Nutrient Release and Grazing Effects by Two Exotic Thiaridae Snails on Periphyton Biomass under Artificial Conditions

By:

Héctor José Esparra-Escalera

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Approved by:

Carlos J. Santos-Flores, Ph.D. President, Graduate Committee	Date
David R. Sotomayor-Ramírez, Ph.D. Member, Graduate Committee	Date
Carlos M. Rodríguez-Minguela, Ph.D. Member, Graduate Committee	Date
Ana Vélez-Díaz, M.Sc. Interim Department Director	Date
John Fernández-Van Cleve, Ph.D. Representative, Graduate Studies	Date

ABSTRACT:

Thiara (*Tarebia*) *granifera* and *Melanoides tuberculata* are invasive parthenogenetic snails that impact food webs in tropical freshwater systems. Due to their high reproductive rate, both snails dominate the community of herbivores during conditions of higher nutrient availability and when the benthic biomass is high. These species should impact the aquatic trophic status by differential grazing and should affect the diversity and abundance of primary producers. Previous research shows knowledge gaps related to how these two snails may independently (or jointly could) influence the periphyton biomass and diversity, as well as niche-related environmental conditions. In this thesis, I examined the nutrient release of both snail species and how this is related to the periphyton biomass as judged by measurements of chlorophyll-*a*, total nitrogen and total phosphorus. Four treatments consisting of a control, *T. (T.) granifera*, *M. tuberculata,* and both species interacting, were set in artificial systems (mesocosms) and replicated four times. Each experiment unit (except control) consisted of 14 adult snails. Nutrient release assays, physico-chemical parameters measured *in-situ* (dissolved oxygen, temperature, pH and turbidity) and standing crop of the periphyton (via chlorophyll-*a* determinations and taxonomic assessment) were evaluated at five different times during 28 to 30 days after the introduction of the snails. Both species were measured (size, length of opercular aperture and spire) at the beginning and at the end of the sampling periods. Born juveniles were quantified at the end of the sampling periods. TN and NO_3-N released by snails varied by treatments and periods of sampling; however, in the interaction of both snails, the concentrations were similar. Among the parameters measured *in-situ*, temperature varied significantly by periods of sampling. DO and pH showed a lineal correlation in presence of *T. (T.) granifera* alone. Also, *T. (T.) granifera*, either alone or interacting with *M. tuberculata,* released more juveniles. *M. tuberculata* showed a significant difference ($p < 0.05$) in rate of

change of the shell length in competition with *T. (T.) granifera*. Both snails showed correlations in external morphological traits (opercular aperture, shell length and spire), especially when each species was kept apart. *T. (T.) granifera* seemed to reduce the periphyton standing crop (lower chl-*a*), probably due to grazing effects; the presence of this snail seemed was related to the dominance by cyanobacteria, such as *Chroococcus, Anabaena,* and species of Microcystaceae. During periods of sampling, the treatment with *M. tuberculata* showed the largest periphyton biomass and the highest proliferation of green algae (Chlorophyta) (as a possible food source), suggesting that it exerts a different grazing pressure on periphyton, when compared to *T. (T.) granifera*. Thus, both Thiarid species seemed to exert different effects on the nutrient concentrations and on the abundance and diversity of the algal species.

RESUMEN:

Thiara (Tarebia) granifera y *Melanoides tuberculata* son caracoles partenogenéticos e invasivos que impactan la red alimenticia en sistemas tropicales dulceacuícolas. Por su alta capacidad reproductiva, ambas especies dominan la comunidad de herbívoros durante condiciones de alta disponibilidad de nutrientes y la biomasa béntica es alta. Estas especies deberían impactar el estado trófico mediante un pastoreo diferencial y deberían afectar la abundancia y biodiversidad de productores primarios. Estudios previos muestran lagunas en conocimiento relacionados a como estos dos caracoles pueden, independientemente o conjuntamente, influenciar la biomasa perifitónica y diversidad, así como las condiciones ambientales relacionadas con el nicho. En esta tesis examiné los efectos de liberación de nutrientes de estos caracoles y cómo esto se relacionó a la biomasa perifitónica, juzgando por mediciones de clorofila-*a*, nitrógeno total y fósforo total. Se utilizaron cuatro sistemas artificiales (mesocosmos), consistiendo en un control, *T. (T.) granifera*, *M. tuberculata* y ambas especies coexistiendo, que fueron replicados cuatro veces. Cada unidad experimental (excepto el grupo control) consistió de 14 caracoles adultos. Ensayos de liberación de nutrientes, parámetros físico-químicos medidos *in-situ* (oxígeno disuelto, temperatura, pH y turbidez) y la productividad perifitónica (mediante clorofila-*a* y evaluación taxonómica) fueron evaluados en cinco diferentes tiempos durante 28 a 30 días después de introducido los caracoles. Ejemplares de ambas especies fueron medidos (tamaño de la concha, largo de la apertura del opérculo y éspira) al principio y al finalizar los períodos de muestreo. El nitrógeno total (TN) y el nitrato (NO_3-N) de los ensayos de liberación de nutrientes por los caracoles variaron entre tratamientos y períodos de muestreo; las concentraciones fueron similares en la interacción de ambos caracoles. De enre los parámetros medidos *in-situ*, solo la temperatura

varió significativamente entre períodos de muestreo. El oxígeno disuelto y pH mostraron una correlación lineal en presencia de *T.* (*T.*) *granifera*. Además, *T. (T.) granifera*, tanto solo o coexistiendo con *M. tuberculata*, liberó mayor cantidad de juveniles. *M. tuberculata* mostró diferencias significativas ($p < 0.05$) en la razón de cambio del largo de la concha en competencia con *T. (T.) granifera*. Ambos caracoles mostraron correlaciones significativas en rasgos morfológicos externos (apertura del opérculo, largo de concha y éspira), en especial cuando ambas especies estaban aparte. *T. (T.) granifera* pareció reducir la productividad perifitónica (clorofila-*a* baja), probablemente por efecto de pastoreo; la presencia de esta especie se relacionó con la dominancia de cianobacterias, tales como *Chroococcus, Anabaena*, así como especies de la familia Microcystaceae. Durante los períodos de muestreo, el tratamiento con *M. tuberculata* mostró los valores más altos de clorofila-*a* y la alta proliferación de algas verdes (Chlorophyta) (posible fuente de alimento), sugiriendo que la especie ejerce una presión de pastoreo diferente sobre el perifitón, en comparación con *T. (T.) granifera*. Por lo tanto, ambas especies de Thiaridae parecen ejercer efectos diferentes sobre las concentraciones de nutrientes y sobre la abundancia y diversidad de especies algales.

DEDICATION:

To my parents: Héctor R. Esparra-López and María del C. Escalera-Martínez

My sister: Sheila M. Esparra-Escalera

To my nephew (almost son): Dariel Vélez-Esparra

In memoriam of Francisca Pérez-Torres
and Víctor Torres

Also, I want to dedicate this work to my family, friends and all professionals who gave me their support during this pathway.

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"Living at risk is jumping from the cliff and building your own wings on the way down."

- *R. Bradbury*

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LIST OF ABREVIATIONS:

Treatment codes	Description
C	Control treatment (No snails in mesocosm)
TTG	Treatment of mesocosm, only with *Thiara (Tarebia) granifera*
MT	Treatment of mesocosm, only with *Melanoides tuberculata*
TTGMT	Treatment of mesocosm with both snail species
TTGMT-TTG	Interaction treatment, but referred to *T. (T.) granifera*
TTGMT-MT	Interaction treatment, but referred to *M. tuberculata*

LIST OF PERIODS OF SAMPLING IN THE MESOCOSMS:

Block code	Period of sampling and date
B1	1: July 5, 2016 (Day 0) 2: July 8, 2016 (Day 3) 3: July 13, 2016 (Day 8) 4: July 20, 2016 (Day 15) 5: August 4, 2016 (Day 30)
B2	1: August 9, 2016 (Day 0) 2: August 12, 2016 (Day 3) 3: August 18, 2016 (Day 9) 4: August 25, 2016 (Day 16) 5: September 6, 2016 (Day 28)
B3	1: September 13, 2016 (Day 0) 2: September 16, 2016 (Day 3) 3: September 22, 2016 (Day 9) 4: September 29, 2016 (Day 16) 5: October 11, 2016 (Day 28)
B4	1: October 6, 2016 (Day 0) 2: October 9, 2016 (Day 3) 3: October 13, 2016 (Day 7) 4: October 22, 2016 (Day 16) 5: November 3, 2016 (Day 28)
B4rep.	1: February 14, 2017 (Day 0) 2: February 18, 2017 (Day 4) 3: February 23, 2017 (Day 9) 4: March 2, 2017 (Day 16) 5: March 14, 2017 (Day 28)

INTRODUCTION AND JUSTIFICATION:

Aquatic ecosystems have been drastically altered and deteriorated by anthropogenic disturbances, such as public welfare, technology and industrialization (Kshirsagar, 2013). Apart of habitat degradation, other factors include the introduction of exogenous species and fluctuation of nutrients caused by consumption and excretion of the extant biota, which also influence the development of primary productivity (Vann, 2002; Moslemi *et al.,* 2012). Due to the biological complexity of aquatic ecosystems and the interaction among many physical and chemical factors, it is difficult to predict the composition and behavior of a community as a result of disturbance. Herbivores depend upon producers for survival, and their grazing activity is the first link of the trophic chain. Besides, producers depend on light availability and nutrients in the water, such as nitrogen (N) and phosphorus (P) concentrations and their ratios, among other factors, to grow. Basically, phytoplankton and periphyton are the communities that include algal and cyanobacteria species that serve as first links on food webs. Phytoplankton includes algal and cyanobacteria species that are suspended in water and are a main available resource for macro-invertebrates or other motile primary consumers (i.e. mosquito larvae and cladocerans, among other micro-crustaceans). On the other hand, periphyton includes algae, bacteria, detritus, micro-invertebrates and other microorganisms that are attached to a substrate, which primary consumers (i.e. snails, small crustaceans, tadpoles, fishes, etc.) can graze upon.

Nutrient concentrations in water are influenced by herbivores as result of grazing; snails are the group of grazers that plays a major role in freshwater systems (Urabe, 1993; Evans-White and Lamberti, 2005). Snails, as well as some macro-crustaceans and fish, are the main organisms that influence the standing crop and the replenishment of periphyton primary community (Brönmark, 1989). Freshwater snails and crustaceans are known to use their

nutritional and structural (grazing) mechanisms to stimulate the growth of mixed biodiversity, such as: cyanobacteria, green algae, diatoms, heterotrophic microbes and detritus (Evans-White and Lamberti, 2005). The impact of invasive snails' species on ecosystems associated with freshwater bodies in tropics is still poorly understood.

The present research was focused on two exotic Thiaridae species: *Thiara (Tarebia) granifera* and *Melanoides tuberculata*; which share similar morphological characteristics. Previous investigations show knowledge gaps in terms of snail grazing behavior, as well as in their impacts on the nutrients and periphyton composition, specifically of primary producers. The study of the interactions of *T. (T.) granifera* and *M. tuberculata* in artificial systems could provide useful information leading to eventual conservation and management for streams and the control of exotic invasive species. Differences appear to exist in nutritional and structural mechanisms between *T. (T.) granifera* and *M. tuberculata,* that influence the N and P cycling (by consumption and nutrient release) and grazing pressure (algal community composition), which these snails may modify the aquatic food webs. In natural streams, it is very difficult to evaluate the impact and subsequent effects of both Thiarid snails due to other target consumers and producers present under field conditions. *T. (T.) granifera* and *M. tuberculata* were kept separate and together in artificial systems (mesocosms). We assumed that N and P nutrients would stimulate a specific community of periphyton, in terms of algal biodiversity, abundance and richness; as well as change in shells dimensions when they are alone or interacting.

CHAPTER 1:
Literature Review

1.1: Background General Overview

Grazers, including snails, are secondary consumers, sensitive to variations in resources of primary production. As a consequence, they need to constantly change their foraging methods (Stelzer and Lamberti, 2002). In lotic or lentic freshwater systems, native or endemic snail species have their own ecological niches that may disappear if exotic species are introduced. The family Thiaridae is composed of certain groups of freshwater snails, some of which are considered exotic, whose shells have a "projectile shape" (Bouchet *et al.*, 2005). Two Thiarid snails' species introduced during the last century in Puerto Rico are *Thiara (Tarebia) granifera* and *Melanoides tuberculata* (Figure 1). Also, these are considered invasive and are suspected to influence nutrient concentrations in some freshwater bodies of Puerto Rico (Vélez, 2014).

Both snail species tend to be similar in morphology. As aquatic gastropods, they have a shell composed of a periostracum and an external layer of $CaCO_3$ and have a gill (ctenidium, with a leaf-like triangular form) in the mantle cavity "lined to blood vessels, muscles and nerves" (Pyron and Brown, 2015). Their gills are composed of filaments that are in charge of diffusion. Foraging is controlled by their higher grazing rates (scrapping the particles available on benthos with their radula) that will increase if the food quality is poor. Based on their osmotic regulation and nutrient release, the accumulation of toxic compounds exerts the proliferation of some species of periphyton (algae and bacteria) and a possible damage to the digestive system of surrounding local macro-fauna (Stelzer and Lamberti, 2002; Pyron and Brown, 2015). The combination of nutrients and grazing mechanisms influence the abundance and biodiversity of benthic algal species that are used as biological indicators of water quality.

A biological indicator is defined as an organism or population sensitive to the effects

Figure 1.1: Ventral view of the shells of *Melanoides tuberculata* (A) and *Thiara (Tarebia) granifera* (B)

of different biotic or abiotic conditions in an environment (Kovacs, 1992). Periphyton is one of the most important biological indicators associated with substrates in aquatic systems and is widely used in studies related to monitoring water quality (Leland and Carter 1985; Newman *et al*., 1985; Cosgrove *et al.*, 2004; Wan-Maznah, 2010). It is composed of a mixture of algae, cyanobacteria, heterotrophic microbes and detritus, attached to different types of substrates (Ekhator, 2010), which exhibits a level of biodiversity (Wan-Maznah, 2010). This community encompasses 90% of total primary production in aquatic environments (Wetzel, 1990; França-Vasconcelos *et al.*, 2013), being diatoms (Bacillariophyta), green algae (Chlorophyta and Charophyta) and cyanobacteria the most important bio-indicators associated to water quality. Also, these communities respond to nutrient concentrations and input levels; thus serving as biological indicators of pollution in water bodies (Kshirsagar, 2013). However, nutrient levels influence periphyton at different parameters: (1) the cell density, (2) ash free dry mass (AFDM), (3) chlorophyll-*a*, and (4) enzymatic activity (Wan-Maznah, 2010); thus, impacting entire aquatic food webs and their

metabolic rates (Downing *et al.,* 1999; Flecker *et al.*, 2002; Elser *et al.*, 2007; Moslemi *et al.*, 2012). Among some of the advantages in the use of periphyton over planktonic organisms (i.e. phytoplankton) in freshwater monitoring are having fixed habitats and doing a quick re-colonization after perturbations, allowing a fast renewal in monitoring (Wan-Maznah, 2010). For grazers, such as snails, crustaceans and tadpoles, periphyton is the most important source of food. Also, analyses of this community focus on the identification of roles in food webs, biomass monitoring and evaluation of water conditions among seasons to open venues for a conservation plan, particularly if the aquatic systems have been severely impacted. Generally, this community passes through three important steps for colonization on benthos: (1) species adaptation, (2) growth of species abundance (if the conditions are favorable), and (3) primary production balance by grazing, nutrient availability, among other biotic and abiotic factors (Rastogi, 2004; Gotelli, 2008; Viggiano-Beltrocco, 2014).

1.2: Thiaridae snails as exotic species in the tropics

Thiara (*Tarebia*) *granifera*, widely known as Quilted Melania, is an invasive snail; originally from south-east Asia (Appleton *et. al.*, 2009), that plays an important role in nutrient fluctuations in tropical and subtropical freshwater systems. If is provided with enough resources, this snail has an ecological advantage over other snail species due to its high reproduction rates and the capacity to give birth fully developed juveniles through parthenogenesis (Miranda *et al*., 2011). Being a grazer, it usually feeds on primary producers and detritus, although it does not feed on terrestrial or aquatic vegetation (Sodeman Jr., n.d). This species is restricted to freshwater but can be found downstream where salinity levels are below 25 ppt (Bolaji *et al*., 2011; Van Oosterhout *et al*., 2013). It has been found in altitudes of 983m in streams and rivers at temperature above 24ºC (Appleton *et al.*, 2009; Miranda *et. al.*, 2011). However, under laboratory conditions it is known to resist temperatures over

16ºC (Tagliarolo *et al.*, 2017). In South Africa, *T. (T.) granifera* is the invertebrate with the greatest impact on benthic communities of natural and artificial freshwater systems, reaching population densities larger than 20,000 individuals/m^2 (Appleton *et al.* 2009).

Van Oosterhout *et al*. (2013) reported that most Caribbean islands, including Puerto Rico, have been invaded by *T. (T.) granifera* and another similar species: *Melanoides tuberculata* (Red-rimmed Melania), which is an Afro-Asian snail (Pilsbry and Bequaert, 1927; França-Vasconcelos *et al.*, 2013). It is presumed that the invasion by both species in the Caribbean was a consequence of aquarium trade (Pointier and McCullough, 1989; Moslemi *et al.*, 2012). However, other hypotheses include the introduction and dispersal by migratory birds, eating and voiding the juveniles in freshwater ponds and rivers (Appleton *et al.,* 2009), as well as spreading by attachment from feet and feathers (Contreras-Arquieta *et al.*, 1995). Due to their short generation time, both species have a rapid expansion rate (Van Oosterhout *et al*., 2013).

Both species exploit the same resources and establish an interspecific competition, which goes in favor of *T.* (*T.*) *granifera* (Naranjo-García *et al.,* 2005; Rangel *et al.*, 2011); *M. tuberculata* is vulnerable to the presence of this snail species (Appleton *et al.*, 2009). Understanding the behavior of different ecological factors (i.e. temperature, conductivity, salinity, pH, dissolved oxygen, among others) is very important, because freshwater snails depend on individual's variation in movement behavior, interactions and spatial distribution upon trophic chain (Snider and Gilliam, 2008). According to Pointier *et al.* (2005), *M. tuberculata* can thrive under different environmental conditions; and no specific association to biotic or abiotic limiting factors has been identified for this species. On the other hand, Silva *et al.* (2010) used *M. tuberculata* to compare different mathematical models based on allometric measurements, concluding that this species has a rapid growth in eutrophic

environments, exerting a competitive pressure to other mollusks species. However, no association of allometric measurements has been described in presence of a related species, such as *T. (T.) granifera* that tends to dominate. Therefore, it is questionable that rapid growth in terms of morphology, and independent of eutrophic environments, will be another mechanism to avoid a complete displacement in an ecological niche. Besides, both species does not exhibit a clear sexual dimorphism; Pino *et al.* (2010) took measurements of 415 specimens of *M. tuberculata*, which between 1.3-3.8% were males, but non-statistical differences were observed between different morphological traits considered, such as: shell length and opercular aperture (length, width and diameter). Also, Chaniotis *et al.* (1980) pointed out that male populations of *T. (T.) granifera* in Puerto Rico does not exceed, in average, of 4.6%.

1.3: Health relevance of Thiarid snails

T. (*T.*) *granifera* was used to control the schistosomiasis in Puerto Rico by 1954, a disease caused by the digenean parasite *Schistosoma mansoni* (Pointier and McCullough, 1989; Moslemi *et al.,* 2012), and consecutively displaced the main intermediate host in freshwater bodies: *Biomphalaria glabrata* (Harry and Aldrich, 1958; Pointier and McCullough, 1989; Vélez, 2014). Both snail species have been found in other Caribbean areas and Florida (Pointier and McCullough, 1989), such as Dominican Republic and Grenada (Ferguson, 1977), Haiti (Robart *et al.*, 1979; Pointier and McCullough, 1989), Venezuela (Chrosciechowski, 1973; Pointier and McCullough, 1989), Dominica (Pointier and McCullough, 1989), and Cuba (Vázquez and Perera, 2010). In Saint Lucia (West Indies), *M. tuberculata* was introduced in 1978 and by 1986 it had served as a biological control of *B. glabrata* (Pointier, 1993). In a few places of Central and South America, both Thiarid snails

are intermediate hosts in the life cycles of digeneans, such as *Clonorchis sinensis* and *Centrocestus formosanus* (Rangel *et al*., 2011), being the last one observed in Puerto Rico but not documented (Sean Locke, *personal communication*; Department of Biology, University of Puerto Rico-Mayagüez). *Paragonimus westernmani* is another parasite that has been related specifically with *T.* (*T.*) *granifera* (Chaniotis *et al.*, 1980), but according to Michelson (1992) "this assertion was wrong." In contrast, *T. (T.) granifera* serves as an intermediate host of the philoptalmid eyefluke (*Philopthalmus gralli*). In Africa, by 2005, this parasite was reported in ostrich farms in Zimbabwe (Mukaratirwa *et al.*, 2005; Appleton *et al.*, 2009). However, Krailas *et al.* (2014) documented the cercariae of 18 species of trematodes obtained from 6,019 of 32,026 specimens of *M. tuberculata* in natural freshwater systems of Thailand; being one of the important vectors associated with human infections. Except for *C. formosanus* and *P. westernmani*, the other mentioned parasites species that use *T. (T.) granifera* or *M. tuberculata* as intermediate hosts are not found in Puerto Rico; nevertheless, the risk of getting any of these diseases is present due to commercial trade of fish or aquatic macro-invertebrates, especially crustaceans.

1.4: Primary productivity, nutrient cycle and water quality

Photosynthetic components of benthos are easy to sample and prepare for analysis and have been widely correlated to specific chemical parameters, such as carbon, oxygen, nitrogen, phosphorus and sulfur (Biggs 1985; Wan-Maznah, 2010). Algae and cyanobacteria are both photoautotrophic (Canter-Lund and Lund, 1995). Whereas, nutrient parameters (N, P) play important roles to supply the needs of producers (Lawes and Gilbert, 1880; Droop, 1974; Hecky and Kilham, 1998; Hutchinson, 1973; Rodríguez and Matlock, 2008). Thus, the characterization of primary productivity is very important in environmental assessment

to indicate any type of change in conditions and impairments, as well as health (Stevenson and Smol, 2015). Zurawell *et al.* (1999) performed several analyses in seven eutrophic lakes of Alberta, Canada, correlating microcystin-LR (MCLR) in tissues of different snail species with phytoplankton biodiversity. MCLR concentration in snail tissues causes damage to human and local-macrofauna organs (i.e. liver and intestines). This toxin is related to trophic status, usually indicating an increased in abundance of taxa such as *Microcystis* (Botes *et al.* 1985; Zurawell *et al.,* 1999), as well as *Anabaena* (Krishnamurthy, 1986; Zurawell *et al.* 1999), *Oscillatoria* (Meriluoto, 1989; Zurawell *et al.* 1999), *Nostoc* (Namikoshi, 1990; Zurawell *et al.*, 1999), and *Chroococcus* (Pearson *et al.*, 2010). Cyanobacteria are usually favored by higher temperatures, low CO_2 and low pH (Jacoby *et al.*, 2000; Chappell, 2012).

The biological diversity of freshwater systems is considered a potential indicator of their quality. An increase in nutrient levels could translate into a decrease in biodiversity, and vice versa, altering the ecosystem balance (Rodríguez-Vargas, 2014). The availability of light and nutrients (N and P concentration, for chlorophyll-*a* production) will regulate the algal biodiversity and abundance. For example, under high N and P concentrations and high light conditions (in open canopies), the algal reproductive activity will be greater, and the chlorophyll-*a* content would increase, which the resulting change in dissolved oxygen (DO) in the water increase and is related with algal reproduction (Kohler, 2010).

1.5: Related studies and research approach

Grazing by freshwater snails is the main factor responsible for the standing crop biomass and primary production in aquatic systems (Brönmark, 1989), which in turn have an impact on the development of benthic communities (Appleton *et al.*, 2009) and in the establishment of a complex ecosystem. Grazers, especially snails, may increase nutrient levels

available for algal growth and metabolic rates when the physical structure of those producers are being altered, as was evaluated by Evans-White and Lamberti (2005) with *Elimia livescens* (a gastropod) and *Orconectes propinquus* (a decapod) in artificial systems. Moslemi *et al.* (2012) evaluated the nutrient cycle (C:N ratios) in tropical streams of Trinidad (West Indies), as consequence of riparian deforestation in the zone, using *T.* (*T.*) *granifera* (due to its abundance) as an indicator of nutrient excretion. The differences in light availability (according to the riparian cover) suggested a heterogeneous distribution of this snail species along the streams. In open canopy sites, *T.* (*T.*) *granifera* density was higher and associated with the mass specific excretion parameters (C:N ratios) and organic matter. Therefore, if deforestation continues, it may facilitate the establishment of other grazing invasive species in these streams, bringing in further habitat degradation. With increased vegetation, the availability of light in benthos was lower (on closed canopy covers), resulting in lower abundance of primary producers, as well as lower abundances of grazers and other types of consumers.

In North America, *Dreissena polymorpha* (Bivalvia: Dreissenidae), well known as zebra mussel, is one of the most studied invasive species and it is used as an ecological indicator of toxicity and water quality (Vilá and García-Berthou, 2010). The presence of this species has been correlated with cyanobacteria over-growths (Raikow *et al.* 2004) attributed to the mussel's nutrient cycling and feeding modes (Bastiviken *et al.* 1998; De Stasio *et al.* 2008; Chappell, 2012). In Puerto Rico, Chappell (2012) studied *Corbicula fluminea* (Bivalvia; Corbiculidae; common name: Asian clam) in the trophic chain in La Plata and Guajataca reservoirs by conducting on-site phytoplankton surveys and independent mesocosm assays supplemented with different substrate types. As a pedal feeder, this clam scrapes organic matter and inorganic particles in benthos and suspends them throughout the

water column (Carregosa *et al.*, 2014; Bertrand *et al.*, 2017). Consecutively, its feeding mechanisms have effects on primary production and the food webs, as well as an impact in dissolved oxygen (DO), bio-mineralization and habitat quality (Kartayev *et al.*, 2007; Bertrand *et al.*, 2017). Estrella-Riollano (2012) identified and determined the mean population density of macro-invertebrates associated with benthos in both reservoirs. Between 2010 and 2011, 95.1% of the abundance of all macro-invertebrates in those reservoirs was represented by Thiarid snails, which included *T. (T.) granifera* and *M. tuberculata*; with dominance by the former species. The mean density of *T. (T.) granifera* in Guajataca reservoir was higher (43,021 ind./m^2; and relative abundance of 77.4%) than in La Plata reservoir (11,590 ind./m^2; and 50.4% of relative abundance). Moreover, Raw *et al.* (2013) demonstrated that snails in the same niche with *T. (T.) granifera* (*M. tuberculata, Assimenia* cf. *capensis* and *Coriandria durbanensis*) in Kwa-Zulu-Natal, South Africa, showed a negative taxis response to chemical signals involved in displacement interactions among these native gastropods.

Water quality depends on different biological, chemical, and physical conditions. Most reservoirs, watersheds and streams in Puerto Rico violate the standards for fecal coliforms, copper (Cu^+), surfactants, turbidity and excess of dissolved oxygen (DO) (Viggiano-Beltrocco, 2014). However, this could be due to the use of standards that are inadequate for identification of water quality. Nitrogen and phosphorus levels, in optimal conditions, need to be lower than 10mg/L and 1mg/L, respectively (PREQB, 2010b). On the other hand, if nutrients (especially from nitrification) are higher they will promote the excess of algae that will incur in more DO during day and low DO at night, toxicity and death of a lot of freshwater animals due to depletion (Viggiano-Beltrocco, 2014).

Competition by freshwater species that are considered invasive could affect a

periphyton biomass that changes constantly, depending on resources available. An intraspecific competition (if food availability is limited) or interspecific competition (if food resources are abundant) will be established if these species are in a same niche. Snails from clade Caenogastropoda (which includes the family Thiaridae) have gills and are limited to stream habitats, where they usually are abundant in riffles for improved oxygen circulation (Pyron and Brown, 2015). In natural systems, *T. (T.) granifera* and *M. tuberculata* often have interspecific interactions, which frequently lead to the displacement of *M. tuberculata* and other native gastropods by *T. (T.) granifera* (MacNeil *et al.* 2013; Miranda *et al*., 2016).

Periphyton has the potential to change the nutrient concentrations of freshwater systems, as well as cause dissolved oxygen fluctuation, following the light availability regimes (Wetzel, 2001; Viggiano-Beltrocco, 2014). Taxonomic composition of periphyton seems to be associated to important relationships with nutrient parameters. Cyanobacteria and colonial green algae often have more nutritional content than other photosynthetic microorganisms (Kohler, 2010). Nevertheless, this composition depends on the establishment of food webs and gastropods play one of the most important roles over algal communities on benthos. Viggiano-Beltrocco (2014) suggested that the presence of *T. (T.) granifera* in the Guanajibo and Río Piedras (both in Puerto Rico) might have limited the periphyton growth and affected nutrient release, but her investigation did not consider the population densities of the species. França-Vasconcelos *et al*. (2013), in freshwater mesocosms, studied biofilm colonization by periphyton, using *M. tuberculata* as the grazer and having a control treatment without snails. The composition of periphytic algae was divided into seven major taxa, being *Chandransia macrospora* (Rhodophyta, Florideophyceae) the main species in these mesocosms. They also found that the algal species richness was higher in the presence

of *M. tuberculata* and the composition changed between the periods of sampling. However, they did not correlate the algal composition with the nutrients released by snails, nor evaluated nutrients in the water or chlorophyll-*a* concentration and did not compare the effects generated by another related species (i.e. *T. (T.) granifera*).

There have been a few studies on the ecology of Thiarid snails in Puerto Rico, however, these have mainly focused on parasitology and medical relevance. Furthermore, those investigations do not show (or assume briefly) the ecological implications, in terms of (a) the nutrient importation that both species exert, (b) the consequences over other local macro-fauna, including the food webs, or (c) the geographical distribution over the island.

The main objective of this thesis was to evaluate the ecology of two invasive snail species used as bio-indicators of nutrient release in artificial systems and how these influences the development of periphyton biomass (mainly focused on primary production) in terms of abundance, species richness and biodiversity. Also, it was expected that differences in nutrient release and grazing between *T. (T.) granifera* and *M. tuberculata* would translate into differences in nutrient availability (N and P) and algal composition over time. As well, the rate of change in size for both snail species was evaluated; either when each species was alone or interacting with each other. It was expected that the rate of change in size for both snail species would differ significantly based on their interaction.

CHAPTER 2:
Materials and Methods

2.1: Raising of freshwater snails

The capture and *a-priori* raising of freshwater snails was necessary for the experiment. Specimens of *Thiara (Tarebia) granifera* and *Melanoides tuberculata* were collected at Quebrada de Oro in Mayagüez, Puerto Rico (Lat. 18.211082 N, Lon. -67.1384881 W). The raising of freshwater snails took place in the Aquatic Biology Laboratory, Department of Biology, at the University of Puerto Rico-Mayagüez. Both species were kept in separate containers to "accustom" them to closed freshwater systems. The containers were provided with water filtered from the creek. *Elodea* sp. was provided as sources of detritus, and aquarium pumps circulated water and provided oxygenation. Also, the containers had light sources above (12 hours x 15 Watts) with controlled photo-period, as well as stream rocks with epilithon for food availability.

2.2. Artificial systems (mesocosms) and experimental design

The treatment selection and experimental design were principally based on the studies conducted by Evans-White and Lamberti (2005), Moslemi *et al.* (2012), Snider and Gilliam (2008) and Chappell (2012), as well as the findings of Viggiano-Beltrocco (2014). The experiment was carried out at the Animal House of the Department of Biology, University of Puerto Rico-Mayagüez (see picture in Appendix 6.9a). Sixteen artificial containers (21cm length x 10cm width x 11cm height; 10.41L capacity) were set in randomized block design, with four treatments and four repetitions per treatment; each repetition was carried out sequentially and not in parallel. The treatments were: *Thiara (Tarebia) granifera* only (TTG), *Melanoides tuberculata* only (MT), the combinations of both snail species (TTGMT), and the control with no snail (C). Treatments with only one species (TTG or MT) had an initial

population of 14 specimens; the treatment with the two species (TTGMT) had an initial population of 7 individuals of each one; if one specimen died it was replaced by another of similar size. Each container had 10L of water. The water was prepared by mixing ¾ parts with twice-filtered water from Quebrada de Oro stream (using the Whatman GF/F filter papers [4.25cm]) and the rest with tap-water. The evaporated water on each container was replaced by adding water frequently (every 3 or 4 days) using the same mix ratio.

Before applying the different treatments, each container needed to have enough nutrients and producers on the provided surfaces (ceramic tiles, 2cm x 2cm), so that during the first three weeks of the experiment no snails were added. After this period, the snails were randomly assigned to each mesocosm according to the treatment level. Filters were removed and cleaned after 3 or 4 days (twice per week) to avoid the establishment of a competing microbial community and the bloom of nutrients into the water. Nutrient release (section 2.3) and physico-chemical parameters (section 2.4) were determined at five times during a 28 to 30 days period, after the snails were put in the containers (**B1**: Days 0, 3, 8, 15 and 30; **B2**: Days 0, 3, 9, 16 and 28; **B3**: Days 0, 3, 8, 16, 28; **B4**: Days 0, 3, 7, 16, 28; **B4rep**: Days 0, 3, 9, 16, 28). Mean densities of juveniles (section 2.8) were determined during the last period of sampling (Day 28 or 30). The light above each mesocosm (17 Watts) was covered with red filters and each container was covered with white nets to avoid the access by small animals, as well as prevent excessively high-water temperatures.

2.3. Nutrient release assays of snails and water sample from each mesocosm

As procedure modified from Moslemi *et al.* (2012), during the 2nd (Day 3 or 4) to 5th sampling periods (Day 28 or 30), 5 adult snails per species were removed from each mesocosm

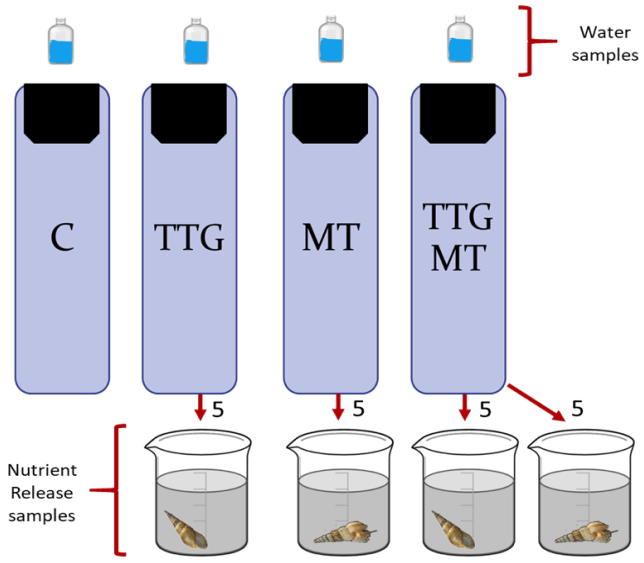

Figure 2.1: Experimental procedure of nutrient release of snails and water samples

(TTG, MT and TTGMT) and separated in beakers per species, with 150 to 175ml of filtered water from each container (see Appendix 6.9c); 50ml were used for total Kjeldahl nitrogen (TKN), nitrate (NO_3-N) and total phosphorus (TP) analyses. After one hour, the snails were removed from the beakers and placed back in their respective mesocosm, the water contained in these beakers were put in amber bottles; sixteen samples were obtained per block. Twenty samples of water (C, TTG, MT and TTGMT) per block were obtained from the five periods of sampling; thirty-six samples per block were sent to the Laboratory of Soil and Water Quality of the UPR-Agricultural Experimental Station. Also, twenty samples of water from each mesocosm (from 1st to 5th sampling period) were obtained (see Figure 2.1). The analyses of N and P compounds from (1) the nutrient release by snails and (2) water samples of each mesocosm were based on the following protocols of water quality. Total Kjeldahl nitrogen concentration (TKN) was determined using the EPA method 351.2. Phosphorus concentration analyses (TP) were performed using EPA method 365.2. Also, NO_3-N was quantified using EPA method 353.1. All samples were preserved with 2 drops of H_2SO_4 (pH < 2). To obtain the total nitrogen (TN) for each sample was obtained by the sum of TKN plus NO_3-N. ANOVAs, using Info Stat ®, were made using Bonferroni tests ($\alpha = 0.05$) to see which chemical

components showed significant differences per treatment, periods of sampling, and blocks, for either nutrient release essays and water samples from mesocosms. Also, interactions (treatments x periods of sampling, and treatments x periods of sampling and block) were included in the ANOVAs. By the nutrient release samples, we established four treatments: TTG, MT, TTGMT-TTG and TTGMT-MT; the last two represents *T. (T.) granifera* and *M. tuberculata*, respectively, in the interaction treatment.

2.4. Parameters measured in-situ

Parameters such as pH, dissolved oxygen (DO), temperature of the water and turbidity were measured *in-situ* in all the containers during the periods of sampling. DO, pH, and temperature were determined using an Oakton pH/DO 300 Series meter. Turbidity was assessed using a LaMotte 2020we meter. The water flow was approximately 2.0-2.2cm/s for each mesocosm and the light intensity of the lamps was 17 Watts. Four Pearson's correlations (one per treatment) in Info Stat ® were performed for the physico-chemical parameters measured, as well as an additional overall correlation, independent of treatments. As well, ANOVA's were performed in InfoStat ® (using Bonferroni tests; $\alpha = 0.05$) to test for the significant differences in mean of the previous parameters measured *in-situ* by treatment, periods of sampling and blocks, as well as interactions (same as nutrient release assays and water samples). For some statistical analysis (i.e. Pearson's correlation), pH was converted to H_3O+ concentrations (M).

2.5. Periphyton sampling

Periphyton sampling was done using a tooth brush to scrap 4 randomly selected tiles from each container and washing the tiles with distilled water up to a 200ml volume (Figure 2.2; Appendix 6.9b). However, the positions of the tiles in each container were considered

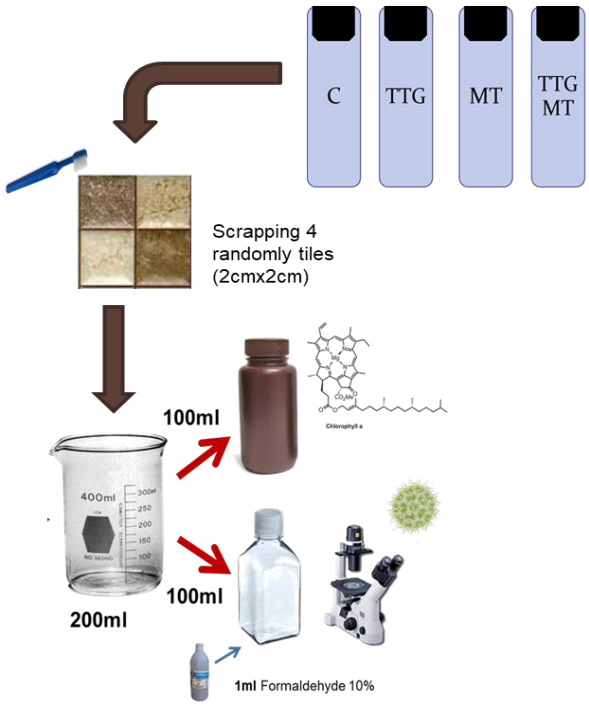

Figure 2.2: Diagram, depicting the procedure used for collecting the samples for chlorophyll-*a* analysis and periphyton taxonomic assessment.

for all the treatments; two tiles were selected close to the water pump, the other two tiles were selected from the other side of the container (more distant from the water pump). Each sample was divided into two sub-samples of 100ml. These were subsequently processed for chlorophyll-*a* and taxa analyses, respectively. The subsamples of taxa analysis were placed in clear amber bottles and preserved with 1ml of 10% formaldehyde; whereas those placed for chl-*a* were transferred to brown amber bottles (analyses were carried out in laboratory the same date of sampling or no later than next day).

2.6. Chlorophyll-a extraction and calculation

Chlorophyll-*a* analyses were carried out, following the protocol of Arar and Collins (1997) in terms of extraction, with modifications based on WOW (2003) for chlorophyll-*a* determination (light spectrum used in spectrophotometer, acidification of samples and

calculated concentrations). To conduct the procedure, the sub-samples obtained from scrapping the tiles for each mesocosm were obtained for totally or partially filtration through Whatman GF/F filter papers (4.25cm), to trap the organic matter. The filter papers were removed from the filter units, folded and cut into pieces that were placed in centrifuge tubes (15ml). The tubes were filled with 10ml of cooled 90% acetone and centrifuged at 5,000 rpm for 10 minutes, using an Eppendorf Centrifuge 5810 R. However, if the pellet had an intense green color, the acetone with chl-*a* biomass contained was transferred into another tube. Thus, samples with the intense green color in the pellet were rinsed with another 5ml of 90% acetone and centrifuged at 3,000 rpm for other 5 minutes and transferred to the tube with the 10ml of acetone with the chl-*a* biomass previously centrifuged. Finally, the supernatants of different sub-samples were cooled at least for one hour and then warmed up (±20ºC) for the reading in the UV-5200 UV/VIS Spectrophotometer to determinate the absorbance of chlorophyll-*a* before acidification. A blank (90% acetone) was used to read the absorbance at 664 nm and 750nm (both parameters at red light spectrum). Subsequently the samples were acidified with 2 drops of HCl (0.1N) were added and then, after two minutes, the absorbance after acidification was determined at 665 nm (wavelength for phaeophytin-*a*) and 750nm, respectively, using again the 90% acetone as a blank. The chl-*a* concentrations (mg/cm^3) were assessed with the following equation:

$$\frac{\mathbf{26.7\ [(}\boldsymbol{Ab}_{\mathbf{664nm}} \mathbf{-}\ \boldsymbol{Ab}_{\mathbf{750nm}}\mathbf{) - (}\boldsymbol{Aa}_{\mathbf{665nm}} \mathbf{-}\ \boldsymbol{Aa}_{\mathbf{750nm}}\mathbf{)]\ V_e}}{\mathbf{L\ V_f}} \qquad [2.1]$$

where 26.7 is the specific absorption coefficient of chlorophyll-*a* (in g/L*cm) in 90% acetone that removes the Mg from the porphyrin ring and changes it into phaeophytin-*a*, A_b is the absorbance before acidification, A_a is the absorbance after acidification, V_e is the volume of acetone in extraction (10ml or 15ml, depending on the intensity of the green color of the pellet in the sub-samples), L is the light path of the glass cuvettes used (of

1cm), and V_f is the volume of sample filtered (for this research, between 60ml-100ml). These concentration values were changed to compare with other investigations performed with natural substrates, making proportions for conversion units (from mg/cm^3 to g/m^2) and obtaining a conversion factor ($10{,}000cm^2$ divided by $16cm^2$, which is the total area scrapped from the four tiles) that is multiplied by the obtained concentration. Pearson's correlations were performed, per treatment, between the chemical compounds measured in water for each mesocosms (TN, TKN, NO_3-N and TP). Finally, ANOVA (using Bonferroni test; $\alpha = 0.05$) was performed, using Info Stat ®, to test for significant differences in chl-*a* concentration by treatment, blocks, and periods of sampling. Also, lineal regressions were performed by TTG and MT, comparing the shape of the distribution of values of chl-*a* with N-compounds (NO_3-N and TKN, respectively). As well, Q-Q plots were performed to verify the normality of the data obtained for chl-*a*, TN, TKN, NO_3-N and TP.

2.7. Periphyton Taxonomic Assessment

Taxonomy of periphyton composition was assessed using an inverted microscope (LEICA DMI 3000 ® and Nikon Eclipse TS100 ®). The procedure was similar as in Viggiano-Beltrocco (2014). A Sedgewick slide was used to facilitate the preliminary analysis. All visual fields were observed in a total magnification of 400x; all the specimens were enumerated and identified to the lowest taxonomic rank possible. The enumeration was performed moving the slide in a zigzag way to change the visual field. A minimum of 200 specimens from different taxa major groups were enumerated and served to determinate the relative abundance of each species by treatment and sampling event. However, colonial Cyanobacteria were considered as an individual. A matrix of all the species found at

different sampling dates was generated to compare the periphyton abundance, biodiversity and richness among treatments. The abundance calculation units were individuals per square centimeter (ind./cm^2) and identifications were performed to the lowest taxonomic rank possible (family, genus or species). A conversion factor was determined (to calculate the total abundance per species) using the following formula:

$$\mathbf{F\ (Asw\ /\ \pi\ rb^2)} \qquad [2.2]$$

where A_{sw} is the Sedgewick chamber rectangular area, r_b is the radius of visual field area, and F is the number of visual fields observed. Then, the number of specimens (S) and the settling volume (V) of each sample were put in the formula below to determinate the abundance for each species found:

$$\frac{\mathbf{S\ (F[Asw\ /\ \pi\ r^2\ ])}}{\mathbf{V}} \qquad [2.3]$$

Finally, to make the values representative of periphyton per area sampled, ind./ml was change to ind./cm^2. This was achieved multiplying by 200ml (volume obtained for chl-*a* and taxonomic analysis), and dividing by 16cm^2 (total area of the 4 tiles scrapped). Also, abundance proportions per treatment were calculated. Diversity in each treatment was obtained using the Shannon-Weaver Index (H'):

$$H' = -\sum_{i=1}^{S} p_i \ln p_i \qquad [2.4]$$

where p_i is the proportion of specimens per species and *S* is the number of all the taxonomic groups found. As well, a summary of all measurements, including the coefficient of variation (C.V.), was performed with Info Stat ®. Based on the results of number of taxonomic groups found per treatment and periods of sampling, similarity and/or differences between the comparisons of communities (or species richness) were evaluated using the Jaccard index

from B1 to B3. B4 was not considered because 13 out of 14 snails were found dead in TTG treatment at third sampling period (day 7). The formula for the Jaccard index is the following:

$$\mathbf{C / (A+B-C)} \qquad [2.5]$$

where A and B are the number of species found only in one particular treatment and C is the number of species found in both compared treatments.

Beyond these analyses, from block 1 to block 3, two Principal Components Analysis (PCA) were performed in PAST ® to compare the periphyton taxonomic groups vs. treatments (C, TTG, MT and TTGMT) by major groups and the lowest taxonomic rank. We used non-parametric ANOVAs, based on Kruskal-Wallis test, to test the statistical differences for Shannon-Weaver, Jaccard, and periphyton abundance. These ANOVAs were performed in Info Stat ®.

2.8. External Morphological Traits of Adult Snails & Mean Density of Juveniles

The 14 adult snails put in each treatment were previously measured, at the beginning (Day 0), and again at the end of sampling (Day 28 or 30) in all the blocks. The dimensions used to measure each snail were the entire shell length, operculum length and shell's aperture length (Pointier *et al.*, 2005). A T-test was performed in Info Stat ® to compare the change in dimensions, within and between each species, between first and last day of sampling of a total of 168 specimens used for the study. Specimens with the apex cracked were not considered for T-test analysis in Info Stat ®.

On the other hand, juveniles from block 1 that were born during the experiment were removed during the experiment because we wanted to have only 14 adult snails in each mesocosm at the beginning of the overall experimentation. Nevertheless, they were not removed from blocks 2 to 4 and were accounted after the last day of sampling. Chi-squares

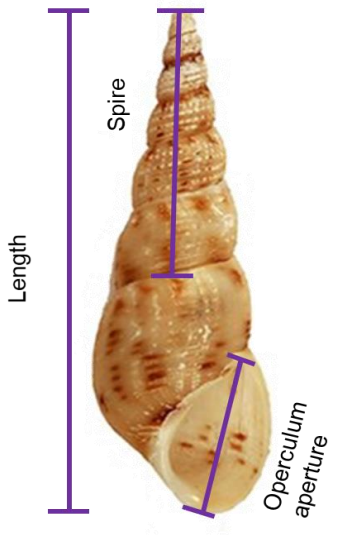

Figure 2.3: Measurements of external morphological traits considered for *Thiara (Tarebia) granifera* and *Melanoides tuberculata*. The second one is represented on this image.

(χ^2) were calculated (comparing TTG/MT, TTG/TTGMT, MT/TTGMT and both species interacting in TTGMT) to see differences in rate of change in sizes (operculum aperture length, shell length and spire length) when species were alone or co-exiting:

$$\chi^2 = \sum \frac{(fo - ft)^2}{ft} \qquad [2.6]$$

where *fo* is the number of the juveniles, and *ft* is the expected theoretical value, where the last one is obtained by the multiplication of *fo* and the total of juveniles accounted by each treatment. Finally, not least important and same as nutrient release, four treatments were established to classify the external morphological traits and mean density of juveniles accounted (TTG, MT, TTGMT-TTG and TTGMT-MT).

CHAPTER 3:
Results

3.1: Relation between physical and chemical parameters and mesocosms

The range and average values for all the parameters measured *in-situ* for each treatment (pH, DO, temperature and turbidity) are described in Table 3.1. Appendix 6.1A summarizes the coefficients of variation (C.V.) of measurements obtained for each parameter by treatment and periods of sampling; values did not exceed from 10.0% for pH, DO and temperature, suggesting a low variability between treatments and sampling periods. However, C.V. of turbidity had a higher variability among the periods of sampling in all the treatments, with a mean value of 320.56% (see ANOVA on Appendix 6.1B). Based on treatments showed values ranged from 164.46% (in C) to 446.00% (in TTG); C.V. in TTGMT was close to the control treatment (Appendix 6.1A). The presence of *T. (T.) granifera* alone seemed to influence the fluctuation in turbidity (far from the mean value). However, turbidity among treatments did not differ statistically probably due to filters in the water pumps that were kept clean the systems. As well, DO and pH different by treatments and periods of sampling ($p > 0.05$). Temperature showed significant differences ($p < 0.05$) only by periods of sampling, showing the highest mean value during the days 15 to 16 (28.7 ^{o}C) and the lowest during the days 28 to 30 (26.5 ^{o}C) (Table 3.1). The overall mean value for temperature was 27.5^{o}C ± 1.48.

Table 3.1: Range and mean values of physical parameters determined *in-situ* in mesocosms.

Parameters	Range	Mean SD	*p*-value (Treatments)	*p*-value (Periods of sampling)	*p*-value (Treatments x Periods of sampling)
Temperature (^{o}C)	22.4-30.2	27.5 ± 1.48	0.5421	0.0263	>0.9999
H_3O+ (M)	1.80 x 10^{-7}-1.82 x 10^{-8}	5.62 x 10^{-8} ± 3.60 x 10^{-8}	0.9563	0.1338	>0.9999
pH	6.74-7.73	7.25 ± 0.13			
DO (mg/L)	4.50-6.55	5.40 ± 0.42	0.5818	0.4406	0.9632
Turbidity (NTU)	0.00-1.48	0.13 ± 0.32	0.6380	0.6105	0.9595

Table 3.2: Pearson's correlation based on overall comparisons between parameters measured *in-situ*

Variable 1	Variable 2	n	Pearson (r)	*p*-value
H_3O+ (M)	DO (mg/L)	77	-0.38	0.0007
H_3O+ (M)	Turbidity (NTU)	81	0.31	0.0046
H_3O+ (M)	Temperature (ºC)	81	-0.14	0.2008
DO (mg/L)	Turbidity (NTU)	77	-0.10	0.3883
DO (mg/L)	Temperature (ºC)	77	0.07	0.5696
Turbidity (NTU)	Temperature (ºC)	85	-0.07	0.5336

In the overall Pearson's correlation analysis (Table 3.2), independent of treatment effects, DO and H_3O+ concentration presented a significant negative lineal correlation ($r = -0.38$; $p < 0.05$), while turbidity and H_3O+ concentration presented a significant positive lineal correlation ($r = 0.31$; $p < 0.05$). DO decreased as H_3O+ concentration increase, and turbidity seems to be increased when H_3O+ concentration increased. Besides, based on Pearson's correlation values (r) (Appendix 6.1C) for the comparisons by treatments among physical parameters measured *in-situ*, only TTG presented statistical differences in correlation between DO and H_3O+ concentration ($r = -0.63$; $p < 0.05$), same as in the overall Pearson's correlation. No association of these parameters measured *in-situ* has been associated to *M. tuberculata* and both species interacting.

Regarding the dissolved nutrients released by snails on sampling assays (TTG, MT, TTGMT-TTG and TTGMT-MT) (Table 3.3; Appendix 6.2), the interaction of treatments/sampling periods, as well as treatments/sampling periods and blocks, for the four chemical components studied, showed non-statistical differences ($p > 0.05$). TN presented significant differences ($p < 0.05$) in treatments, periods of sampling, and repetitions (B2 different from B1, B3 and B4). Based on treatments, TTG had the greatest median value of TN (1.58mg/L; mean values range: 0.52-2.84mg/L) and was significantly different from MT

Table 3.3: Median values of chemical concentrations obtained (TKN, TN, NO_3-N and TP), based on nutrient release assays by Thiarid snails in their respective treatments (TTG, MT, TTGMT-TTG and TTGMT-MT).

Treatment	TKN	NO_3-N*[1]	TN*	TP
	(mg/L)			
TTG	0.53 a[2]	0.89 a	1.58 a	0.03 a
MT	0.37 a	0.39 a	0.90 b	0.02 a
TTGMT-TTG	0.43 a	0.53 a	1.01 ab	0.03 a
TTGMT-MT	0.31 a	0.52 a	0.99 b	0.03 a

[1] * means statistical differences by sampling periods
[2] Equal letters mean no statistical differences by treatment

Table 3.4: Median values of chemical concentrations obtained (TKN, TN, NO_3-N and TP), based on water samples of the respective treatments (C, TTG, MT, and TTGMT).

Treatment	TKN	NO_3-N*[1]	TN*	TP
	(mg/L)			
C	0.31 a[2]	0.25 a	0.54 a	0.02 a
TTG	0.31 a	0.79 a	1.06 a	0.02 a
MT	0.18 a	0.28 a	0.69 a	0.02 a
TTGMT	0.36 a	0.48 a	0.88 a	0.03 a

[1] * means statistical differences by sampling periods
[2] Equal letters mean no statistical differences by treatment

and TTGMT-MT; but not from TTGMT-TTG. Besides, TTGMT-TTG was not significantly different from MT or TTGMT-MT ($p > 0.05$). MT presented the lowest median value of TN concentration (0.90mg/L; mean values range: 0.54-2.25mg/L). On the other hand, NO_3-N showed significant differences based on the periods of sampling; the mean values ranged from 0.85 to 1.71 mg/L and increased from day 3 to day 28 to 30 (2^{nd} to 5^{th} period of sampling). Also, ANOVA (Appendix 6.2) did not show significant differences for TKN and TP, when comparing TTG with MT and between species interacting (TTGMT-TTG and TTGMT-MT), as well as for sampling periods. Nutrient release assays values were normally distributed in a lineal regression (see Appendix 6.5).

The same previous chemical components were also evaluated in the water samples (Table 3.4; Appendix 6.4). Non-statistical differences ($p > 0.05$) were observed for treatments and interactions (treatments/periods of sampling and treatments/periods of sampling/blocks)

for these chemical compounds. However, TN showed significant differences ($p < 0.05$) by periods of sampling, ranging the median values from 0.54 to 1.06mg/L, and increasing from day 0 to day 28-30 (1^{st} to 5^{th} period of sampling). NO_3-N showed significant differences ($p < 0.05$), based on periods of sampling and repetitions (B2 from B4), ranging the median values from 0.25 to 0.79 mg/L, and increasing concentration from day 0 to days 28-30 (1^{st} to 5^{th} period of sampling). TKN and TP did not differ based on periods of sampling and blocks. Chemical concentrations of the water indicated that values are normally distributed in a lineal regression (see Appendix 6.8). Comparing the median values obtained, some nutrient release values (using five adult snails for one hour and filtered water from each mesocosm) were higher than nutrient values obtained from the water (except TP). The water pumps with filters and the close artificial systems influenced the lowest values of nutrients. Therefore, the presence of one or both Thiarid snails represents an alteration of nutrients fluctuation in the water that was controlled for this experimental design.

3.2: Chlorophyll-a vs. nutrients

Chlorophyll-*a* concentrations (Figure 3.1) were determined in presence of one and both snail species in their respective treatments. Results were compared with control treatment. Figure 3.1, as well as ANOVA from Appendix 6.19A, showed significant differences in treatments (C similar to MT, as well as similarity between treatments with snails; $p < 0.05$) and blocks (B1 similar to B2; B3 similar to B4; B4 similar to B4rep; $p < 0.05$); but none for the sampling periods ($p > 0.05$). Control treatment had the highest mean values for chl-*a* (1.33 g/m^2). TTGMT had the greatest variability (C.V. = 148.56), followed by TTG (C.V. = 138.48) (see Appendix 6.19B for summary of measurements), which values in both treatments were similar. However, considering treatments besides TTG, the results suggest that (in presence of

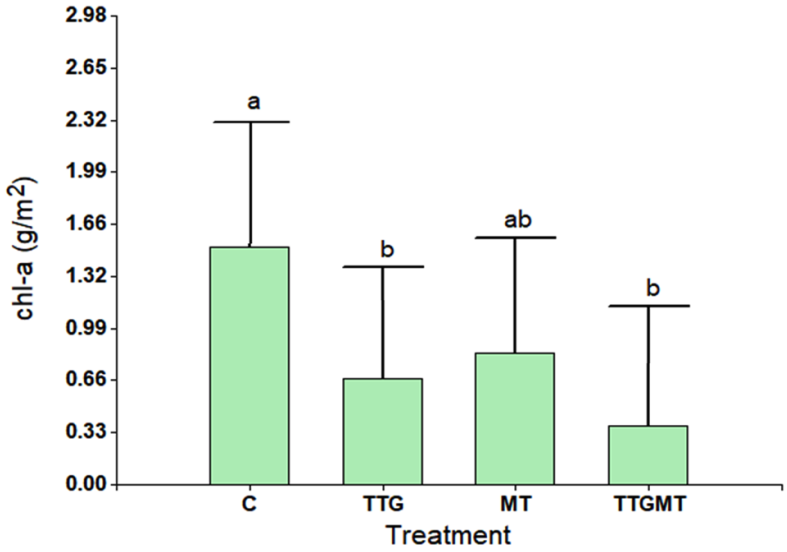

Figure. 3.1: Mean concentration of chlorophyll-*a* values at different treatments (C, TTG, MT and TTGMT)

Table 3.5: Pearson's correlations based on overall comparisons between chlorophyll-*a* and chemical components (TN, TP, NO_3-N and TKN).

Variable 1	Variable 2	n	r	p-value
Chlorophyll-*a*	TN	80	-0.22	0.0535
Chlorophyll-*a*	TP	77	0.10	0.3856
Chlorophyll-*a*	NO_3-N	80	-0.34	0.0021**
Chlorophyll-*a*	TKN	80	0.23	0.0410*

*Significant correlation ($p < 0.05$); **highly significant correlation ($p < 0.005$)

snails) periphyton biomass changed, as well as food quantity in both treatments were similar. availability (i.e.: due to grazing effects).

In the overall Pearson's correlation, the chl-*a* presented a negative lineal correlation with NO_3-N ($r = -0.34$; $p < 0.05$), and a positive lineal correlation with TKN ($r = 0.23$; $p < 0.05$), that presented a positive lineal correlation (Table 3.5). Chl-*a* decreased in proportion to the increase in NO_3-N concentration, as well as seemed to increase when TKN value increased. TKN is a quantitative determination of organic nitrogen and NH_4, where the last one is the easiest component to oxidase in the biochemical processes of photosynthesis. For

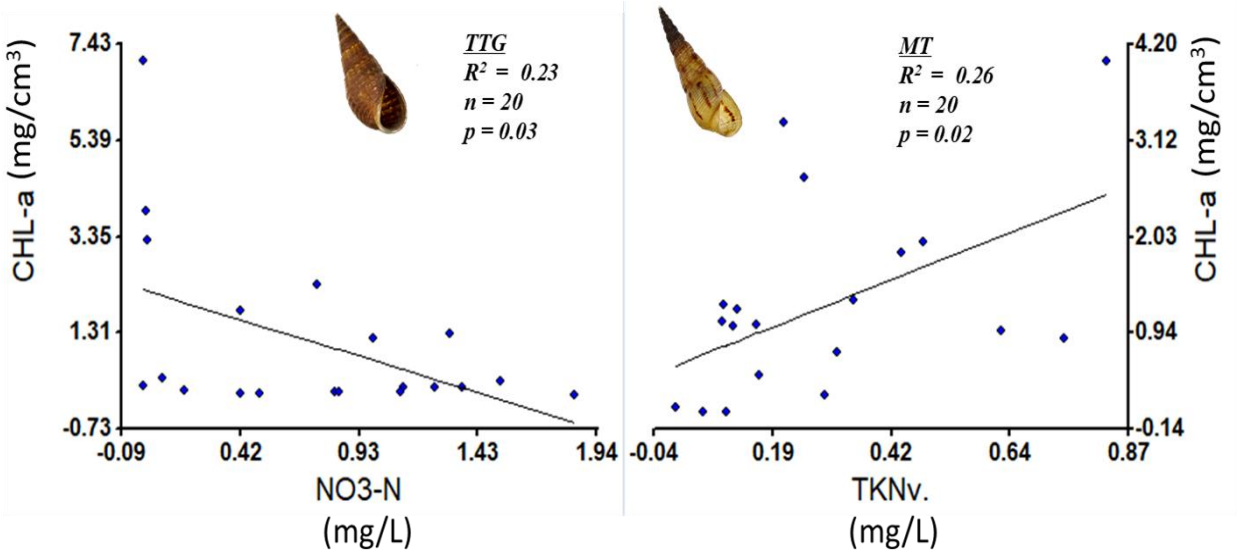

Figure 3.2: Lineal regressions of TTG and MT, correlating the chl-*a* value with the chemical components that showed significant differences ($p < 0.05$) in Pearson's correlations (NO_3-N and TKN, respectively).

TTGMT and control treatment, no significant correlation was observed between any N or P compounds and chl-*a* (Appendix 6.19C). Lineal regressions that showed a significant correlation (TTG and MT) are in Figure 3.2. Chl-*a* biomass decreased in TTG and it was related to an increase in NO_3-N ($r^2 = 0.23$; $p = 0.03$), perhaps by the N-fixing performed by cyanobacteria, that represented the 86% of mean abundance (see Figure 3.4 in section 3.3), and nitrification. Therefore, around 23% of the decrease in chl-*a* biomass was related to the increase in NO_3-N, the other 77% is associated to other variables. Otherwise, TKN had the most influence for the increase of chl-*a* in MT ($r^2 = 0.26$; $p = 0.02$), perhaps, related to the assimilation and ammonification of nitrogen components dissolved in the water. Thus, around 26% of the increase in chl-*a* is related to an increase in TKN, the other 74% is associated to other variables; but in both cases, presumably, the effect of grazing is the principal fact.

3.3: Periphyton abundance, biodiversity and composition

Around 53 major periphyton groups were identified during the study. Appendix 6.18 shows the list of species found, including the unknown ones, divided by treatment. Besides, Shannon-Weaver indexes (Figure 3.3) indicated the level of biodiversity of taxa major groups found in the experiment. Taxonomic analyses of periphyton from the four treatments are described in Appendix 6.20b, and the following major taxa were observed: Cyanobacteria, Chlorophyta, Bacillariophyta, Amoebozoa, Charophyta, Ciliophora, Cryptophyta, Euglenozoa, Fungi, Ochrophyta, and Rotifera (Figure 3.4). The primary producers' composition was as follows: Bacillariophyta (31.25%), Charophyta (4.17%), Chlorophyta (31.25%), Cyanobacteria (27.08%), Cryptophyta (2.08%), and Euglenozoa (4.17%). The periphyton comparisons in treatments with snails and control group did not show significant differences in terms of biodiversity (Shannon-Weaver index) ($p > 0.05$), based on non-parametric ANOVA, using Kruskal-Wallis test (Appendix 6.20). Shannon-Weaver index considers the abundance of taxonomic groups identified and their proportion. If H' is close to

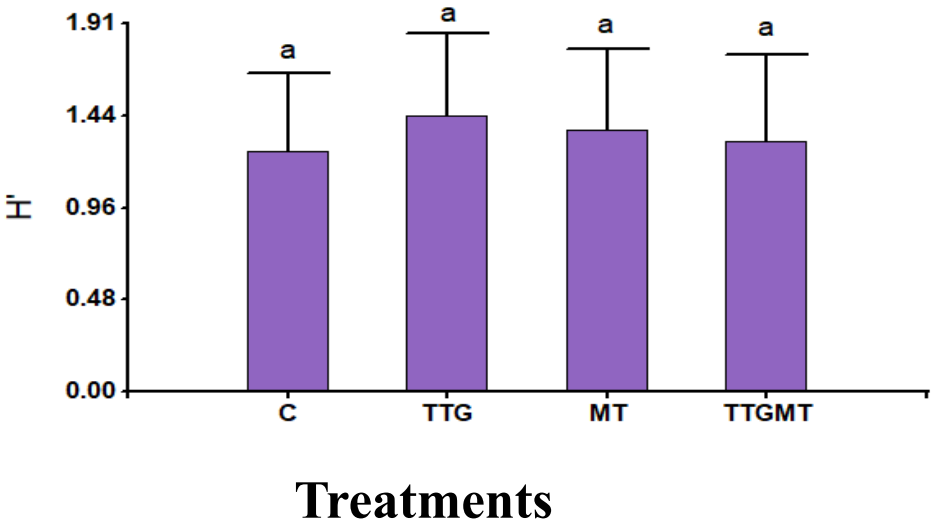

Figure 3.3: Mean values of Shannon-Weaver diversity indexes, divided by treatments.

0, a very low diversity is indicated. The mean H' values obtained in all the treatments were between 1.25 and 1.44 (Figure 3.5). The highest value was reported from TTG-B4 in the 3rd period of sampling (H' = 2.24); the lowest was reported from TTGMT-B1 at 2nd period of sampling (H' = 0.27) (Appendix 6.3). Based on the repetitions made, for control treatment obtained (H' values) ranged for 0.52 to 2.04; TTG for 0.76 to 2.24; MT for 0.69 to 2.03; and TTGMT for 0.27 to 2.05 (Appendix 6.3). These values respond to a very low biodiversity, where Cyanobacteria dominated in all treatments (see Figure 3.4).

Periphyton abundance (ind./cm^2) was determined for the taxa groups found in all treatments and periods of sampling. Species of *Anabaena*, Microcystaceae, *Chroococcus,* and colonial coccoid Chlorophyceae were well represented for all the treatments. Based on

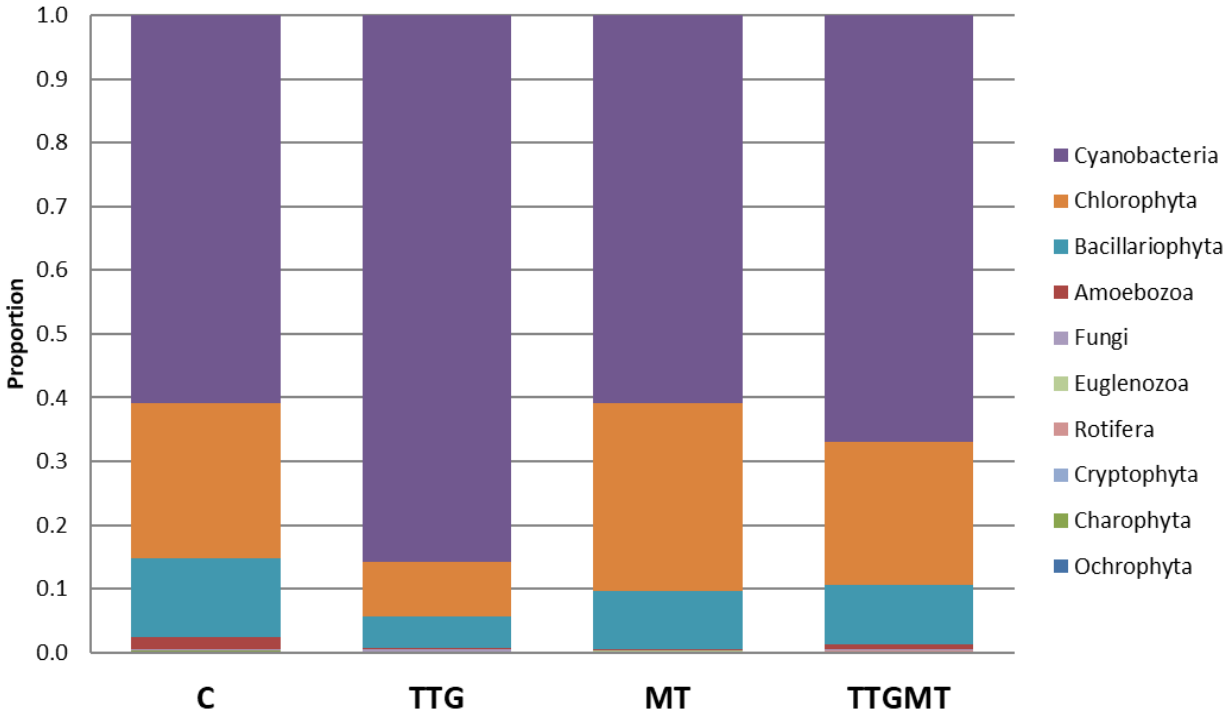

Figure 3.4: Abundance proportions obtained for each taxonomic major group by treatments (C, TTG, MT, TTGMT).

Kruskal-Wallis test (Non-parametric ANOVA) differences were obtained ($p < 0.05$) by treatments and major taxa groups (Appendix 6.22A). In terms of major groups proportions (Figure 3.4), Cyanobacteria exhibited around 0.61 of total abundance proportion, followed by Chlorophyta with 0.24, Bacillariophyta with 0.13, Amoebozoa with 0.01, and the rest of the groups identified exhibited a 0.01 in total (Ochrophyta, Charophyta, Cryptophyta, Rotifera and Fungi). Besides, TTG showed a proportion of 0.86 in Cyanobacteria, followed by Chlorophyta with 0.08, Bacillariophyta with 0.05, and the rest of the groups identified with 0.01 in total (Amoebozoa, Cryptophyta, Rotifera and Fungi). Also, proportions in MT were 0.61 for Cyanobacteria (similar to control treatment), 0.29 for Chlorophyta, 0.09 for Bacillariophyta, and 0.01 for the rest of the groups (Amoebozoa, Charophyta, Euglenozoa and Fungi). Additionally, TTGMT was the only treatment that had species of all the major groups identified; exhibiting a proportion of 0.67 for Cyanobacteria, 0.22 for Chlorophyta, 0.09 for Bacillariophyta and 0.03 for the rest of the groups. In an overall comparison between treatments, more abundance of Cyanobacteria and Bacillariophyta were observed in TTGMT with median values of 3.86×10^5 ind./cm^2 and 2.83×10^4 ind./cm^2, respectively; Chlorophyta were more abundant on MT with a median value of 5.14×10^4 ind./cm^2 (see Appendix 6.22A). As well, periphyton abundance composition in treatments with snails was different (Appendix 6.22B and 6.22C).

Principal Component Analyses (PCA) were performed to relate the treatments and major groups described on the taxonomic survey of periphyton, employing for all the samplings, using the abundance values by major groups (Figure 3.5), treated to the lowest taxonomic rank possible (Figure 3.6). The major groups were: Amoebozoa, Ochrophyta, Charophyta, Chlorophyta, Cyanobacteria, Cryptophyta, Bacillariophyta, Rotifera, Euglenozoa, and Fungi. The PC1 constituted 97.53% of variance (Figure 3.5). When

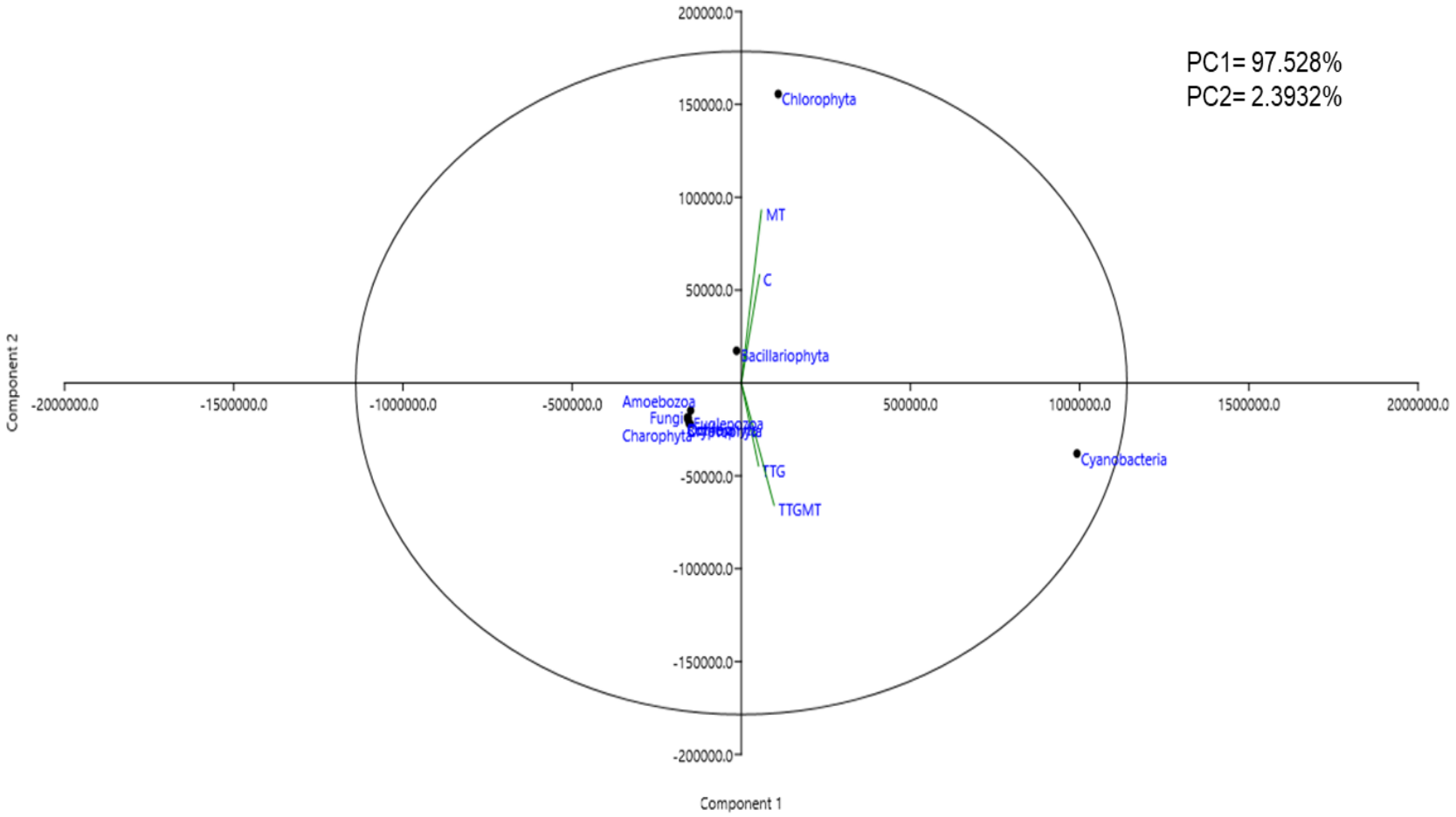

Figure 3.5: Principal Components Analysis (PCAs), comparing the overall major groups identified in the periphyton taxonomic assessment performed.

proportions of the principal components are higher (longer lines), the treatment exerts more pressure for a specific group in the periphyton. The proportions of the PC1 were dominated by TTGMT. The PC2, that constituted the 2.39% of variance, showed the highest negative proportion in TTGMT and higher positive proportion in MT; corresponding lines in the graph are opposite. Therefore, related to periphyton major groups, Chlorophyta was more related in the pressure exerted by MT and control treatment; opposite to Cyanobacteria that were more related to the pressure exerted from TTGMT and TTG. The rest of the groups were well distributed over the different treatments.

On the other hand, the PCA related to periphyton, identified to the lowest taxonomic rank (Figure 3.6), showed that PC1 constituted the 91.47% of variance, which value was dominated by TTGMT. The PC2 constituted the 4.61% of variance; this value was negatively dominated by TTGMT, followed by TTG, and positively dominated by MT. Therefore, unknown Chlorophyceae was more related in the pressure exerted from MT, opposite to Microcystaceae (cf. *Anacystis* sp.) and *Anabaena* sp. that were more related to the pressure exerted from TTGMT and TTG. The rest of the groups were distributed equally over the different treatments, with a small difference in other Cyanobacteria species, such as *Chroococcus* sp. and *Synechococcus* sp. that were most related to the pressure exerted from TTGMT and TTG. As in the PCA of the major groups, unknown colonial Chlorophyceae were more abundant in the presence of *M. tuberculata* alone, and Cyanobacteria species were more abundant in the presence of both snail species.

Jaccard's proportions were obtained based on the comparisons between mean proportions of periphyton groups by treatments, Block 4 was not chosen for these analyses due to lack of TTG treatment. The higher values (close to 1) indicated a higher similarity

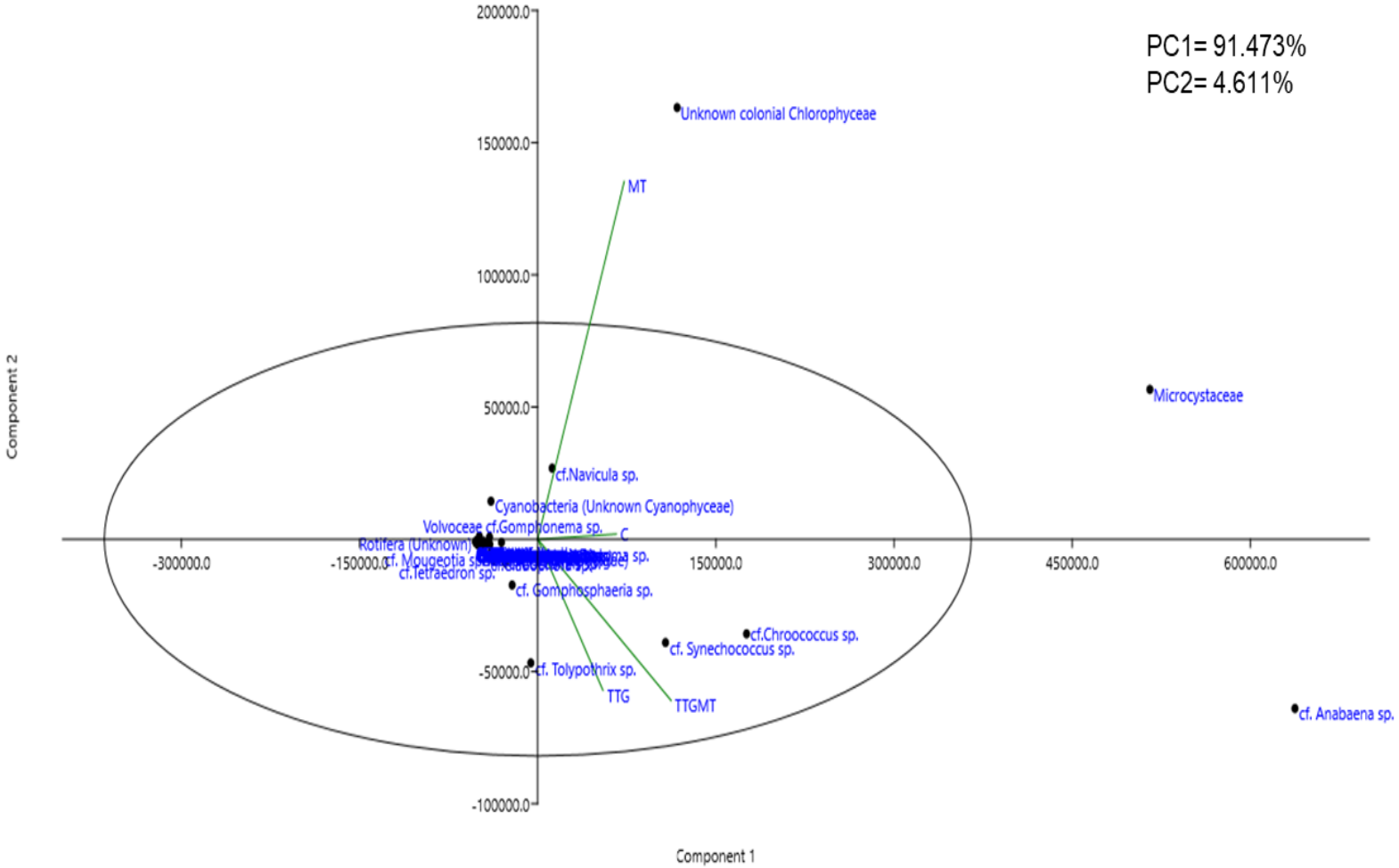

Figure 3.6: Principal Components Analysis (PCAs), comparing the overall taxa identified (to the lowest taxonomic rank possible) in the periphyton assessment performed.

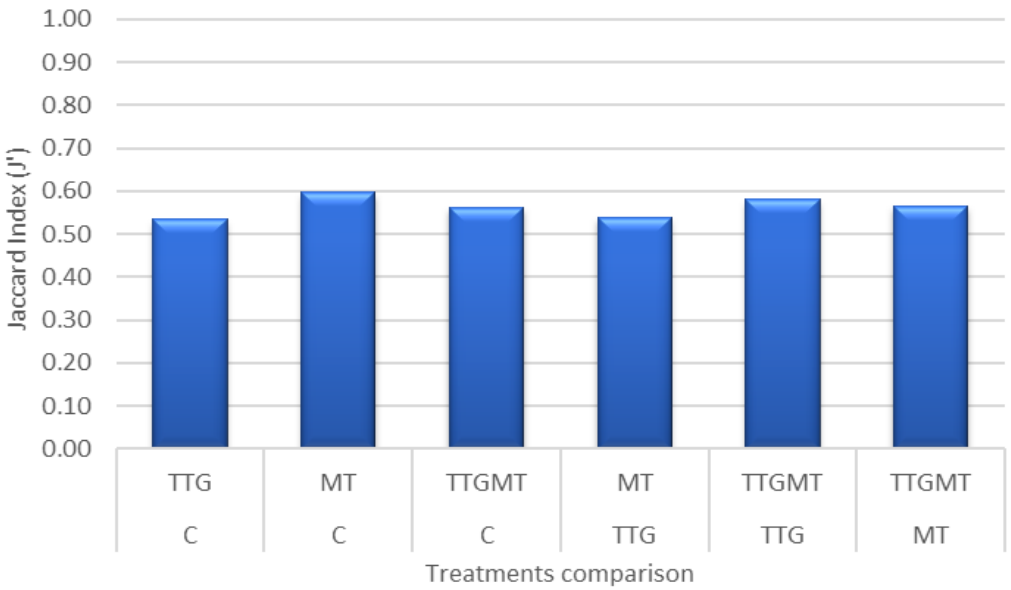

Figure 3.7: Jaccard's proportion of similarity between treatments.

between the periphyton taxonomic assessments; on the other hand, the lowest values proportions (close to 0) indicate a low degree of similarity. Six comparisons by treatment were performed (Figure 3.7); all the comparisons were between 0.53 and 0.60, with no statistical differences in species composition based on non-parametric ANOVA (using Kruskal-Wallis test) (Appendix 6.21). These results suggest that differences in species composition in control treatment and treatments with snails were not significantly different. Although the algal abundances in treatments were relatively different by taxonomic major groups (Appendix 6.22), the number of species shared was similar between treatments.

3.4: Mean density of juveniles and morphological traits of Thiaridae snails

Juveniles were produced in all the treatments (Table 3.6). *T. (T.) granifera* showed a higher density of juveniles in both treatments (TTG and TTGMT). A χ^2 was performed (Table 3.7) to see if mean density of juveniles differed between species alone or together. Based on the χ^2 analysis, the null hypotheses were rejected, either comparing densities of both species' juveniles (TTG and MT) and when comparing the sum of both species' juveniles obtained in

Table 3.6: Total number of juveniles of *T. (T.) granifera* and *M. tuberculata* obtained from each mecososm at the 5th period of sampling from their respective treatments.

Treatment	Total of Juveniles quantified
TTG	152
MT	31
TTGMT-TTG	31
TTGMT-MT	11

Table 3.7: Chi-square values (χ^2) calculated, with their respective theoric number (χ^2 tab.) according to degrees of freedom (df). B4 and B4rep of TTG were not considered for this analysis.

Treatment	χ^2 calc.	χ^2 tab.	Null-hypothesis
TTG and MT (df = 1)	0.53	3.84	Rejected
TTG and TTGMT* (df = 1)	8.51	3.84	Accepted
MT and TTGMT* (df = 2)	6.27	5.99	Accepted
Species together (TTGMT) (df = 2)	1.98	5.99	Rejected

* For χ^2 analyses, the total juveniles obtained for both Thiarid species were considered to compare with TTG and MT.

TTGMT (A and B). On the contrary, when comparing the juveniles obtained from TTG and MT with the total of juveniles obtained in TTGMT (AB), the null hypotheses were accepted. Therefore, both Thiarid species seemed to differ in their reproductive potential when they were alone in their respective treatment. Also, the numbers of juveniles tended to be higher when both species were together and possibly competing for resources.

All the adult snails that were used during the experimental design were measured before and after the time lapse of the experiment for each block. The criteria of snails' shells (Shell length size, opercular aperture length and spire) presented correlations in proportion

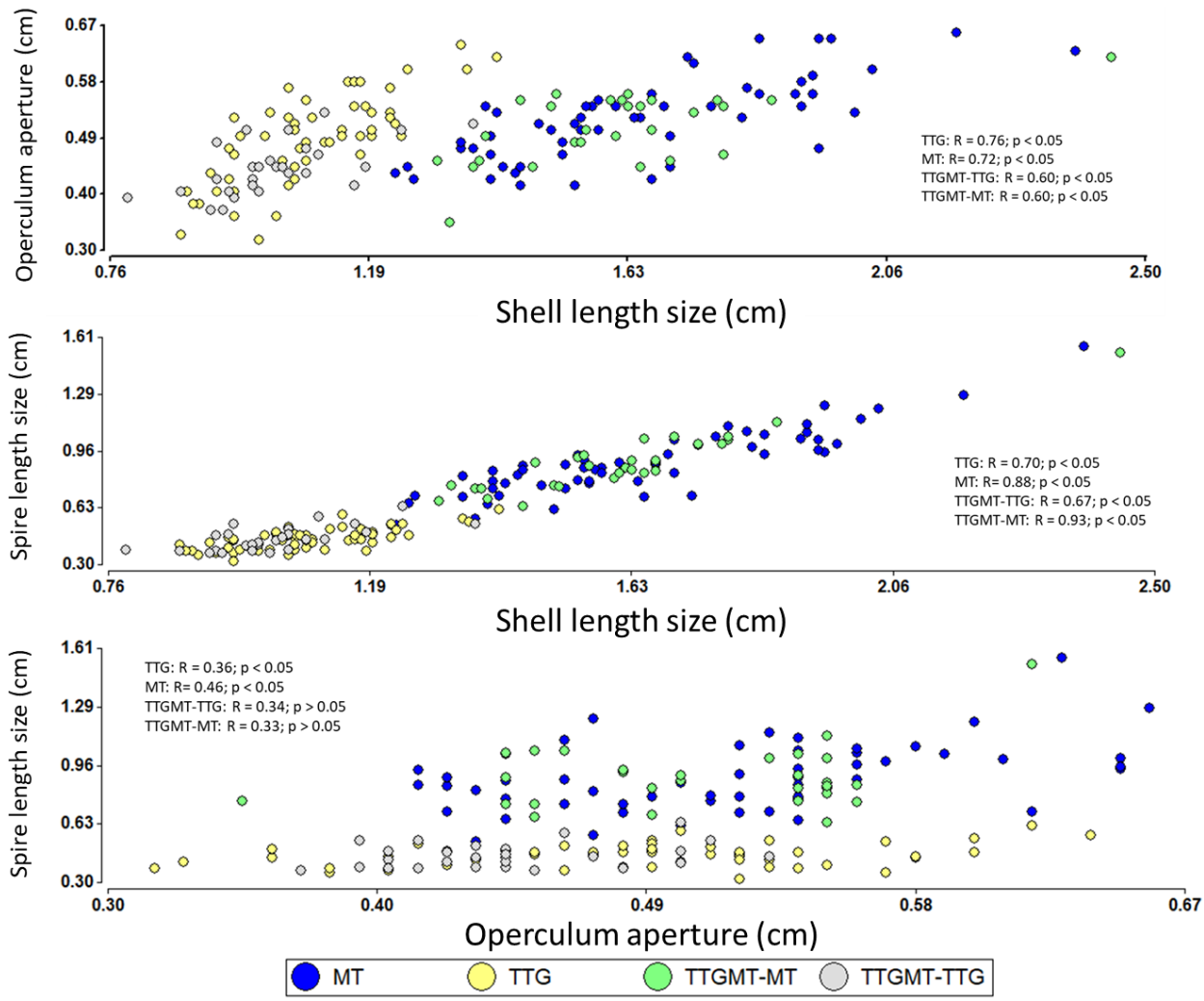

Figure 3.8: Correlation of shells measurements (shell length size, opercular aperture and spire) for Thiarid snails.

Table 3.8: Mean of change in growth (cm) of *T. (T.) granifera* and *M. tuberculata*, based on length of shell, operculum aperture and spire, of all the specimens measured for the experiment.

Species	Treatment	Change Length	Change Operculum Aperture - (cm)	Change Spire
T. (T.) granifera	TTG	0.06 ± 0.02	0.07 ± 0.03	0.05 ± 0.02
	TTGMT	0.03 ± 0.02	0.03 ± 0.03	0.02 ± 0.02
M. tuberculata	MT	0.02 ± 0.02*	0.05 ± 0.01	0.06 ± 0.01
	TTGMT	0.12 ± 0.02*	0.06 ± 0.01	0.08 ± 0.01

* means statistical difference in external morphological trait.

to the sizes of each structure of the shells between treatments (Figure 3.8). All the treatments with snails presented correlation ($p < 0.05$) with operculum aperture and length size (TTG: $r = 0.76$; MT: $r = 0.72$; TTGMT-TTG: $r = 0.60$; TTGMT-MT: $r = 0.60$); as well as length size and spire (TTG: $r = 0.70$; MT: $r = 0.88$; TTGMT-TTG: $r = 0.67$; TTGMT-MT: $r = 0.93$). Spire and opercular aperture presented statistical correlation ($p < 0.05$) only in treatments with species alone (TTG: $r = 0.36$; MT: $r = 0.46$), which suggest that both structures grow disproportionally in TTGMT when there is competition for the same resources.

The means for shell length size of *T. (T.) granifera* and *M. tuberculata* were 1.10 cm and 1.65 cm, respectively. On the other hand, mean shell lengths of both species interacting were 1.04 cm, for *T. (T.) granifera*, and 1.61 cm for *M. tuberculata*. Opercular aperture mean values were 0.48cm for *T. (T.) granifera* and 0.52cm for *M. tuberculata*, while for both species interacting were 0.44 cm and 0.51 cm, respectively. Mean value for spire was 0.45 cm, for *T. (T.) granifera,* and 0.89cm for *M. tuberculata*; while both species interacting showed mean values of 0.46 cm and 0.90 cm, respectively.

When comparing treatments with species alone vs. together, *M. tuberculata* showed significant differences in shell length ($p < 0.05$) (Appendix 6.6). The highest change in average length of *M. tuberculata* was 0.12 cm on TTGMT (Table 3.8). For *T. (T.) granifera* alone, no significant differences were shown in the three shell traits measured, when comparing with *T. (T.) granifera* in TTGMT. Therefore, *M. tuberculata* seemed to focus its metabolism to increase external morphological traits instead of reproduction. It is assumed that this behavior is shown when it is competing with *T. (T.) granifera* for resources.

CHAPTER 4:
Discussion

The presence of invasive macro-invertebrate species in freshwater systems may change the ecological balance with the alteration of food webs and cause the subsequent displacement of native or endemic macro-fauna (Park, 2004; Santos and Eskinazi-Sant'Anna, 2010). *Thiara (Tarebia) granifera* and *Melanoides tuberculata* seemed to promote the development of diverse algal species, some of which produce toxic substances. However, the primary composition depends also of which physico-chemical parameters may influence their development on periphyton; as well as the combination of those factors with both species alone or interacting may influence the number of born developed juveniles and contrast with their external morphological traits.

By altering their specific excretion ratios, exotic or invasive organisms are capable of changing the assemblages of macro- and microbiota and, thus, affect nutrient recycling in aquatic ecosystems (Arnott and Vanni, 1996; Hall Jr. *et al.*, 2003; McIntyre *et al.*, 2008; Moslemi *et al.*, 2012). Therefore, the presence of both Thiarid snails in natural freshwater systems implies important and understudied effects related to toxicity and the health of other living organisms, as well as open venues for conservation and management.

4.1: Physico-chemical parameters and chlorophyll-a

Temperature, DO, and pH are among the most important parameters that influence the dissolved nutrients, trophic chains and the biotic composition of aquatic ecosystems. In this research, mesocosms' temperatures were above 25ºC, pH above 7, and dissolved oxygen (DO) above 5.0 mg/L. Temperature and pH are two factors that were associated with a great population density of *T. (T.) granifera* in estuaries of South Africa (Miranda *et al.*, 2012). In

treatment with *T. (T.) granifera* alone, DO and [H_3O^+] were negatively correlated ($p < 0.05$). Drastic changes in DO changes should affect the snails' metabolism and potentially modify the trophic chains (Mäkelä and Oikari, 1992). Studies with *C. fluminea*, such as those by Kartayev *et al.* (2007), mention that great fluctuations of DO were caused by the removal of photosynthetic species on benthos by pedal feeding of this particular mollusk. *T. (T.) granifera* and *M. tuberculata* use the nutrient and grazing mechanisms described by Evans-White and Lamberti (2005) for a snail (*Elimia livescens*) and a crustacean (*Orconectes propinquus*). Hence, freshwater snails, such as the target species in this study, need oxygen for their metabolic purposes (Oloyede *et al.*, 2016), which is one important product released by primary producers. The high plasticity shown by *T. (T.) granifera* for extreme and simultaneous fluctuations in pH and DO in the mesocosms, suggest a high potential of this species to colonize natural areas, where local macro-fauna do not represent competition, and even displace the native or endemic fauna from these areas, or even other snails that are being considered invasive, such as *M. tuberculata*. Pointier *et al.* (2005) suggested that none of the abiotic parameters evaluated had correlation with the presence of *M. tuberculata*. *Biomphalaria glabrata*, intermediate host of *S. mansoni*, was displaced in a lot of rivers and watersheds of Puerto Rico in the presence of both Thiarid snails, but especially by *T. (T.) granifera* (Vélez, 2014).

Thiarid snails may increase dissolved nutrients significantly in a short period of time. Three weeks before the initiation of the experiment, the water pumps were circulating in each mesocosm and allowing for the establishment of periphyton communities based on the nutrients available in the water. Although we used closed systems, the four treatments established for this research may help to contrast the nutrient cycling with and without the two freshwater invasive snail species and give hints about what should happen. Viggiano-

Beltrocco (2014) documented the presence of *T. (T.) granifera* in Guanajibo river, where median values for TN and TP were 0.43 to 0.46 mg/L and 0.03 to 0.04 mg/L, respectively. Meanwhile, in our TTG treatment, values of TN and TP concentrations in the water were 1.06 mg/L and 0.02 mg/L, respectively. Chappell (2012) and Estrella-Riollano (2012) also documented great densities of *T. (T.) granifera* in two reservoirs in Puerto Rico: La Plata and Guajataca. Although Chappell (2012) evaluated the TKN in Guajataca and La Plata reservoirs (median values of 0.80 mg/L and 0.50 mg/L, respectively), it is presumed that TN mean values (not determined in their studies) were above 1.00 mg/L in both reservoirs. Likewise, mean values of TP were 0.02 mg/L and 0.05 mg/L (in Guajataca and La Plata reservoirs, respectively). Perhaps our mesocosms seem to mimic better what is happening in more closed systems such as reservoirs than in streams; in reservoirs, the nutrient retention times are longer, and water with dissolved nutrients within our mesocosms were continuously re-circulated. In terms of nutrient released by the snails in our study, TN presented statistical differences by treatment and periods of sampling ($p < 0.05$); NO_3-N only presented differences by periods of sampling. In comparison with control, *T. (T.) granifera* seemed to exert a different pressure in its release of nutrients, but further studies are required to examine this possibility. Besides, water nutrients from each artificial system showed non-significant differences per treatment for all the chemical determinations. In some streams of Trinidad (West Indies), the largest mean population densities of *T. (T.) granifera* were obtained in sites that were not covered by vegetation (deforested areas) (Moslemi *et al.*, 2012). The availability of light and nutrients influenced the development of more cyanobacteria than green algae or diatoms, which instead influenced the displacement of other local macro-fauna.

Even N-fixing in cyanobacteria can use NH_4^+, NO_2^- and NO_3^- (and NO_3-N) for their development and reproduction, but NH_4^+ seems to be the most important source since it is

easier to assimilate (Ochoa de Ada *et al.*, 1996; Von Rückert and Giani, 2004); requiring less investment of energy (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) in light independent phase (Wetzel, 2001). NH_4^+ is oxidized into NO_3^-, which could explain the significant differences in concentration of NO_3-N shown among periods of sampling in our study, and suggests that competition between snails' species might play an important role in nutrients availability for the periphyton, especially proliferation of cyanobacteria (Martiny *et al.*, 2009; Berube *et al.*, 2015; Shilova *et al.*, 2017). Cyanobacteria may regulate the concentrations of nitrogen compounds through fixation, uptake, assimilation and other metabolic routes (Shilova *et al.*, 2017). These species tend to grow in presence of NO_3^-; oxidized from NH_4^+ (chemical component that is mostly excreted by snails) (Martiny *et al.*, 2009; Berube *et al.*, 2015; Shilova *et al.*, 2017). Other cyanobacteria (i.e. *Synechococcus* sp.), diatoms and heterotrophic bacteria have been shown to use NO_3^- as their primary N source (Allen *et al*., 2006; Casey *et al*., 2007; Collier *et al*., 2012; Shilova *et al*., 2017).

Grazing effects should influence the nutrient release by snails and primary productivity on periphyton. For instance, snails may change their foraging methods to stabilize their environment. If the chl-*a* concentrations were low, snails could have had increased nutrient releases, influencing the development of algae when days passed, making their food available for survival in the near future (Evans-White and Lamberti, 2005). The availability of resources at different seasons may change the competition effects, consequently with the alteration of the direction of change caused by competition (Riley and Dybdahl, 2015). The biomass of primary producers may influence the diversity of fauna in aquatic ecosystems, especially if these organisms are being affected by invasive species whose food habits usually are non-specific and if these same species excrete higher nutrient concentrations to develop another type of primary community. The use of invasive species as bio-indicators of pollution or

habitat loss is a great challenge for researchers who need to understand the level of impact (Kennard *et al*., 2005; Vilá and García-Berthou, 2010).

In Guanajibo river, where the presence of *T. (T.) granifera* was documented (Viggiano-Beltrocco, 2014), mean chl-*a* biomass in natural substrates was 0.02 g/m^2. That result is different fromthe findings of this study; from TTG treatment in enclosed spaces, the mean chl-*a* was 0.67 g/m^2. The other treatments (including the control group) showed values close or higher than those in TTG. It seems that chl-*a* biomass in enclosed freshwater systems (vs. natural habitats) provided higher availability of resources for both species.

Chl-*a* biomasses in treatments with snails were lower than control treatment ($p < 0.05$), probably due to the grazing effects that both snail species exerted. Cyanobacteria dominated in terms of relative abundance in all treatments, comprising around 86% of relative abundance in treatment TTG and 60-67% in all other treatments. However, in MT, nitrogen seemed to have stimulated the proliferation of other types of microalgae; in this case, mostly Chlorophyta (around 29% of the abundance composition). Thus, chl-*a* concentration was correlated to NO_3-N in the presence of *T. (T.) granifera*, while chl-*a* was correlated to TKN in the presence of *M. tuberculata*. Food availability for many activities, such as foraging, can be limited by a snail's own modes of feeding and locomotion (Raw *et al*., 2016). *T. (T.) granifera* seems to exert a higher grazing pressure, but this was not tested in our study. Rotifers were identified in the samples and these animals are also grazers; but they were in very low abundances and might have had no significant effects on chl-*a* content, as well as on diversity of primary producers. On the other hand, if the nutrients released by this invasive freshwater snail influence the growth of more cyanobacteria, even above that of Chlorophyta or Charophyta, this will translate into a lower biodiversity, affecting the food quality of the periphyton. As well, this could bring a great alteration to the aquatic ecosystem performance, as pointed out

by Rodríguez-Vargas (2014).

4.2. Taxonomic Abundance and Biodiversity of Periphyton

Around 53 different periphytic taxa were identified in this research. Based on the nutrient and structural (grazing) mechanisms used by a snail (*Elimia livescens*) and a crustacean (*Orconectes propinquus*), as described by Evans-White and Lamberti (2005), Moslemi *et al.* (2012) pointed out that *T. (T.) granifera* has different nutrient release and grazing strategies that will vary based on the environment. If the grazing pressure of an invasive species is higher, the effect will be the increase of nutrient release rates, especially the nitrogen compounds that algae (mostly cyanobacteria) use for metabolic purposes. Viggiano-Beltrocco (2014) related *T. (T.) granifera* with the periphyton growth limitation in Guanajibo river (located at south-western Puerto Rico), based on the higher grazing and nutrient supply that they exert; however, no monitoring of population density was performed. In our experiment, it was not clear to what degree biodiversity attributes (i.e. Shannon-Weaver index, H') changes due to these Thiarid species alone or together. However, as Evans-White and Lamberti (2005) suggested, if the biodiversity is low, herbivores tend to regulate their nutrient mechanism and grazing effects to increase this biodiversity and, thus, obtain resources from periphyton.

Proliferations of cyanobacteria and colonial green algae were found in all the treatments, although the relative abundances of the algal groups differed among them. Besides, a higher grazing pressure on periphyton by *T.* (*T.*) *granifera* could have caused the subsequent stimulation of cyanobacterial growth, such as: Microcystaceae, *Anabaena* sp.*, Synechococcus* sp.*, Chroococcus* sp., among others; with the concomitant reduction of microalgae diversity, as eukaryotic algae were presumably and preferentially removed. These cyanobacterial groups

are bio-indicators of toxicity in streams (Canter-Lund and Lund, 1995; Zurawell *et al*., 1999), and can be harmful for local macro-fauna and humans. As we observed, NO_3-N concentrations were significantly correlated with chl-*a* values in TTG. However, NO_3-N is more related to chl-*a* biomass than other nitrogen components (Wetzel, 2001).

On the other hand, colonial Chlorophyceae (and Chlorophyta in general) grew in the four treatments; more notably in MT and control treatment. Through nutrient release, *M. tuberculata* might had increased the green algae content to satisfy its dietary needs; as well, its grazing seems to be lower than in *T. (T.) granifera*. Raw *et al*. (2016) made a research in sub-tropical coastal lakes in South Africa, evaluating the diet of *M. tuberculata* with stable isotopes. It was considered as a "generalist deposit feeder" that feeds on microalgae present in periphyton and detritus (Madsen, 1992; França-Vasconcelos *et al*., 2013). Songtham *et al.* (2005) suggested that *M. tuberculata* can be carnivore (Coat *et al.* 2009). However, *Cladophora* sp., *Ceratophyllum* sp., and benthic microalgae dominated in the diet composition of this snail species, based on C_{13} and N_{15} analyses (Raw *et al*., 2016). *Cladophora* sp. was identified in some samples of our research; nevertheless, it always had a low abundance.

Biofilms (microalgae and plants or plant detritus) are main resources available for primary consumers since the beginning of the development of food webs (Coat *et al*., 2009; Lefrançois *et al.*, 2010). However, water quality can be evaluated using algae to provide information about environmental change in a short period of time based on nutrient availability (Bellinger and Sigee, 2015). For example, species of *Microcystis* (Cyanophyceae, Microcystaceae) are good bio-indicators of high nutrients concentrations or eutrophic status in freshwater systems (Bellinger and Sigee, 2015).

The development of an algal community on substrates depends mostly on abiotic factors and the fauna composition in aquatic systems. Moreover, snails and small crustaceans

can control the abundance and biodiversity on benthos. Lefrançois *et al.* (2010) also used stable isotopes to relate nine river biofilms with the type of diet of freshwater shrimps (Palaemonidae, Atyidae, and Xiphocarididae), as well as fishes (Gobiidae and Eleotridae) that are consumed by people in Guadeloupe. Their result suggested that diatoms are abundant on epilithon and are usually the main food habit of these crustaceans. They also found that cyanobacteria species proliferation was promoted on benthos with the increased release of nitrogen compounds exerted by these decapods. Based on stable isotopes analyses, Atyid shrimps (which had a great population over the other freshwater shrimps, mollusks and fishes) reached around 85% of the epilithic biofilm (in diet) on their respective ecological niche. On the other hand, mollusks, which had a low population density compared with shrimps on these rivers, reached around 32% of the epilithic biofilm in their respective ecological niche. Therefore, the degree of impact on periphyton composition will depend on the abundance of organisms that instead depend partially or exclusively on the food availability in benthos.

Algal species richness was slightly similar among the treatments of our study; but abundances of the taxonomic groups varied accordingly to the presence or absence of both Thiarid species. In terms of major groups, we observed differences in abundance for Cyanobacteria, Bacillariophyta and Chlorophyta among the four treatments. In terms of relative abundances, although Cyanobacteria were more dominant in all the treatments but more so in TTG, Chlorophyta microalgae had an increased relative abundance in treatment MT. Nevertheless, whether higher abundance of cyanobacteria prevails with low abundance of eukaryotic microalgae needs further studies (Lefrançois *et al.*, 2010).

Anabaena sp., *Chroococcus* sp., and Microcystaceae were usually abundant ($> 10^4$ individuals/cm^2) in all the treatments with presence of snails. Pollution of freshwater caused by cyanobacteria may limit the amount of water for recreation or consumption (Chorus and

Bartram, 1999; Lefrançois *et al.*, 2010). As well, levels of cyanotoxins (i.e. MCLR) in current biofilms need to be a critical point of study in a future (Lefrançois *et al.*, 2010). Grazers (in this case, snails) may control the nutrients in the water, but at a certain point they depend on these substances to develop a primary community for subsistence.

4.3: Mean density of juveniles and external morphological traits of Thiarid snails

The establishment of Thiarid species in aquatic systems might bring a drastic change in the entire ecosystem, beginning with the primary community on benthos and, following next, the trophic chain. In the research by Moslemi *et al.* (2012), the mean population density of *T. (T.) granifera* went higher when the canopy cover was low, assuming a higher light availability that influenced the photosynthetic communities that served as the first link of food webs. In these streams of Trinidad-West Indies, places with more resources available had higher densities of *T. (T.) granifera*.

On the other hand, Facon and David (2006) described the parthenogenesis response of Thiarid snails based on obligate apomixis (very similar to asexual reproduction in plants); besides, sexual reproduction rate is low. Our results in the artificial systems showed that the number of juveniles born in one month of sampling was different among treatments; higher when both species were apart in their respective mesocosms than interacting, thus rejecting the null hypothesis of χ^2_{obs} being lower than χ^2_{tab}. The interaction, or the lack of it, between both Thiarid species could have influenced the number of born juveniles in these mesocosms. Before the 42 adult snails were set into their respective treatments, they were collected and put in different containers to acclimate them to enclosed aquatic systems. Based on concluding remarks by Facon and David (2006), aside from parthenogenesis, the success of these invasive snails in freshwater systems is mainly due to their viviparity (suggesting higher possibilities

to survive) and to their great adaptation to anthropogenic disturbances. Although no sex had been determined at the beginning of our experiment and many studies about these Thiaridae species have been focused on population dynamics. Male has a very low proportion in natural streams as was pointed by Pino *et al.* (2010) in their study at Lurin river, Peru, and by Chaniotis *et al.* (1980) in Puerto Rico. When the freshwater systems have more resources available (periphyton and chl-*a* biomass), these snails invest more energy in reproduction, obtaining higher density values, such as 152 juveniles from three blocks in TTG. Therefore, if the primary productivity rate on benthos is low when *T. (T.) granifera* and/or *M. tuberculata* colonize an aquatic environment, either of them will tend to release higher concentrations of N and P to promote the establishment of periphyton (amount of food) and, through grazing, these snails may control the algal biodiversity (and, thus, food quality). Thus, if their grazing effects become intense, snails seem to release more nutrients with time to promote or remove species related to their "preferred" primary community. In Indonesia, under eutrophic conditions *T. (T.) granifera* showed a great plasticity, having shell lengths over 2.0 cm, and high fecundity with 203 embryonic shells into the brood pouch from a single specimen (Isnaningsih *et al.*, 2017). In mesocosms or enclosed spaces, both Thiarid snails seem to be under stress conditions, in contrast with natural systems where they are acclimatized to riffles.

When primary resources are limited and the fluctuation of nutrients (as time passes) are higher, the number of born juveniles should be lower, resulting in a contrasting primary community to that where the numbers of born juveniles are higher. These behaviors suggest that adult Thiarid snails, prior to reproduction, need to be in optimal primary resources condition to give birth developed juveniles. In natural freshwater systems, food availability in zones with higher availability of light to the benthos produce a greater abundance of primary producers; in turn, the number of *T. (T.) granifera* tends to be lower in zones that have a greater

canopy cover and less availability of light (Moslemi *et al.*, 2012). All the treatments in our research were subjected to similar conditions. In MT, relative abundance of green algae was higher than in the other two treatments with snails (TTG, TTGMT). Green algae are considered as of higher quality as a food source than cyanobacteria; so, we expected to find more juveniles born in treatment MT. However, it turned to be the opposite and *T. (T.) granifera*, either alone or co-exiting with *M. tuberculata*, always released more juveniles.

Rangel *et al.* (2011) described the geographical distribution of both Thiarid snails in lakes, streams and watersheds of Tabasco, Mexico. In a lot of the samplings in which they found both species, *T. (T.) granifera* dominated over *M. tuberculata* in terms of relative abundance. Both species have the same type of reproduction and compete for similar resources, but *T. (T.) granifera* does not show a total exclusion phenomenon over *M. tuberculata*. As *T. (T.) granifera* could have been exerting a higher pressure, and since it is known to have a better adaptation to diverse aquatic conditions, it is reasonable that it would have had a higher relative abundance of born juveniles than *M. tuberculata* in our experiment. In freshwater natural systems, both species have heterogeneous distributions (De la Vega *et al.*, 2003; Facon and David, 2006; Snider and Gilliam, 2008; Miranda *et al.*, 2011).

Fenchel and Kofoed (1976), in Copenhagen, Denmark, evaluated the differences in snail size of three mud snail species (*Hydrobia ulvae*, *H. ventrosa*, and *H. neglecta* from family Hydrobiidae; all of which are parthenogenetic) in estuaries, in terms of interspecific and intraspecific competition. Their principal food habits are living diatoms and they have shown diverse forms of co-existence in different types of species combinations. The results of that study suggested significant differences in size frequency in correlation with their grazing and availability of diatoms in interspecific and intraspecific competitions. Size frequencies tended to be similar if they ate from the same substrate; similar to our results obtained with interacting

Thiarid species. If the competition is intraspecific, the number of juveniles will be low.

Temperature affects the densities of macro-invertebrates, as studied recently by Tagliarolo *et al.* (2017) with Thiaridae species, bivalves, crustaceans and dipterans. If water temperatures are close to 30ºC or above, the organisms invest more energy for metabolic purposes and reproduction rates decrease. In our study, temperature showed significant differences among samplings ($p < 0.0263$) but not among treatments within sampling periods.

Our experimental snails were measured at the beginning and after the fifth period of sampling in each block. The principal purpose was to evaluate the differences in size attributes for both species kept separate or interacting. Based on the external morphological traits described at section 2.8, the comparisons between treatments did not show significant differences in terms of growth change, except in shell length for *M. tuberculata* alone and interacting with *T. (T.) granifera*. Fenchel and Kofoed (1976) did not relate the shell size of the Hydrobiidae snails in their study, but it could be used as another parameter of intraspecific or interspecific competition. Farani *et al.* (2015) correlated the different morphological structures of *M. tuberculata* specimens, both for juveniles and adults, exposed to different salinity conditions to see their growth and survival rates. As observed, the length of shells grew in proportion to the operculum aperture size. As well, Crowl and Schnell (1990), in natural freshwater systems of Oklahoma, determined the effects of DO on snail species from the area, pointing out that oxygenated water had effects on snail size. Based on our results, both species alone, without co-existing, showed some significant positive correlations ($p < 0.05$) based on (1) shell length size vs. opercular aperture (for TTG: $r = 0.76$; MT: $r = 0.72$), (2) length size vs. spire (for TTG: $r = 0.70$; MT: $r = 0.88$), and (3) opercular aperture vs. spire (for TTG: $r = 0.36$; MT: $r = 0.46$). On the other hand, both species interacting showed significant correlation between (1) spire and shell length (for TTGMT-TTG: $r = 0.67$; TTGMT-

MT: r = 0.93) and (2) between opercular aperture and shell length (for TTGMT-TTG: r = 0.60; TTGMT-MT: r = 0.60). However, it is interesting that rate of change in shell length of *M. tuberculata* in TTGMT was larger than in *M. tuberculata* alone (MT), which seems to be an adaptation in their morphology in the presence of a competitor, such as *T. (T.) granifera*. Otherwise, further studies or more repetitions are needed to determine if the rate of change in shell length of interacting *M. tuberculata* is part of an adaptation to regulate their energy in consumption and nutrient release.

CHAPTER 5:
Concluding Remarks and Recommendations

5.1: Concluding remarks:

This research contrasts the behavior of both Thiarid species about what happens in mesocosms, in terms of nutrient release, physico-chemical parameters, reproduction, size attributes, and periphyton biomass, either when they are alone or interacting. Also, these topics go beyond clinical relevance, giving a greater importance to the ecological part, mainly focused on the relationship between nutrients and the establishment of certain species of primary producers in the periphyton. If this first link of food webs is affected, the rest of the trophic chain will be affected in a staggered way. Thiarid snails (*T. (T.) granifera* and *M. tuberculata*) are being considered as invasive species in a lot of tropical places, especially in Africa based on clinical relevance. Other animals that co-exist with one or both snail species may need to modify their food habits or become displaced to other areas in the aquatic ecosystem to guarantee their subsistence. Based on our results, *T. (T.) granifera* and *M. tuberculata* seem to differ in their nutrient release behavior and their effects on periphyton biomass. Some points that may contribute to future studies of both Thiarid species as bioindicator of water quality are the following:

- DO and pH were positively correlated to the presence of *T. (T.) granifera* alone. However, this species has been shown to have a better adaptation to abiotic factors or disturbances than *M. tuberculata*. Extreme values for both parameters are implicated with impact in natural systems. Indeed, *T. (T.) granifera* tends to colonize areas that local stream macro-fauna; thus, sometimes facing little competition.
- NO_3-N is the chemical component that seems to be mostly influenced by the presence of *T. (T.) granifera* in mesocosms (based on the analyses of nutrient released by snails

and water samples of the mesocosms). According to Wetzel (2001), NO_3-N is associated with higher chl-*a* biomass, in most of cases by nitrification of nitrogen components, such as NH_3 and NH_4^+. Mesocosms with *T. (T.) granifera* alone showed lower chlorophyll-*a* biomass with the increase of NO_3-N values; both components showed a negative lineal correlation ($p < 0.05$), where higher grazing effects might be the cause. Nitrogen levels for this treatment were associated to more proliferation of species of cyanobacteria. Besides, according to Pointier *et al.* (2005) *M. tuberculata* does not show association with the abiotic factors under *in-situ* conditions; but in this study, chl-*a* concentration was correlated with TKN in presence of this snail.

- Both Thiarid snails may control the abundance of periphyton community. More species of cyanobacteria proliferated in presence of *T. (T.) granifera*, specifically those that may injure the digestive tract of aquatic macro-fauna or even humans (i.e. *Anabaena* sp., *Chroococcus* sp. and Microcystaceae, among others). Despite of the differences in abundance, periphyton did not show differences in biodiversity (H') and species composition (J') (regardless of the presence of one or both Thiarid species at their respective treatments).
    - The growth of Chlorophyta species, as primary food source, were higher in presence of *M. tuberculata*. This snail species exerted more pressure on the abundance of that microbial group. Therefore, based on the results, the effect of the grazing pressure of *T. (T.) granifera* appears to be different from that of *M. tuberculata*.
    - In presence of *M. tuberculata*, concentrations of chl-*a* were higher and related to TKN concentration (in a positive lineal correlation). Colonial Chlorophyceae were strongly associated with the presence of this snail

species. Whether this snail may promote more food source due to nutrient release or to its grazing pressure (lower than that of *T. (T.) granifera*) needs further study.

- Density of juveniles was higher for *T. (T.) granifera*, either alone and co-existing with *M. tuberculata*; although the number of juveniles released in the interaction treatment was lower than the one released from species alone. Interspecific competition might play an important role in the ecology of both snails; if *T. (T.) granifera* needs more food resources (independent of its plasticity), the presence of *M. tuberculata* compensate this fact and both species could invest more energy in metabolism than in the development of juveniles. In natural systems, *T. (T.) granifera* seems not to cause a complete displacement of *M. tuberculata*, as pointed out by Rangel *et al.* (2011).
- Changes in shell length were larger for *M. tuberculata* interacting with *T. (T.) granifera* than the species (*M. tuberculata*) alone. This could be one mechanism that *M. tuberculata* employs to avoid the exclusion or displacement in presence of *T. (T.) granifera*. This shell trait increased disproportionally to other external morphological traits (i.e.: opercular aperture and spire). Further studies are required to determine if changes in shell length are another mechanism to co-exist and avoid displacement by *T. (T.) granifera.*

5.2: Recommendations

- In a future study, conducted in natural systems, it will be important to select different zones where *T. (T.) granifera* and *M. tuberculata* will be alone or together. This will provide a better understanding about their ecology, in terms of nutrient cycling, effects

on primary productivity, and their behavior in natural systems. In observational terms, both species are abundant in a lot of water bodies of Puerto Rico. Some research papers and theses mention that these exotic freshwater snails have an impact in food webs and are also of clinical relevance, but none of these investigations have addressed the degree of impact on trophic chains and water quality in Puerto Rico.

- One limitation of this experiment was the water contained and constantly restored of into the mesocosms. Both Thiarid snails, in natural freshwater systems, are more abundant in riffles. For instance, PVC tubes for the design of artificial freshwater systems will be better to repeat this experiment. Also, snail measurements should be performed *in-situ* in each period of sampling. If calcium levels are low, shells will tend to erode; some of the snails measured at the last period of sampling had their shell apex cracked. Therefore, it is suggested to include calcium content analyses. Other external morphological traits that were not considered in our study (i.e. opercular aperture width and length of the last spin of the shell) should be explored too.
- The use of another water source, apart from Quebrada de Oro, will be helpful for further studies in the ecology of these Thiarid snails. The proliferation of more Cyanobacteria than other taxa major groups, including in the control treatment, suggest that these producers were already abundant in the water source.
- Future studies must include Puerto Rico as a critical point to study Thiarid snails. Facon and David (2006) described in detail the morphs and phylogenies of 40 populations world-wide of *M. tuberculata* (in South Asia, Africa, Cuba, southern North America, and Central and South America, but did not include Puerto Rico).
- A repetition of this experiment by block design should be conducted at the same time

to avoid bias in terms of the abundance of periphyton major groups. Due to limit of space and materials, the experiment was divided into 4 blocks and 4 samplings seasons (4 different months).

- Evaluate the periphyton composition in the shells of both species and relate it with natural substrates. Filamentous algae use shells as an additional substrate to develop a primary community and use snails as a transportation mechanism in freshwater systems.
- Laboratory techniques, such as stable isotopes (C_{13} and N_{15}), will serve as additional methods to evaluate gut content in both snail species.
- Determine if cyanobacteria will serve as a food source for these Thiarid snails, without damaging their digestive tract. Further studies need to be performed to evaluate this possibility. However, neglecting the block 4 (and 4rep.), a few specimens from blocks 1 to 3 were replaced due to death.
- MCLR (Microcystin-LR) analysis for snails and other macroinvertebrates that share the same ecological niche with one or both Thiarid species will provide information about the clinical relevance of this toxin in Puerto Rico.
- Evaluate the use of other pigments, such as chlorophyll-*b*, chlorophyll-*c*, zeaxanthin, fucoxanthin and alloxanthin, as diagnostic pigments of microalgae present in periphyton.
- The identification of snails (and other invasive macro-invertebrates species), as well as the determination of how nutrient cycling influences the algal content in every trophic status (oligotrophic, mesotrophic or eutrophic), in terms of N/P concentrations, will serve to understand one important part of the ecological complexity and the

impacts that these will create on fauna, flora and mankind (Vila and García-Berthou, 2010). Furthermore, the rates of spread of invasive species in freshwater habitats, compared to those in marine and terrestrial habitats are poorly known.

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6. APPENDICES:

Appendix 6.1: A. Summary of parameters measured *in-situ* along the periods of sampling per treatment (pH, DO, temperature and turbidity); **B.** ANOVA of parameters measured *in-situ*, comparing the treatments, periods of sampling and blocks; **C**. Pearson's correlations based on the values obtained from the variables of study (temperature, pH, dissolved oxygen (DO) and turbidity) per treatment.

A.

Treat.	Variable	n	Mean	D.E.	Var(n-1)	Var(n)	S.E.	CV	Mín	Máx
C	X Temp.	5	26.90	0.87	0.75	0.60	0.39	3.23	26.10	28.20
C	X pH	5	7.23	0.11	0.01	0.01	0.05	1.54	7.11	7.35
C	X DO	5	5.38	0.19	0.04	0.03	0.09	3.60	5.14	5.59
C	X turb.	5	0.21	0.12	0.01	0.01	0.05	56.92	0.04	0.34
MT	X Temp.	5	27.94	0.94	0.89	0.71	0.42	3.37	26.80	29.00
MT	X pH	5	7.30	0.13	0.02	0.01	0.06	1.81	7.18	7.51
MT	X DO	5	5.49	0.20	0.04	0.03	0.09	3.58	5.26	5.68
MT	X turb.	5	0.06	0.05	2.3E-03	1.9E-03	0.02	76.88	0.00	0.13
TTG	X Temp.	5	27.06	0.86	0.73	0.59	0.38	3.16	26.20	28.30
TTG	X pH	5	7.26	0.13	0.02	0.01	0.06	1.81	7.09	7.39
TTG	X DO	5	5.31	0.12	0.01	0.01	0.05	2.22	5.18	5.48
TTG	X turb.	5	0.11	0.19	0.04	0.03	0.08	175.58	0.01	0.45
TTGMT	X Temp.	5	27.64	0.91	0.83	0.67	0.41	3.30	26.60	28.70
TTGMT	X pH	5	7.24	0.14	0.02	0.02	0.06	1.98	7.08	7.45
TTGMT	X DO	5	5.34	0.21	0.05	0.04	0.10	3.99	5.01	5.55
TTGMT	X turb.	5	0.17	0.10	0.01	0.01	0.04	58.56	0.05	0.28

B.

Analysis of variance

Temperature (°C)

Variable	N	R^2	Adj R^2	CV
Temperature (C)	80	0.52	0.00	7.22

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	90.78	58	1.57	0.40	0.9972
Treat.	8.74	3	2.91	0.74	0.5421
Block	15.21	3	5.07	1.28	0.3065
Per. Sampling	54.24	4	13.56	3.43	0.0263
Treat.*Per. sampling	3.98	12	0.33	0.08	>0.9999
Treat.*Block*Per. sampling..	8.62	36	0.24	0.06	>0.9999
Error	83.07	21	3.96		
Total	173.85	79			

Test:Bonferroni Alpha:=0.05 LSD:=2.20461

Error: 3.9557 df: 21

Per. sampling	Means	n	S.E.		
5	26.52	16	0.50	A	
3	26.88	16	0.50	A	B
2	27.31	16	0.50	A	B
1	28.23	16	0.50	A	B
4	28.74	16	0.50		B

pH**

Variable	N	R^2	Adj R^2	CV
pH	76	0.57	0.00	4.97

Analysis of variance table (Sequential SS)

S.V.	SS	df	MS	F	p-value
Model.	2.93	58	0.05	0.39	0.9962
Treat.	0.04	3	0.01	0.10	0.9563
Block	1.53	3	0.51	3.91	0.0273
Per. sampling	1.06	4	0.27	2.04	0.1338
Treat.*Per. sampling	0.11	12	0.01	0.07	>0.9999
Treat.*Block *Per. sampling.	0.19	36	0.01	0.04	>0.9999
Error	2.22	17	0.13		
Total	5.14	75			

Test:Bonferroni Alpha:=0.05 LSD:=0.35116

Error: 0.1303 df: 17

Block	Means	n	S.E.		
4	7.04	20	0.08	A	
2	7.31	20	0.08	A	B
3	7.34	20	0.08	A	B
1	7.42	16	0.09		B

DO (mg/L)

Variable	N	R^2	Adj R^2	CV
DO (mg/L)	72	0.63	0.00	10.00

Analysis of variance table (Sequential SS)

S.V.	SS	df	MS	F	p-value
Model.	7.85	55	0.14	0.49	0.9741
Treat.	0.59	3	0.20	0.67	0.5818
Block	0.94	3	0.31	1.08	0.3859
Per. sampling	1.16	4	0.29	0.99	0.4406
Treat.*Per. sampling	1.23	12	0.10	0.35	0.9632
Treat.*Block *Per. sampling..	3.93	33	0.12	0.41	0.9855
Error	4.67	16	0.29		
Total	12.51	71			

Turb. (NTU)

Variable	N	R^2	Adj R^2	CV
Turb. (NTU)	80	0.57	0.00	320.56

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	4.63	58	0.08	0.47	0.9866
Treat.	0.29	3	0.10	0.57	0.6380
Block	1.60	3	0.53	3.18	0.0851
Per. sampling	0.46	4	0.12	0.68	0.6105
Treat.*Per. sampling	0.75	12	0.06	0.37	0.9595
Treat.*Block*Per. sampling..	1.52	36	0.04	0.25	0.9999
Error	3.53	21	0.17		
Total	8.16	79			

** Values of H_3O^+ concentrations were not recognized by the Info Stat program in a parametric ANOVA due to such low values. pH values were used to determine the statistical difference in treatments and sampling periods.

C.

Correlation coefficients

Treatment = c

Variable(1)	Variable(2)	n	Pearson	p-value
H3O (M)	DO (mg/L)	23	-0.29	0.1870
H3O (M)	Turb. (NTU)	24	0.25	0.2392
H3O (M)	Temperature (C)	24	-0.10	0.6552
DO (mg/L)	Turb. (NTU)	23	3.5E-03	0.9875
DO (mg/L)	Temperature (C)	23	-0.05	0.8350
Turb. (NTU)	Temperature (C)	25	0.19	0.3673

Treatment = MT

Variable(1)	Variable(2)	n	Pearson	p-value
H3O (M)	DO (mg/L)	18	-0.32	0.1884
H3O (M)	Turb. (NTU)	19	0.35	0.1466
H3O (M)	Temperature (C)	19	0.03	0.9148
DO (mg/L)	Turb. (NTU)	18	0.01	0.9829
DO (mg/L)	Temperature (C)	18	-0.24	0.3299
Turb. (NTU)	Temperature (C)	20	-0.15	0.5284

Treatment = TTG

Variable(1)	Variable(2)	n	Pearson	p-value
H3O (M)	DO (mg/L)	18	-0.63	0.0051
H3O (M)	Turb. (NTU)	19	0.22	0.3567
H3O (M)	Temperature (C)	19	-0.38	0.1096
DO (mg/L)	Turb. (NTU)	18	-0.33	0.1783
DO (mg/L)	Temperature (C)	18	0.24	0.3466
Turb. (NTU)	Temperature (C)	20	-0.18	0.4417

Treatment = TTGMT

Variable(1)	Variable(2)	n	Pearson	p-value
H3O (M)	DO (mg/L)	18	-0.26	0.3070
H3O (M)	Turb. (NTU)	19	0.45	0.0558
H3O (M)	Temperature (C)	19	0.12	0.6255
DO (mg/L)	Turb. (NTU)	18	-0.07	0.7753
DO (mg/L)	Temperature (C)	18	0.28	0.2559
Turb. (NTU)	Temperature (C)	20	-0.20	0.3939

Appendix 6.2: ANOVA from B1 to B4 (and B4 rep.) of TKN, NO_3-N, TN and TP values of the snail's nutrient release and the comparison of treatments per dates of sampling.

TKN

Variable	N	R^2	Adj R^2	CV
TKN	64	0.88	0.45	60.25

Analysis of variance table (Sequential SS)

S.V.	SS	df	MS	F	p-value
Model.	9.91	49	0.20	2.05	0.0711
Treat/Snailsp.	0.86	3	0.29	2.91	0.0718
Block	3.87	4	0.97	9.82	0.0005
Per sampling	0.18	3	0.06	0.62	0.6137
Treat/Snailsp.*Per samplin..	0.93	9	0.10	1.05	0.4491
Treat/Snailsp.*Block*Per s..	4.06	30	0.14	1.37	0.2699
Error	1.38	14	0.10		
Total	11.29	63			

Test:Bonferroni Alpha:=0.05 LSD:=0.47665

Error: 0.0986 df: 14

Block	Means	n	S.E.		
1	0.33	16	0.10	A	
2	0.47	16	0.10	A	
3	0.49	16	0.10	A	
4	0.55	12	0.12	A	
4rep	1.51	4	0.21		B

NO3-N

Variable	N	R^2	Adj R^2	CV
NO3-N	64	0.82	0.18	69.64

Analysis of variance table (Sequential SS)

S.V.	SS	df	MS	F	p-value
Model.	13.52	49	0.28	1.28	0.3144
Treat/Snailsp.	1.19	3	0.40	1.84	0.1861
Block	2.56	4	0.64	2.98	0.0566
Per sampling	6.84	3	2.28	10.60	0.0007
Treat/Snailsp.*Per samplin..	0.32	9	0.04	0.16	0.9951
Treat/Snailsp.*Block*Per s..	2.62	30	0.09	0.41	0.9812
Error	3.01	14	0.22		
Total	16.53	63			

Test:Bonferroni Alpha:=0.05 LSD:=0.50315

Error: 0.2151 df: 14

Per sampling	Means	n	S.E.		
2	0.28	16	0.15	A	
3	0.48	16	0.15	A	
4	0.75	16	0.15	A	B
5	1.15	16	0.15		B

TN

Variable	N	R^2	Adj R^2	CV
TN	64	0.91	0.60	33.71

Analysis of variance table (Sequential SS)

S.V.	SS	df	MS	F	p-value
Model.	23.27	49	0.47	2.97	0.0148
Treat/Snailsp.	4.02	3	1.34	8.37	0.0020
Block	3.40	4	0.85	5.30	0.0082
Per sampling	7.45	3	2.48	15.52	0.0001
Treat/Snailsp.*Per samplin..	0.76	9	0.08	0.53	0.8315
Treat/Snailsp.*Block*Per s..	7.64	30	0.25	1.59	0.1799
Error	2.24	14	0.16		
Total	25.51	63			

Test:Bonferroni Alpha:=0.05 LSD:=0.43415

Error: 0.1601 df: 14

Treat/Snailsp.	Means	n	S.E.			
MT	0.90	16	0.13	A		
TTGMT-MT	1.10	16	0.13	A		
TTGMT-TTG	1.16	16	0.13	A	B	
TTG	1.59	16	0.13		B	

Test:Bonferroni Alpha:=0.05 LSD:=0.60741

Error: 0.1601 df: 14

Block	Means	n	S.E.			
1	0.93	16	0.13	A		
4	0.94	12	0.15	A	B	
3	1.16	16	0.13	A	B	
2	1.44	16	0.13		B	C
4rep	2.04	4	0.26			C

Test:Bonferroni Alpha:=0.05 LSD:=0.43415

Error: 0.1601 df: 14

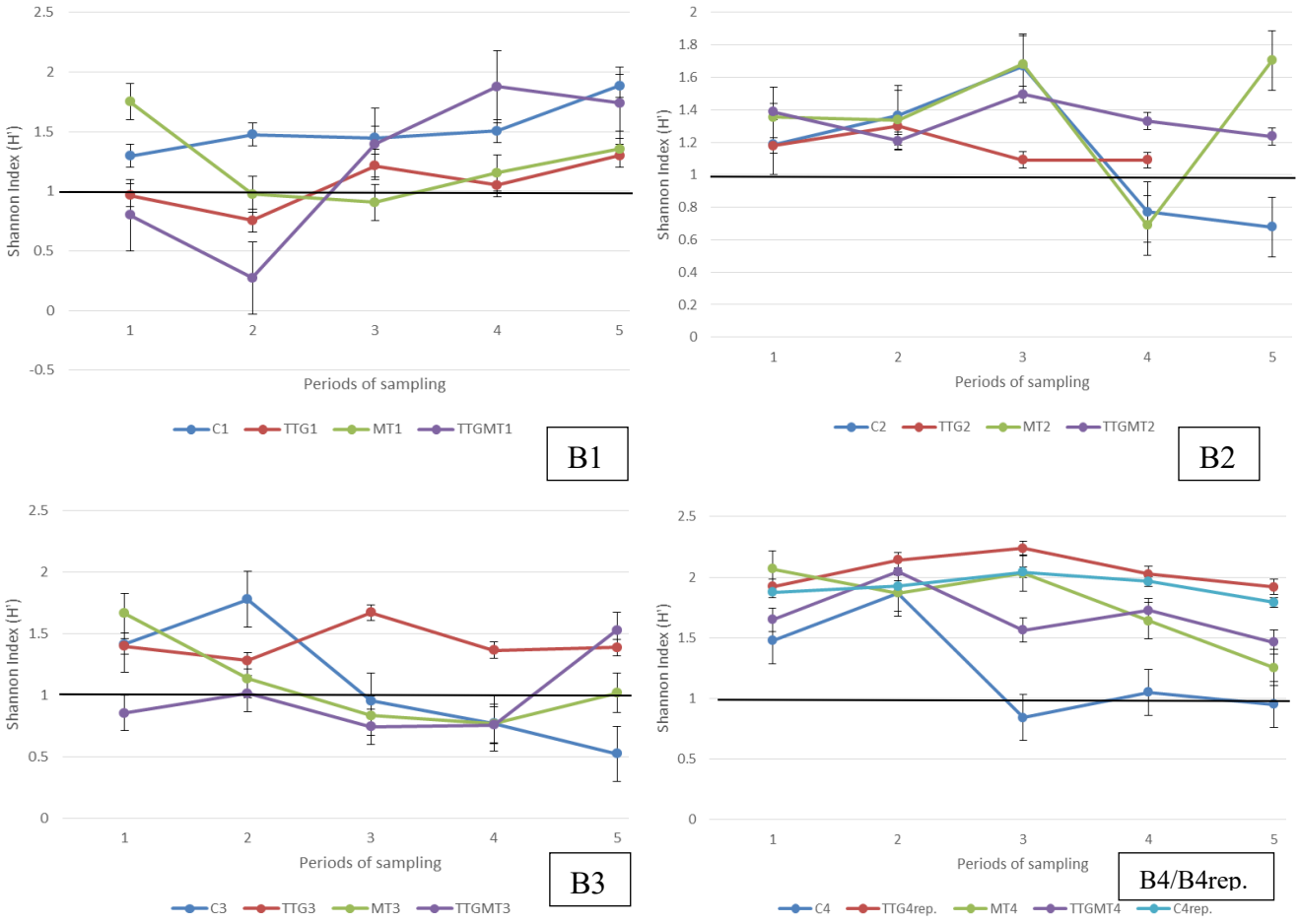
Per sampling	Means	n	S.E.		
2	0.85	16	0.13	A	
3	0.92	16	0.13	A	
4	1.27	16	0.13	A	B
5	1.71	16	0.13		B

TP

Variable	N	R^2	Adj R^2	CV
TP	64	0.76	0.00	73.66

Analysis of variance table (Sequential SS)

S.V.	SS	df	MS	F	p-value
Model.	0.03	49	5.5E-04	0.93	0.6004
Treat/Snailsp.	1.2E-03	3	4.0E-04	0.66	0.5876
Block	1.7E-03	4	4.3E-04	0.72	0.5949
Per sampling	4.8E-04	3	1.6E-04	0.27	0.8480
Treat/Snailsp.*Per samplin..	0.01	9	8.6E-04	1.44	0.2622
Treat/Snailsp.*Block*Per s..	0.02	30	5.3E-04	0.90	0.6160
Error	0.01	14	6.0E-04		
Total	0.04	63			



Appendix 6.3: Shannon-Weaver diversity indexes for all the samples obtained per treatment, separated by blocks. Bars in each point indicate the standard error (S.E). Note that 5th period of sampling of TTG-B2 is the unique sample that were not considered for this analysis due to a very low account of periphyton.

Appendix 6.4: ANOVA from B1 to B4 (and B4 rep.) of TKN, NO3-N, TP and TN values of water samples of each mesocosm and the comparison of treatments per dates of sampling.

TKN.

Variable	N	R^2	Adj R^2	CV
TKN	80	0.57	0.00	84.17

Analysis of variance table (Sequential SS)

S.V.	SS	df	MS	F	p-value
Model.	1.92	58	0.03	0.48	0.9844
Treatment	0.04	3	0.01	0.20	0.8959
Period of sampling	0.10	4	0.03	0.37	0.8304
Block	0.23	3	0.08	1.13	0.3581
Treatment*Period of sampli..	0.31	12	0.03	0.37	0.9591
Treatment*Period of sampli*B	1.24	36	0.03	0.50	0.9654
Error	1.44	21	0.07		
Total	3.36	79			

NO3-N

Variable	N	R^2	Adj R^2	CV
NO3-N	80	0.72	0.00	95.43

Analysis of variance table (Sequential SS)

S.V.	SS	df	MS	F	p-value
Model.	13.46	58	0.23	0.93	0.6000
Treatment	1.41	3	0.47	1.88	0.1635
Period of sampling	6.65	4	1.66	6.67	0.0013
Block	2.85	3	0.95	3.81	0.0253
Treatment*Period of sampli..	0.85	12	0.07	0.28	0.9857
Treatment*Period of sampli.B	1.71	36	0.05	0.19	>0.9999
Error	5.23	21	0.25		
Total	18.70	79			

Test:Bonferroni Alpha:=0.05
LSD:=0.55333
Error: 0.2492 df: 21

Period of sampling	Means	n	S.E.		
2	0.25	16	0.12	A	
1	0.26	16	0.12	A	
3	0.41	16	0.12	A	
4	0.67	16	0.12	A	B
5	1.01	16	0.12		B

Test:Bonferroni Alpha:=0.05
LSD:=0.45969
Error: 0.2492 df: 21

Block	Means	n	S.E.		
4	0.29	20	0.11	A	
1	0.41	20	0.11	A	B
3	0.60	20	0.11	A	B
2	0.79	20	0.11		B

TP

Variable	N	R^2	Adj R^2	CV
TP	77	0.81	0.19	79.17

Analysis of variance table (Sequential SS)

S.V.	SS	df	MS	F	p-value
Model.	0.04	58	6.2E-04	1.31	0.2707
Treatment	1.6E-03	3	5.3E-04	1.12	0.3669
Period of sampling	2.8E-03	4	6.9E-04	1.47	0.2520
Block	3.3E-03	3	1.1E-03	2.37	0.1048
Treatment*Period of sampli..	0.01	12	4.2E-04	0.90	0.5661
Treatment*Period of sampli..	0.02	36	6.4E-04	1.35	0.2511
Error	0.01	18	4.7E-04		
Total	0.04	76			

TN

Variable	N	R^2	Adj R^2	CV
TN	80	0.62	0.00	77.56

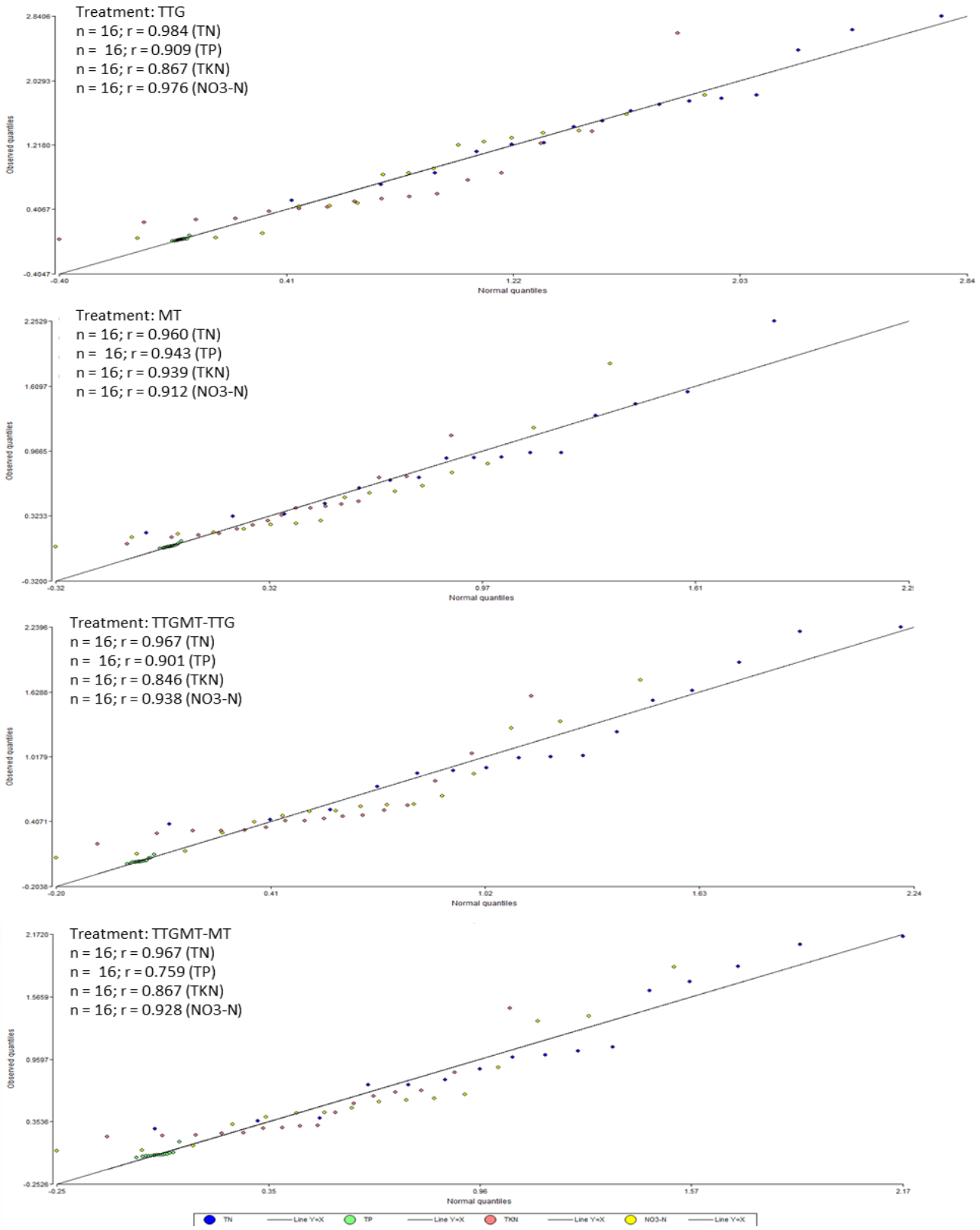
Analysis of variance table (Sequential SS)

S.V.	SS	df	MS	F	p-value
Model.	14.44	58	0.25	0.60	0.9383
Treatment	1.58	3	0.53	1.26	0.3145
Period of sampling	5.54	4	1.38	3.31	0.0299
Block	2.32	3	0.77	1.85	0.1699
Treatment*Period of sampli..	1.66	12	0.14	0.33	0.9737
Treatment*Period of sampli..	3.34	36	0.09	0.22	>0.9999
Error	8.79	21	0.42		
Total	23.23	79			

Test:Bonferroni Alpha:=0.05 LSD:=0.71696

Error: 0.4184 df: 21

Period of sampling	Means	n	S.E.		
1	0.55	16	0.16	A	
2	0.62	16	0.16	A	B
3	0.74	16	0.16	A	B
4	0.98	16	0.16	A	B
5	1.27	16	0.16		B



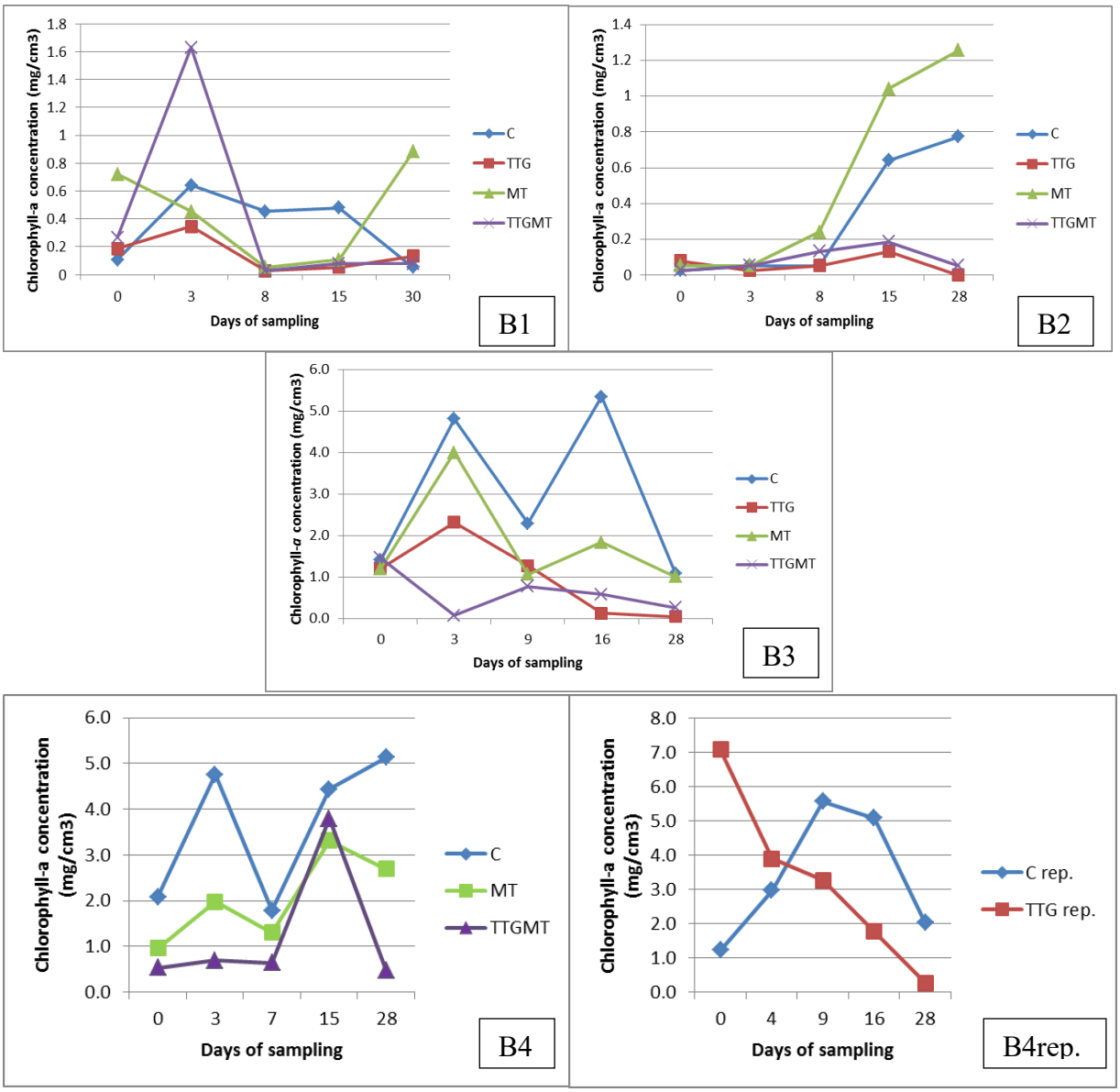
Appendix 6.5: Q-Q Plots, showing the normal distribution of nutrient release assays (TN, TKN, NO_3-N and TP) for the snails, separated by treatment.

Appendix 6.6: T-test of change in snails' dimensions between first and last period of sampling (length, spire and operculum aperture).

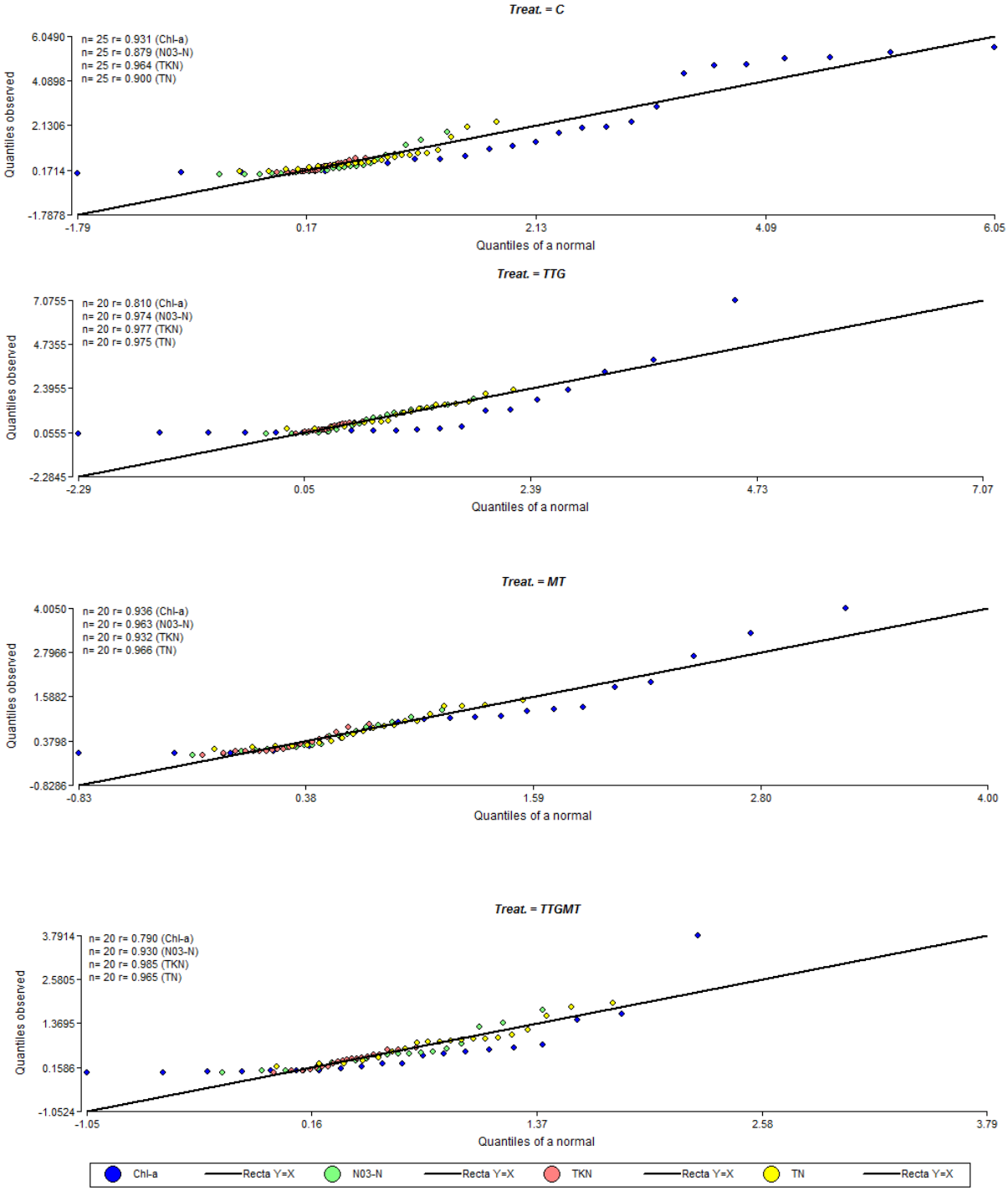
Clasific	Variable	Group 1	Group 2	n(1)	n(2)	LI(95)	LS(95)	pHomVar	T	df	p-value
Treatment*Species	R.C Length	{MT:M. tuberculata}	{TTGMT:M. tuberculata}	3	2	-0.18	-0.02	0.5941	-3.85	3	0.0309
Treatment*Species	R.C Length	{TTG:T. granifera}	{TTGMT:T. granifera}	3	4	-0.08	0.14	0.1830	0.73	5	0.4961

Clasific	Variable	Group 1	Group 2	n(1)	n(2)	LI(95)	LS(95)	pHomVar	T	dfp-value
Treatment*Species	R.C. Spire	{MT:M. tuberculata}	{TTGMT:M. tuberculata}	3	2	-0.24	0.09	0.7043	-1.45	3 0.2433
Treatment*Species	R.C. Spire	{TTG:T. granifera}	{TTGMT:T. granifera}	3	4	-0.01	0.06	0.8740	2.04	5 0.0970

Clasific	Variable	Group 1	Group 2	n(1)	n(2)	LI(95)	LS(95)	pHomVar	T	df	p-value
Treatment*Species	R.C Operc	{MT:M. tuberculata}	{TTGMT:M. tuberculata}	3	2	-0.14	0.05	0.4677	-1.60	3	0.2081
Treatment*Species	R.C Operc	{TTG:T. granifera}	{TTGMT:T. granifera}	3	4	-0.04	0.14	0.8233	1.35	5	0.2345



Appendix 6.7: Chlorophyll-*a* concentration obtained during the five periods of sampling, from B1 to B4 (and B4rep.), for all the treatments of study (Blue = C; Red = TTG; Green = MT; Purple = TTGMT).



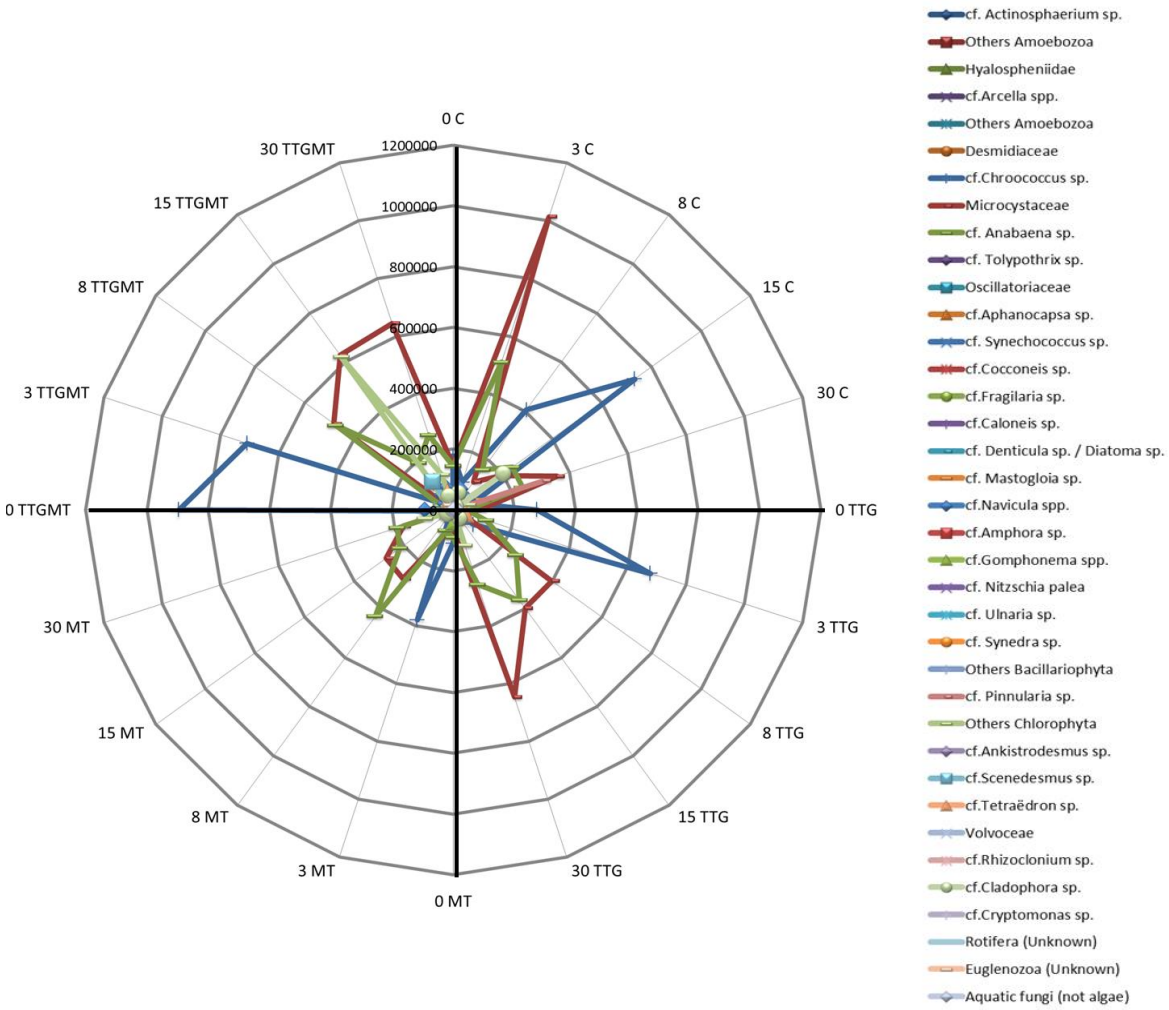
Appendix 6.8: Q-Q plots, showing the normal distribution of chl-*a* with chemical compounds (TN, TKN, NO_3-N and TP) separated by treatments.

A

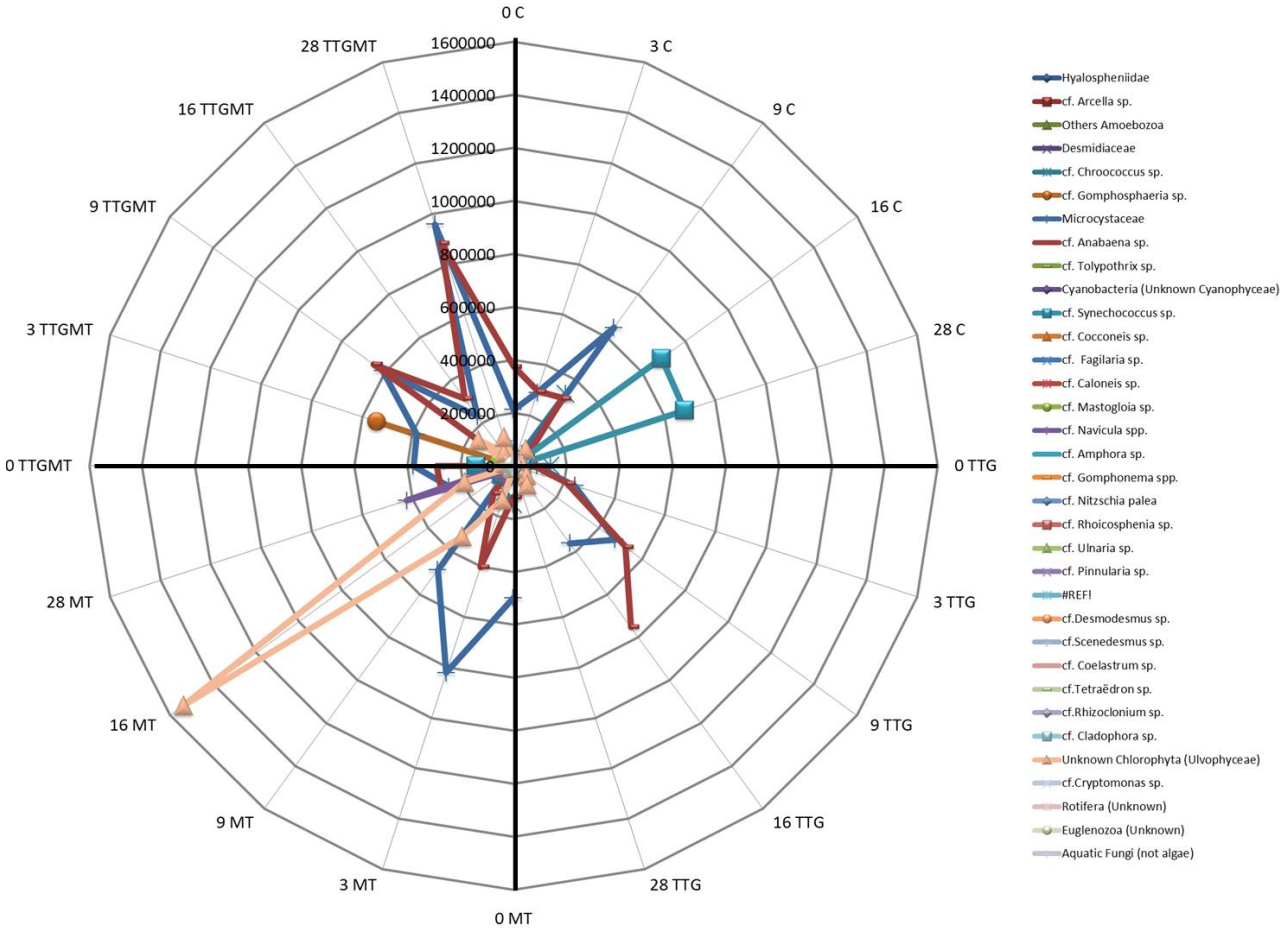
B

C

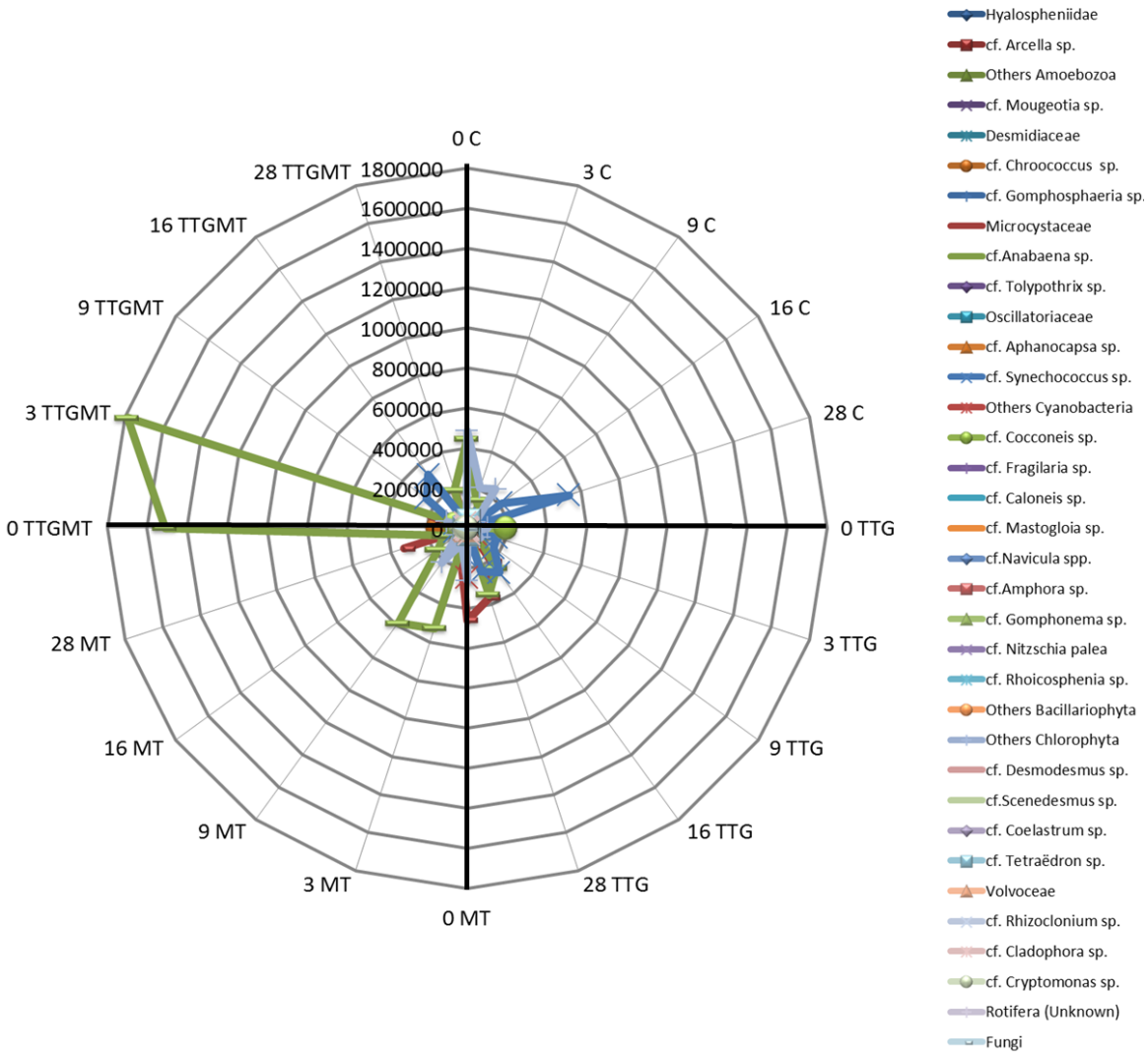
Appendix 6.9: **A.** Picture of experimental design of mesocosms; **B.** Picture of procedure for chl-*a* extraction and taxa analyses of periphyton, scrapping 4 randomly tiles **C.** Picture of nutrient release assays for Thiarid snails



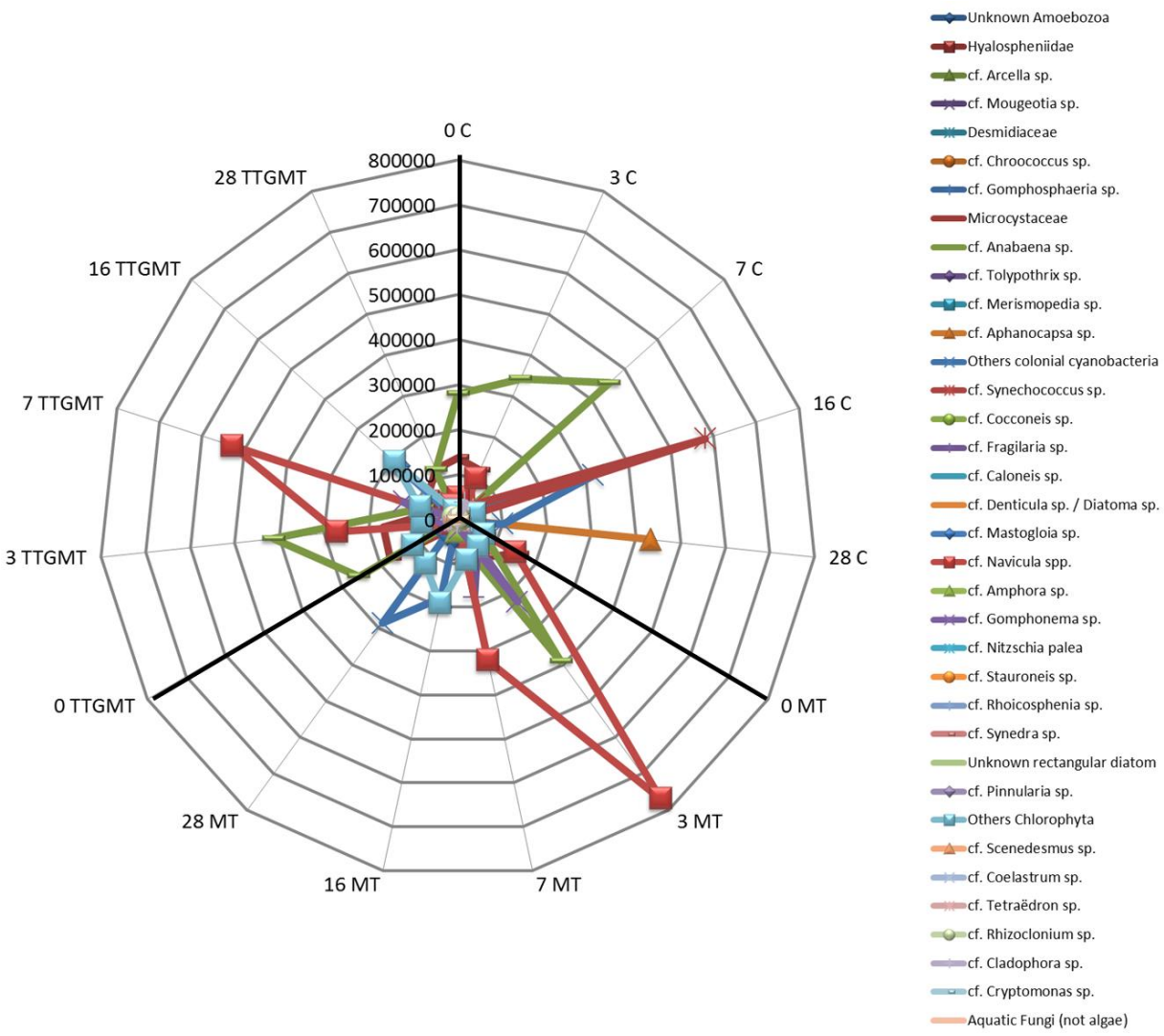
Appendix 6.10: Taxonomic composition and abundance of periphyton found per treatments and periods of sampling at B1 (ind./cm^2) [Right-upper corner = C; Right-inferior corner = TTG; Left-inferior corner = MT; Left-upper corner = TTGMT]



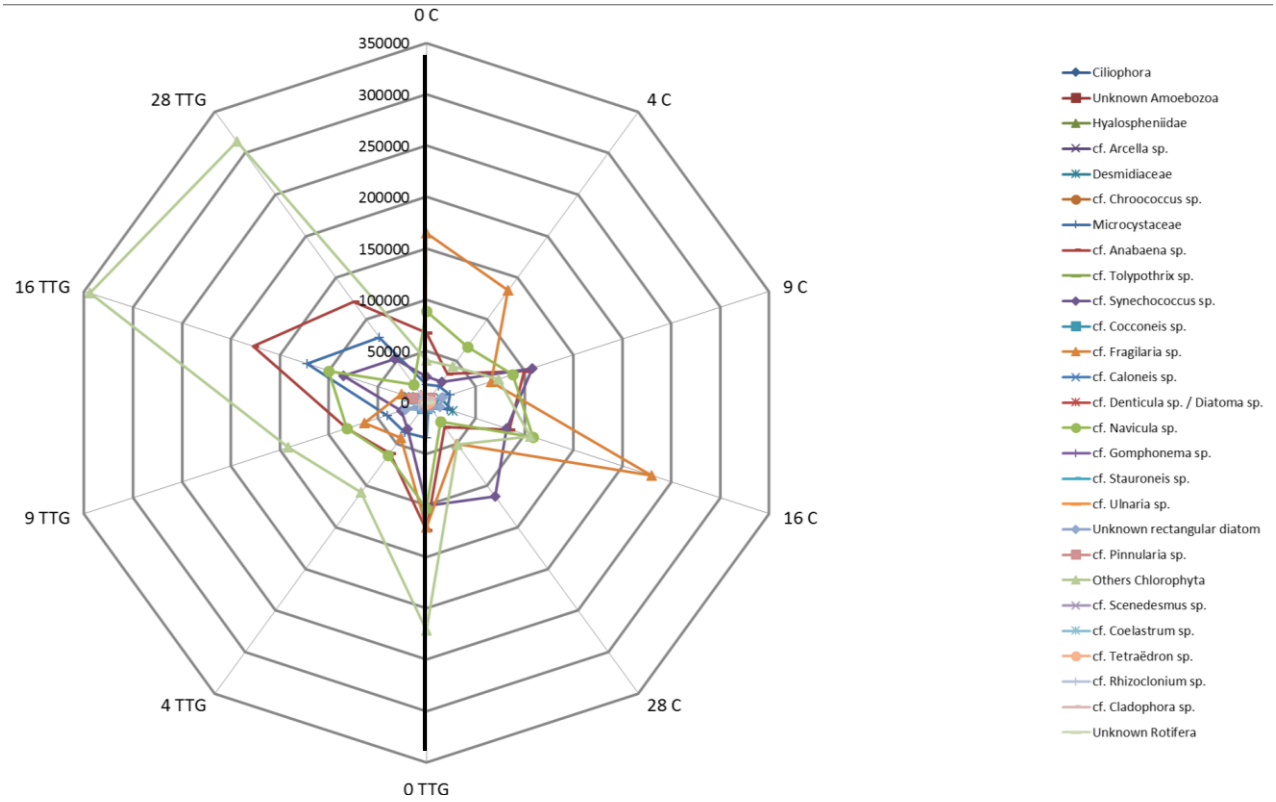
Appendix 6.11: Taxonomic composition and abundance of periphyton found per treatments and periods of sampling at B2 (ind./cm^2) [Right-upper corner = C; Right-inferior corner = TTG; Left-inferior corner = MT; Left-upper corner = TTGMT]



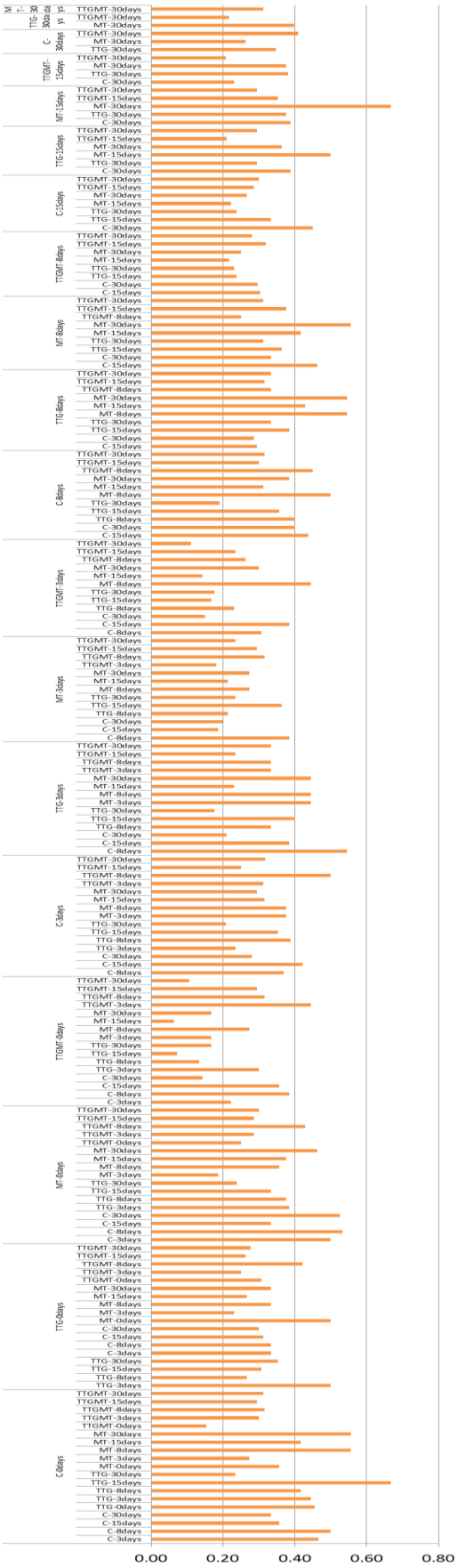
Appendix 6.12: Taxonomic composition and abundance of periphyton found per treatments and periods of sampling at B3 (ind./cm^2) [Right-upper corner = C; Right-inferior corner = TTG; Left-inferior corner = MT; Left-upper corner = TTGMT]



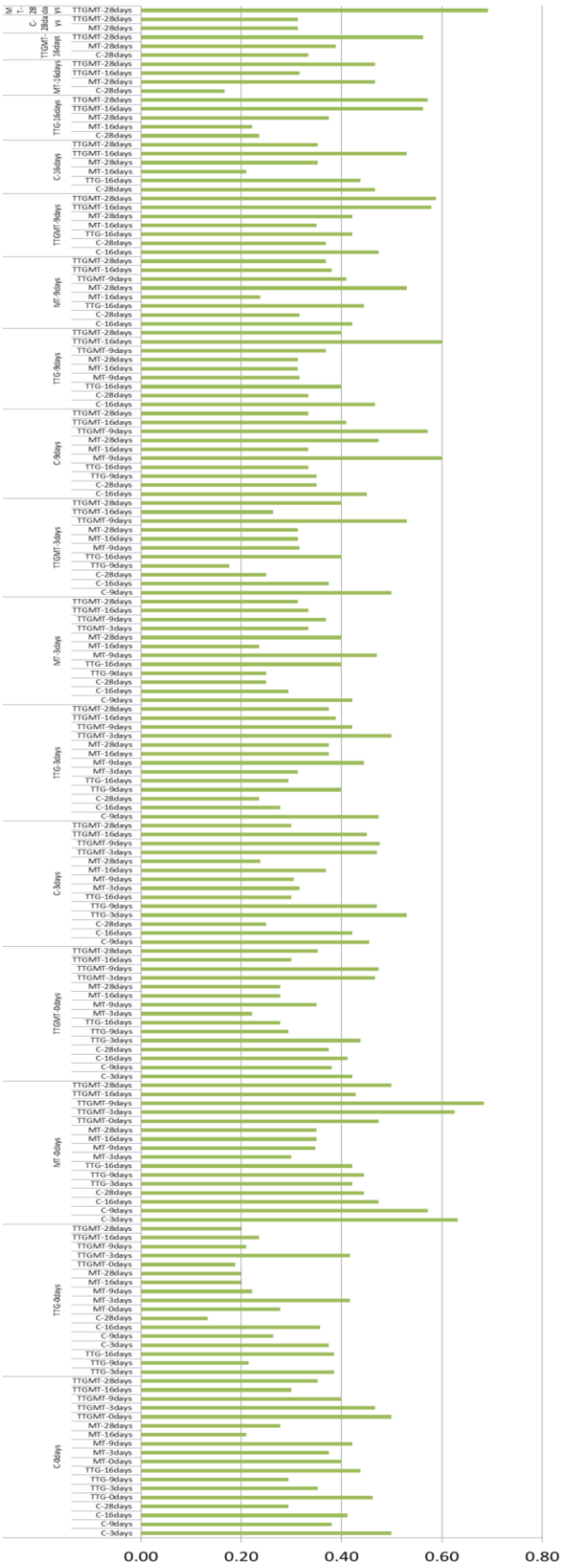
Appendix 6.13: Taxonomic composition and abundance of periphyton found per treatments and periods of sampling at B4 (ind./cm^2) [Right-upper corner = C; Inferior corner = MT; Left-upper corner = TTGMT]



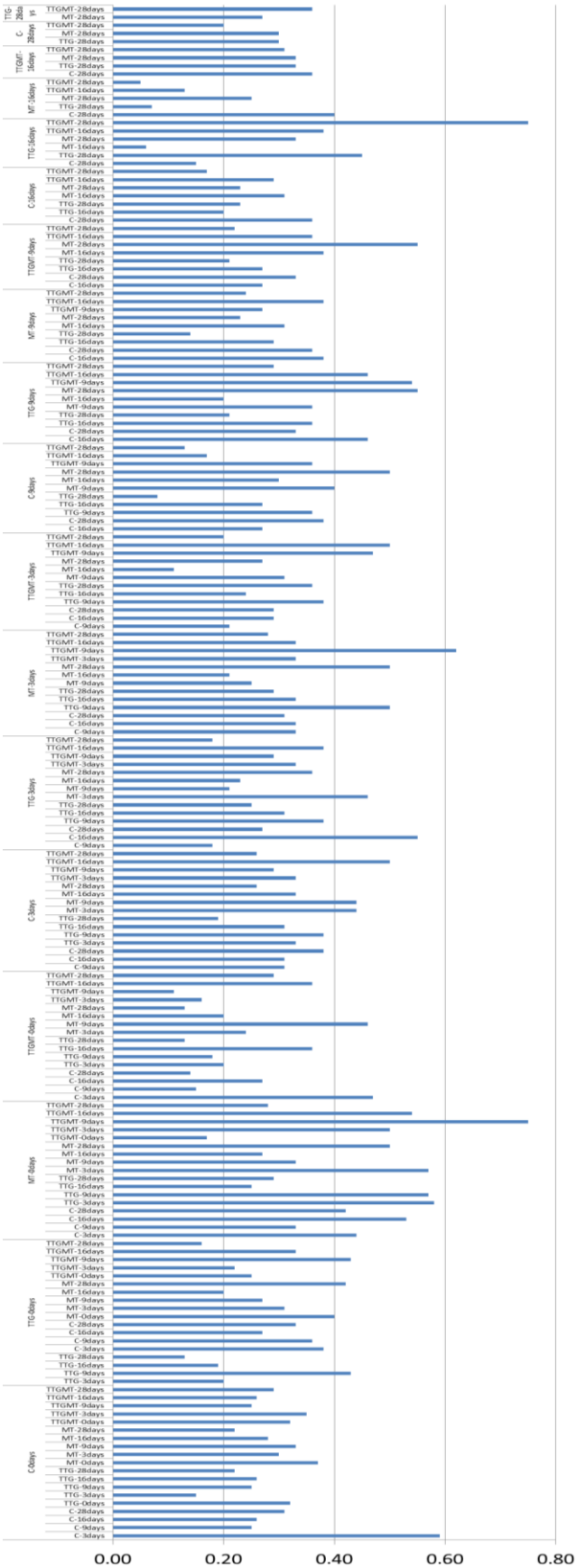
Appendix 6.14: Taxonomic composition and abundance of periphyton found per treatments and periods of sampling t B4rep. (ind./ml) [Left = TTG; Right = Control]



Appendix 6.15: Jaccard indexes of all the comparisons between samples per treatment and periods of sampling at B1.



Appendix 6.16: Jaccard indexes of all the comparisons between samples per treatment and periods of sampling at B2. TTG-28days was not take into account for this analysis.



Appendix 6.17: Jaccard indexes of all the comparisons between samples per treatment and periods of sampling at B3.

Appendix 6.18: List of all the taxonomic groups found in the periphyton samples per treatment [C, TTG, MT, TTGMT].

Taxa Phylum/ Kingdom	Class	Family	Genus/Species	Treatment levels			
				C	TTG	MT	TTGMT
Ciliophora			Unknown	X			
Ochrophyta	Marista	Actinophrydae	cf. *Actinosphaerium sp.*	X	X		X
Amoebozoa			Unknown (water drop-like)	X	X	X	X
Amoebozoa	Tubulinea	Hyalospheniidae	Unknown	X	X	X	X
Amoebozoa	Tubulinea	Arcellinidae	cf. *Arcella sp.*	X	X	X	X
Charophyta	Conjugatophycea	Zygnemataceae	cf. *Mougeotia* sp.				X
Charophyta	Conjugatophyceae	Desmidiaceae	Unknown	X	X	X	X
Cyanobacteria	Cyanophyceae	Chroococcaceae	cf. *Chroococcus* sp.	X	X	X	X
Cyanobacteria	Cyanophyceae	Gomphosphaeriaceae	cf. *Gomphosphaeria sp.*		X		X
Cyanobacteria	Cyanophyceae	Microcystaceae	cf. *Anacystis* sp.	X	X	X	X
Cyanobacteria	Cyanophyceae	Nostocaceae	cf. *Anabaena* spp.	X	X	X	X
Cyanobacteria	Cyanophyceae	Tolypothrichaceae	cf. *Tolypothrix* sp.	X	X	X	X
Cyanobacteria	Cyanophyceae	Oscillatoriaceae	cf. *Phormidium*	X		X	X
Cyanobacteria	Cyanophyceae	Merismopediaceae	cf. *Merismopedia sp.*	X			
Cyanobacteria	Cyanophyceae	Merismopediaceae	cf. *Aphanocapsa sp.*		X	X	X
Cyanobacteria	Cyanophyceae	Nostocaceae	Unknown large cyanobacteria (hair-like with heterocysts)	X	X	X	X
Cyanobacteria	Cyanophyceae		Unknown colonial rounded cyanobacteria	X	X	X	X
Cyanobacteria	Cyanophiceae		Unknown colonial rounded cyanobacteria (origami-like)		X	X	
Cyanobacteria	Cyanophyceae	Synechococcaceae	cf. *Synechococcus* sp.	X	X	X	X
Bacillariophyta	Bacillariophyceae	Cocconeidaceae	cf. *Cocconeis* sp.	X	X	X	X
Bacillariophyta	Bacillariophyceae	Fragilariaceae	cf. *Fragilaria* sp.	X	X	X	X
Bacillariophyta	Bacillariophyceae	Naviculaceae	cf. *Caloneis* sp.	X	X	X	X
Bacillariophyta	Bacillariophyceae	Bacillariaceae / Tabellariaceae	cf. *Denticula sp. / Diatoma sp.*	X	X	X	X
Bacillariophyta	Bacillariophyceae	Mastogloiaceae	cf. *Mastogloia* sp.	X			
Bacillariophyta	Bacillariophyceae	Naviculaceae	cf. *Navicula* spp.	X	X	X	X
Bacillariophyta	Bacillariophyceae	Catenulaceae	cf. *Amphora* sp.	X	X	X	X
Bacillariophyta	Bacillariophyceae	Gomphonemataceae	cf. *Gomphonema* spp.	X	X	X	X
Bacillariophyta	Bacillariophyceae	Bacillariaceae	cf. *Nitzschia palea*	X	X	X	
Bacillariophyta	Bacillariophyceae	Stauroneidaceae	cf. *Stauroneis sp.*	X	X		
Bacillariophyta	Bacillariophyceae	Rhoicospheniaceae	cf. *Rhoicosphenia sp.*	X		X	X
Bacillariophyta	Bacillariophyceae	Ulnariaceae	cf. *Ulnaria sp.*	X	X	X	X
Bacillariophyta	Bacillariophyceae	Fragilariaceae	cf. *Synedra sp.*	X			X
Bacillariophyta	Bacillariophyceae		Unknown rectangular diatom	X	X	X	X
Bacillariophyta	Bacillariophyceae	Pinnulareaceae	cf. *Pinnularia sp.*	X	X	X	X
Chlorophyta	Chlorophyceae		Unknown filamentous green algae		X		
Chlorophyta	Chlorophyceae		Unknown filamentous green algae (hair like)	X	X	X	X

Appendix 6.18 (Cont.)

Taxa Phylum/ Kingdom	Class	Family	Genus/Species	Treatment levels			
				C	TTG	MT	TTGMT
Chlorophyta	Chlorophyceae		Unknown solitary rounded green algae	X	X	X	X
Chlorophyta	Chlorophyceae		Unknown colonial green algae without mucilage	X	X	X	X
Chlorophyta	Chlorophyceae		Unknown colonial green algae with mucilage			X	
Chlorophyta	Chlorophyceae		Unknown colonial rounded green algae (cells attached together-like tiles)	X	X	X	X
Chlorophyta	Chlorophyceae	Selenastraceae	cf. *Ankistrodesmus* sp.				X
Chlorophyta	Chlorophyceae	Selenastraceae	cf. *Desmodesmus* sp.	X			
Chlorophyta	Chlorophyceae	Scenedesmaceae	cf. *Scenedesmus* sp.	X	X	X	X
Chlorophyta	Chlorophyceae	Scenedesmaceae	cf. *Coelastrum sp.*	X			
Chlorophyta	Chlorophyceae	Hydrodictyaceae	cf. *Tetraëdron* sp.	X	X	X	X
Chlorophyta	Chlorophyceae	Volvocaceae	Unknown	X	X	X	X
Chlorophyta	Ulvophyceae	Cladophoraceae	cf. *Rhizoclonium* sp.	X	X	X	X
Chlorophyta	Ulvophyceae	Cladophoraceae	cf. *Cladophora sp.*	X	X	X	X
Cryptophyta	Cryptophyceae	Cryptomonadaceae	cf. *Cryptomonas* sp.	X	X	X	X
Rotifera			Unknown (1 sp.)	X	X	X	X
Euglenozoa			Unknown (1 sp.)			X	X
Euglenozoa			cf. *Trachelomonas sp.*				X
Fungi			Unknown Aquatic Fungi (1 sp.)	X	X	X	X
			TOTAL:	44	40	40	43

Appendix 6.19: **A.** ANOVAs of chlorophyll-*a* values obtained by each mesocosm from B1 to B4/B4 rep. and the comparison of treatments per dates of sampling; **B.** Summary of measurements for chlorophyll-*a* values separated by treatment; **C.** Pearson's correlations between N and P chemical compounds, in relation to chlorophyll-*a* concentration, separated by treatment.

A.

Analysis of variance

Variable	N	R^2	Adj R^2	CV
chl-a (g/m^2)	70	0.85	0.40	90.67

Analysis of variance table (Sequential SS)

S.V.	SS	df	MS	F	p-value
Model.	62.37	52	1.20	1.88	0.0767
Block	32.27	4	8.07	12.63	0.0001
Period of sampling	3.70	3	1.23	1.93	0.1633
Treatment	9.17	3	3.06	4.78	0.0135
Period of sampling*Treatme..	3.61	9	0.40	0.63	0.7589
Block*Period of sampling*T..	13.64	33	0.41	0.65	0.8612
Error	10.86	17	0.64		
Total	73.22	69			

Test:Bonferroni Alpha:=0.05 LSD:=1.00916

Error: 0.6386 df: 17

Block	Means	n	S.E.		
2	0.19	16	0.22	A	
1	0.21	16	0.22	A	
3	1.05	16	0.22	A	B
4	1.64	14	0.24		B
5	1.94	8	0.31		B

Test:Bonferroni Alpha:=0.05 LSD:=0.80649

Error: 0.6386 df: 17

Period of sampling	Means	n	S.E.	
5	0.60	17	0.21	A
3	0.71	18	0.21	A
4	1.07	17	0.21	A
2	1.15	18	0.21	A

Test:Bonferroni Alpha:=0.05 LSD:=0.80965

Error: 0.6386 df: 17

Treatment	Means	n	S.E.		
TTGMT	0.37	16	0.22	A	
TTG	0.67	18	0.21	A	
MT	0.83	16	0.22	A	B
C	1.51	20	0.20		B

B.

Summary statistics

Treatment	Variable	n	Mean	S.D.	Var(n-1)	Var(n)	S.E.	CV	Minimum	Maximum	Median
C	chl-a conc. (g/m^2)	25	1.33	1.25	1.55	1.49	0.25	93.63	0.02	3.47	0.88
MT	chl-a conc. (g/m^2)	20	0.76	0.68	0.47	0.44	0.15	90.12	0.03	2.50	0.64
TTG	chl-a conc. (g/m^2)	21	0.62	0.86	0.74	0.70	0.19	138.48	0.00	2.74	0.12
TTGMT	chl-a conc. (g/m^2)	20	0.37	0.55	0.30	0.29	0.12	148.56	0.02	2.37	0.17

C.

Correlation coefficients

Treatment = C

	TKNv.	NO3-N	TP	TN	CHL-a
TKNv.	1.00	0.48	2.4E-03	0.02	0.48
NO3-N	0.17	1.00	0.19	2.2E-09	0.08
TP	0.65	-0.31	1.00	0.88	0.42
TN	0.51	0.93	-0.04	1.00	0.22
CHL-a	0.17	-0.40	0.20	-0.29	1.00

Treatment = TTG

	TKNv.	NO3-N	TP	TN	CHL-a
TKNv.	1.00	0.54	0.28	0.06	0.32
NO3-N	0.15	1.00	0.91	4.1E-11	0.03
TP	-0.25	-0.03	1.00	0.68	0.39
TN	0.43	0.96	-0.10	1.00	0.11
CHL-a	0.23	-0.48	0.20	-0.37	1.00

Treatment = MT

	TKNv.	NO3-N	TP	TN	CHL-a
TKNv.	1.00	0.79	0.96	0.01	0.02
NO3-N	0.07	1.00	0.09	3.1E-06	0.62
TP	0.01	0.39	1.00	0.16	0.90
TN	0.59	0.84	0.32	1.00	0.11
CHL-a	0.51	0.12	-0.03	0.37	1.00

Treatment = TTGMT

	TKNv.	NO3-N	TP	TN	CHL-a
TKNv.	1.00	0.63	0.56	0.18	0.36
NO3-N	-0.11	1.00	0.47	3.4E-08	0.32
TP	0.15	-0.18	1.00	0.64	0.27
TN	0.31	0.91	-0.12	1.00	0.57
CHL-a	0.21	-0.23	0.27	-0.13	1.00

Appendix 6.20: A. Summary of measurements for Shannon-Weaver Indexes; B. Non-Parametric ANOVA of Shannon-Weaver Indexes obtained, performed with Kruskal-Wallis Test, and comparing the Shannon Indexes per treatments, periods of sampling and repetitions.

A.

Block	Treat	Variable	n	Media	D.E.	Var(n-1)	Var(n)	E.E.	CV	Mín	Máx
1	C	H'	5	1.52	0.22	0.05	0.04	0.10	14.31	1.30	1.89
1	MT	H'	5	1.23	0.34	0.12	0.09	0.15	27.65	0.91	1.75
1	TTG	H'	5	1.06	0.21	0.05	0.04	0.10	20.23	0.76	1.30
1	TTGMT	H'	5	1.22	0.67	0.45	0.36	0.30	55.21	0.27	1.88
2	C	H'	5	1.13	0.41	0.17	0.14	0.18	36.41	0.68	1.67
2	MT	H'	5	1.35	0.41	0.17	0.13	0.18	30.34	0.69	1.70
2	TTG	H'	4	1.16	0.10	0.01	0.01	0.05	8.44	1.09	1.30
2	TTGMT	H'	5	1.33	0.12	0.01	0.01	0.05	8.71	1.21	1.50
3	C	H'	5	1.09	0.51	0.26	0.20	0.23	46.41	0.52	1.78
3	MT	H'	5	1.08	0.36	0.13	0.10	0.16	32.83	0.77	1.67
3	TTG	H'	5	1.42	0.15	0.02	0.02	0.07	10.35	1.28	1.67
3	TTGMT	H'	5	0.98	0.32	0.11	0.08	0.14	33.06	0.75	1.53
4	C	H'	5	1.24	0.43	0.18	0.15	0.19	34.43	0.84	1.87
4	MT	H'	5	1.77	0.34	0.11	0.09	0.15	18.91	1.25	2.07
4	TTG	H'	5	2.05	0.14	0.02	0.02	0.06	6.76	1.92	2.24
4	TTGMT	H'	5	1.69	0.22	0.05	0.04	0.10	13.10	1.46	2.05

B.

Kruskal Wallis Test

Variable	Treat	Period	N	Means	S.D.	Medians	H	p
H'	C	1	4	1.34	0.13	1.36	15.25	0.7064
H'	C	2	4	1.62	0.24	1.63		
H'	C	3	4	1.23	0.39	1.20		
H'	C	4	4	1.02	0.35	0.91		
H'	C	5	4	1.01	0.61	0.82		
H'	MT	1	4	1.71	0.29	1.71		
H'	MT	2	4	1.33	0.39	1.24		
H'	MT	3	4	1.36	0.59	1.29		
H'	MT	4	4	1.06	0.44	0.96		
H'	MT	5	4	1.33	0.28	1.30		
H'	TTG	1	4	1.37	0.41	1.29		
H'	TTG	2	4	1.37	0.57	1.29		
H'	TTG	3	4	1.55	0.52	1.44		
H'	TTG	4	4	1.38	0.45	1.23		
H'	TTG	5	4	1.54	0.34	1.39		

H'	TTGMT	1	4	1.17	0.41	1.12
H'	TTGMT	2	4	1.14	0.73	1.11
H'	TTGMT	3	4	1.30	0.38	1.45
H'	TTGMT	4	4	1.42	0.50	1.53
H'	TTGMT	5	4	1.49	0.21	1.50

Appendix 6.21: Non-Parametric ANOVA for Jaccard Indexes, performed with Kruskal-Wallis Test.

Kruskal Wallis Test

Variable	Column1	Column2	N	Means	S.D.	Medians	H	p
Prop.	C	MT	3	0.60	0.07	0.59	1.65	0.8930
Prop.	C	TTG	3	0.53	0.05	0.55		
Prop.	C	TTGMT	3	0.56	0.10	0.59		
Prop.	MT	TTGMT	3	0.57	0.06	0.55		
Prop.	TTG	MT	3	0.54	0.12	0.50		
Prop.	TTG	TTGMT	3	0.58	0.09	0.55		

Appendix 6.22: A. Non-Parametric ANOVA (Kruskal-Wallis test) of periphyton abundance values obtained by major taxa groups; **B.** Non-parametric ANOVA (Kruskal-Wallis test) of periphyton abundances, comparing TTG and TTGMT; **C.** Non-parametric ANOVA (Kruskal-Wallis test) of periphyton abundances, comparing MT and TTGMT.

A.

Kruskal Wallis Test

Variable	Treat	Major group	N	Means	S.D.	Medians	df	H	p
ind.	C	Amoebozoa	3	4277.37	2645.01	5686.72	39	84.27	<0.0001
ind.	C	Bacillariophyta	3	30850.63	31780.31	24987.74			
ind.	C	Charophyta	3	493.27	854.37	0.00			
ind.	C	Chlorophyta	3	60000.40	41268.89	78288.54			
ind.	C	Cryptophyta	3	140.93	129.17	169.12			
ind.	C	Cyanobacteria	3	149721.96	158321.97	122831.70			
ind.	C	Euglenozoa	3	0.00	0.00	0.00			
ind.	C	Fungi	2	824.47	568.04	824.47			
ind.	C	Ochrophyta	1	591.93	0.00	591.93			
ind.	C	Rotifera	3	84.56	111.86	42.28			
ind.	MT	Amoebozoa	3	563.74	686.43	232.54			
ind.	MT	Bacillariophyta	3	24410.14	30303.30	9746.35			
ind.	MT	Charophyta	3	479.18	740.31	105.71			
ind.	MT	Chlorophyta	3	79369.77	86523.43	51413.02			
ind.	MT	Cryptophyta	3	0.00	0.00	0.00			
ind.	MT	Cyanobacteria	3	163284.61	143951.68	216666.10			
ind.	MT	Euglenozoa	3	183.22	317.34	0.00			
ind.	MT	Fungi	2	528.51	747.42	528.51			
ind.	MT	Ochrophyta	1	0.00	0.00	0.00			
ind.	MT	Rotifera	3	0.00	0.00	0.00			
ind.	TTG	Amoebozoa	3	507.37	589.27	232.54			
ind.	TTG	Bacillariophyta	3	8075.58	8211.22	4228.08			
ind.	TTG	Charophyta	3	0.00	0.00	0.00			
ind.	TTG	Chlorophyta	3	14617.06	10899.36	13106.94			
ind.	TTG	Cryptophyta	3	105.70	183.08	0.00			
ind.	TTG	Cyanobacteria	3	145657.90	127310.37	200210.60			
ind.	TTG	Euglenozoa	3	0.00	0.00	0.00			
ind.	TTG	Fungi	2	824.47	328.86	824.47			
ind.	TTG	Ochrophyta	1	0.00	0.00	0.00			
ind.	TTG	Rotifera	3	176.17	235.73	84.56			
ind.	TTGMT	Amoebozoa	3	1557.33	824.83	1585.52			
ind.	TTGMT	Bacillariophyta	3	31569.40	16434.93	28327.90			
ind.	TTGMT	Charophyta	3	3382.44	4219.79	1226.13			
ind.	TTGMT	Chlorophyta	3	35707.93	19441.95	30019.11			

ind.	TTGMT	Cryptophyta	3	972.45	439.39	1226.13
ind.	TTGMT	Cyanobacteria	3	277702.53	238665.73	386781.50
ind.	TTGMT	Euglenozoa	3	2466.36	2305.09	2832.79
ind.	TTGMT	Fungi	2	813.90	1151.03	813.90
ind.	TTGMT	Ochrophyta	1	338.24	0.00	338.24
ind.	TTGMT	Rotifera	3	112.75	195.28	0.00

B.

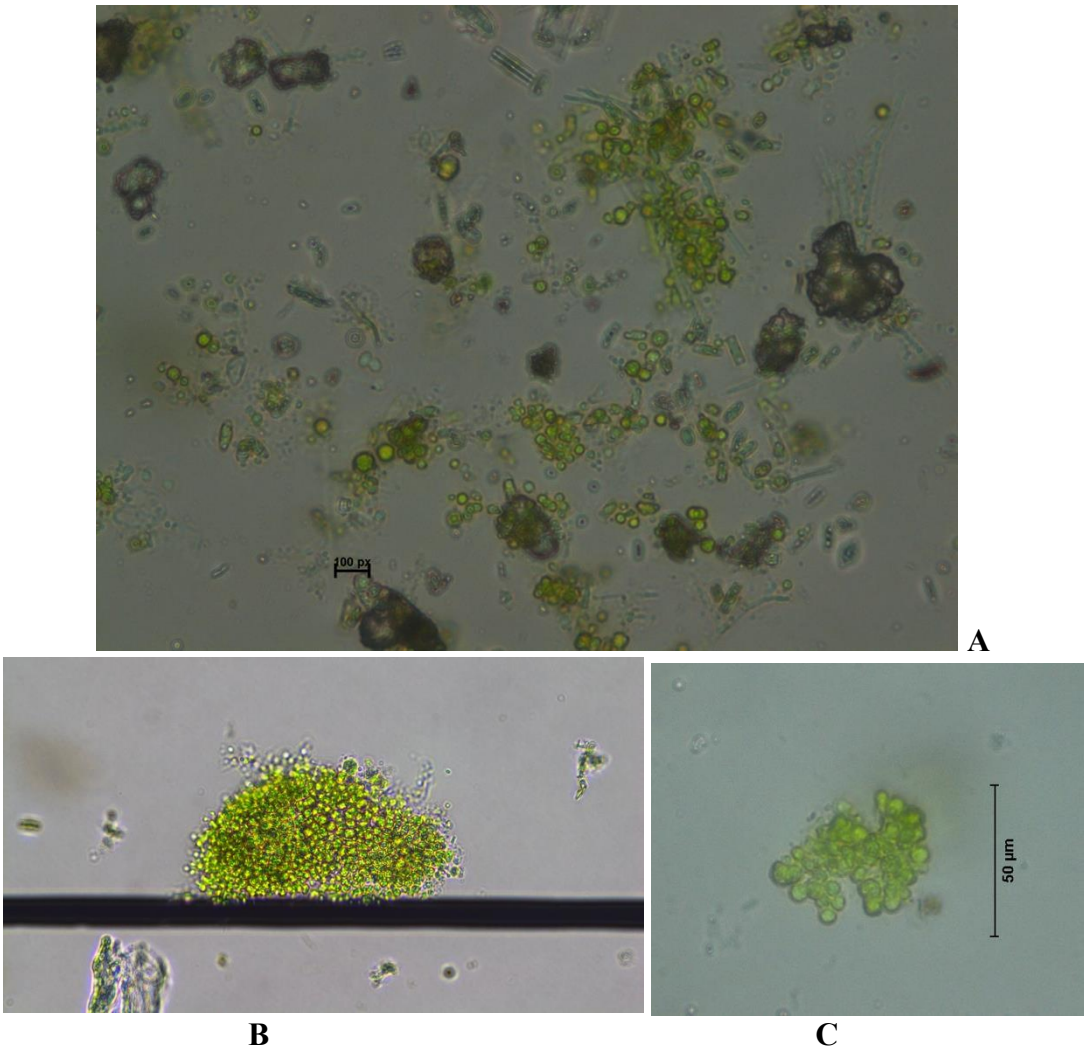
Kruskal Wallis Test

Variable	Treat	Major group	N	Means	S.D.	Medians	df	H	p
ind.	TTG	Amoebozoa	3	507.37	589.27	232.54	19	41.48	0.0017
ind.	TTG	Bacillariophyta	3	8075.58	8211.22	4228.08			
ind.	TTG	Charophyta	3	0.00	0.00	0.00			
ind.	TTG	Chlorophyta	3	14617.06	10899.36	13106.94			
ind.	TTG	Cryptophyta	3	105.70	183.08	0.00			
ind.	TTG	Cyanobacteria	3	145657.90	127310.37	200210.60			
ind.	TTG	Euglenozoa	3	0.00	0.00	0.00			
ind.	TTG	Fungi	2	824.47	328.86	824.47			
ind.	TTG	Ochrophyta	1	0.00	0.00	0.00			
ind.	TTG	Rotifera	3	176.17	235.73	84.56			
ind.	TTGMT	Amoebozoa	3	1557.33	824.83	1585.52			
ind.	TTGMT	Bacillariophyta	3	31569.40	16434.93	28327.90			
ind.	TTGMT	Charophyta	3	3382.44	4219.79	1226.13			
ind.	TTGMT	Chlorophyta	3	35707.93	19441.95	30019.11			
ind.	TTGMT	Cryptophyta	3	972.45	439.39	1226.13			
ind.	TTGMT	Cyanobacteria	3	277702.53	238665.73	386781.50			
ind.	TTGMT	Euglenozoa	3	2466.36	2305.09	2832.79			
ind.	TTGMT	Fungi	2	813.90	1151.03	813.90			
ind.	TTGMT	Ochrophyta	1	338.24	0.00	338.24			
ind.	TTGMT	Rotifera	3	112.75	195.28	0.00			

C.

Kruskal Wallis Test

Variable	Treat	Major group	N	Means	S.D.	Medians	df	H	p
ind.	MT	Amoebozoa	3	563.74	686.43	232.54	19	40.19	0.0024
ind.	MT	Bacillariophyta	3	24410.14	30303.30	9746.35			
ind.	MT	Charophyta	3	479.18	740.31	105.71			
ind.	MT	Chlorophyta	3	79369.77	86523.43	51413.02			
ind.	MT	Cryptophyta	3	0.00	0.00	0.00			
ind.	MT	Cyanobacteria	3	163284.61	143951.68	216666.10			
ind.	MT	Euglenozoa	3	183.22	317.34	0.00			
ind.	MT	Fungi	2	528.51	747.42	528.51			
ind.	MT	Ochrophyta	1	0.00	0.00	0.00			
ind.	MT	Rotifera	3	0.00	0.00	0.00			
ind.	TTGMT	Amoebozoa	3	1557.33	824.83	1585.52			
ind.	TTGMT	Bacillariophyta	3	31569.40	16434.93	28327.90			
ind.	TTGMT	Charophyta	3	3382.44	4219.79	1226.13			
ind.	TTGMT	Chlorophyta	3	35707.93	19441.95	30019.11			
ind.	TTGMT	Cryptophyta	3	972.45	439.39	1226.13			
ind.	TTGMT	Cyanobacteria	3	277702.53	238665.73	386781.50			
ind.	TTGMT	Euglenozoa	3	2466.36	2305.09	2832.79			
ind.	TTGMT	Fungi	2	813.90	1151.03	813.90			
ind.	TTGMT	Ochrophyta	1	338.24	0.00	338.24			
ind.	TTGMT	Rotifera	3	112.75	195.28	0.00			

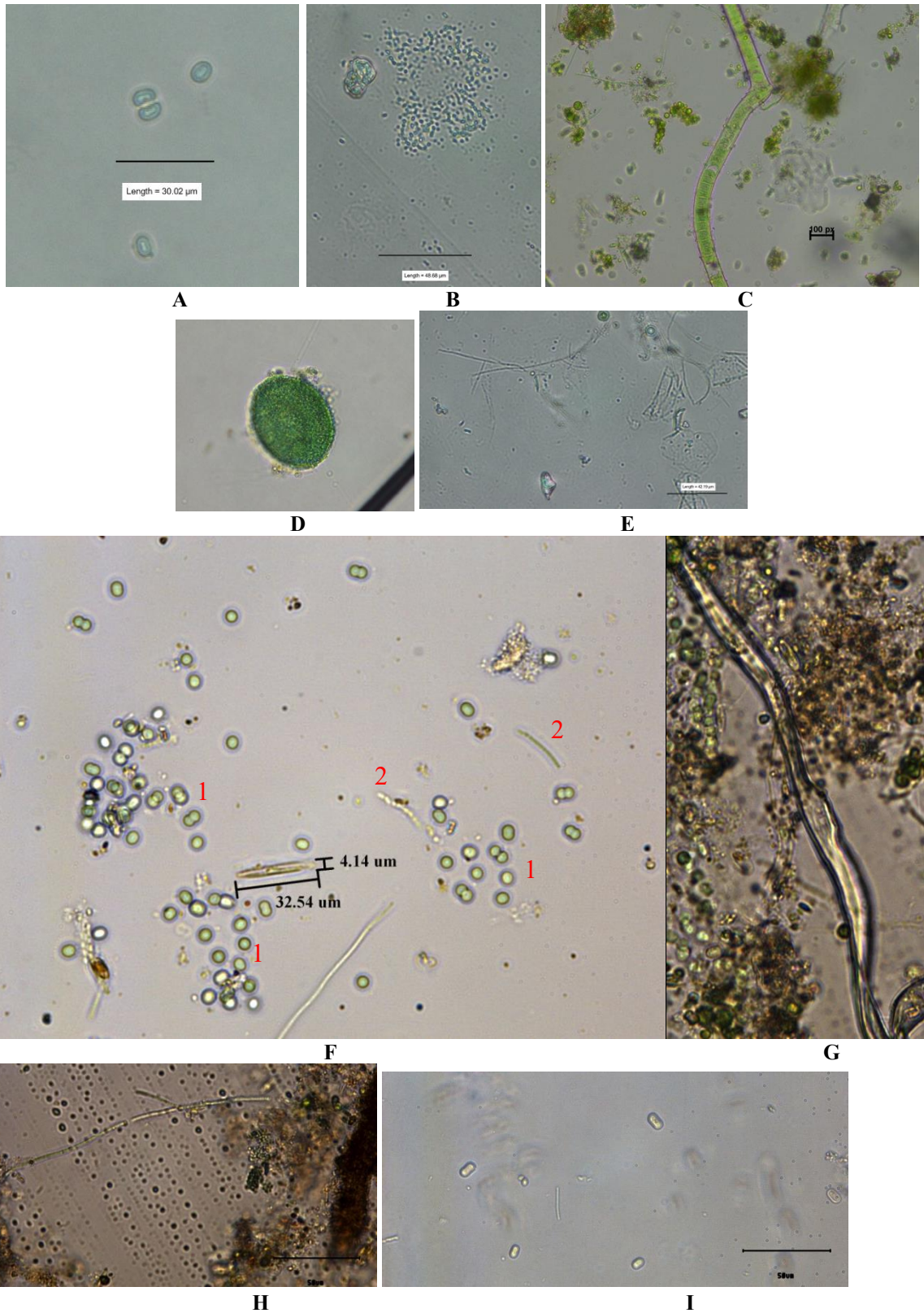


A

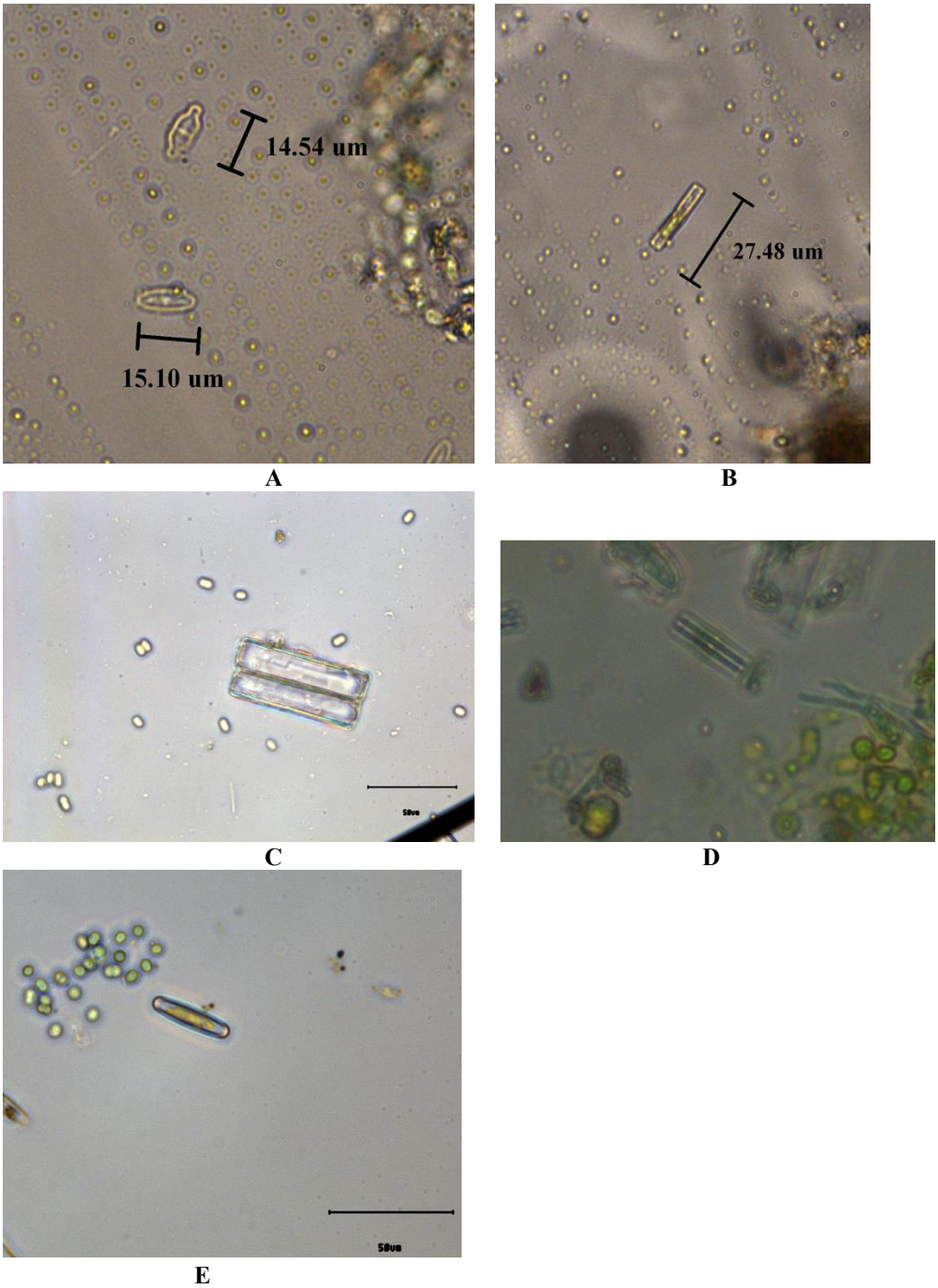
B

C

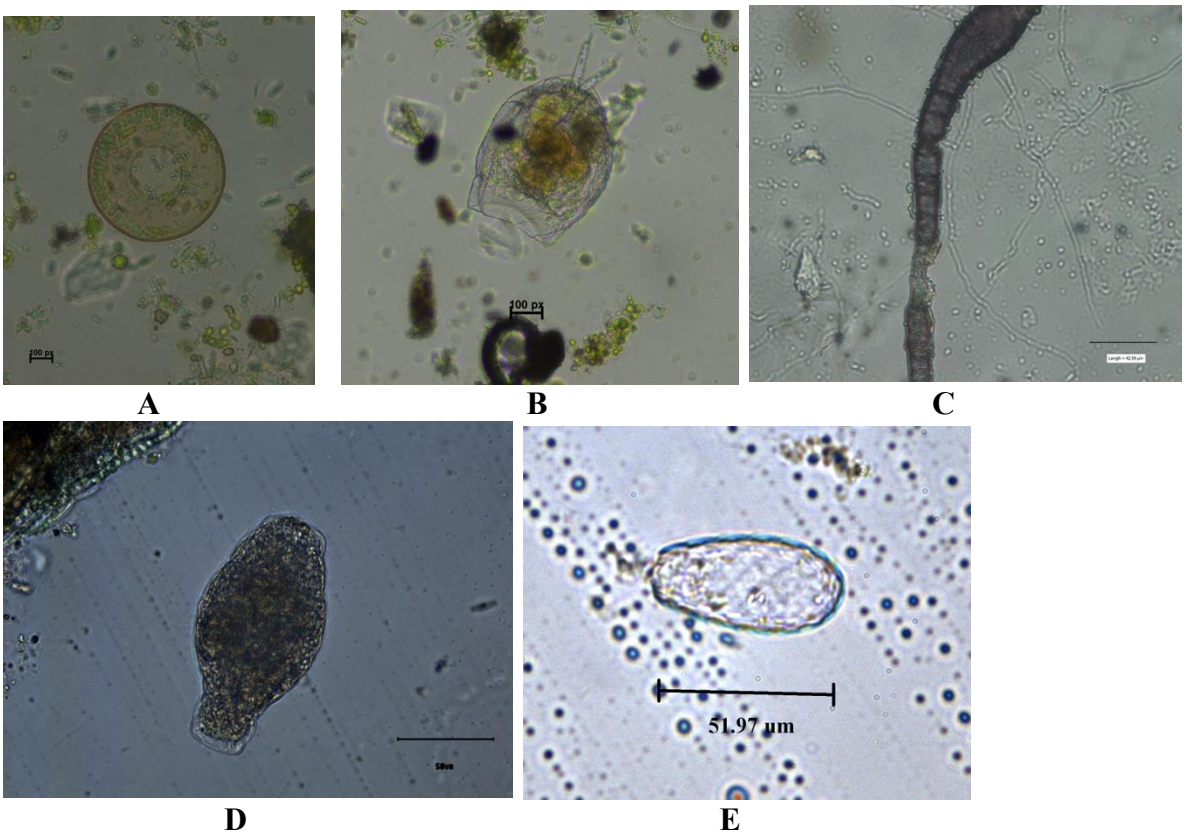
Appendix 6.23: Some common species of Chlorophyta: A. Unknown colonial green algae (Chlorophyceae), co-existing with other diatoms and cyanobacteria around (20x); B. Unknown colonial green algae (Chlorophyceae) with cells attached together (40x); C. Unknown colonial green algae



Appendix 6.24: Some common species of Cyanobacteria: A. *Synechococcus* sp. (40x); B. Microcystaceae (cf. *Anacystis* sp.) (40x); C. Oscillatoriaceae (cf. *Phormidium* sp.) (20x); D. *Aphanocapsa* sp. (40x); E. Filaments of *Anabaena* sp. (40x); F. (1) *Chroococcus* sp. in different colonies and (2) *Anabaena* sp.; G. *Tolypothrix* sp.; H. Oscillatoriaceae (center); I. *Synechococcus* sp. (Oval cells)



Appendix 6.25: Some common species of Bacillariophyta: A. *Caloneis* sp. (up) and *Cocconeis* sp. (down); B. *Gomphonema* sp.; C. Unknown rectangular diatoms (Bacillariophyceae); D. *Fragilaria* sp.; E. *Navicula* sp. (center) with coccoid Cyanobacteria (left)



Appendix 6.26: Other species found in samples: A. Amoebozoa (*Arcella* sp.) (20x) ; B. Rotifera (20x); C. Possible hypha of an aquatic fungi (40x); D. Another Rotifera (40x); E. Amoebozoa (Hyalospheniidae)

Appendix 6.27: Ecology and description of the most abundant algae in mesocosms

Taxon	**Description**
Anabaena (Nostocaceae)	This genus was one of the most abundant Cyanobacteria in all the treatment. They are identified by their filaments and intercalary heterocytes and cylindrical akinetes (Komárek and Johansen, 2015), as well as unbranched trichomes (Whitton, 2008). Most of the species identified to this genus are specific from benthos, and their reproductive mechanism occurs by two forms: (1) trichome fragmentation in heterocysts, and (2) akinete production (Komárek and Johansen, 2015). However, they are able to increase in abundance without the use of N-fixation (under diazotrophic

	conditions; using NH_4^+) (Golden and Yoon, 2003; Malatinszky *et al.*, 2017). As well, certain parts of the filamentous algae can transform their cells into heterocysts, irreversibly, which provide a low rate of DO in the aquatic environment and subsequently the nitrogenase activation in N-fixation (Golden and Yoon, 2003; Malatinszky *et al.*, 2017). Some species may form gas vacuoles that will create great water-blooms in freshwater systems (Canter-Lund and Lund, 1995).
Chroococcus (Chroococcaceae)	This cyanobacterial genus is mainly described from freshwater habitats (Komárek and Anagnostidis 1999; Wood *et al.*, 2017). They are recognized by their single cells or small colonies, in which cells have a hemispherical shape and are surrounded by mucilage (Wood *et al.*, 2017). Usually forms aggregations of 2 to 16 cells (Whitton, 2008). Their reproduction occurs via colony fragmentation and by binary fission (Komárek and Johansen, 2015) "in more than one plane in subsequent generations" (Golubić, 1967; Kováčik *et al.*, 2011). Also, they present ecological adaptations with differences in pigmentation types, depending of temperature and light intensity (Kováčik *et al.*, 2011).
Microcystaceae	This family, as well part of phylum Cyanobacteria, includes some species (i.e. *Microcystis*) related to toxic compounds that can affect animals and humans. It is found in colonies, which are irregularly arranged cells covered by mucilage, sometimes being in clusters (Komárek and Johansen, 2015). Most of these species are toxic; some species are not well studied yet, but are mainly common in eutrophic waters (Komárek and Johansen,

	2015). Species of this group may produce gas vacuoles that can form water-blooms in the surface of water bodies (Canter-Lund and Lund, 1995). In the summer season, during higher temperatures, they may increase in abundance, causing blooms. Previously, during spring season, they colonize the water column; in the fall, cells go to the bottom, being part of the sediments on benthos (Agha-Frías, 2013).
Colonial Coccoid Chlorophyta (Chlorophyceae)	For this research, some colonial Chlorophyceae (most of them coccoids) were identified as unknowns, but in general, their ecology should be very similar of other species of Chlorophyceae identified, such as: *Ankistrodesmus* sp., *Desmodesmus* sp., *Scenedesmus* sp., *Coelastrum* sp., *Tetraëdron* sp., among others. These species do not produce a resistant wall in their cells. As bio-indicators, they are not as useful as Bacillariophyta and Cyanobacteria species, based on their limited fossil record in terms of climate change (Bellinger and Sigee, 2015), but more useful in terms of water eutrophication (Santos, *personal communication*). Also, they are found solitary, in subaerial zones (Shubert and Gärtner, 2015). Usually, are found on benthos, being one of the primary components of the plankton or attached to substrates and available for grazers needs. This is a pioneer group that colonizes substrates in a short period of time (Shubert and Gärtner, 2015).