analyses: chlorophyll-*a* and ash free dry mass (AFDM), explained in detail in Chapter 1.

Several considerations were taken into account at the time of substrate collection. First, all the substrates were installed with the same side facing upward to the water surface. Therefore, if at the time of sampling a substrate was not facing upwards, it was discarded. Second, the substrates were located only where continuous flow was observed and at a distance from stream bank greater than 0.5 m. Third, although the substrates were installed in an orderly way, blocks were randomly selected for sampling.

Temporal variation of periphyton biomass as an indicator of trophic status

An accrual time of 7 days was chosen based on results obtained in the first section. From February 2013 to June 2013, artificial substrates were installed on various occasions to establish temporal variability for an accrual time of 7 days. At each sampling date, twelve artificial substrates were installed with the intention of collecting four replicates. However, in some sampling dates several concrete blocks were either lost by the current or turned, affecting the initially exposed surface; and in some occasions vandalism was observed. Hence, the number replicates at each sampling date varied (i.e. from 3 to 4 replicates/sampling event) as well as the number of sampling events on each study site. For RM site, six sampling events were completed, while at RG and RP sites eight and five sampling dates were assessed. The sampling events gathered were conducted during both the dry and wet season at all sites.

Environmental and biological parameters

The following physical parameters were measured *in situ:* dissolved oxygen (DO), pH, specific conductance, and temperature using YSI Professional Pro (YSI, Inc., Yellow Springs, OH.). Stream flow velocity was measured with a flow probe (Global Water Instrumentation, College Station, TX). The total stream width was divided in segments, and thus base flow was calculated as the sum of the product of width, depth and water velocity measured for each segment.

Water samples were collected from the surface using a 1L polyethylene clear bottle, preserved on site (36N H_2SO_4 @ pH$\leq$ 2) and transported in a cooler with ice for chemical characterization at the Soil and Water Quality Laboratory located at the Río Piedras Agricultural Experimental Station in Río Piedras, Puerto Rico. Unfiltered water samples were analyzed for total Kjeldahl nitrogen (TKN; EPA 351.2) and total phosphorous (TP; method 365.4). Filtered samples (0.45 µm filter) were assessed for nitrate-N (NO_3-N; method 353.2. Total nitrogen (TN) was the sum of TKN and NO_3-N concentrations. Hardness and the following minerals: Al, B, Ca, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Si, and Zn were analyzed in the Soil and Water Laboratory in University of Georgia, U.S.A.

Periphyton biomass samples were analyzed for chlorophyll-a and ash free dry mass (AFDM) at the Chemistry Laboratory of the Agronomy and Soils Department in University of Puerto Rico, Mayagüez, Puerto Rico. The autotrophic index was calculated as the ratio of the

AFDM to chlorophyll-a. The procedures for the analyses mentioned above are explained in detailed in Chapter 1.

Also, periphyton growth rate (R) was calculated as:

$$R = \frac{(Chl-a)_i \; - (Chl-a)_{i-1}}{T_i - T_{i-1}}$$

where $(\text{Chl-a})_i$ is the chlorophyll-a concentration accumulated at accrual time *i* (in days), $(\text{Chl-a})_{i-1}$ is the chlorophyll-a yielded at accrual time *i-1* divided by the difference in *T* accrual days.

Data Analysis

Statistical analyses were conducted using InfoStat[4] version 2013 (InfoStat software, National University of Córdoba, Argentina). All values were log-transformed prior the statistical analyses to homogenize variances and the Shapiro-Wilks test was used to determine if the data followed normal distribution.

The periphyton biomass was analyzed, as chlorophyll-a concentration, based on the effects of three factors: study sites, accrual days and seasons. In order to evaluate potential interactions between the three factors, an analysis of variances (ANOVA) was generated. The interactions are combinations of the levels from each factor. The levels were: 3 study sites (RM, RG, and RP), 3 accrual days (3, 7, and 10 days), and 2 seasons (dry and wet) for a 3x3x2 factorial ANOVA model. Significant means were separated using Tukey statistical test. The optimal periphyton accrual time was identified based on the accrual day when no interaction between season and study site was observed.

To guarantee that the optimal accrual day was selected properly, two additional analyses were assessed. First, I analyzed periphyton growth rate (R) using a 2-way ANOVA model that

[4] http://www.infostat.com.ar/

compares effects between accrual days and seasons without discrimination of study sites. Second, I determined the optimal accrual day based on the day that had the highest growth rate.

To explain the temporal and spatial variability on 7-day periphyton biomass, simple linear regressions were generated with each nutrient component (TKN-N, NO_3-N, TN and TP) and environmental parameters (pH, DO, specific conductance, and temperature). Stepwise regression was performed to examine if more than one independent variable was an important determinant with the 7-day periphyton biomass. A linear regression model was generated to assess the relationship between the periphyton biomass colonized for seven days and the periphyton biomass in natural substrates that were collected on the same day.

The mean daily discharge in m^3/s was gathered from the USGS monitoring network website[5]. This discharge was converted into volume per day (m^3/day) so that the mean volume accumulated for the 7 days antecedent to the sampling date was computed. Linear regressions were generated between 7-day periphyton biomass and volume accumulated to explain temporal variations caused by water discharge.

[5] http://pr.water.usgs.gov/

RESULTS

Determination of the periphyton optimum accrual time

The study sites (factor A, 3 sites) were assessed for periphyton biomass growth by collecting artificial substrates after 3, 7, and 10 days (factor B, 3 accrual days) of colonization during a dry and a wet season (factor C, 2 seasons). The triple interaction was significant ($F=14.84$, $p<0.01$) which means that the effect between first and second factors at a given level of the third factor is not the same as the two-way interaction in the other level of the third factor (Macchiavelli, 2014). To simplify the interpretation of the data, the interaction used for the purposes of comparison was the effect between season and accrual day at each study site (Table 5).

Table 5: Analysis of variance of the 3x3x2 factorial to evaluate periphyton biomass accumulated in artificial substrates. The analysis of variance is conducted with the log transformed values of chlorophyll-a concentration.

Variable	N	R^2	R^2 Adjusted	CV
LOG10_chla	54	1	0.98	12.33

Source of variation	SS	d.f	MS	F	p-value
Model	17.05	17	1	141.09	<0.0001
Study Site (A)	6.46	2	3.23	454.55	<0.0001
Accrual Days (B)	9.53	2	4.77	670.46	<0.0001
Season (C)	0.15	1	0.15	21.03	0.0001
A*B	0.17	4	0.04	6.1	0.0007
A*C	0.01	2	0.0045	0.64	0.5336
B*C	0.3	2	0.15	21.25	<0.0001
A*B*C	0.42	4	0.11	14.84	<0.0001
Error	0.26	36	0.01		
Total	17.3	53			

At RM site, the effect of season was significant only at an accrual time of 3 days ($p<0.01$). Río Guanajibo did not show significant differences in periphyton biomass between seasons at any of the accrual days. Río Piedras showed significant seasonal differences at 3 and 10 accrual days, while on day 7 no statistical differences were observed (Table 6).

Table 6: Mean periphyton biomass at each accrual day for the dry and the wet season. Complete Tukey comparison test of the 3x3x2 factorial ANOVA is presented on Appendix 8.

		Chlorophyll-a (mg/m^2)		
Study Site[1]	Season	**3**	**7**	10
RM	dry[1]	0.24 a	3.29 b	3.74 b
	wet	0.61 a *[3]	3.42 b	3.72 b
RG	dry	1.85 a	7.10 b	13.04 c
	wet	1.67 a	12.88 b	15.31 b
RP	dry	1.57 a	24.74 b	27.04 b *
	wet	5.20 a *	26.10 c	14.08 b

[1]RM is Río Mameyes; RG is Río Guanajibo; RP is Río Piedras
[2]Common letters within a row indicates no statistical difference between accrual days ($\alpha<0.05$)
[3]*Differences between seasons for each accrual day within each study site ($\alpha<0.05$)

The trend of the extent of the effect of periphyton biomass growth was similar in all the study sites in both seasons. Río Mameyes showed the same growth pattern in both seasons in which the periphyton biomass after 3 days was significantly lower than after 7 and 10 days, while the chlorophyll-a between 7 and 10 days were not statistically different. Río Piedras showed the same pattern as RM at the dry season (i.e. 3days< 7days=10 days). However, during the wet season the periphyton significantly differed among accrual days with maximum biomass occurring after day 7 and then decreasing after 10 days. In contrast, RG exhibited the highest periphyton biomass at day 10 in both seasons; in the dry season all the accrual days differed between each other but in the wet season no differences were observed between days 7 and 10. On five of six experimental trials, the peak biomass was reached at day 7 (Table 6).

An additional approach was assessed to confirm the optimal accrual time. The two-way ANOVA for periphyton growth rate showed that the highest growth rate occurred after 7 days of incubation at both seasons followed by 10 days and the growth rate after 3 days was the slowest in all rivers (Appendix 13).

The Autotrophic Index ratio in RM exhibited statistical differences among accrual days with the ratio being higher at 3 accrual days for both seasons. Seasonal differences were observed between days 3 and 10 (Table 7). The heterotrophs or organic debris dominated within the first 3 days, and later the community shifted to be dominated by algae with no significant differences of AI ratio between 7 and 10 accrual days. In RG, the AI ratio did not show statistical differences within each season at any of the accrual times. However, seasonal differences of AI ratio were observed at 7 days with higher values in the dry season. At RP, significant differences between seasons were observed for 3 and 10 accrual times. Only during the dry season, the AI ratio at day 3 was significantly higher than the following accrual days.

Table 7: Autotrophic index (AI) for each accrual day for the dry and wet season

Study Sites[1]	Season	3	7	10
RM	dry[2]	12,121 b*[2]	878 a	795 a *
	wet	2,056 c	816 b	520 a
RG	dry	421 a	509 a	384 a
	wet	340 a	309 a *	355 a
RP	dry	3,093 b *	175 a *	205 a
	wet	372 a	152 a	386 a *

[1]RM is Río Mameyes; RG is Río Guanajibo; RP is Río Piedras
[2]Common letters within a row indicates no statistical difference between accrual days (α<0.05)
[3]*Differences between seasons for each accrual day within each study site (α<0.05)

Temporal variations of periphyton biomass as indicator of trophic status

Each river had different number of experimental trials completed, with 6, 8, and 5 replicates for RM, RG, and RP, respectively, for a total of 19 observations of 7-day periphyton biomass. The mean 7-day chlorophyll a value exhibited significant differences (F=14.66; p<0.01) between the study sites (Table 8). Throughout the sampling period, RP site showed the greater variability in periphyton biomass, while RM site had the least variability. The periphyton biomass fluctuated between sampling dates in RM site from 2.09 to 5.88 (CV=16%) with mean value of 3.46 mg Chla/m^2, in RG site from 4.27 to 12.88 (CV=22%) with mean value of 7.36 mg Chla/m^2, and in RP site from 5.55 to 26.30 (CV=33%) with mean value of 17.02 mg Chla/m^2.

Comparison of the mean benthic chlorophyll-a obtained from artificial and natural substrates was conducted by Tukey statistical test. In RM, the mean 7-day periphyton biomass value was significantly higher than the mean value obtained from the natural substrates. Statistically, the difference is significant ($F=9.72$; $p<0.01$) but the means differed by approximately 1 mg Chla/m^2 suggesting that the difference may not be of important value. Contrastingly, at RG the mean natural benthic chlorophyll a values was 3x higher than ($F=14.29$; $p<0.01$) mean values of the 7-day periphyton biomass (Table 8). Río Piedras showed no statistical differences between substrates.

Table 8: Mean benthic chlorophyll-a accrued on the concrete blocks and natural rocks from the streambed at each study site.

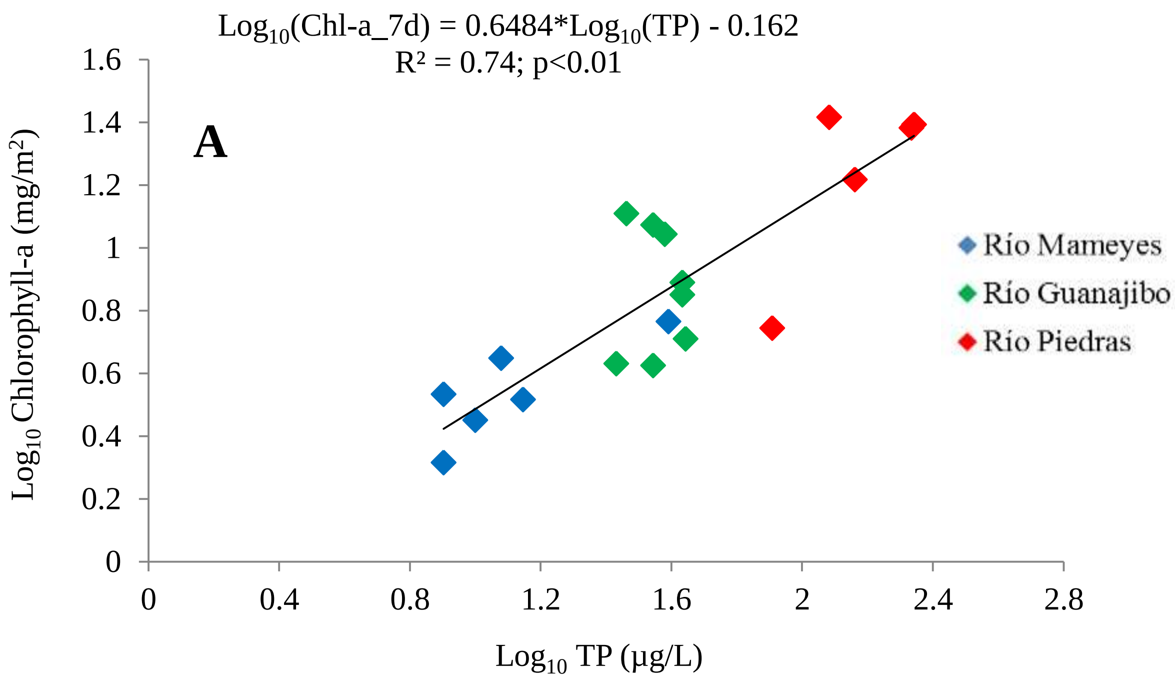
Study site[1]	7-day[2]	Natural[3]
	-------mg/m^2------	
RM[4]	3.46 b	2.16 a
RG	7.36 a	21.70 b
RP	17.02 a	27.40 a

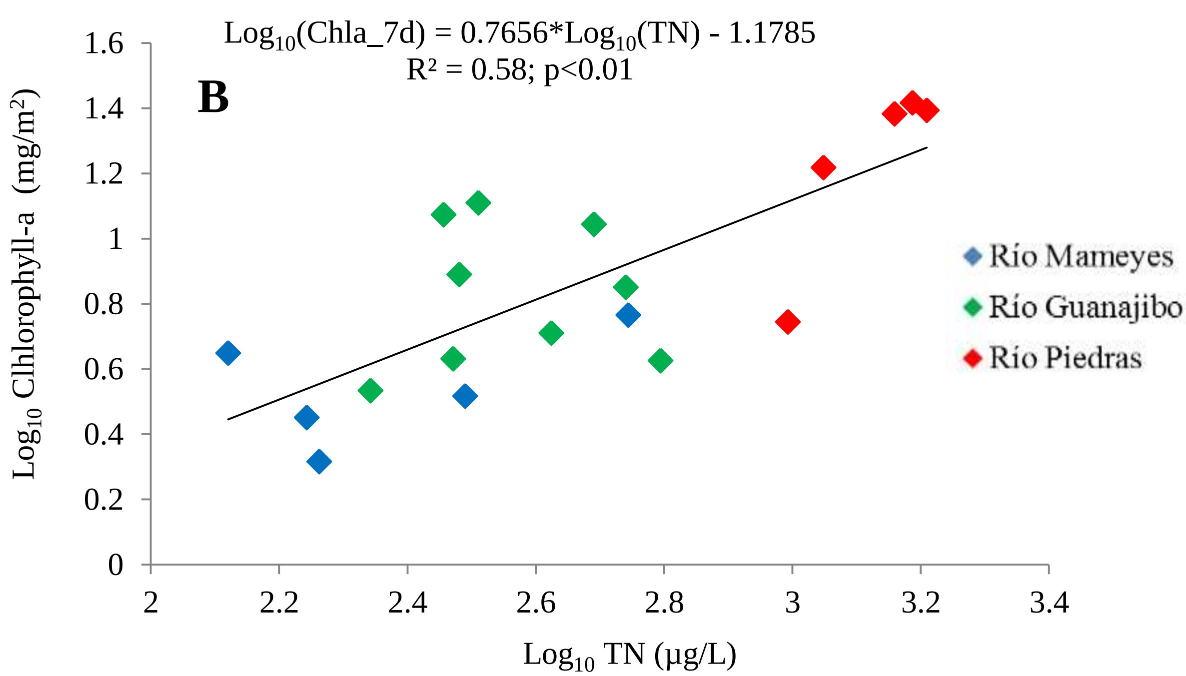
[1]RM is Río Mameyes; RG is Río Guanajibo; RP is Río Piedras;
[2]7-day refers to the periphyton biomass accrued for 7 days on the concrete blocks;
[3]Natural refers to the natural rocks in the river;
[4]Common letters within a row indicates no statistical difference between substrates ($\alpha<0.05$)

The significant differences ($p<0.01$), observed in the 7-day chlorophyll-a values between study sites, followed the same gradient as the nutrient conditions with greater periphyton biomass in the nutrient enriched site (RP), followed by the moderate nutrient enriched site (RG) and lowest values in the least nutrient enriched study site (RM). Total P ($R^2=0.74$; $p<0.01$) and

TN (R^2=0.58; p<0.01) were the most influential factors explaining variations of 7-day periphyton biomass with both showing significant positive relations with periphyton biomass (Figure 6).

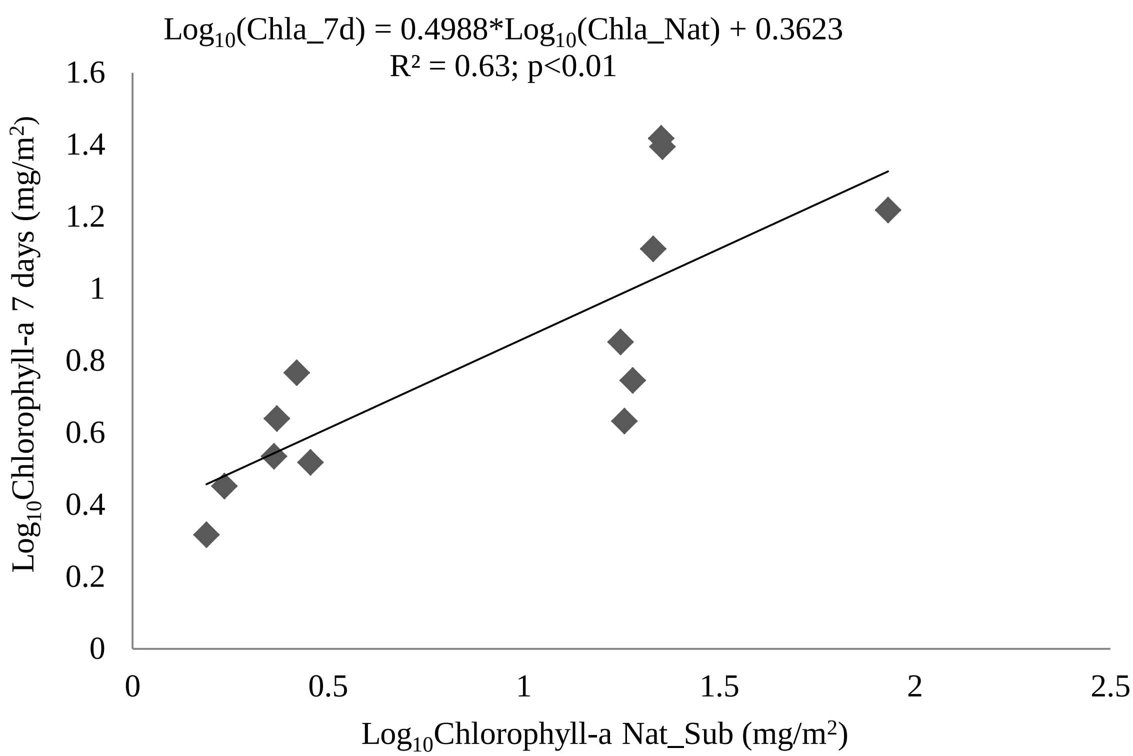
Figure 6: Periphyton biomass accumulated for 7 days (Chla_7d) as a function of A) TP and B) TN.





A regression analysis was conducted to describe the association between periphyton biomass accrued in artificial substrates and the periphyton biomass found in natural substrates. Only five sampling dates coincided during which periphyton biomass colonized for 7 days and periphyton biomass from natural substrates were harvested on the same day. Nonetheless, the regression explained 84% of periphyton biomass variability ($F=22.8$; $p<0.05$). An additional linear regression model (Figure 7) was generated with additional sampling dates (N=13) by including observations of 7-day and natural periphyton biomass harvested on dates with no more than a week apart and no flood events occurring within that time frame; this model resulted in a significant positive relationship ($p<0.01$) explaining 63% of the periphyton biomass variability.

Figure 7: Regression of chlorophyll-a levels accrued for 7 days in relation to natural levels from rocks.



Environmental Parameters

The environmental parameters measured *in situ* with the YSI instrument are summarized in Table 9. All the study sites exhibited significant differences in mean water temperature between seasons ($P<0.05$) in which higher temperature was observed in the wet season at all sites. Water pH was highest in RG showing significant differences between season ($p<0.01$) with higher pH in the dry season, while no significant difference was observed in RM and RP between seasons. Specific conductance showed significant differences between seasons for RG ($p<0.01$) and RM ($p<0.01$) with higher values in the dry season; RP showed no statistical differences between seasons ($p=0.1268$). For dissolved oxygen, RP showed weak statistical differences between seasons ($p=0.0375$) while no statistical differences were obtained in RM and RG.

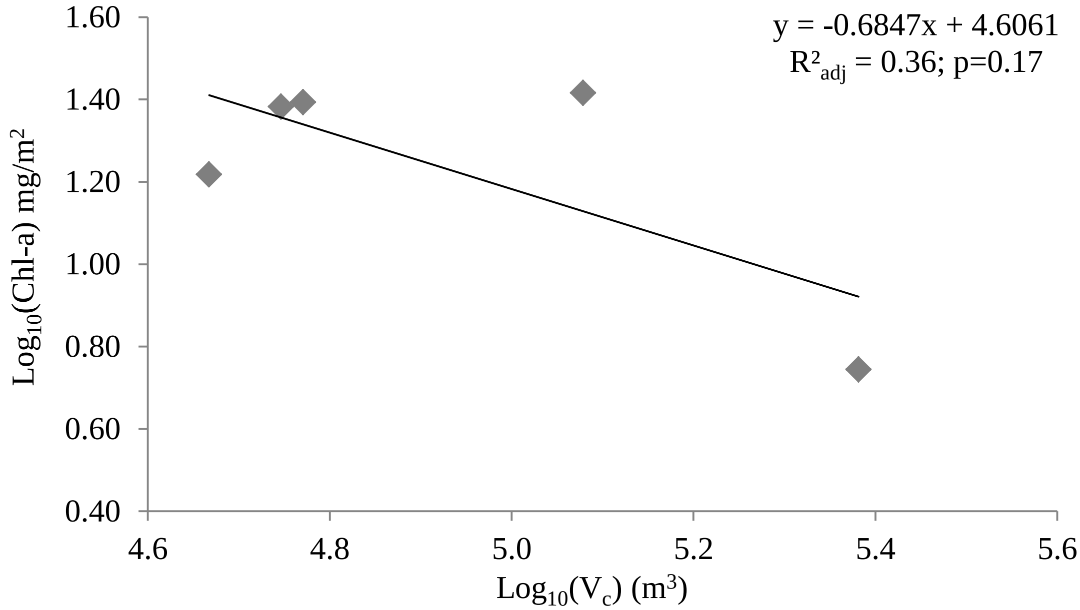
Table 9: Environmental parameters measured *in situ* for each study site in both seasons.

Study site[1]	Season	pH	Temperature °C	DO mg/L	Specific conductance µS/cm
RM	dry	7.88	21.7	8.21	100.1*
	wet	7.78	23.8*	8.23	81.8
RG	dry	8.78*[2]	24.6	7.96	528.0*
	wet	8.35	27.9*	8.25	390.4
RP	dry	7.82	25.2	6.69	424.8
	wet	7.88	26.7*	7.16*	398.9

[1]RM is Río Mameyes; RG is Río Guanajibo; RP is Río Piedras
[2]*Differences between seasons within each study site ($\alpha<0.05$)

The flood discharges were evaluated in terms of the volume accumulated during the seven days prior to the artificial substrates installment. In RP site, a negative linear correlation explained 36% of the periphyton biomass variability but the correlation did not exhibit statistical significance ($p=0.17$; $\alpha<0.05$). Although, no statistical significance was found, it is visually notable that 7-day cumulative discharge volumes higher than $2.0\text{x}10^5$ m^3 can decrease periphyton biomass (Figure 8). In the other two study sites, no significant relationship was achieved.

Figure 8: Periphyton biomass accrued for seven days in function of the cumulative volume (V_c) during that period for Rio Piedras site.



Chemical characterization

Nutrient concentrations (i.e. NO_3-N, TN and TP) differed significantly among study sites with RM exhibiting the lowest nutrient concentrations while RP the highest. Total P differed significantly ($p<0.01$) between seasons for RP, with higher concentrations being observed in the dry season. On the other hand, NO_3-N concentrations were higher during the wet season ($p<0.01$). In RG, poor statistical differences were obtained between seasons for TP ($F=5.17$; $p=0.0355$). Total nitrogen showed significant differences among study sites ($F=108.8$, $p<0.01$), however within sites the TN values did not differ between seasons.

Table 10: Mean values of the nutrient concentrations for each study site separated by season.

Study site[1]	Season	TKN-N	NO_3-N	TN	TP
		-------------------mg/L------------------			
RM	dry[2]	0.120	0.055	0.164	0.010
	wet	0.130	0.073	0.190	0.011
RG	dry	0.123	0.319	0.460	0.045*
	wet	0.129	0.268	0.430	0.031
RP	dry	0.422	0.745	1.198	0.174*
	wet	0.281	0.929*	1.289	0.100

[1]RM is Río Mameyes; RG is Río Guanajibo; RP is Río Piedras
[2]*Differences between seasons within each study site ($\alpha<0.05$)

Elemental chemical analysis was performed on several water samples gathered on sampling dates corresponded to 7-days periphyton biomass was collected (Table 11). Río Guanajibo showed the highest values in hardness, Mg, and Si; RP had the highest concentrations of Fe, Na and K, and RM had the lowest values for all the minerals. In all study sites, the following minerals were below detection limit: B, Cr, Cu, Mo, Ni, and Zn.

Table 11: Water samples, taken on sampling dates that artificial substrates were gathered, were analyzed for the following ions and hardness. Results for B, Cr, Cu, Mo, Ni, and Zn were BDL at all sites.

Observations[1]	n=4	n=7	n=5
Study Site[2]	RM	RG	RP
Hardness	35.73 (4.67) a[3]	221.71 (57.17) b	163.46 (16.97) b
Al	0.05 (0.00) a	0.05 (0.00) a	0.394 (0.251) b
B	0.01 (0.003) a	0.033 (0.01) b	0.03 (0.00) b
Ca	10.4 (1.34) a	22.33 (5.07) b	46.34 (5.11) c
Fe	0.068 (0.035) a	0.05 (0.00) a	0.864 (0.46) b
K	0.62 (0.032) a	0.68 (0.13) a	2.23 (0.25) b
Mg	2.38 (0.322) a	40.3 (11.06) b	11.59 (1.15) a
Mn	0.025 (0.00) a	0.03 (0.00) a	0.097 (0.06) b
Na	7.16 (0.482) a	9.06 (2.21) a	24.76 (1.87) b
Si	0.25 (0.00) a	1.08 (1.01) a	0.40 (0.331) a

[1]Number of samples (observations) taken for each site
[2]RM is Río Mameyes; RG is Río Guanajibo; RP is Río Piedras
[3]Common letters within a row indicate no statistical differences between study sites (α<0.05).

DISCUSSION

Determination of optimal periphyton accrual time

The time period selected for periphytic biomass incubation in this study primarily includes the first two stages of the growth rate model described by Biggs (2000a) (Figure 5). In this study, the initial colonization occurred within three days of exposure and then exponential growth continued until near day 7. Within a week of exposure, periphytic community reached peak biomass and then the biomass declined or stabilized due to: algal succession, death rate begins, or scouring by the stream flow or grazing. Biggs (2000a) described that the peak biomass is reached before any disturbance occurs. A disturbance can be described as any factor that causes a decline in biomass either as foreign agent to the community (i.e. consumers), physical perturbations (i.e. floods) or autogenic succession.

A seven day incubation period was identified as the optimal periphyton biomass accrual time for stressor-response diagnosis. The evidence indicates that in most instances the peak biomass at the three sites was reached at 7 days during both seasons. This is further supported by results of periphyton growth rate in five of six instances. Therefore, it would be advantageous for practical purposes to assess periphyton biomass sampling events within seven days.

The forested stream, RM, showed similar growth pattern in both seasons with peak biomass being reached after 7 days and AI ratio being inversely related to the chlorophyll-a levels. This suggests that at the beginning organic detritus from vegetation predominate in the substrata and later primary production colonizes the substrate until it reaches a quasi-steady state (Table 7). Similar trends were observed in an unenriched river studied by Biggs (1988) where the AI was stable beyond accrual times of four weeks.

Seasonal variability was observed in RM only in periphyton biomass accrued for three days being higher during the wet season (Table 6). This significant difference may be due to contrasts in nutrient concentrations reported between seasons at that accrual time. The TP values reported were 0.006 for the dry and 0.017 mg/L for the wet season. Also, the TN values contrasted substantially from 0.146 mg/L in the dry season (mean: 0.164 mg N/L) to 0.261 mg/L in the wet season (mean: 0.190 mg N/L). In addition, the flow was higher in the wet season with mean stream flow 2.33 m^3/s as the dry season (mean was 1.26 m^3/s), which could explain the increase in nutrient concentrations. Given the highly forested land cover in RM basin, the nutrient supply comes mostly from vegetation degradation, organic detritus and from soil desorption from sediments. The observed growth pattern may indicate that the periphytic community is regulated primarily by nutrients and that stream flow enhances their nutrient supply within a range limit. It is noteworthy to mention that the stream geomorphology of RM plays an important role in periphyton assemblages due to the large obstructions (large cobbles and stones) that can assist in a stable streambed by being resilient to flow disturbances (Biggs and Smith, 2002).

I hypothesized that in streams differing in nutrient concentrations, the periphyton biomass accumulation would vary accordingly to the same gradient. The observed results support my assumptions, as significant differences were observed between study sites. Río Guanajibo had higher growth rates than RM but lower than RP, following the nutrient concentration levels. In RG, the periphyton growth seemed to occur in a non-lineal pattern, due to the fact that chlorophyll-a values for the 10 day accrual were greater than the 7 day values, although statistical differences were only observed during the dry season. However, up until the tenth day the algal biomass did not reach comparable biomass values as that of natural levels or declined, suggesting that the death rate had not yet started. This study site is located in a drier region where the effects of scouring by flood disturbances are less frequent. Therefore, the periphyton growth limitation is more commonly associated with grazing by *Tarebia granifera* and nutrients supply. When the samplings were performed, visible grazers were in many cases observed on the surface of the substrates, though no monitoring of population density was performed.

The nutrient enriched RP site had a more peculiar and highly variable growth pattern. This site was not affected by changes in nutrient availability but has physical limitations due to its geomorphology and hydrology regime. The streambed has a crucial influence on the periphytic community foundation. The periphyton biomass accrued after 3 days differed significantly between seasons with the chlorophyll-a levels in the wet season (5.20 mg/m^2) being higher than in the dry season (1.57 mg/m^2). In the latter season, nutrient concentrations were higher suggesting that this was not the limiting factor between seasons. A few kilometers upstream from sampling site, a construction site was observed most likely contributing to nutrients and sediments loading into the river during the first sampling dates of the dry season trials. The sediment loading was possibly contributing to the significant lower algal biomass accrued given that suspended sediments interfere with light penetration and also when sediments precipitate, the algal assemblage availability can be affected. No other causal variable was affected by seasons nor was a physical disturbance reported. The algal biomass accrued at 7 days did not show significant differences between seasons, although the 10-day periphyton biomass was significantly different between seasons. Differences for the latter accrual time were most likely caused by a storm event, resulting in a discharge 2x greater than the base flow, that occurred in the wet season before sampling for the tenth accrual day. The storm event possibly caused significant sloughing on the biofilm attached to the substrate.

In contrast to RM, the geomorphology at RP has a streambed mainly dominated by sediments and rocks not heavy enough to resist storm events. This type of streambed is likely to create turbulence when a flood event occurs, causing suspended particles to reduce the algal richness and abundance (Biggs and Smith, 2002). When a storm event occurs, the water becomes turbid and the streambed changes drastically resulting in scouring of the periphyton mat. Lake (2000) states that strong flood disturbances exert high shear forces that suspend sediments, move and distribute stream bottom, remove plants, move detritus and displace biota. To avoid flood disturbances that could wash away the concrete blocks, it would be preferable to sample during the dry season.

Temporal variation of periphyton biomass as an indicator of trophic status

According to the results in this study, one week is sufficient time for periphyton community to assemble on the substrate before organisms begin to decay and a new succession begins. In the humid tropics, the frequency of precipitation that can cause high discharges is unpredictable. Flow-generated disturbances can vary in duration, intensity, spatial extent, and predictability (Lake, 2000). In this study several rainfall events caused setbacks for collecting artificial substrates given that on occasions the stream flow was strong enough to remove anchored substrates. In several sampling dates, artificial substrates were collected prior to or after the seven days of incubation to prevent loss of samples or due to security measures; the results from these samples were not accounted for analysis. Primary succession occurring in artificial substrates has a short-term colonization and it is easier to identify the factor that is limiting the community development. If we are to establish relationships between nutrient thresholds and biological conditions, it is crucial to understand what are the most influential factors affecting or promoting algal growth.

At RP, discharges volumes proved to be a determinant factor on periphyton biomass accumulation. For instance, the 7-day periphyton biomass collected during June yielded the lowest value (5.55 mg/m^2), while for the other four sampling events the 7-day biomass ranged from 16 to 26 mg $Chla/m^2$ (Appendix 9). This result (June, 21, 2013) was obtained after a high discharge event that occurred two days prior to the sampling date. Likewise during the month of July, concrete blocks were installed, however, another high discharge event occurred and all the substrates were lost. In fact, on the day in which the concrete blocks were supposed to be harvested, a periphyton sample from natural substrate was taken resulting in chlorophyll-a biomass (0.12 mg/m^2) significantly below the mean chlorophyll-a determined in natural substrates (mean: 27.40 mg/m^2) for this stream site (Table 8).

In order to quantitatively correlate periphyton biomass and discharge volume, a linear regression analysis was performed using 7-day periphyton biomass and the accumulated volume within the seven days at RP site. The model explained 36% of the variation in periphyton biomass; although no statistical significance was obtained (Figure 8). However, it can be said that a 7-day cumulative discharge volume greater than $2.0x\ 10^5\ m^3$ seemed to have the capacity to detach benthic algae from substrate. These results confirm the vulnerability that the natural periphyton community experiences at urban humid tropical streams given that the streambed structure and the water flow force cause a negative effect on algal attachment to the substrate. In the other two sites, RM and RG, the 7-day algal biomass accumulation did not vary significantly by effects of discharge volumes likely due to: 1) no significant flow events reported during periphyton colonization period; 2) the anchoring of the substrates was more stable in these two sites than in RP site; or 3) visually the streambed structure of RM and RG has a larger proportion of rocks than the RP site which has more areas with gravel and sand.

At RM site, the 7-day periphyton biomass on artificial substrate reached mean values comparable to that accrued in natural substrates (Table 8). Three possible factors may explain this result. Primarily, the RM site exhibits low nutrient concentrations that are the most influential factors on regulating periphyton growth. Secondly, the composition and characteristics of the natural substrates in RM are smoother in texture than the concrete block which has a greater porous surface giving a stronger algal attachment. Additionally, it is possible that periphyton community succession in unenriched streams have slower recovery after discharge events in conjunction with the limiting nutrient supply that habitually presence this site.

Periphyton biomass accrued in RG showed the least variability throughout the sampling period. The maximum 7 accrual day periphyton biomass value in artificial substrate (12.8 mg/m^2) was below to the mean chlorophyll-a from natural substrate (21.70 mg/m^2). These results suggest that the seven accrual days may not be enough time to benthic algae reach similar peak biomass from values observed under natural conditions. Also, nutrients were not found to be a limiting factor given that no positive correlation was obtained between 7-day periphyton biomass and nutrients for this particular stream site.

An evaluation of stressor-response relationship with data from the three rivers combined revealed a strong effect of nutrient on periphyton biomass growth. Total P and TN explained 74% and 58%, of the total variation in chlorophyll-a, respectively. These results demonstrate that response of benthic algae to nutrients is significant and that it can be measured within seven days of colonization.

The quantitative relationship generated between the 7-day and natural periphyton biomass at the three rivers could assist on predictions to determine trophic status. The linear regression model that included more robust data sets (N=13) resulted in the following linear equation (Figure 7):

$$\mathrm{Log}_{10}(\mathrm{Chla\_7d}) = 0.4988 * \mathrm{Log}_{10}(\mathrm{Chla\_Nat}) + 0.3623;\ r^2 = 0.63 \qquad [2.1]$$

where Chla_7d is the chlorophyll-a concentration from the 7-day periphyton biomass and Chla_Nat is the chlorophyll-a concentration from natural substrates. Dodds et al. (1998) suggested trophic boundaries for nutrients and benthic biomass based on chlorophyll-a using a frequency distribution approach. The mean chlorophyll-a suggested as the meso-eutrophic boundary value was 70 mg/m^2. If I substitute 70 mg/m^2 in equation [2.1], the resulting chlorophyll-a concentration of 7-day periphyton in artificial substrate would be 19.2 mg/m^2.

Predictions of periphyton biomass were conducted to find at what nutrient concentration the periphyton biomass incubated for 7 days would yield 19.2 mg/m^2 using the following linear equation (Figure 6A):

$$\mathrm{Log}_{10}(\mathrm{Chl\_7d}) = 0.6484 * \mathrm{Log}_{10}(\mathrm{TP}) - 0.162;\ r^2 = 0.74 \qquad [2.2]$$

The resulting TP concentration was 0.169 mg/L. In Puerto Rico, Sotomayor-Ramírez and Martínez (2013) suggested numeric nutrient criteria of 0.160 mg TP/L. Contrastingly, current water quality standard established for streams in Puerto Rico is 1.0 mg TP/L. The predicted (0.169 mg P/L) and the suggested value (0.160 mg TP/L) are closer to suggested values in other parts of the continental U.S. Recently, numeric nutrient thresholds were proposed in five regions of the state of Florida. For the regions of Panhandle east and Peninsula the numeric criteria were: 0.18 and 0.12 mg P/L, respectively (Florida DEP, 2012).

For TN criteria, the linear equation (Figure 6B) generated was:

$$\mathrm{Log}_{10}(\mathrm{Chl\_7d}) = 0.7656 * \mathrm{Log}_{10}(\mathrm{TN}) - 1.1785;\ r^2 = 0.58 \qquad [2.3]$$

The predicted TN concentration based on the 19.2 mg/m^2 estimated from the periphyton biomass accrued for 7 days was 1.64 mg N/L resulting in a very similar value as that in Sotomayor-Ramírez and Martínez (2013) whom suggested a 1.70 mg N/L as the threshold for impaired waters.

Environmental and chemical characteristics

In this study, the sampling seasons were identified *a priori* as the "dry" and "wet" season based on the historical cumulative precipitation for each month, considering if it was below or above the mean annual precipitation, respectively. Data were gathered *a posteriori* to confirm that the classification was appropriate. As mentioned in Materials and Methods, the mean daily discharge at each sampling point was based on data gathered from USGS monitoring stations. The mean daily discharge was computed for the months from January to April as the dry season and from June to October 2013 as the wet season. For the dry and wet seasons, the mean daily discharge was 1.26 and 2.33 m^3/s in RM, 0.019 and 0.89 m^3/s in RG and 0.21 and 0.65 m^3/s in RP, respectively, calculated based on the USGS monitoring stations records. The seasonal mean stream flow is observed to be higher in the wet season than in the dry season at all study sites supporting the *a priori* identification of seasons.

As it was expected, water temperature differed between seasons showing higher temperatures in the wet season at all study sites. All other *in situ* parameters (pH, DO, and electrical conductivity) remained reasonably consistent between seasons in base flow conditions. However, significant differences were obtained between sites as discussed below.

Specific conductance have been closely related with nutrient supply and consequently with algal biomass (Potapova et al., 2004; Chetelat et al., 1999; Gualtero-Leal et al., 2010). Río Guanajibo and RP showed similar conductivity values despite the fact that RG has significantly lower nutrient concentrations than RP water. These results can be attributed to that in most part of the RG basin is serpentine which contributes to the alkaline pH, elevated hardness and Mg values and thus high conductivity levels. The specific conductivity levels observed in RP is more likely associated with suspended solids and nutrient concentrations.

As it was expected, RM showed the lowest nutrient concentrations followed by RG, and RP. The most anthropogenic intervened site had the highest concentrations. In general, the three sites sustained similar concentrations between seasons though higher maximum concentrations were seen in the wet season for RM and RG, while RP showed higher maximum concentrations during the dry season due to an urban construction (improvements in UMET buildings) being conducted at that time. The construction could have caused an increase sediment inputs rate contributing to higher TP concentration and lower DO levels in the same season. The forested stream site, RM exhibits baseline or minimal nutrient concentrations, and thus is considered a reference stream. In fact, Gualtero-Leal (2007) studied benthic algal diversity and biomass as indicators in five reference streams which included RM as one of her study sites. Though no statistical comparison was conducted, similar trends were observed between the mentioned study sampled during 2006 to this present study for the physical, chemical and biological conditions. Therefore, it could be assumed that no significant changes in terms of land cover from the upper watershed have occurred.

CONCLUSIONS

The results in this study demonstrated that the periphyton growth rate significantly varies among streams with contrasting nutrient concentrations by exhibiting higher growth rates at the highly nutrient enriched stream. Seven accrual days was identified as an optimal time to assess periphyton biomass in establishing trophic status in tropical streams. The periphyton biomass accrued for seven days on artificial substrates yielded positively significant correlations with TN (R^2=0.58; $p<0.01$) and TP (R^2=0.74; $p<0.01$) suggesting that quantitative relationships exists between stressors and biological variables. The use of artificial substrates as periphyton growth media can be used to identify streamwater conditions given by the biological responses attributed to events occurring within the time exposed. Therefore, in rapid bioassessments periphyton biomass on artificial substrates may be employed as a biological indicator of the trophic status and the ecological conditions of streams.

Most of the studies that have evaluated the use artificial substrates for periphyton biomass accumulation have used longer incubation times than the accrual times that I used in this study. Due to recurrent physical disturbances in the humid tropics, longer exposure times than those evaluated in this study may not be adequate due to loss of substrates from recurring flood events. The temporal fluctuations observed within each stream were more likely to be caused by physical episodic discharge events rather than nutrients given that the latter did not vary significantly over time. Further studies should be conducted to estimate the discharge capacity needed to deplete the periphyton community attachment. Besides physical disturbances, the time for periphyton community to reach peak biomass depends on the nutrient availability that regulates the algal growth rate.

Chapter 3

BIODIVERSITY OF THE PERIPHYTON COMMUNITY ON ARTIFICIAL SUBSTRATES IN THREE STREAMS OF PUERTO RICO

INTRODUCTION

In Puerto Rico, ecological studies on freshwater or stream benthos have focused mainly on the characterization of macroinvertebrate community, especially aquatic insects (Ramírez and Hernández-Cruz, 2004; De Jesús-Crespo and Ramírez, 2011). Studies on the taxonomic composition of the periphyton community have emphasized on cyanobacteria, diatoms and green algae (Gardner, 1927; Bryan, 2001; Santos-Flores, 2001, respectively). Gualtero et al. (2010) and Sotomayor-Ramírez et al. (2011) have reported on the relationships between primary productivity characteristics and water quality conditions in reference streams.

In general, the most common benthic algae groups are green algae (Chlorophyta), diatoms (Heterokontophyta), cyanobacteria (Cyanophyta), and red algae (Rhodophyta). The filamentous cyanobacteria contain heterocyst which are the cells that fix N in aerobic conditions and cause the most problems of eutrophication in standing waters where they form large mats and dense blooms (Cushing and Allan, 2001). Diatoms have the particular characteristic of possessing a hard cell wall made from silica, called frustule, and they can be present either in the plankton or benthic community. The green algae are an extremely large and morphologically diverse group and many species are flagellated, enabling them to move from the benthos to the planktonic zone, while the red algae are more sensitive and none of the species is planktonic (Wetzel, 2001).

Chetelat et al. (1999) reported that green algae composed the highest portion of periphyton biomass, averaging 47%, among sites from Canada followed by diatoms, red algae and cyanobacteria with 27, 19 and 7%, respectively. In New Zealand, Biggs and Smith (2002) found that diatoms made up the largest taxonomic group followed by cyanobacteria, green algae, red algae, and only one taxon for green-yellow algae (Xanthophyta). Black et al. (2011) performed taxonomical measurements for coarse (rocks) and fine (sand) substrate samples identifying diatoms as the most abundant taxa when TN and TP were elevated. Sotomayor-Ramírez et al. (2011) reported a total of 129 algal species in reference streams of Puerto Rico, being diatoms the most diverse group (72%) followed by green algae (16%), cyanobacteria (11%) and only one species of Euglenophyta in one of the rivers.

High levels of nutrients can alter the taxonomic composition of the periphyton community causing shifts from nutrient sensitive to tolerant species which may cause changes in the macroinvertebrate community from native to invasive species (Fairchild et al., 1985; Lavoie et al., 2004; Suren et al., 2003; Wan-Omar, 2010). Chetelat et al. (1999) found shifts in dominance from sensitive (*Cocconeis* sp.) to tolerant (*Melosira* sp.) diatom species and suggested that nutrients indirectly affected diatom composition by stimulating the growth of filamentous biomass, thereby increasing the surface availability for colonization of epiphytic taxa. The presence of the cyanobacterium known to indicate good water quality (i.e. *Homoeothrix* sp.), the diatom *Achnanthes biasolettina* which is found in oligo-mesotrophic streams of moderate to high electrolyte content, and many sediment tolerant species from the genus *Nitzschia* were observed in Río Mameyes (Gualtero-Leal, 2007). The genera *Cladophora*, *Spirogyra* and *Melosira* have been found where there is an ample range of nutrient availability.

This study will evaluate the periphyton community composition and structure at a phylum taxonomic level. The periphyton community was colonized from artificial substrates that were exposed for 3, 7 and 10 days in order to describe the community succession in a short term period.

OBJECTIVES

- Evaluate the relative abundance of the periphyton community colonizing on artificial substrates to a phylum taxonomic level
- Assess for temporal changes in the periphyton community structure and composition in three rivers of differing nutrient statuses.

MATERIALS AND METHODS

Study sites

Periphyton samples were collected from three streams with distinct physical and chemical characteristics as described in detail in Chapter 1. Río Mameyes site (RM) is within the Subtropical Rain Forest and Lower Montane Rain Forest life zones (Gualtero-Leal, 2007). The sampling site sub watershed is covered by more than 99% of forest and pastures and less than 0.03% of urban areas (Table 1). Río Guanajibo (RG) is a third order stream within the Guanajibo watershed and it is located at the southwestern region of the Island as part of the Subtropical Wet Forest and the Subtropical Moist Forest life zones (Sotomayor-Ramírez et al., 2011; Ewel and Whitmore, 1973). The Río Guanajibo (RG) sub watershed comprises a mixed distribution of land uses including forests and pastures (81%), agricultural lands (13%), and urban zones (5.8%). Lastly, the third study site, Río Piedras (RP), is located in the city of San Juan and the watershed falls within the Subtropical Moist Forest lifezone. The stream runs from south to north and its headwaters originate at the mountains of the Trujillo Alto municipality (300 m.a.s.l). The northern part of the watershed is highly populated mostly concentrated in the riparian zone (Appendix 1C). The urbanized area covers 49% of the catchment area, 50% of forests and pastures and the remaining percentage being attributed to Las Curías reservoir. PREQB (2008) identified the Río Piedras watershed as impaired for violations to coliforms, turbidity and dissolved oxygen criteria.

Río Mameyes site was considered a reference site based on its land cover and nutrient concentrations with median values of 0.010, 0.180, and 0.062 mg/L of total phosphorus (TP), total nitrogen (TN) and nitrate as nitrogen (NO_3-N), respectively (Table 3). The nutrient concentrations in RG are of an intermediate level with higher values than RM site. Median concentrations of this river are 0.038 mg TP/L, 0.456 mg TN/L and 0.287 mg NO_3-N/L. Río Piedras represents the nutrient enriched stream with median concentrations of 0.121 mg TP/L, 1.22 mg TN/L and 0.821 mg NO_3-N/L.

Field method

The experiment to determine the optimal accrual day (Chapter 2) consisted of using artificial substrates as media to colonize periphyton for incubation periods of 3, 7 and 10 days. Field trials for the said experiment were conducted twice over a ten month period covering both the dry season and the wet season. Additional assessments of artificial substrates incubated for 7 days were performed at two study sites only (RM and RP site) amidst the growth rate experiments. At each accrual day, three samples were collected in 500 mL amber bottles as described in Chapter 2. The three samples were divided in sub-samples initially for biomass analyses (chlorophyll-a and ash free dry mass). For the current assessment, one of the three samples was selected randomly to be divided in a separate sub-sample for taxonomical analysis. The 100 mL sub-sample was preserved *in situ* with 10% formaldehyde or 2% of glutaraldehyde in a 250mL amber bottle. The preserved sub-sample was transported in a cooler to the Aquatic Sciences Laboratory at the Biology building in University of Puerto Rico, Mayagüez Campus. From this point onwards, the 100 mL sub-sample will be referred as sample.

Laboratory analysis

The microscopy analysis of the taxa count for the periphyton samples was conducted by the student Héctor Esparra. An initial screening of each sample was performed in a simple microscopic slide by adding one aliquot of the homogenized sample and examining it at 400X magnification using an ocular microscope (Olympus Company). In order to determine the necessary volume for further assessment, the preliminary analysis consisted of identifying a minimum of 150 organisms to verify if the sample had sufficient organisms for the subsequent enumeration analysis.

The Utermöhl technique is designed for the identification and enumeration of species using an inverted microscope. The Utermöhl chamber is composed of a cylinder fixed to a base plate where the determined volume is allowed to settle for 24 hours. After 24 hours, all the biological material is settled to the bottom and supernatant water is discarded to keep the organisms in the base plate. The cylinder is removed from the base and a coverslip is placed on the Utermöhl chamber plate to protect the settled volume. The Utermöhl chamber slide was examined for organism count observed at 400X or 1000X magnifications using a Leica DM3000B inverted microscope equipped with a Micrometrics camera of 5 megapixels and SE Premium software Version 2.9. The magnifications varied depending on the visual field observed; the visual field area in 400X magnification is 0.0314 mm^2 and 0.1995 mm^2 with the 1000X magnification. The settling volume for the Utermöhl chamber varied among samples, ranging from 3 mL for the most concentrated samples up to 50 mL for the samples with the least number of organisms. The organism count was performed once for each sample (i.e. one replicate/sample).

Data analysis

An overall periphyton community composition and structure description based on number of genera per phylum were determined. The relative abundance of genus taxa per phylum was calculated at each accrual day and study sites. The periphyton community structure, which characterizes the proportion contributing each phylum of the total abundance, was evaluated for each accrual day and at each study site. Within each study site, the periphyton community structure and composition was described for each accrual day to evaluate if differences existed within 10 days of accrual.

In order to assess the total community density on each sample, the field count was based on the number of the taxa units (i.e. individuals) observed in the Utermöhl chamber base plate, instead of the taxon cell count. The total field count as individuals per volume was calculated with the following formula:

$$\text{Field Count:} \quad \mathrm{Ind./mL} = \frac{\mathrm{CBPA} \times \mathrm{N}}{\mathrm{VFA} \times \mathrm{N_{VFO}} \times \mathrm{V_s}} \qquad [3.1]$$

where CBPA is the chamber base plate area, N is the number of individuals counted in a single visual field, VFA is the visual field area, N_{VFO} is the number of visual fields observed and V_s is the settling volume. The counted individuals were based on defined area in the surface of the artificial substrate. The total community density per sample was calculated as individuals count per area (Ind./mm^2) with the following formula:

$$\text{Community density:} \quad \mathrm{Ind./mm^2} = \frac{\text{Field count}}{\mathrm{A}} \times \mathrm{V} \qquad [3.2]$$

where the Field count is the number of individuals per volume (Ind./mL) calculated in equation [3.1], V is the volume of the sub-sample (100 mL), A is the field area 1057.695 mm^2. This area corresponds to the PVC ring used to scrub the biofilm from the surface of three artificial substrates.

The Shannon-Weaver index for each sub-sample was calculated with the following formula:

$$H = -\sum \frac{n_i}{N} \ln\left(\frac{n_i}{N}\right) \qquad [3.3]$$

The n_i/N is the relative abundance where n_i refers to the number of individuals for a given i taxa level and N is the total number of organisms in the sample. Therefore, the Shannon Weaver index (H) is the sum of the product between the relative abundance and the natural logarithm of the relative abundance for each taxon. For all the samples of the same accrual day, the diversity index was calculated to the genus level.

Additionally, the relative abundance was generated using InfoStat Software (2013 version) according to the number of organisms per phylum as well as the total community density at phylum taxonomic level for an overall community structure for each study site and accrual day. A two-way ANOVA statistical analysis was conducted to find differences between study sites and accrual days on the mean community density using the Tukey comparison statistical test. In order to determine if relationships exist between nutrients and periphyton community abundance, Pearson correlations and simple linear regressions were evaluated.

RESULTS

A total of 21 samples consisting of the periphyton community developed on artificial substrate exposed for 3, 7 and 10 days were examined. The Río Piedras site had more samples analyzed which included three sampling dates of each accrual day. The number of samples varied among sites as well as the number of samples assessed for each accrual day (Appendix 10).

Overall periphyton community composition and structure

Overall, a total of 77 taxa were identified on all rivers. Twenty-one taxa were identified only to phyla level, and 43 to genus level. We were unable to classify 9 taxa by neither genus nor phyla and 4 heterotrophs. The latter includes one protist (*Astramoeba radiosa*), one rotifer, one larva of the Chironomidae, and an unidentified filamentous fungus; these individuals were identified only once and their limited occurrence was observed over a 10 days period. Seven taxa were recognized to species level including *Amphora exigua*, *Denticula occidentalis, Cocconeis apiculata*, *C. singulata*, *Oscillatoria obtusa*, *Rhaphoneis crucifera* and *Ulnaria ulna*. Tofacilitate comparison, results will be discussed at a genus level. Eleven individuals, at the genus level were common among study sites; ten genera were Heterokontophyta (diatoms) and one cyanobacterium (*Anabaena* sp.) (Table 12).

Table 12: Common genera found at all the study sites

Taxa
Amphora
Anabaena *
Caloneis
Cocconeis
Cymbella
Epithemia

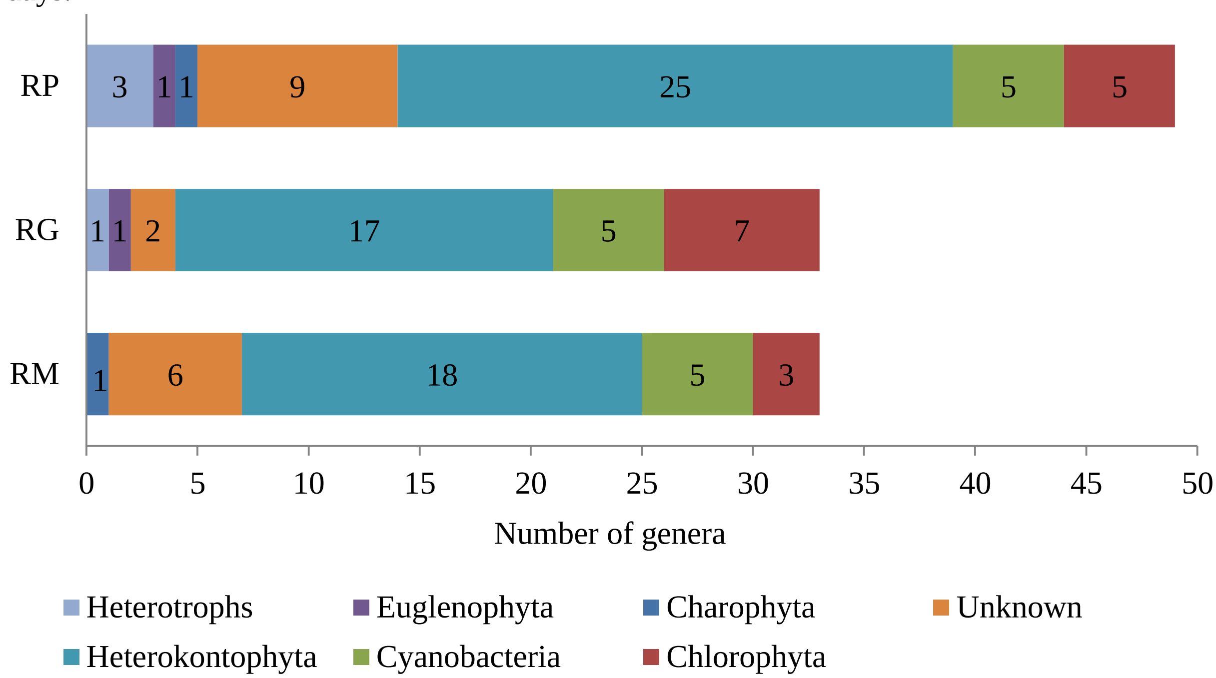
Fragilaria
Gomphonema
Gyrosigma
Navicula
Synedra

*genus Cyanobacteria

Periphyton community structure at each study site

The number of organisms per phyla or group varied among study sites (Figure 9). The total periphyton richness included 46 algae taxa and 3 heterotrophs for the nutrient enriched site RP, 32 algae taxa and one heterotroph for the RG site, and 33 algae genera for the unenriched site (Appendix 11).

Figure 9: Periphyton community structure in the three study sites from samples incubated for ten days.



Heterokontophyta was the most abundant and diverse phylum in the three sites with 18 genera (54.5%) in RM, 17 (53.1%) in RG, and the highest number of genus, 25 taxa (51%) in RP site. Five genera of Cyanobacteria were observed contributing 15.2, 15.6 and 10.2% of relative abundance for RM, RG, and RP site, respectively. On the other hand, the Chlorophyta genera were more abundant in RG site representing over 21% of the total richness and 6.25% of unknown genera. The latter composed over 18% in both RM and RP site. Río Guanajibo site exhibited higher evenness in the community structure followed by RP, and RM site with lowest evenness of algal groups (Table 13).

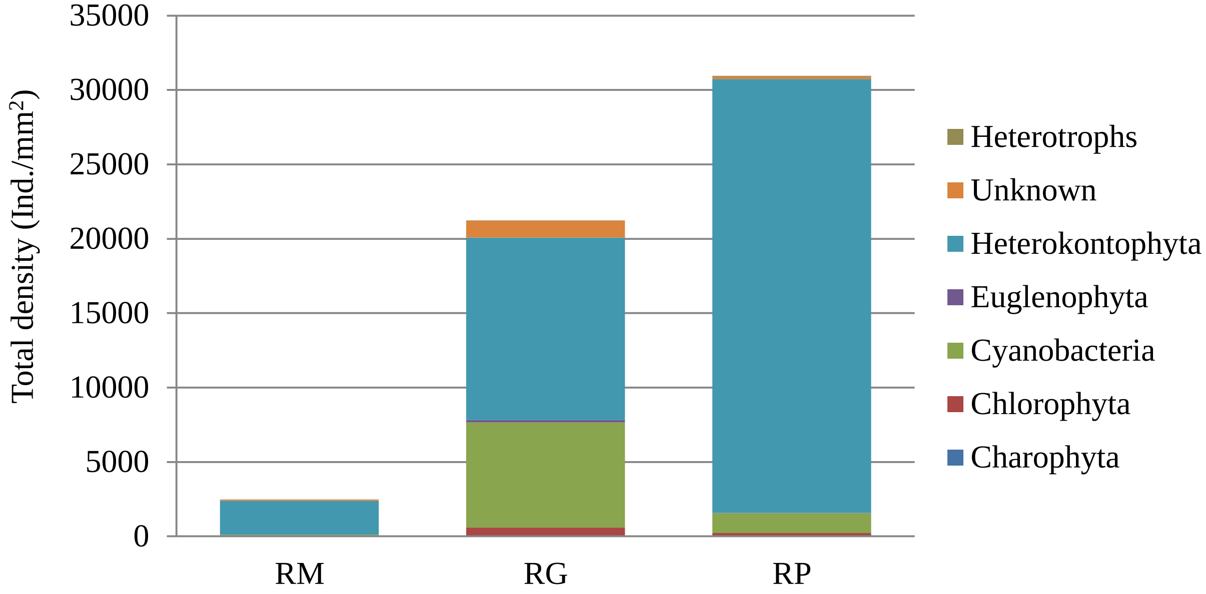
Table 13: Shannon-Weaver index for the three study sites for ten days of colonization

Study site[1]	Shannon-Weaver Index
RM	1.74
RG	1.99
RP	1.81

[1]RM is Río Mameyes; RG is Río Guanajibo; RP is Río Piedras

In terms of phylum density, RG site exhibited diatoms with 57.6% of the total density, followed by 33.2% of Cyanobacteria, 5.4% of unknown species, 2.8% of Chlorophyta, and 0.9% of Euglenophyta. Río Mameyes and RP site exhibited similar community structure with over 91.1 and 94.3% of diatoms density, respectively. The remaining percentage was distributed by Cyanobacteria with 2.7% in RM and 4.3% in RP site, 1.0% in RM and 0.63% in RP of Chlorophyta and unknown individuals contributed 4% in RM and 0.53% in RP site (Figure 10). Additionally, RM site exhibited 1.2% of Charophyta and RP exhibited less than 0.3% of Charophyta, Euglenophyta and heterotrophs combined.

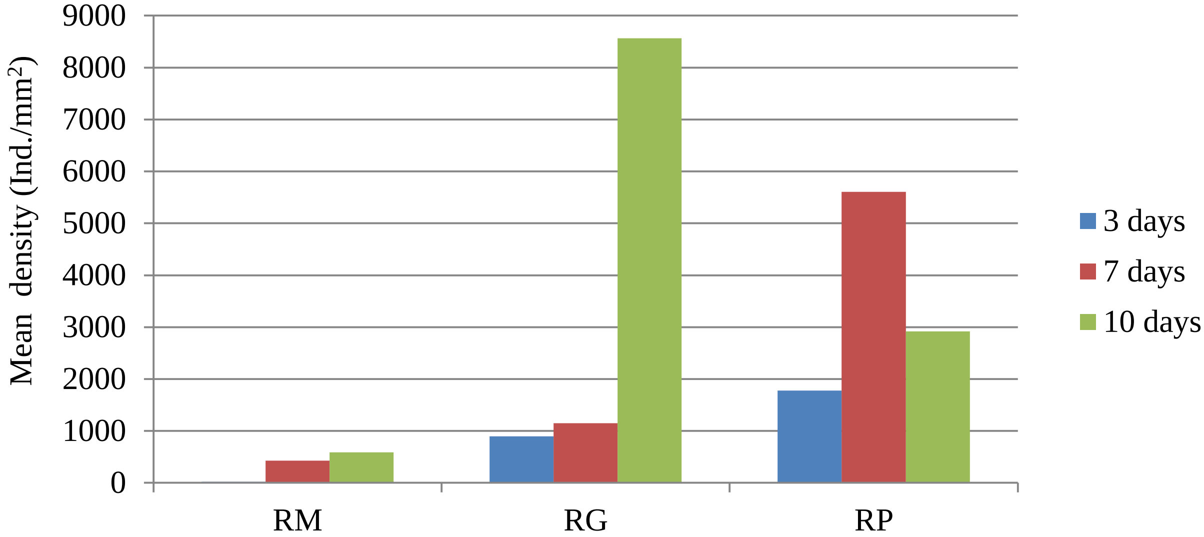
Figure 10: Periphyton community structure based on the total density at phyla level for the three study sites



Temporal variations in periphyton community composition

In a period of ten days the study sites exhibited minimal albeit some changes in total community density following the trends observed with the benthic chlorophyll-a values. Although no statistical differences were found between accrual days for RP ($F=0.41$; $p=0.679$), results show that the highest mean community density was observed at 7 days while the highest mean density in RG and RM was observed at 10 days (Figure 11). In RM, the mean density at 3 days was significantly lower than at 7 and 10 days (Table 14). The community in RM site reached the greatest phyla diversity at 7 days with twenty-nine genera, of which sixteen were diatoms (with almost 70% of *Navicula* spp.), three genera of Cyanobacteria and Chlorophyta, one Charophyta and six (2%) unknown species. Thus, the community genera evenness at this time was the lowest with Shannon index of 1.55, due to the large number of *Navicula* individuals (Table 15). The number of individuals of the said genus decreased at 10 days and the presence of more unknown species emerged increasing evenness (Appendix 12A).

Figure 11: Mean periphyton community density for the three study sites at each accrual day. (Statistical ANOVA analysis detailed on Table 14).



In contrast, RG site showed higher diversity after 7 days but with lower number of genera of each phylum. At day 7, twenty genera were observed more evenly distributed resulting in a 1.93 diversity index. The community composition included six genera of Chlorophyta (19 individuals, 5.0%), nine Heterokontophyta (268 individuals; 57%), three Cyanobacteria with 43 individuals (19.6%) of which 90% were *Anabaena* sp. taxa. Also, one *Euglena* sp. individual and one unknown species were recognized (Appendix 12B). After ten days, the abundance of phylum Chlorophyta individuals decreased to 2.9%, diatoms abundance decreased (23.8%), the Cyanobacteria increased to 56.4%, mostly *Anabaena* sp. genus, and the genus *Euglena* sp. and the unknown species increased from 1 to 5 and to 30 individuals, respectively. Based on community mean total density per accrual day, the RG site exhibited significant higher total density ($F=13.46$; $p<0.01$) than the other study sites (Table 14).

Table 14: Mean periphyton density (Ind./mm^2) for each accrual day in the three study sites

Study site[1]	3 days	7 days	10 days
RM[2]	19 a*[3]	403 b	587 b
RG	840 a	1138 a	7950 b*
RP	513 a	1735 a	1506 a

[1]RM is Río Mameyes; RG is Río Guanajibo; RP is Río Piedras
[2]Common letters within a row indicates no statistical difference between accrual days ($\alpha<0.05$)
[3]*Differences between study sites within each accrual day ($\alpha<0.05$)

In terms of Shannon-Weaver index, Río Piedras exhibited similar trends as RG site with the highest index observed at 7 days, and decreased at 10 days (Table 15). However, this site showed uneven structure at 7 accrual days resulting in relative abundances of 91.8% Heterokontophyta, 7% contribution of Chlorophyta and Cyanobacteria and below 1% of Euglenophyta, heterotrophs and unknown species. The latter increased to 5% abundance at 10 accrual days; however, no statistical differences on total density were achieved amongst accrual days. Based on phylum relative density, Cyanobacteria density declined from 7 to 10 days (Appendix 12C).

Table 15: Shannon-Weaver Index in each accrual day for the three study sites

Study site[1]	3 days	7 days	10 days
RM	1.88	1.55	1.71
RG	1.51	1.93	1.89
RP	1.57	1.79	1.54

[1]RM is Río Mameyes; RG is Río Guanajibo; RP is Río Piedras

Pearson correlations were assessed with no consideration on the effect of the study sites and the accrual days. Significant positive correlations between benthic chlorophyll-a biomass and the mean community density were achieved ($r=0.93$; $p<0.01$). Also, the nutrients TP ($R^2=0.75$; $p=0.02$) and TN ($R^2=0.71$; 0.034) explained most of the variations in the mean community density for all the samples analyzed (Appendix 3). Simple linear regressions were generated with significant correlations between mean density at each accrual day for all study sites and TP (R^2= 0.50; p=0.02) and TN (R^2=0.43; p=0.034) as well as mean chlorophyll-a (R^2=0.85; $p<0.01$) as a function of mean community density (Appendix 14).

DISCUSSION

Overall periphyton community structure and composition

The community composition, in general, showed that diatoms were the most abundant and diverse phylum at all study sites, as well as being the first colonizers on the substratum. Gualtero-Leal (2007) reported diatoms as the most abundant species in five reference streams of Puerto Rico. This was somewhat expected given the phylum richness on a large array on habitats of diverse water quality conditions, hence they tend dominate benthic algae communities (Wehr and Sheath, 2003). Additionally, genus abundance of green algae (Chlorophyta) and Cyanobacteria largely contributed to the total richness followed by several taxa of unknown classification. Four heterotrophs from different phylum classification were observed only once. Within ten days of periphyton colonization, the consumers' limited occurrence may indicate that heterotrophs contribute a higher proportion to the community when the algal biomass density is at a higher degree of development.

Periphyton community structure

The diversity index in RG site achieved the highest value among the study sites (Table 13), possibly attributed to the tendency observed at each accrual day having a more even distribution phyla density. Overall, the periphyton community was dominated by diatoms and cyanobacteria mostly composed of the filamentous cyanobacteria *Anabaena* sp. The latter genus is able to fix N_2 and their prevalence appears to be greater in low flow conditions and thus may produce surface blooms (Baker and Baker, 1979). The RG site was the only stream to show in several occasions algal blooms around the stream shoreline, and the mean flow was 0.21 m^3/s during the ten month sampling period. Río Piedras site has the highest nutrient concentrations and thus the community exhibited low taxon evenness with an overall community dominated by

Navicula spp., *Cocconeis* spp., and *Gomphonema* spp. and many taxa were observed only once. Dominance of tolerant species to eutrophic habitats tends to reduce community diversity (Griffith et al., 2005).

Temporal variations in periphyton community composition

The periphyton community density varied over time within each stream site. The forested stream, RM site, had the lowest mean total density of all sites exhibiting a non-significant increasing trend with accrual time. This result is associated with the chlorophyll-a levels obtained for the same samples analyzed in the previous chapter (Table 6). Nutrient concentrations at this site may be contributing to the overall low periphyton density. The community reached a peak biomass density and the highest number of organisms at 7 days of accrual. The organism counts in this stream site are only few counts per visual field assuming limitations in periphyton growth rates that can be pointed to nutrient supply, although no statistical correlations were accomplished. Hence, further investigations should be conducted to determine the effects of nutrients on periphyton richness as in Biggs and Smith (2002).

Río Guanajibo site exhibited a significant, though not statistically different, increase over time reflected in the community structure and density. Diatoms dominated at the beginning but over time a shift to Cyanobacteria occurred after 10 days. Also, the *Euglena* spp. density increased along with Chlorophyta as well as unknown species. This site is the only stream showing Cyanobacteria taking over the numerical dominance of the community. Río Guanajibo is located in a heterogeneous land use watershed, exhibited moderate nutrient conditions, low base flow, pH values over 8.0, and high magnesium and silicon concentrations compared to the other streams evaluated (Table 11). Additionally, this site receives discharges from a water treatment plant a few kilometers upstream which may contribute labile organic carbon components that may influence the primary productivity (Dr. Gustavo Martínez, personal communication). However, in this study it was not possible to find a qualitative linkage that could explain the abundance of cyanobacteria in RG site in contrast to the other sites.

The nutrient enriched stream site, RP, was the most intensely sampled with three sampling dates for each accrual day. These samples were taken between January and October, from periphyton samples collected in the growth rate assessment detailed in Chapter 2. Periphyton total density of each sample was averaged for each accrual day resulting in substantial variability within samples of the same accrual day. The total density at 10 accrual days resulted in lower count of individuals per area than at 7 days. Examining the sampling dates, a significant flood event occurred at two of the 10 day sample dates. This fact could explain the decrease in total density of periphyton community between 7 and 10 days (Biggs and Smith, 2002; Lake, 2000). The effect of flooding caused a decrease in diatoms (*Navicula*, *Fragilaria*) and a slight increase on *Anabaena* sp. individuals were observed.

Mean community density of individuals per accrual day at each study site showed a significant positive relationship with nutrients following the same tendencies as the relationships with biomass reported in the previous chapters. These results are indicative that nutrients are a major influential factor in regulating periphyton community dynamics and that the chlorophyll a concentration will be correspondent of the community density (Appendix 14C). This study was able to perform the analysis based on number of individuals per area, however, an analysis of cell count per area would be more precise to determine a more specific relationships given that some species exhibit multiple cells within an individual such as filamentous green algae contrary to diatom species which can be present as single cell occupying much less area.

CONCLUSIONS

The dynamics of the periphyton succession within a ten-day period may not be long enough time to detect significant changes in community structure. Heterokontophyta dominated the first succession in the three streams, which was expected given that diatoms are one of the most diverse phyla with a broad number of species that can inhabit a wide array of environmental conditions. The results in this study showed what could be the beginning of dominance shift from diatoms to cyanobacteria individuals in the periphyton community structure for the moderately nutrient enriched stream, Río Guanajibo. Several characteristics were particular of this site including base flow, the elevated hardness and magnesium concentrations, and a water treatment plant located upstream from the sampling site that could have been loading constituents that were not analyzed in this study. The latter may possibly be discharging elevated carbon that may influence the dominance of Cyanobacteria. The algal density was positively associated to nutrient concentrations, following the same tendencies as of the periphyton biomass observed on previous chapters. Further research with a more comprehensive analysis of the taxonomic richness and diversity should be conducted to identify differences between benthic algae community structure exposed to a gradient of nutrient status, as well as to identify possible species indicators of trophic status. Streams with similar physical and chemical parameters as Río Guanajibo should be studied to define the influential variable that causes changes in the community. The use of artificial substrates reduces the effect of sloughing caused by floods and limits the succession dynamics if their use is for short time periods. Additionally, the comparison of periphyton community colonized on natural substrates and on artificial substrates could be assessed to describe the community structure and composition in different growth phases.

REFERENCES

Anping, L., L. Kwan-sing P., and H. Zhangli. 2011. Comparison of two sampling methods when studying periphyton colonization in Lam Tsuen River, Hong Kong, China. Chinese J. Ocean. and Limnol. 29:141-149.

Baker, A. L., and K.K. Baker. 1979. Effects of temperature and current discharge on the concentration and photosynthetic activity of the phytoplankton in the upper Mississippi River. Freshwater Biology 9:191–198.

Barinova, S. 2011. Algal diversity dynamics, ecological assessment, and monitoring in the river ecosystems of the Eastern Mediterranean. Nova Science Publishers. 369 p.

Bellinger B.J., Cocquyt, C. and C.M. O'Reilly. 2006. Benthic diatoms as indicators of eutrophication in tropical streams. Hydrobiologia 573: 75-87.

Biggs, B.J.F. 1985. The use of periphyton in the monitoring of water quality. In R D Pridmore and A.B. Cooper (eds.), Biological monitoring in freshwaters: Proceedings of a seminar. Water and Soil Miscellaneous Publication 82. Wellington, New Zealand: Ministry of Works and Development, pp. 117-142

Biggs, B.J.F. 1988. Artificial substrate exposure times for periphyton biomass estimates in rivers. New Zealand Journal of Marine and Freshwater Research. 22: 507-515.

Biggs, B.J.F. 2000a. New Zealand periphyton guidelines: detecting, monitoring and managing enrichment streams. Prepared for the Ministry for the Environment, Christchurch, June 2000. Available at: http://www.mfe.govt.nz/publications/water/nz-periphyton-guide-jun00.pdf

Biggs, B.J.F. 2000b. Eutrophication of streams and rivers: dissolved nutrient-chlorophyll relationships for benthic algae. J. N. Am. Benthol. Soc. 19: 17-31.

Biggs, B.J.F, and M.E. Close. 1989. Periphyton biomass dynamics in gravel bed rivers: the relative effects of flows and nutrients. Freshwater Biol. 22: 209-231.

Biggs, B.J.F, and R.A. Smith. 2002. Taxonomic richness of stream benthic algae: Effects of flood disturbance and nutrients. Limnol. Oceanogr. 47(4): 1175-1186.

Biggs B.J.F, and S. Stokseth. 1996. Hydraulic habitat preferences for periphyton in rivers. Regulated Rivers: Research and Management, 12: 251-261.

Blair, A., Sanger, D., Holland, F.A., White, D., Vandiver, L., and S. White. 2010. Storm water runoff-modeling impacts of urbanization and climate change. Proceedings of the 2010 South Carolina Water Resources Conference, held October 13-14, 2010, at the Columbia Metropolitan Convention Center

Black R.W., P.W. Moran, and J.D. Frankforter. 2011. Response of algal metrics to nutrients and physical factors and identification of nutrients thresholds in agricultural streams. Environ. Monit. Assess., 175: 397-417.

Boisson, J.C., and Y. Perrodin. 2006. Effects of road runoff on biomass and metabolic activity of periphyton in experimental streams. Journal of Hazardous Materials. 132: 148-154.

Bothwell, M. 1985. Phosphorus limitation of lotic periphyton growth rates: An intersite comparison using continuous-flow troughs (Thompson River system, British Columbia) Limnol. Oceanogr. 30: 527-542.

Bryan, B. 2001. An annotated survey of common periphytic diatoms of the Río Mameyes, Northeast Puerto Rico. USDA Forest Service. International Institute of Tropical Forestry, Río Piedras, Puerto Rico.

Carpenter, S.R., Caraco, N.F, Correll, D.L, Howarth, R.W., Sharpley, A.N, and V.H. Smith. 1998. Nonpoint pollution of surface waters with phosphorous and nitrogen. Ecological Applications. 8: 559-568.

Cattaneo, A., and M. Amireault. 1992. How artificial are artificial substrata for periphyton?. J.N. Am. Benthol. Soc. 11: 244-256.

Chambers, P.A., McGoldrick, D.J., Brua, R.B., Vis, C., Culp, J.M., and G.A. Benoy. 2012. Development of environmental thresholds for nitrogen and phosphorus in streams. J. Environ. Qual. 41:7-20.

Chetelat J., Pick, F.R., Morin, A, P.B. and Hamilton. 1999. Periphyton biomass and community composition in rivers of different nutrient status. Can J. Fish. Aquat. Sci., 56: 560-569

Cushing, C.E., and J.D. Allan. 2001. Streams: Their Ecology and Life. Academic Press. 366 pp.

Danilov R.A. and N. Ekelund. 2001. Comparison of usefulness of three types of artificial substrata (glass, wood, and plastic) when studying settlement patterns of periphyton in lakes of different trophic status. J. Microbiological Methods. 45:167-170.

Davidson, Kwen, R.J., Tett, P., Bresnan, E., Harrison, P., McKinney, A., Milligan, S., Mills, D., Silke, J., and A.M. Crooks. 2012. Harmful algal blooms: How strong is the evidence that nutrient ratios and forms influence their occurrence?. Estuarine, Coastal and Shelf Sc. 115: 399-413.

De Jesús-Crespo, R., and A. Ramírez. 2011. Effect of urbanization on stream physicochemistry and macroinvertebrate assemblages in a tropical urban watershed in Puerto Rico. J. N. Am. Benthol. Soc., 30(3): 739-750.

Dixon, J.B. 1989. Kaolin and serpentine group minerals. In J.B. Dixon and S.B. Weed (eds.) Minerals in soil environments. 2nd Ed. SSA Book Series 1. Soil Sci. Soc. Amer. Madison, WI, USA. Pp.467-526.

Dodds, W.K., Smith, V.H., and B. Zander. 1997. Developing nutrient targets to control benthic chlorophyll levels in streams: A case study of the Clark Fork River. Wat. Res., 31: 1738-1750.

Dodds, W.K., Jones, J.R., and E.B. Welch. 1998. Suggested classification of stream trophic state: distributions of temperate stream types by chlorophyll, total nitrogen and phosphorus. Wat. Res. 32: 1455-1462.

Dodds, W., and E. Welch. 2000. Establishing nutrient criteria in streams. J. Am. Benthol. Soc. 19: 186-196.

DRNA, 2004a. Cuenca del Río Guanajibo. P.R. Departamento de Recursos Naturales y Ambientales website [accessed on: October 10, 2012].

DRNA, 2004b. Cuenca del Río Mameyes. P.R. Departamento de Recursos Naturales y Ambientales website [accessed on October 7, 2012].

DRNA, 2004c. Cuenca del Río Piedras. P.R. Departamento de Recursos Naturales y Ambientales website [accessed on October 25, 2012]

Ewel J.J. and J.L. Whitmore. 1973. The ecological life zones of Puerto Rico and the U.S. Virgin Islands. Forest Service Research Paper. ITF-18. Institute of tropical forestry. USDA., Rio Piedras, P.R.

Fairchild, G.W., Lowe, R.L., and W.B. Richardson. 1985. Algal periphyton growth on nutrient diffusing substrates: an in situ bioassay. Ecology 66:465 472.

Florida Department of Environmental Protection (DEP), 2012. Implementation of Florida's Numeric Nutrient Standards. Submitted to EPA on April, 2013 Gardner, N. L.1927. New Mixophyceae from Porto Rico. Memories New York Botanical Garden, 7:1-144.

Gardner, N. L.1927. New Mixophyceae from Porto Rico. Memories New York Botanical Garden, 7:1-144.

Gotelli, N.J. 2008. A primer of ecology. 4th ed. Sinauer Associates. 291 p.

Granéli, E., Vidyarathna, N., Funari, E., Cumaranatunga, P.R.T, and R. Scenati. 2011. Can increases in temperature stimulate blooms of the toxic benthic dinoflagagellate *Ostreopsis ovata*?. Harmful Algae 10: 165-172.

Griffith, M.B., Hill, B., McCormick, F., Kaufmann, P., Herlihy, A., and A. Selle. 2005. Comparative application of indices of biotic integrity based on periphyton, macroinvertebrates, and fish to southern Rocky Mountain streams. Ecological Indicators: 117-136.

Gualtero-Leal, Diana. 2007. Composición y abundancia de las algas bénticas de cinco sistemas lóticos de Puerto Rico. University of Puerto Rico-Mayagüez campus. Thesis dissertation.

Gualtero-Leal, D., Martínez, G.A., Santos-Flores, C., and D. Sotomayor-Ramírez. 2010. Benthic algal diversity and biomass as indicators to establish reference streams in Puerto Rico. Verh. Internat. Verein. Limnol., 30: 1567-1572.

Hauer, F.R., and G.A. Lamberti. (editors). 2006. Methods in Stream Ecology, Second Edition. Elsevier, Amsterdam.

Hill, B., Herlihy, A., Kaufmann, P., DeCelles, S., and M. Vander Borgh. 2002. Assessment of streams of the eastern United States using a periphyton index of biotic integrity. Elsevier Science Ltd. Ecological Indicators. 325-338.

Kevern, N.R., Wilhm, J.L., and G.M. Van Dyne. 1966. Use of artificial substrata to estimate the productivity of periphyton. Limnol. Oceanog. 11: 499-502.

Lake, P.S. 2000. Disturbance, patchiness, and diversity in streams. J.N. Am. Benthol. Soc. 19: 573-592.

Lavoie, I., Vincent, W.F., Pienitz, R., and J. Painchaud. 2004. Benthic algae as bioindicators of agricultural pollution in the streams and rivers of southern Québec (Canada). Aquat. Ecosystem Health. 7(1): 43-58.

Macchiavelli, R. (2014). Notas de clase de Biometría Avanzada. http://academic.uprm.edu/rmacchia/agro6600/agro6600.pdf

Mesner, N., and G. Paige. 2011. Best management practices monitoring guide for stream systems. Regional Water Program. University of Wyoming. 52 pp.

Metcalf, J.S., and G.A. Codd. 2014. Cyanobacteria toxins (cyanotoxins) in water. 2014. Foundation for Water Research, UK. 45 pp.

Murdock J., Roelke, D., and F. Gelwick. 2004. Interactions between flow, periphyton, and nutrients in a heavily impacted urban stream: implications for streams restoration effectiveness. Ecological Engineering. 22: 197-207.

Nordin, R. N, 1985. *Water Quality Criteria for Nutrients and Algae (Technical Appendix)*. British Columbia Ministry of the Environment, Victoria, BC. 104 pp.

Odum, H.T. 1956. Primary production in flowing waters. Limnol. Oceanog. 1: 102-117.

Ortiz-Zayas, J.R., Lewis, W., Saunders III, J.F McCutchan Jr. J.M., and F.N Scatena. 2005. Metabolism of a tropical rainforest stream. N. Am. Benthol. Soc., 24(4): 729-783.

Potapova, M., Charles, D., Ponader, K. and D. Winter. 2004. Quantifying species indicator values for trophic diatom indices: a comparison of approaches. Hydrobiologia 517: 25-41.

Puerto Rico Environmental Quality Board. (PREQB). 2008. Puerto Rico Water Quality Inventory and List of Impaired Waters. 2002 305(b)/303(d). Estado Libre Asociado de Puerto Rico.

Puerto Rico Environmental Quality Board. (PREQB) 2010a. 305(b)/303(d) Integrated Report: Plans and Special Projects Division. Evaluation and Strategic Planning Area. Revised September 2010. Estado Libre Asociado de Puerto Rico, San Juan, P.R.

Puerto Rico Environmental Quality Board. (PREQB). 2010b. Puerto Rico Nutrient Standard Plan. September 2010-Review. Available at: http://water.epa.gov/scitech/swguidance/standards/criteria/nutrients

Puerto Rico Environmental Quality Board (PREQB) 2010c. Reglamento de estándares de calidad de agua de Puerto Rico. Versión enmendada marzo 2010. Estado Libre Asociado de Puerto Rico. San Juan, PR. 97 pp.

Puerto Rico Environmental Quality Board. (PREQB) 2012. Assessment Methodology for 305(b)/303(d) Integrated Report 2012 cycle. Plans and Special Projects Division. Evaluation and Strategic Planning Area. Estado Libre Asociado de Puerto Rico, San Juan, P.R.

Quinn, J.M., and B.W. Gilliland. 1989. The Manawatu River cleanup-Has it worked? Transactions of the Institution of Professional Engineers, New Zealand. 16:22-26.

Ramírez, A. 2008. Tropical stream conservation. *In* David Dudgeon (ed.) Tropical stream ecology. San Diego: Academic Press. 285 p.

Ramírez, A, and L. Hernández-Cruz. 2004. Aquatic insect assemblages in shrimp-dominates tropical streams, Puerto Rico. Biotropica 36(2): 259-266.

Ramírez, A., Engman, A. Rosas, K.G., Pérez-Reyes, O., and D. Martinó-Cardona. 2011. Urban impacts on tropical island streams: Some key aspects influencing ecosystem response. Urban Ecosyst. 11 pp..

Rastogi, V.B. 2004. Modern biology. Vol II. 7th ed. Pitambar Publishing Company LTD. New Delhi.

Santos-Flores, C. J. 2001.Taxonomy and distribution of the freshwater micro-crustaceans and green algae of Puerto Rico, three contributions to American cladocerology, and a bibliography on West Indian limnology. PhD Thesis University of Wisconsin, Madison, USA.

Sekar, R., Venugopalan, V.P., Nandakumar, K., Nair, K., and V. Rao. 2004. Early stages of biofilm succession in a lentic freshwater environment. Hydrobiologia. 52: 97-108.

Smucker, N.J., Becker, M., Detenbeck, N.E., and A.C. Morrison. 2013. Using algal metrics and biomass to evaluate multiple ways of defining concentration-based nutrient criteria in streams and their ecological relevance. Ecological Indicators 32: 51-61.

Sotomayor, D., and G. Martínez. 2013. Narrative to describe how numeric reference criteria and numeric nutrient criteria were obtained for rivers of Puerto Rico. Unpublished manuscript submitted to PREQB on July 16 2013. Supplements to Sotomayor et al. (2011).

Sotomayor-Ramírez, Martínez, D. G., and L. Pérez-Alegría. 2011. Determination of numeric nutrient criteria in rivers and streams of Puerto Rico. Final Report submitted to PREQB as per Agreement 000080395 between UT Batelle, LLC Oak Ridge National Laboratory and University of Puerto Rico, Agricultural Experiment Station. 58 pp with appendices.

Suren A.M., Biggs, B.J., Kilroy, C., and L. Bergey. 2003. Benthic community dynamics during summer low-flows in two rivers of contrasting enrichment. New Zealand Journal of Marine and Freshwater Research 37:53-70.

Tuchman, M.L., and R.J. Stevenson. 1980. Comparison of clay tile, sterilized rock, and natural substrate diatom communities in a small stream in Southeastern Michigan, USA. Hydrobiologia. 75:73-79.

Uriarte, M., Yackulic, C.S., Lim, Y., and J.A. Arce-Nazario. 2011. Influence of land use on water quality in a tropical landscape: a multi-scale analysis. Landscape Ecol. 26:1151-1164.

USEPA. 1995. Drinking water regulations and health advisories. Office of Water, U.S. Environmental Protection Agency, Washington, DC. EPA 822-R-95-001.

USEPA. 1998. *National Strategy for the Development of Regional Nutrient Criteria*. Office of Water, U.S. Environmental Protection Agency. EPA 822-R-98-002.

USEPA, 2000. Nutrient Criteria Technical Guidance Manual. Rivers and Streams. USEPA, Office of Water. EPA-822-B-00-002.

USEPA, 2013. National rivers and streams assessment 2008-2009: A collaborative survey (Draft). EPA/841/D-13/001. Washington D.C. Available at: http://water.epa.gov/type/rsl/monitoring/riverssurvey/upload/NRSA0809_Report_Final_508Compliant_130228.pdf

Tien, C., Wu, W.H., Chuang, T.L and C.S. Chen. 2009. Development of river biofilms on artificial substrate and their potential for biomonitoring water quality. Chemosphere, 76: 1288-1295.

Wang, H., Hondzo, M., Xu, C., Poole, V., and A. Spacie. 2003. Dissolved oxygen dynamics of streams draining an urbanized and an agricultural catchment. Ecological Modelling. 160: 145-161.

Wan-Omar, W.M. 2010. Perspective on the use of algae as biological indicators for monitoring and protecting aquatic environments, with special reference to Malaysian freshwater ecosystem. Tropical Life Sciences Research, 21:51-67.

Wehr, J.D., and R. Sheath. (editors) 2003. Freshwater algae of North America: Ecology and classification. Academic Pres.

Welch, B. E. 1992. *Ecological Effects of Wastewater*. Chapman and Hall, London.

Welschmeyer, N. 1994. Fluorometric analysis of chlorophyll *a* in the presence of chlorophyll *b* and pheopigments. Limnol. Oceanogr. 39(8): 1985-1192.

Wetzel, Robert. 2001. Limnology: Lake and River Ecosystems. 3rd Edition. Elsevier Science and Technology.

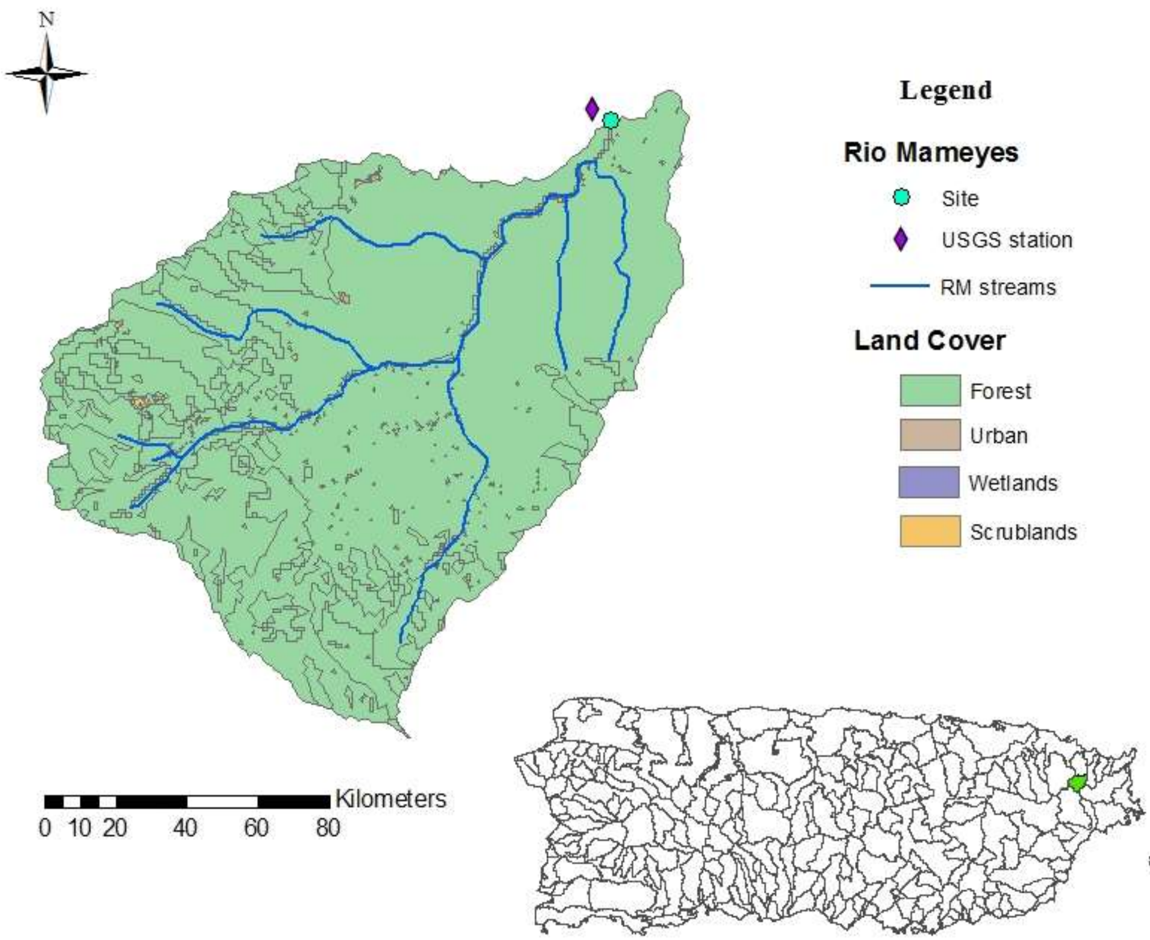
Withers, P.J.A., and H.P. Jarvie. 2008. Delivery and cycling of phosphorus in rivers: A review. Sci. Total. Environ. 400: 379-395.

Zheng, L., and M.J. Paul. 2008. Effects of eutrophication on streams ecosystems. [Online]. Tetra Tech, Inc. Available at http://n-steps.tetratech-ffx.com/PDF&otherFiles/literature_review/Eutrophication%20effects%20on%20streams.pdf

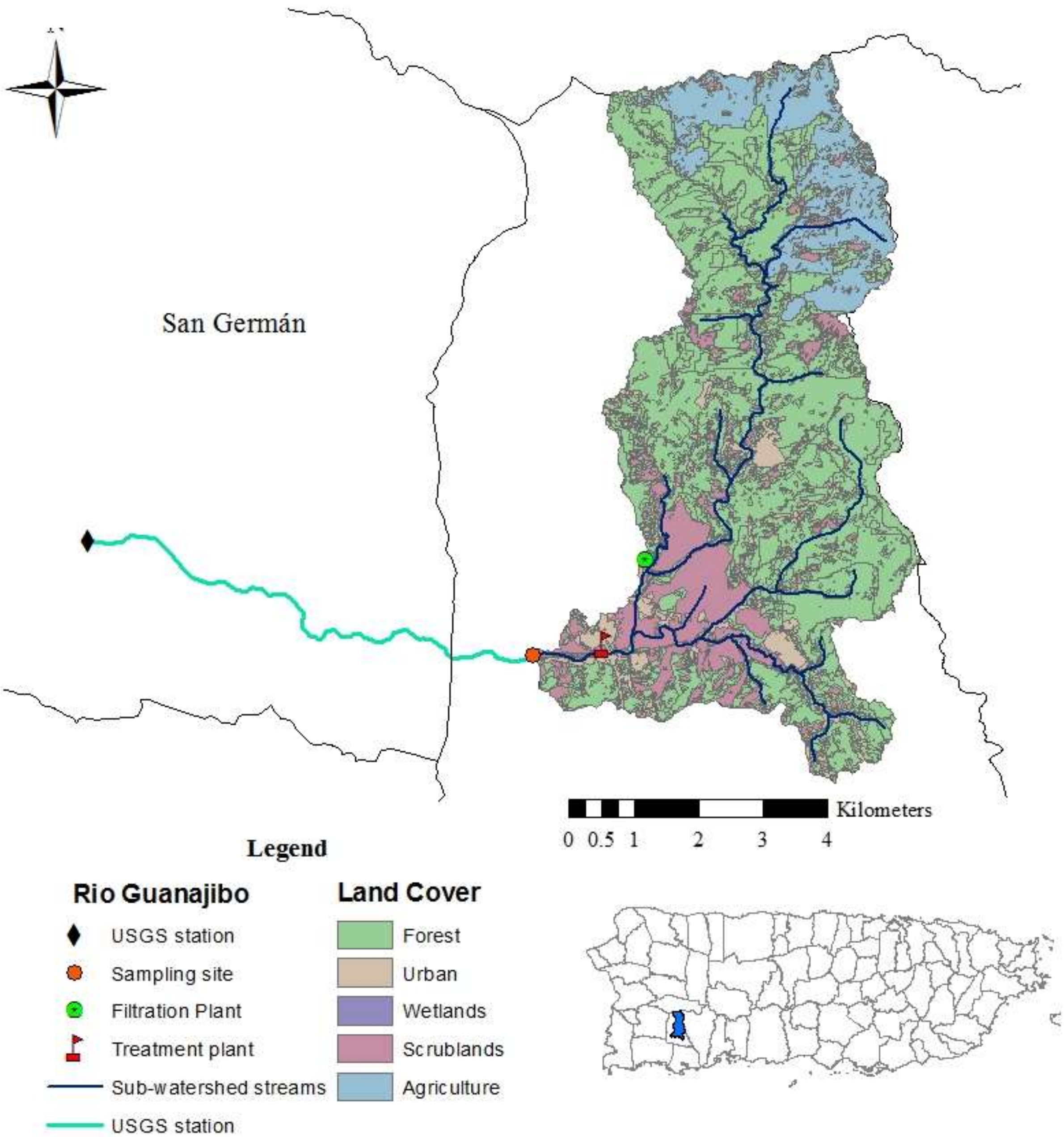
APPENDICES

Appendix 1: GIS based maps of the three sub watersheds of the study sites a) Río Mameyes, b) Río Guanajibo and c) Río Piedras.

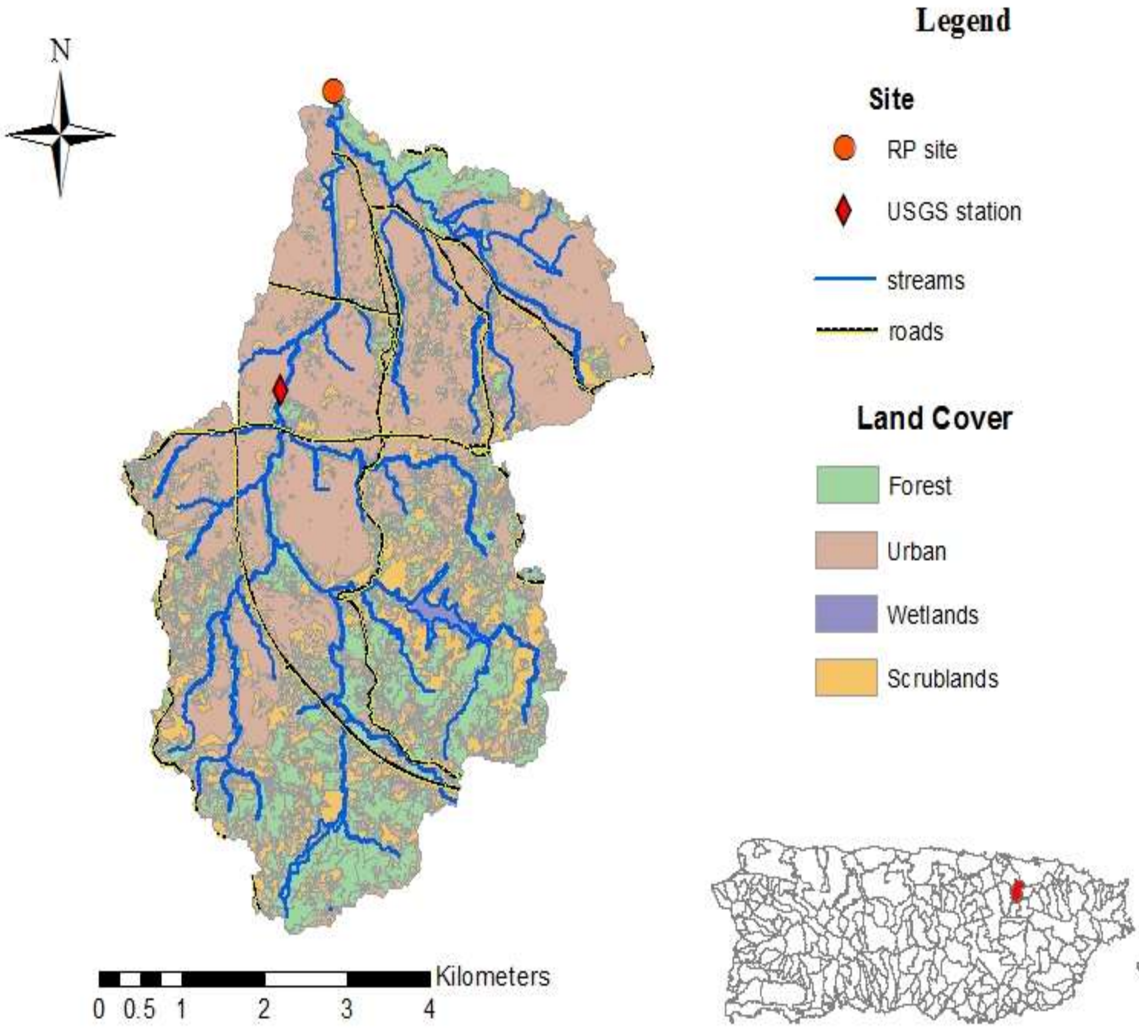
A



B

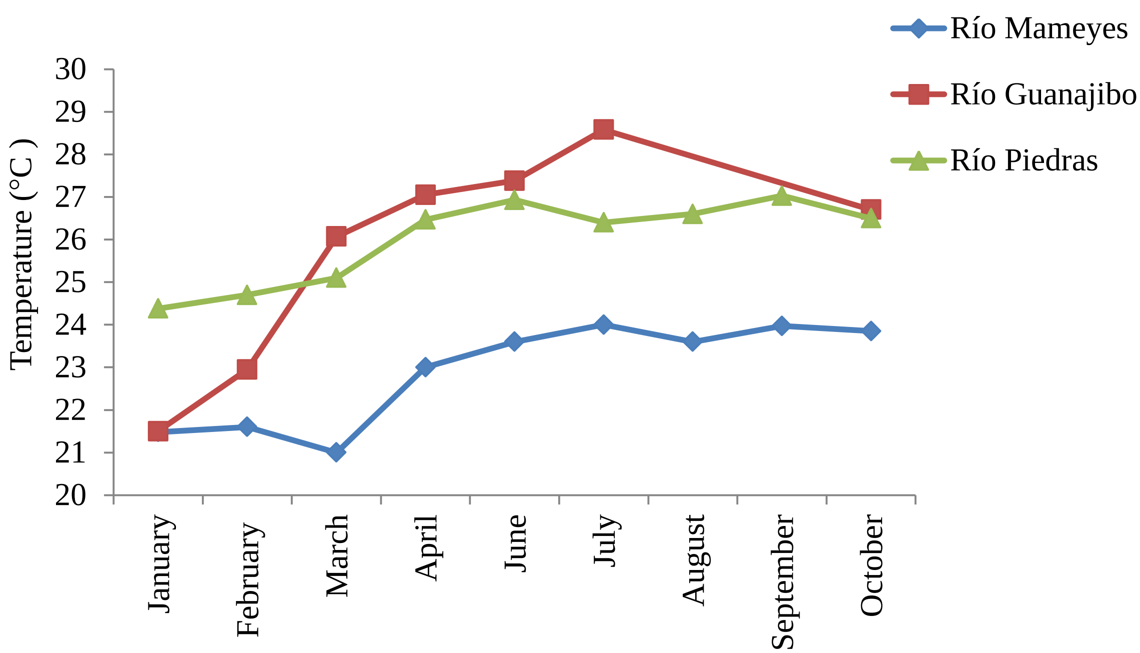

San Germán
Kilometers
0 0.5 1 2 3 4
Legend
Rio Guanajibo
USGS station
Sampling site
Filtration Plant
Treatment plant
Sub-watershed streams
USGS station
Land Cover
Forest
Urban
Wetlands
Scrublands
Agriculture

C

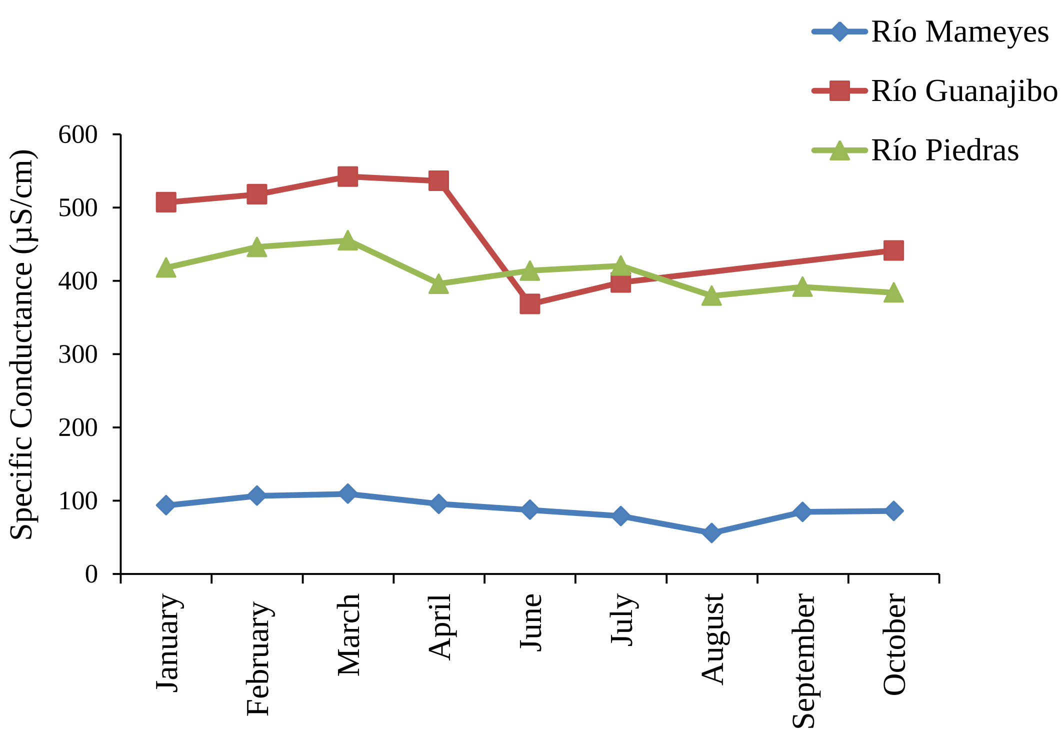

N
Legend
Site
RP site
USGS station
streams
roads
Land Cover
Forest
Urban
Wetlands
Scrublands
Kilometers
0 0.5 1 2 3 4

Appendix 2: Temporal variation of physical and chemical parameters for each study site a.) temperature, b) specific conductance, c) pH, d) DO (mg/L), e) TP, f) NO_3-N, g) TN

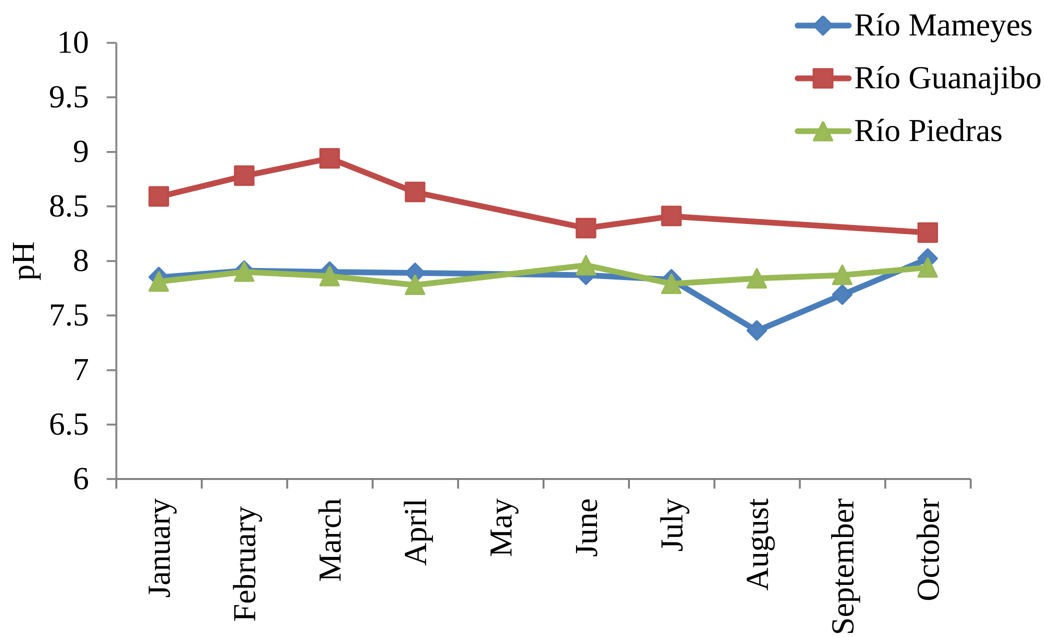
A



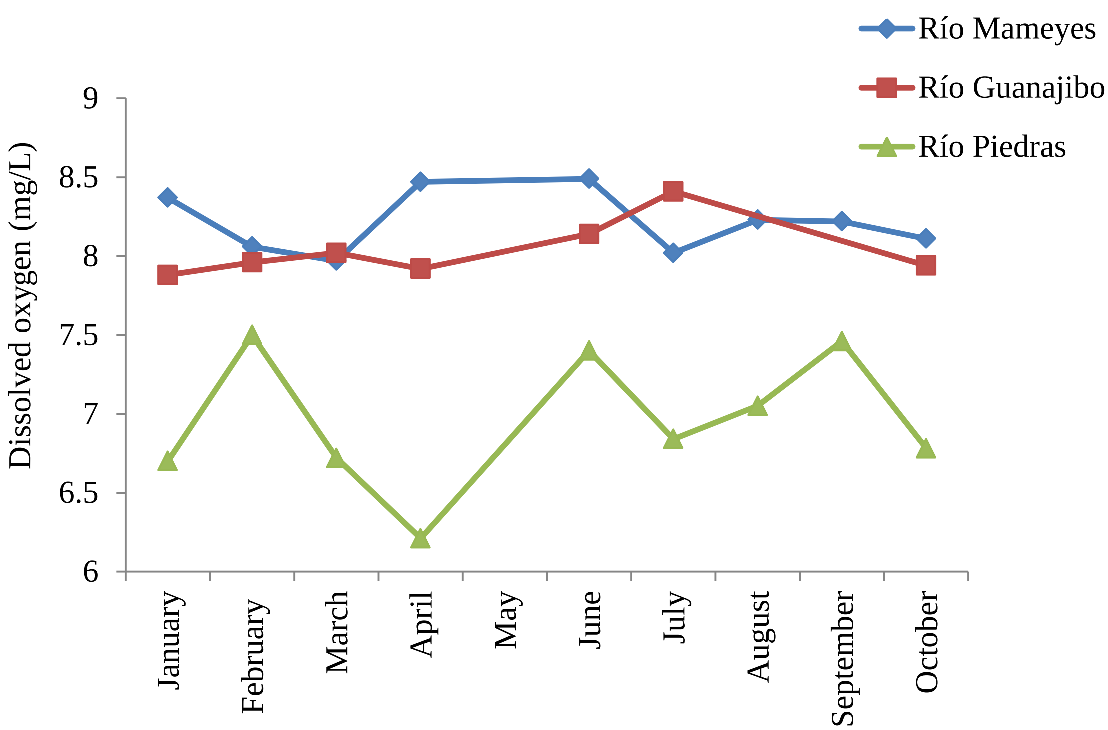
B



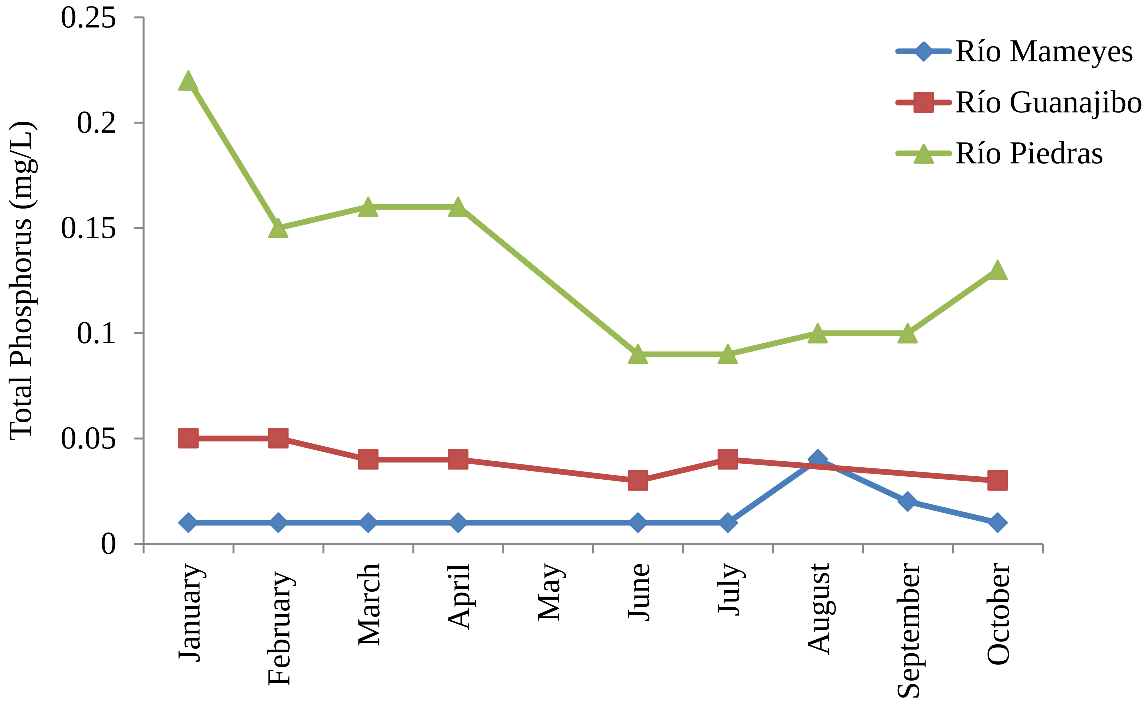
C


Río Mameyes
Río Guanajibo
Río Piedras
10
9.5
9
8.5
8
7.5
7
6.5
6
pH
January
February
March
April
May
June
July
August
September
October

D

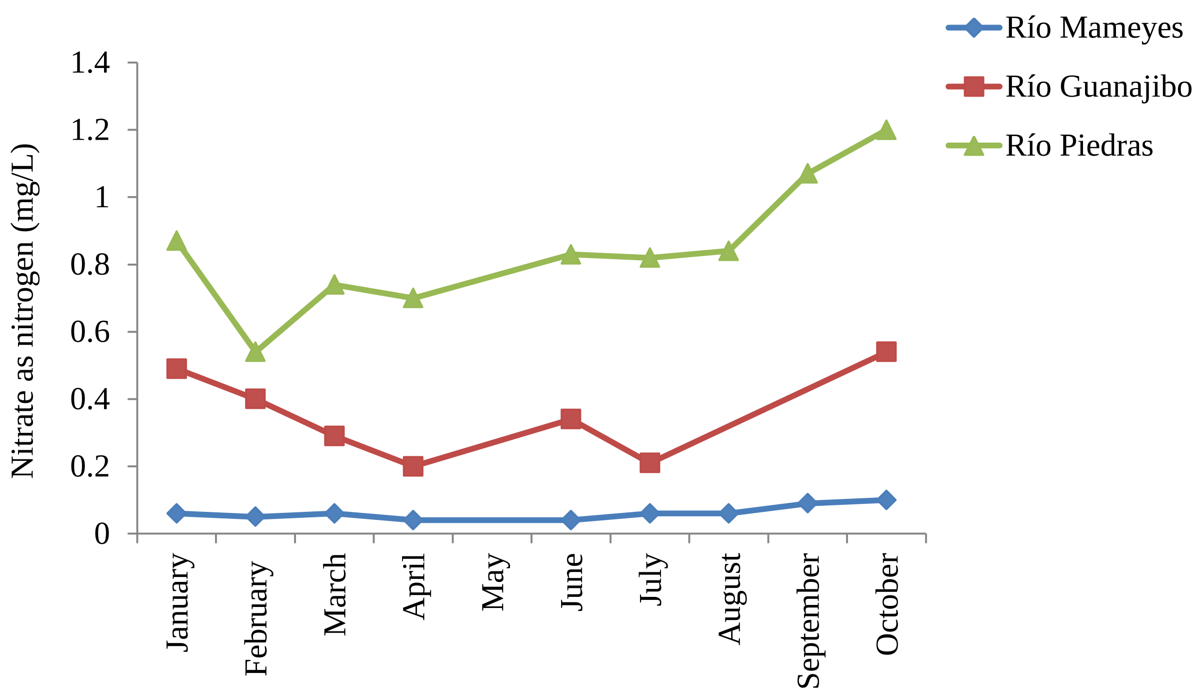

Río Mameyes
Río Guanajibo
Río Piedras
9
8.5
8
7.5
7
6.5
6
Dissolved oxygen (mg/L)
January
February
March
April
May
June
July
August
September
October

E



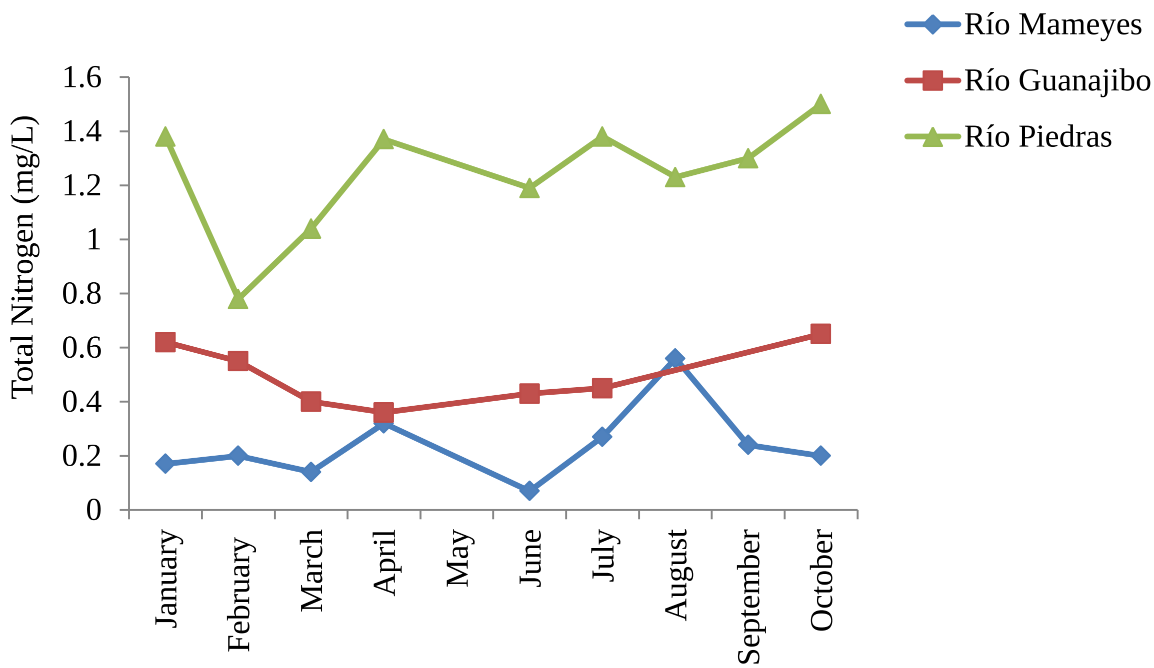
0.25
0.2
0.15
0.1
0.05
0
Total Phosphorus (mg/L)
January
February
March
April
May
June
July
August
September
October
Río Mameyes
Río Guanajibo
Río Piedras

F



1.4
1.2
1
0.8
0.6
0.4
0.2
0
Nitrate as nitrogen (mg/L)
January
February
March
April
May
June
July
August
September
October
Río Mameyes
Río Guanajibo
Río Piedras

G



Appendix 3: Pearson correlation comparing physical, chemical and biological parameters

Variable(1)	Variable(2)	Pearson	p-valor
Chla	AFDM	0.94	<0.0001
Chla	NO_3-N	0.75	0.0001
Chla	TN	0.59	0.0044
Chla	TP	0.79	<0.0001
Chla	Temperature	0.68	0.0007
Chla	Sp. conductance	0.92	<0.0001
AFDM	NO_3-N	0.70	0.0004
AFDM	TN	0.47	0.0304
AFDM	TP	0.68	0.0007
AFDM	Temperature	0.77	<0.0001
AFDM	Sp. conductance	0.89	<0.0001
AI	NH_4-N	-0.46	0.0344
TP	Temperature	0.49	0.0001
TP	Sp. conductance	0.78	<0.0001
TP	DO	-0.71	<0.0001
TN	Temperature	0.51	<0.0001
TN	Sp. conductance	0.70	<0.0001

TN	DO	-0.68	<0.0001
NO_3-N	Temperature	0.54	<0.0001
NO_3-N	Sp. conductance	0.83	<0.0001
NO_3-N	DO	-0.68	<0.0001
Sp. conductance	Temperature	0.60	<0.0001
Sp. conductance	DO	-0.40	0.0021
Sp. conductance	pH	0.54	<0.0001
Total density (Ind./mm^2)	Chla	0.93	0.0002
Total density (Ind./mm^2)	TP	0.75	0.0196
Total density (Ind./mm^2)	TN	0.71	0.0338

Appendix 4: Date on which periphyton biomass represented outliers from Río Piedras site.

Sampling Date	Outliers values	2-day V_c[1]
7/24/2013	1.13 mg/m^2	3.25 $x10^5$ m^3
8/2/2013	0.25 mg/m^2	3.98 $x10^5$ m^3

[1]2-day-V_c means the cumulative volume discharged 2 days before sampling date.

Appendix 5: Evaluation of the cumulative volume of several days before sampling date for periphyton biomass

Days	Exponential	Lineal
	-----adjusted R^2-----	
1	0.71	0.75
2	0.95	0.88
3	0.92	0.81
5	0.86	0.8
7	0.69	0.65
10	0.58	0.51

Appendix 6: Benthic chlorophyll-a from natural substrates and the corresponding TP concentration at the most nutrient enriched site studied, Río Piedras

TP	Chlorophyll-a
mg/L	mg/m2
0.118	76.82
0.125	107.99
0.176	22.66
0.152	85.45
0.097	19.01
0.098	34.44
0.091	17.33
0.099	23.46
0.121	22.5

Appendix 7: Pictures of the study sites: a) Río Mameyes (RM), b) Río Guanajibo (RG) and c) Río Piedras (RP)

A

B

C

Appendix 8: Tukey comparison tests tables of periphyton biomass on artificial substrates

A) Tukey test comparison for the principal effect of study sites.

α=0.05 DMSS=0.06869; Error: 0.0071 d.f: 36

Study site	Mean	n	S.E.			
RM	0.22	18	0.02	A		
RG	0.79	18	0.02		B	
RP	1.05	18	0.02			C

B) Tukey test comparison of principal effect on accrual days

α=0.05 DMS=0.06869; Error: 0.0071; d.f. 36

Accrual days	Mean	n	S.E.		
3	0.09	18	0.02	A	
7	0.96	18	0.02		B
10	1.0	18	0.02		B

C) Tukey test comparison of principal effect on seasons

α=0.05 DMS=0.04653*;* Error: 0.0071 d.f:36

Season	Mean	n	S.E		
dry	0.63	27	0	A	
wet	0.74	27	0		B

D) Tukey test comparison between two factors: study sites and accrual days.

α=0.05 DMS=0.16048; Error: 0.0071 d.f: 36

Study site	Accrual days	Means	n	S.E						
RM	3	-0.42	6	0.03	A					
RG	3	0.24	6	0.03		B				
RP	3	0.45	6	0.03			C			
RM	7	0.51	6	0.03			C			
RM	10	0.57	6	0.03			C			
RG	7	0.97	6	0.03				D		
RG	10	1.15	6	0.03					E	
RP	10	1.28	6	0.03					E	F
RP	7	1.4	6	0.03						F

E) Tukey test comparison between two factors: accrual days and seasons.

α=0.05 DMS=0.11956; Error: 0.0071 d.f: 36

Season	Accrual days	Means	n	S.E.	
dry	3	-0.06	9	0.03	A

wet	3	0.24	9	0.03	B
dry	7	0.92	9	0.03	C
wet	10	0.96	9	0.03	C
wet	7	1.01	9	0.03	C
dry	10	1.03	9	0.03	C

F) Tukey statistic test comparing interactions between season and study site.
α=0.05 DMS=0.11956; Error: 0.0071; d.f: 36

Season	Study site	Means	n	S.E.	
dry	RM	0.15	9	0.03	A
wet	RM	0.29	9	0.03	B
dry	RG	0.74	9	0.03	C
wet	RG	0.83	9	0.03	C
dry	RP	1	9	0.03	D
wet	RP	1.09	9	0.03	D

G): Triple interaction effect between the three factors.
α=0.05 DMS=0.25815; Error: 0.0071 d.f.: 36

Season	Study site	Accrual days	Medias	n	E.E.	
Dry	RM	3	-0.63	3	0.05	A
Wet	RM	3	-0.22	3	0.05	B
Dry	RP	3	0.2	3	0.05	C
Wet	RG	3	0.22	3	0.05	C
Dry	RG	3	0.27	3	0.05	C D
Dry	RM	7	0.51	3	0.05	D E
Wet	RM	7	0.52	3	0.05	D E
Dry	RM	10	0.56	3	0.05	E
Wet	RM	10	0.57	3	0.05	E
Wet	RP	3	0.71	3	0.05	E F
Dry	RG	7	0.85	3	0.05	F G
Wet	RG	7	1.09	3	0.05	G H
Dry	RG	10	1.12	3	0.05	H
Wet	RP	10	1.14	3	0.05	H I
Wet	RG	10	1.18	3	0.05	H I J

Dry	RP	7	1.39	3	0.05	I	J
Wet	RP	7	1.42	3	0.05		J
Dry	RP	10	1.43	3	0.05		J

Appendix 9: Mean benthic chlorophyll-a values of 7-days accrued on artificial substrates for A) Río Mameyes, B) RíoGuanajibo and C) Río Piedras.

	Río Mameyes	Río Guanajibo	Río Piedras
	--------------------------	mg/m^2	----------------------
	3.29	7.10	24.74
	4.46	7.76	16.52
	5.83	11.07	24.14
	2.07	5.14	5.55
	2.83	11.84	26.10
	3.42	4.28	
		4.22	
		12.88	
Mean values	3.5	7.4	17.02
95% confidence interval	(2.37-5.04)	(5.03-10.76)	(7.58-38.24)

Appendix 10: Number of samples analyzed at each study site for each accrual day. Each sample taken from different sampling dates had one replicate analyzed.

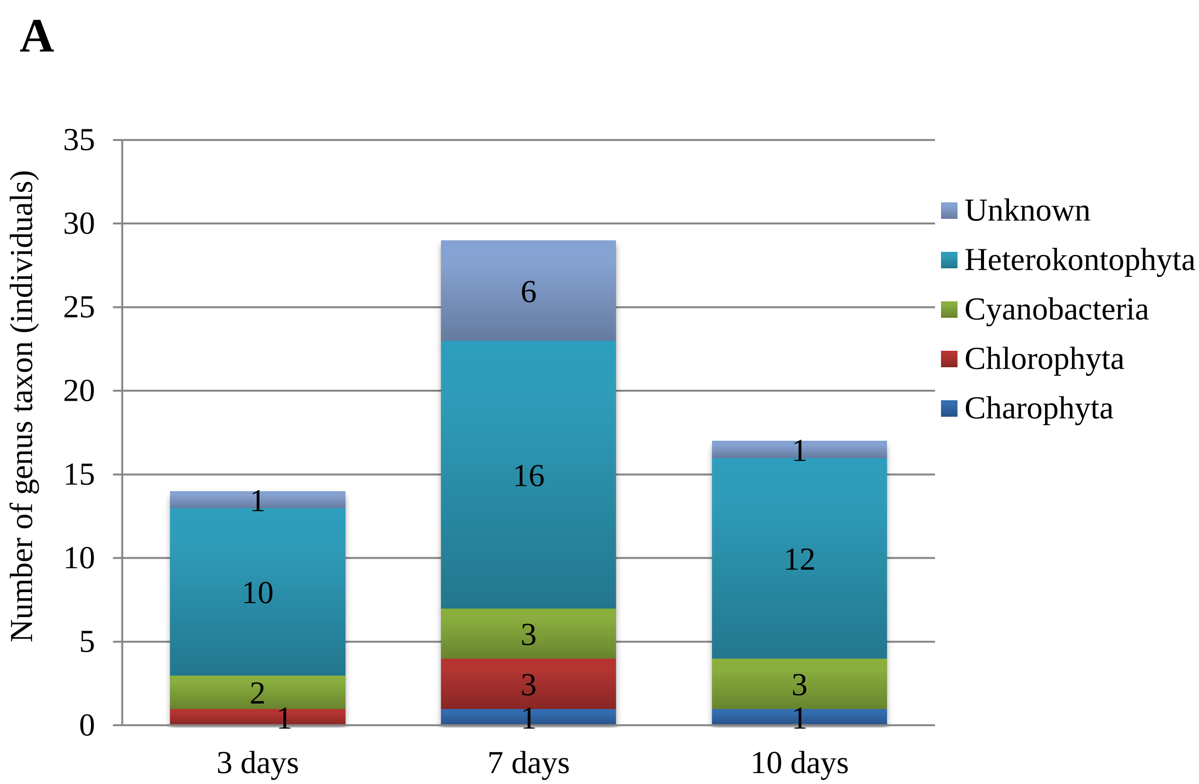
	3 days	7 days	10 days	Overall
	Number of samples analyzed			Total
RM	1	3	2	6
RG	2	2	2	6
RP	3	3	3	9

Appendix 11: Number of genera per phylum identified in the three study sites

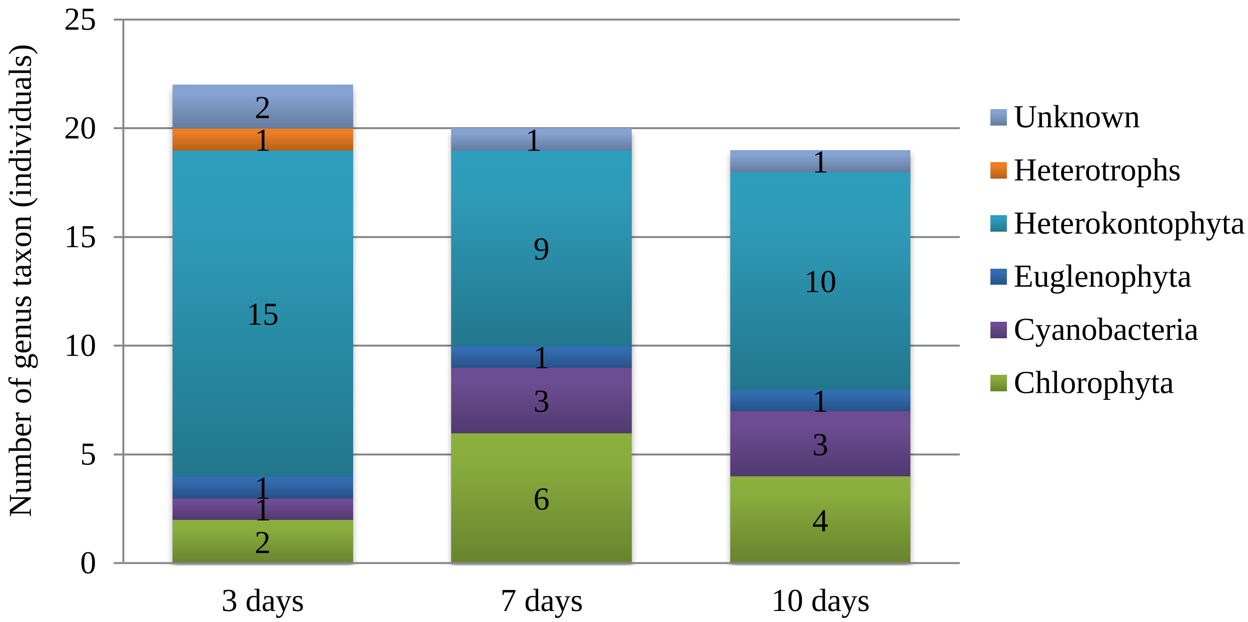
		RM	RG	RP
Heterokontophyta	*Achnanthes*		X	
	Amphora	X	X	X
	Caloneis	X	X	X
	Cocconeis	X	X	X
	Cyclotella	X		X
	Cymbella	X	X	X
	Denticula		X	
	Dinobryon		X	
	Diploneis			X
	Epithemia	X	X	X
	Fragilaria	X	X	X
	Gomphonema	X	X	X
	Gonyostomum		X	
	Gyrosigma	X	X	X
	Mastogloia	X		
	Melosira		X	X
	Navicula	X	X	X
	Nitzschia	X		
	Pinnularia	X		X
	Pleurosigma			X
	Rhaphoneis			X
	Stauroneis		X	
	Synedra	X	X	X
	Ulnaria			X
	Unknown (colonial)	4	1	2
	Unknown			6
Chlorophyta	*Ankistrodesmus*			X
	Chaetophora			X
	Cladophora		X	X
	Cosmarium	X	X	
	Eudorina		X	
	Pandorina		X	
	Protococcus		X	
	Rhizoclonium			X
	Scenedesmus		X	
	Selenastrum		X	
	Ulothrix	X		
	Unknown (colonial)	1		
	Unknown (filamentous)			1
Cyanobacteria	*Anabaena*	X	X	X

	Gonyostomum		X	
	Oscillatoria		X	
	Tolypothrix	X		X
	Unknown (filamentous)			
	Unknown (colonial)	X		
	Unknown	1		1
	Unknown	1	2	2
Euglenophyta	*Euglena sp.*		X	X
Charophyta	*Mougeotia*	X		X
Unknown	Unknown	6	2	10
Total richness		33	32	47

Appendix 12: Number of genera taxa classified by phylum or group for each accrual day at the three study sites a) RM, b) RG and c) RP

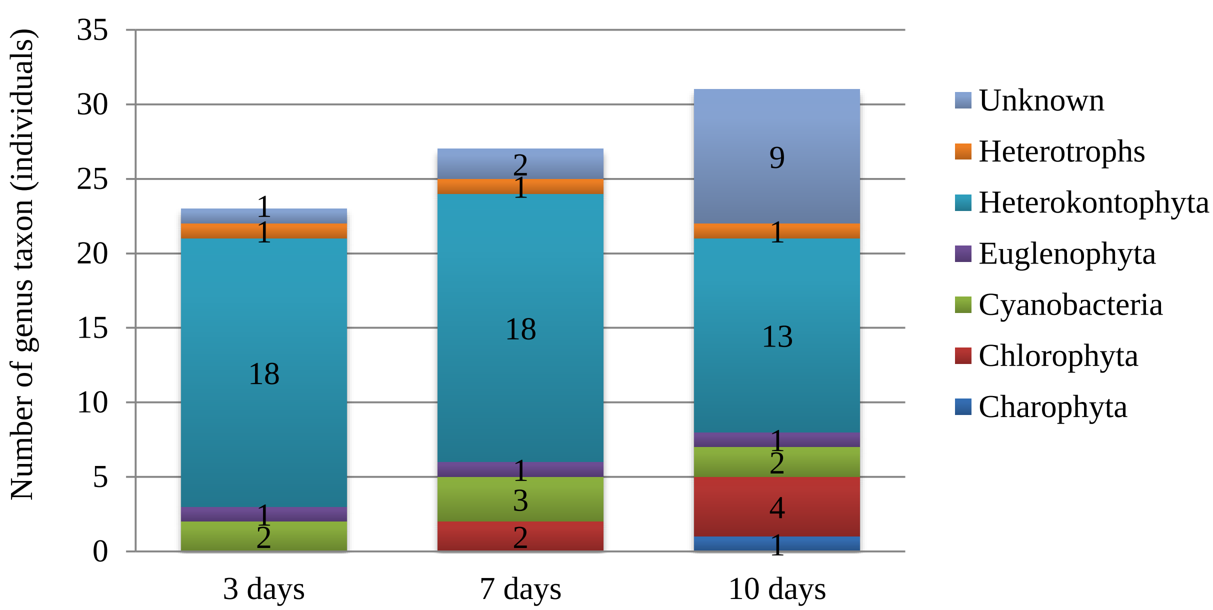


B



Number of genus taxon (individuals)
25
20
15
10
5
0
3 days
7 days
10 days
2
1
15
1
1
2
1
9
1
3
6
1
10
1
3
4
Unknown
Heterotrophs
Heterokontophyta
Euglenophyta
Cyanobacteria
Chlorophyta

C



Number of genus taxon (individuals)
35
30
25
20
15
10
5
0
3 days
7 days
10 days
1
1
18
1
2
2
1
18
1
3
2
9
1
13
1
2
4
1
Unknown
Heterotrophs
Heterokontophyta
Euglenophyta
Cyanobacteria
Chlorophyta
Charophyta

Appendix 13: analysis of variance for the periphyton growth rate

A) Two-way ANOVA for periphyton growth rate

Variable	N	R^2	R^2 Adj	CV
LOG10_R	54	0.29	0.21	433.81

V.S	S.S	d.f	M.S	F	p-valor
Model	2.93	5	0.59	3.86	0.0051
Accrual days (A)	2.50	2	1.25	8.22	0.0008
Season (B)	0.17	1	0.17	1.15	0.2895
A*B	0.26	2	0.13	0.85	0.4338
Error	7.3	48	0.15		
Total	10.24	53			

B) Tukey test for comparison between accrual days for periphyton growth rate.

α=0.05 DMS=0.31440; Error: 0.1521 d.f.: 48

Accrual Days	Mean	n	E.E.		
3	-0.39	18	0.09	A	
10	-4.50E-04	18	0.09		B
7	0.12	18	0.09		B

C) Tukey test comparison between seasons for periphyton growth rate

α=0.05 DMS=0.21342; Error: 0.1521 d.f.: 48

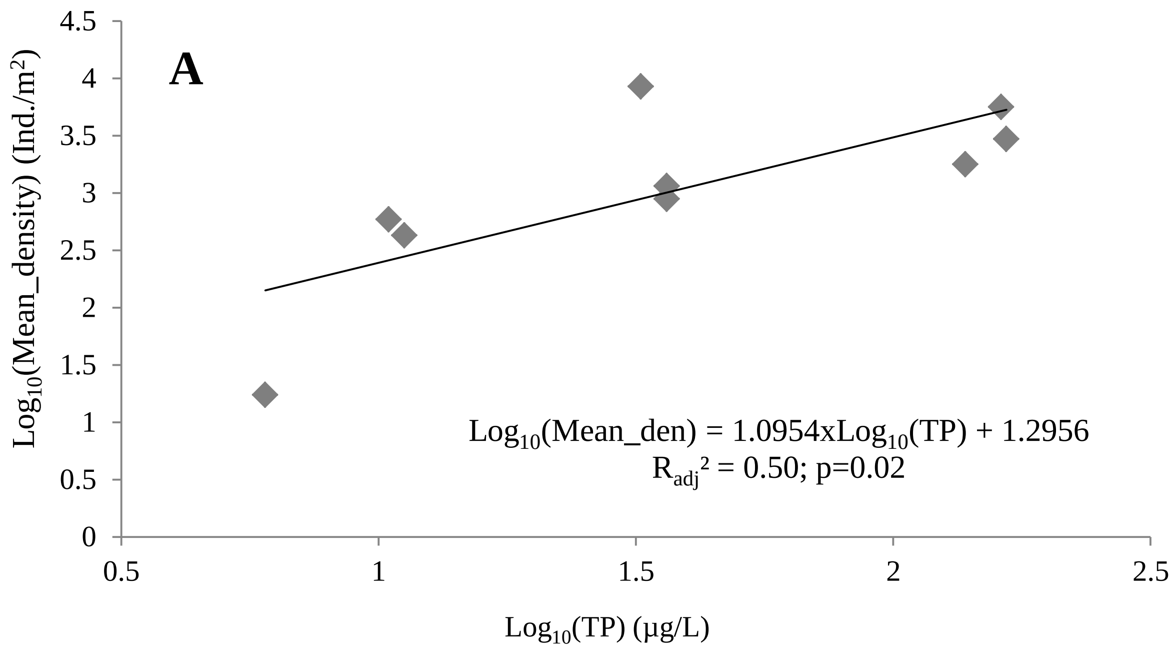
Season	Mean	n	S.E.	
dry	-0.15	27	0.08	A
wet	-0.03	27	0.08	A

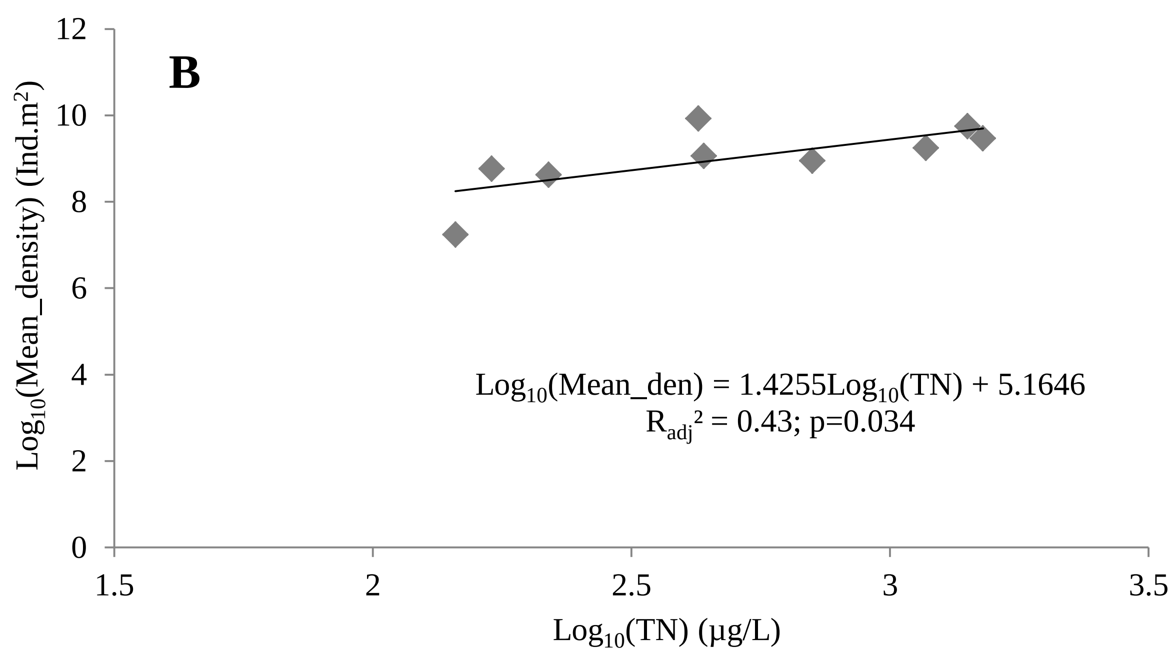
D) Tukey test for comparison of the interaction between accrual days and season for periphyton growth rate

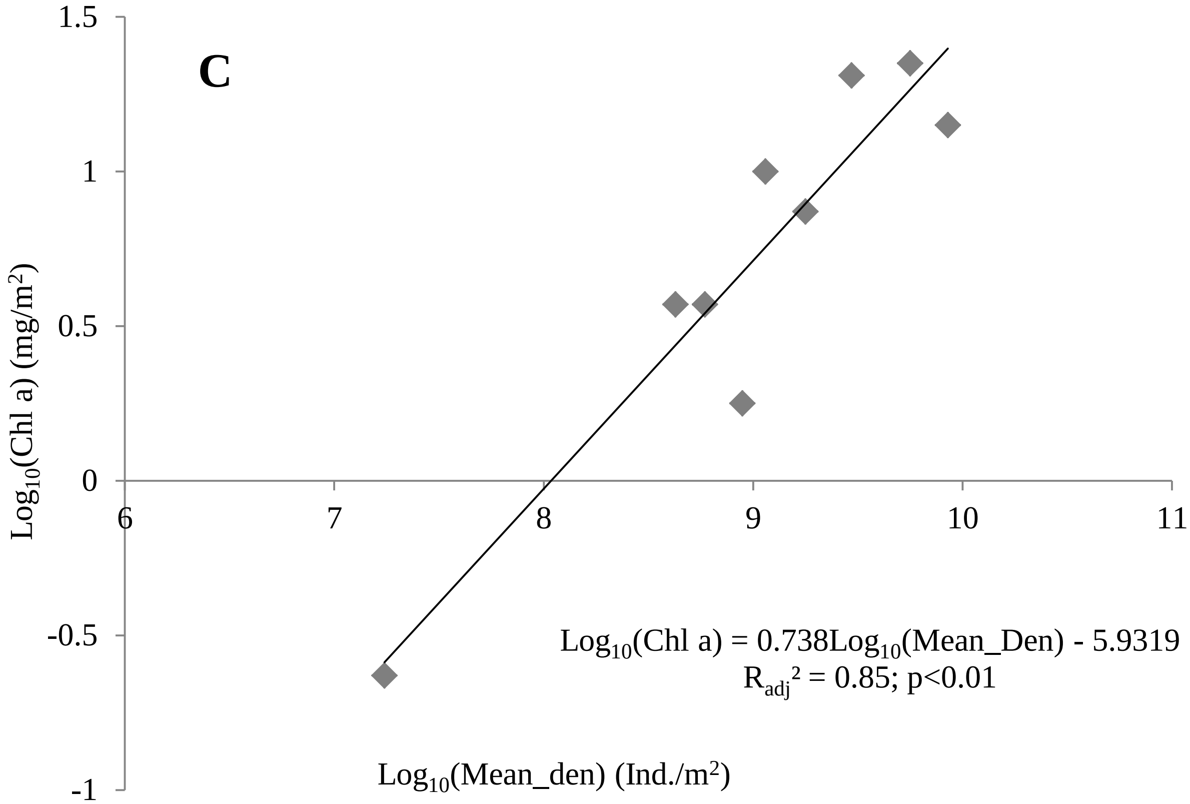
α=0.05 DMS=0.54564; Error: 0.1521 d.f.: 48

Accrual days	Season	Mean	n	S.E.		
3	dry	-0.53	9	0.13	A	
3	wet	-0.24	9	0.13	A	B
10	wet	-0.02	9	0.13	A	B
10	dry	0.02	9	0.13		B
7	dry	0.07	9	0.13		B
7	wet	0.16	9	0.13		B

Appendix 14: Simple linear regressions between mean community density and A) TP, B) TN and C) Chlorophyll a






C
1.5
1
0.5
0
-0.5
-1
6
7
8
9
10
11
Log10(Chl a) (mg/m2)
Log10(Chl a) = 0.738Log10(Mean_Den) - 5.9319
Radj2 = 0.85; p<0.01
Log10(Mean_den) (Ind./m2)