HEVELIM SERRÃO DE LIMA

Carbon flux in the microbial epipelagic food chain in a meso-oligotrophic coastal ecosystem: Mamanguá- RJ and Ubatuba-SP

Dissertação apresentada ao Instituto Oceanográfico da Universidade de São Paulo como parte dos requisitos para obtenção do título de Mestre em Ciências, Programa de Oceanografia, área de Oceanografia Biológica.

Orientador: Prof. Dr. Frederico Pereira Brandini

São Paulo

2019

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Versão corrigida

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ABREVIATIONS LIST

CO_2	Carbon Dioxide
GPP	Gross Primary Productivity
NPP	Net Primary Productivity
RESP	Respiration
NCP	Net Community Productivity
POC	Particulate Organic Carbon
DO	Dissolved Oxygen

ABSTRACT

In oligotrophic regions the food web is longer, and the primary Carbon biomass production comes from the photosynthesis of pico-autotrophic organisms. Heterotrophic bacteria also represents an important role in these food chain, where most of the energy flows within the steps of the microbial loop before it reaches higher trophic levels. This study investigated the main origin of organic carbon accumulated by the second trophic level. Mortality and growth rates were estimated using *in situ* microcosm incubations. *Synechococcus* and heterotrophic bacteria represented more than 40% of the microbial community. Dilution technique applied in three experiments conducted in Ubatuba. It was quantified grazing and growth rate of heterotrophic bacteria, pico- and nanoplankton. Heterotrophic bacteria grazing was 53.85 μgC.L-1 per day , 2.53 μgC.L-1 per day and 3.00 μgC.L^{-1}. The autotrophic biomass of carbon grazed 2.26 μgC.L-1 per day for *Synechococcus* and 35.97 μgC.$L^{-1}$for picoeukaryotes in the second experiment. *Synechococcus* grazed biomass was 1.14 μgC.L-1 per day and Picoeukaryotes grazed biomass was 50.46 μgC.L^{-1}. The results of the experiments showed the flux of Carbon from both heterotrophic and autotrophic bacteria to grazers of the second trophic level was not as important as the flux of Carbon from picoeukarytes to grazers.

Keywords: Grazing, Carbon biomass, Heterotrophic bacteria, picoplankton, oligotrophic, Brazilian coast

RESUMO

Em regiões meso-oligotróficas marinhas, a cadeia alimentar é distribuída em maior número de níveis tróficos e a produção primária de biomassa de carbono é oriunda da fotossíntese dos organismos do picofitocoplâncton. As bactérias heterotróficas representam um papel importante nesta cadeia alimentar, onde a maior parte da energia flui através dos níveis tróficos microbianos até atingir os níveis superiores. Desta forma, este estudo investigou a principal origem do carbono orgânico acumulado pelo segundo nível trófico. A mortalidade e as taxas de crescimento foram estimadas usando incubações em microcosmos *in situ* (Mamanguá e Ubatuba). *Synechococcus* e bactérias heterotróficas representaram mais de 40% da comunidade microbiana. em Ubatuba Técnica da diluição foi aplicada em três experimentos realizados. Foi quantificado a herbivoria e a taxa de crescimento de bactérias heterotróficas, pico e nanoplâncton. O consumo de bactérias heterotróficas foi 53,85 µgC.L-1, 2,53 µgC.L-1 per day e 3,00 µgC.L-1. A biomassa autotrófica de carbono consumida foi 2,26 µgC.L-1 per day para *Synechococcus* e 35,97 µgC.L-1 per day para picoeucariotos no segundo experimento. A biomassa de *Synechococcus* foi 1,14 µgC.L-1 per day e a biomassa de Picoeukaryotes foi de 50,46 µgC.L-1. Os resultados dos experimentos mostraram que o fluxo de carbono das bactérias heterotróficas e autotróficas para os herbívoros do segundo nível trófico não foi tão importante quanto o fluxo de carbono de picoeucariotos repassado para os consumidores.

Palavras-chave: pastejo, biomassa de carbono, bactéria heterotrófica, picoplâncton, oligotrófico, Costa Brasileira

SUMARY

1. INTRODUCTION

Planktonic food web works as a driver of the major energy flux of the oceanic ecosystem and, as a consequence it represents the main dynamic pool of the global carbon cycle. Autotrophic organisms are responsible for the uptake of carbon dioxide (CO_2) from the atmosphere and also transference of organic carbon to the deep ocean (STEINBERG & LANDRY, 2017). They are the initial reservoir of organic carbon that accumulates the energy consumed by primary consumers, as ciliates and flagellates. These by instance are a source of carbon for the higher trophic levels. Part of the carbon that is not fixed and incorporated as biomass by the photosynthetic organisms returns as CO_2 to the ecosystem by the respiration of the planktonic community (STOCK *et al.*, 2014; STEINBERG & LANDRY, 2017).

The majority of this biogenic carbon occupies the lowest trophic levels and enters the planktonic system through two distinct biogeochemical pathways: (i) phytoplanktonic primary production (=autotrophic) and (ii) microbial heterotrophic production. Both sustaining specific planktonic food web and occurring simultaneously in the pelagic habitat under different proportions depending on nutrients availability, which is controlled by the hydrographic regime (FASHAM, 2003).

Oceanographic literature defines primary production as new and regenerated production. This definition was described for the first time by Dugdale and Goering (1967) associating primary production with the available form of the inorganic nitrogen. New production depends on the influx of nutrients, such as nitrate and nitrite to the euphotic zone by physical processes, such as upwellings, riverine inputs and atmospheric sources. The regenerated production is sustained exclusively by dissolved organic matter from excretion of the planktonic food web, such as ammonium that is recycled by heterotrophic bacteria in the euphotic zone to inorganic nutrients becoming available to primary producers. This transference of dissolved organic carbon to the higher trophic levels in the heterotrophic food web through bacteria, nanoflagellates and ciliates is defined as *microbial loop* (AZAM *et al.* 1983; WORDEN *et al.*, 2015).

The total fixation of energy provided by photosynthesis is defined as gross primary productivity (GPP). The difference between GPP and respiration rate of this autotrophic community is known as net primary productivity (NPP), and it is considered as the amount of new biomass available for consumption by heterotrophic organisms. Heterotrophic plus autotrophic respiration represents the total community respiration (RESP). Moreover, GPP minus R is defined as net community productivity (NCP) (BEGON *et al.,* 2006). This balance between primary production and respiration of the community determines if the oceans are a sink or a source of atmospheric CO_2. In other words, the oceans are considered a sink if GPP>RESP removing CO_2 from the atmosphere, and then there is more carbon being fixed. On the other hand, they are considered a source if GPP<RESP contributing to the addition of CO_2 into the atmosphere, releasing more carbon than it is being fixed (ANDERSSON & MACKENZIE, 2004; WORDEN *et al.*, 2015).

NCP in the open ocean is 90% supported by nutrient regenerated production which part is produced by small grazers and heterotrophic bacteria. In addition, bacterial production accounts for around of 20% of NCP (BEGON *et al.,* 2006). Comparing marine with terrestrial ecosystems, plant biomass is ~ 600 – 1,000 Gt C $year^{-1}$ and most of it is stored as wood, and then this organic matter can be stored in forests for decades to centuries. However, in marine ecosystems NPP is ~ 40 Gt C $year^{-1}$ fixed by phytoplankton, considering the ocean in a steady state mode. This marine carbon is rapidly fixed, and the majority is consumed throughout the food web or sinks from the surface to the interior ocean (FASHAM, 2003; SCHLESINGER, 2005).

The oceans are a combination of these autotrophic and heterotrophic production systems that depends on the hydrography and nutrients availability in the euphotic zone. Upwellings regions, for example, bring nutrient-rich waters from deep layers towards the surface water increasing the primary productivity (SARMIENTO & GRUBER, 2006). Moreover, in these regions most of the biomass production is from the new production and the microbial loop does not play the most important role. In fact, the classic food web composed by phytoplankton and zooplankton link is the major responsible for the transfer of energy in these upwelling regions (CUSHING, 1989; BEGON *et al.,* 2006). On

the other hand, the main oceanic space coincides with the subtropical oligotrophic basins where regenerated production dominates. The waters over the Brazilian shelf, for example, are composed by western boundary currents as part of the anticyclonic subtropical gyre in the South Atlantic. This water mass is formed by warm and nutrient-poor tropical waters advected by the Westerlies towards the western side of the Atlantic basin (BRANDINI *et al.,* 1997)

In oligotrophic regions the food web is longer, and the primary Carbon biomass production comes from the photosynthesis of (mainly) pico-autotrophic prokaryotes (0.2 - 2µm) specially by cyanobacteria of the genera *Prochlorococcus* and *Synechococcus* with smaller contribution of the pico- and nano eukaryotes (respectively, 0.2 - 2µm and 2 -20µm) from diverse taxonomic classes. Heterotrophic bacteria also represent an important role in these food chain, where most of the energy flows within the steps of the microbial loop before it reaches higher trophic levels (CUSHING, 1989; FENCHEL, 2008). The dominance of picoplankton can be justified by their small size and large surface area per unit volume leading to a more efficient nutrient uptake in these poor nutrient regions where small flagellates are the main grazers of the picoplankton (AGAWIN *et al.,* 2000; CALBET, 2008; FENCHEL, 2008). Heterotrophic nanoflagellates (flagellated protozoa <20 µm) consume a significant portion of picoplankton production. According to O'Neil (1999) these nano-sized flagellates can remove 35-100% of the total production of *Synechococcus* per day.

Bacterial marine biomass is directly related to the concentration of phytoplankton released products, mainly Carbon and reduced Nitrogen compounds (e.g., urea, ammonia). This biomass can be represented by free or attached bacteria that respond differently to the activity of the phytoplankton cells. According to Abreu *et al.* (1992) free bacteria increases together with phytoplankton production, while attached bacteria are almost constant independent of phytoplankton concentration, then due to the higher nutrient availability on the particles that they are attached to, they are often less affected by nutrient limitation (ABREU *et al.,* 1992; HORNICK *et al.*, 2016). The number of free bacteria is controlled by heterotrophic nanoplanktonic flagellates (AZAM *et al.,* 1983; STEINBERG & LANDRY, 2017).

The lower concentration of nutrients in oligotrophic waters limits photosynthetic growth. However, some protists are mixotrophic, then representing an ecological strategy as producers or consumers in the planktonic food web, and then often play both roles in relation to energy flux. (WORDEN *et al.* 2015). In other words, pigmented cells and photosynthetically activated are also able of consuming a prey to complete their energetic demand (THINGSTAD *et al.* 1996; WORDEN *et al.* 2015). The mixotrophy is common in the planktonic system that is considered a fusion between autotrophy and heterotrophy. Prey ingestion may serve as a source of energy and carbon when photosynthesis is limited by light availability (PERNTHALER, 2005; WORDEN *et al.* 2015). Based on the material released in each feeding behavior, predation releases CO_2 and photosynthesis captures CO_2. This trophic role shifting acts opportunistically to capture resources through predation, reshaping their ecosystem roles and food web dynamics (PERNTHALER, 2005).

The microbial food chain can be controlled according to the ecological concepts of top-down or bottom-up controls. A food web is characterized as top-down if the biomass of prokaryotes in the water column is limited by the competition of carbon and nutrients and the biomass of herbivores protists is limited by predation (PERNTHALER, 2005). On the other hand, bottom-up controlled is when the microbial standing-stock is controlled by mortality, so protists mortality is caused by starvation based on the concentration of the prokaryote's cells in the ecosystem. Therefore, top-down control by herbivores protists or bottom-up control by carbon availability and nutrients are related to the general production of the ecosystem (THINGSTAD *et al.* 1996; PERNTHALER, 2005).

The study of the energy flux within the microbial food web is an excellent way of understanding the lowest trophic levels and their importance in this exchange of energy. Hirose *et al.* (2007) analyzed growth and grazing mortality rates of picophytoplankton by the dilution technique in a bay of the Uwa sea Japan affirming that are yet limited information about picophytoplankton in coastal waters. Therefore, this study investigates the main origin of the particulate organic carbon (POC) in the water column (Figure 1).

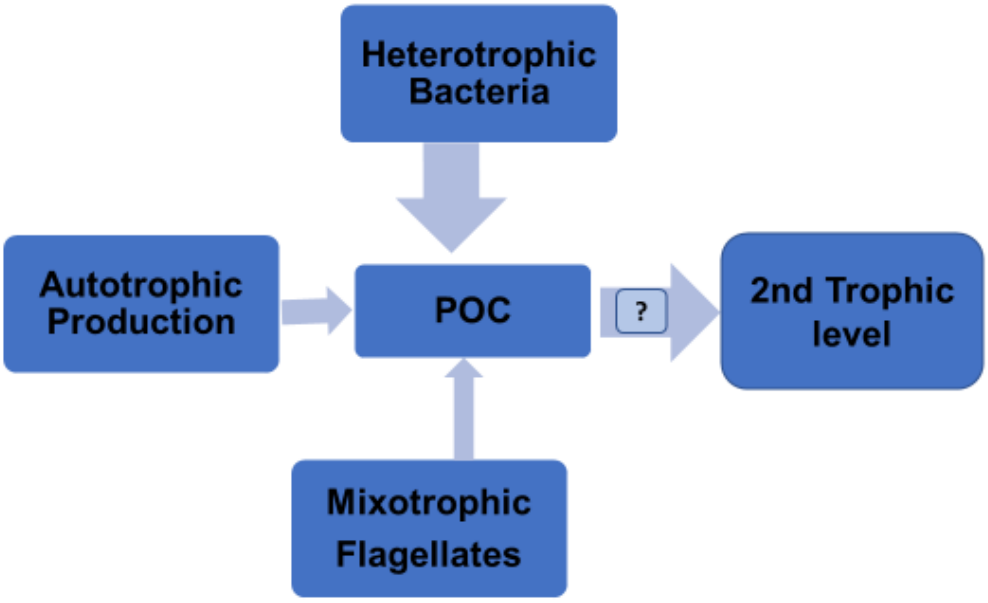

Figure 1: flux chart of the origins of particulate organic carbon (POC) in the microbial food web

2. HYPOTHESIS

Heterotrophic bacteria are more relevant in this energy flux and are the major responsible of the carbon biomass accumulated by nanoplankton of the second trophic level.

3. OBJECTIVES

This investigation aims to quantify the flux of carbon consumed by the second trophic levels in the pelagic microbial food web of the coastal meso-oligotrophic ecosystem of State of São Paulo.

3.1 Specific Objectives

- Determine the carbon biomass from picoautotrophs and heterotrophic bacteria to microzooplankton of the second trophic level and the most important.

- Provide consistent data about the energy flux at low trophic levels in order to feed conceptual models of pelagic microbial food web that may be applied to the meso-oligotrophic coast of the State of São Paulo.

4. METODOLOGY

4.1 Study area and in situ experiment

The study was conducted in the northern coast of São Paulo and southern coast of Rio de Janeiro as representative ecosystems of the southeastern Brazilian coast. These areas were chosen based on earlier studies that characterized it as meso-oligotrophic systems (AIDAR *et al.,*1993; MESQUITA & FERNANDES, 1996). The pilot experiment herewith labeled as Exp^{MMG} was conducted in Mamanguá Ría[1] which is part of Ilha Grande coastal environment localized in the south coast of Rio de Janeiro state. Two other experiments Exp^{Uba1} and Exp^{Uba2} were conducted in the Enseada of Flamengo in Ubatuba, São Paulo (Figure 2).

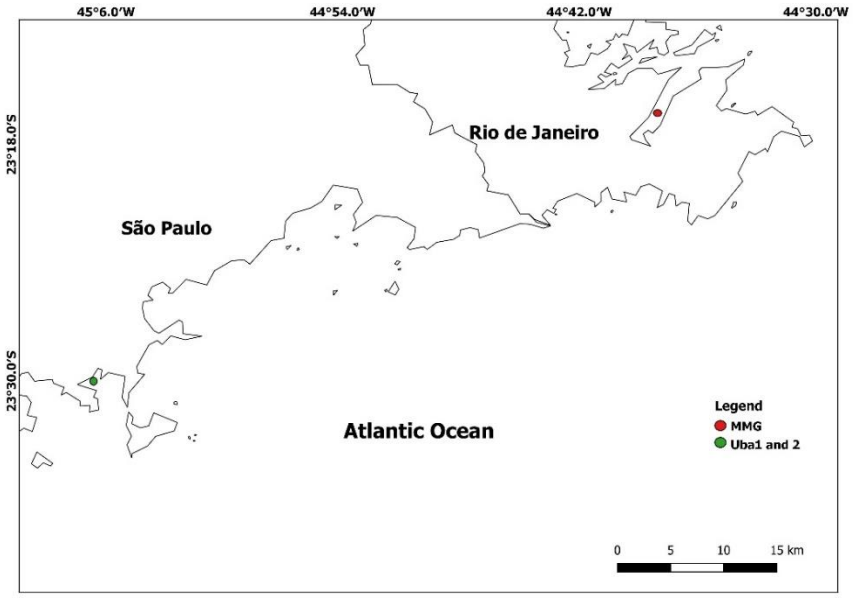

Figure 2. Map of the study areas. MMG: first experiment in Mamanguá Ría; Uba1 and 2: Ubatuba´s experiment

1. The term ria comes from the geomorphological classification of an estuary that is among non-glacial mountains.

Plankton growth and mortality rates were estimated using *in situ* microcosm incubations. Microcosms are closed systems of <20L that can be easily manipulated allowing the researcher to extrapolate the experimental results for the study area.

Exp^{MMG} was conducted with 5L glass bottles filled with marine water pre-filtered in 5µm and 60µm nylon nets. The filtration separated producers from consumers, allowing the picophytoplankton growth without nanoplankton consumers in the <5µm filtered water. Microcosms were protected by an aluminum frame (Figure 3) that was fixed at around 5m depth for 12 hours. Water samples were collected from both microcosms every 2 hours for biological and chemical analyses. Physical parameters from the environment were obtained with EXO2 YSI Inc. (USA) multiparameter only in this experiment. The experiment started at 11:20 am on 6th of October, 2017 and ended at 11:20pm of the same day.

Figure 3: Illustration of the preparation and the ongoing experiment

Additional experiments were labeled Exp^{Uba1} (3rd and 4th of April 2018) and Exp^{Uba2} (Exp^{Uba2a} 11th and 12th of June; Exp^{Uba2b} 13th and 14th of June 2018) The dilution technique (LANDRY & HASSETT, 1982) revised by Caron (2001) was employed to estimate grazing of nano-sized cells on both pico-cyanobacteria and heterotrophic bacteria in all experiments in Ubatuba. By using this method, it was possible to infer growth and grazing mortality of phytoplankton and bacteria from observed changes in population density during incubations of different dilutions of natural seawater over time.

Three assumptions must be taken:

- The growth rate (μ) of the organism is not directly affected by the dilution effect on the populational density, and on the presence or absence of another organism.
- The probability of an organism be consumed is a direct function of the prey and predator encounter. The number of preys ingested by a given consumer is linearly related to prey density.
- The mortality rate (m) or grazing coefficient is indicative of the grazing losses in each dilution bottle dependent on the absolute abundances of consumers, which in turn are dependent on the degree of the dilution (D_i) with filtered water.

$$k_i = \mu - (m \text{ x } D_i) \quad (1)$$

k_i is the apparent growth rate in each dilution bottle and follows the first assumption if concentrations of nutrients remain approximately constant. Also, It is a consequence of the growth rate (μ) of the organisms, mortality rate (m) and relative abundances of consumers. Then, K_i is calculated for each dilution bottle following this equation,

$$\mathrm{k}_i = (\ln t_f - \ln t_0)/\,(\text{incubation time}) \quad (2)$$

Which t_0 is the density of the organisms in the beginning of the experiment and t_f is the density at the end of the incubation. This calculation assumes exponential growth of the organisms and then plotted against the fraction of unfiltered water in each bottle. The mortality rate (m) is obtained from the slope of the regression and growth rate (μ) from the interception of the y-axis that should decline in direct proportion to the dilution effect on consumer density (LANDRY & HASSETT,1982; CARON, 2001).

The dilution experiments were set up with seawater obtained at the same site, then it was filtered through a GF/F 47mm filter in order to obtain the following dilutions of the natural unfiltered seawater: 1:0 (100% unfiltered seawater); 3:1 (75%); 1:1 (50%); and 1:3 (25%). These dilutions were conditioned in plastic bottles maintained *in situ*, in duplicate over 18h in Exp^{Uba1} and triplicate over 24h in Exp^{Uba2}. Aliquots were collected right after the preparations of the dilutions and at the end of the experiment for chemical and biological analyses (Figure 4).

Figure 4: Dilution technique experimentation setting.

In order to quantify the gross growth and grazing in biomass of carbon it was applied a linear regression model. First, the net growth was obtained by the following formula:

$$\text{Net growth rate} = (\mu gC_f / \mu gC_i) - 1 \quad (3)$$

μgC_f = final density of cells in biomass of carbon

μgC_i = initial density of cells in biomass of carbon

The net growth rate was plotted against the fraction of unfiltered water in each bottle as it was done in the dilution technique. The interception of the y-axis is the prediction of growth rate in a hypothetical 0% dilution without grazing. Multiplying the growth rate in the 0% by the initial density of cells in each dilution it was obtained the final biomass of carbon without grazing, then final biomass of carbon with grazing was calculated by the product of growth rate in each dilution with the initial density of cells also per dilution. Thus, grazing was calculated by the difference between final biomass of carbon without grazing and final biomass with grazing.

In order to determine the importance of prokaryotes in the microbial food web, abundance of heterotrophic bacteria, *Synechococcus* spp., autotrophic pico- and nanoeukaryotes were determined by flow cytometry in all experiments according to Marie *et al.* (2014). Analyses were conducted by Attune NxT® cytometer, equipped with blue excitation laser (488nm). Emitted light was collected through the following set of filters: 488/10 band pass for side scatter (SSC), 530/30 band pass for SYBR green I fluorescence (BL1), 585/42 band pass for orange phycoerythrin fluorescence (BL2), and 695/40 band pass for red chlorophyll fluorescence (BL3). Samples of 1.5 mL were collected to cryotubes, preserved with 0.1% glutaraldehyde (final concentration), flash-frozen in liquid nitrogen and stored at −80°C until analyses. Heterotrophic bacteria analyses were conducted with addition of SYBR Green I (Invitrogen Life Technologies, USA) into the remaining sample after autotrophs analyses, and incubated in the dark at room temperature for 15 minutes (Marie *et al*., 1999). Aliquots were fixed

with formaldehyde 2% and analyzed under inverted light microscope in order to obtain the overview of the most important grazers.

Phytoplankton and bacterioplankton cell abundance (cell.mL^{-1}) were converted into carbon biomass (µgC·L^{-1}) using constant cell-to-carbon conversion factors according to Buitenhuis *et al.* (2012), 255 fgC·cell^{-1} for *Synechococcus* spp. and 2,590 fgC·cell^{-1} for picoeukaryotes, and according to Lee and Fuhrman (1987) 20 fgC·cell^{-1} for heterotrophic bacteria. There is not robust conversion factor for nanophytoplankton biomass then it was not calculated.

Samples for nutrients analyses were retrieved from all microcosms experiments in the beginning and at the end of incubation period (except for Exp^{MMG} that samples were taken after 2 and 4 hours of experiment) to obtain concentrations of nitrate, nitrite and phosphate. Water was filtered through Whatman GF/F filters using low vacuum pump. The filtered water was frozen at −20 ◦C until laboratorial analyses with an auto-analyzer A3 from Seal Analytical.

Chlorophyll-*a* extraction was conducted in triplicate by filtering 350mL with GF/F 47mm filters in the beginning and at the end of the experiments. The extraction followed the method described by WELSCHMEYER (1994). Photosynthetic rates were estimated with the light-and-dark O_2 technique described by Strickland and Parsons (1972). The water was sampled at night, then it was kept in thermos water gallons throughout the night in order to diminish the O_2 saturation due to the total community respiration. The water was homogenized carefully in order to avoid bubbles inside the production flasks during the incubation and underestimation of final dissolved oxygen (DO) concentration. A total of 4 bottles (2 light and 2 dark) in Exp^{Uba1} and 8 bottles (4 light and 4 dark) in Exp^{Uba2} were filled over the top. The dark bottles were totally covered to avoid sunrays inside and consequently only respiration took place inside them. On the other hand, in light bottles both photosynthesis and respiration were estimated. These production flasks were maintained *in situ* in Exp^{Uba1} for 11 hours from 4:34am to 3:30pm, and Exp^{Uba2a} for 24 hours starting at 10pm and Exp^{Uba2b} starting at 11:15pm.

5. RESULTS

In the Exp^{MMG} incubation the average temperature, salinity and dissolved oxygen were respectively, 24°C, 30.9 psu and 7mg/L. In both <60µm and <5µm microcosms concentrations of nitrite and phosphate did not vary throughout the incubation except for nitrite at 5:20pm in the <60µm that showed an increase. Nitrate concentration also remained similar in both microcosms with the exception of T12 showing an abnormal increased characterizing some contamination or errors in the analysis (Table 1).

Table 1: Concentration of nutrients (µM) inside of each microcosm throughout 12 hours experiment

<60µm				
Sampling		Nitrite	Nitrate	Phosphate
T0	11:20am	0.03	1.27	0.75
T2	1:20pm	0.02	0.85	0.62
T6	5:20pm	0.1	1.91	0.59
T12	11:20pm	0.05	4.56	0.6
<5µm				
Sampling		Nitrite	Nitrate	Phosphate
T0	11:20am	0.03	1.01	0.61
T2	1:20pm	0.02	0.51	0.65
T6	5:20pm	0.03	2.71	0.77
T12	11:20pm	0.08	8.71	0.73

*µM

Throughout the incubation *Synechococcus* and heterotrophic bacteria represented more than 40% of the microbial community abundance. Their mean densities throughout the whole experiment in the <5µm microcosm were respectively, 244,060 ± 52,588 cell.mL^{-1} and 246,005 ±53,025 cell.mL^{-1}. In the <60µm filtered water cell densities of *Synechococcus* were 293,186 ±49,311 cell.mL^{-1} and densities of heterotrophic bacteria were 250,003 ±16,732 cell.mL^{-1}. There were small variations in the nanoplankton´s density in both microcosms with 5,366 ±1,660 cell.mL^{-1} in the <5µm filtered water and 6,904±1,239 cell.mL^{-1} in the <60µm. They represented less than 10% of the total of the microbial cell throughout the experiment. Picoeukaryotes density in the <60µm microcosms were 18,959 ± 1,555 cell.mL^{-1} similar concentrations were found in the <5µm

microcosms with 18,960± 1,679 cell.mL^{-1}. Yet, they also represented <10% throughout the experiment (Figure 7).

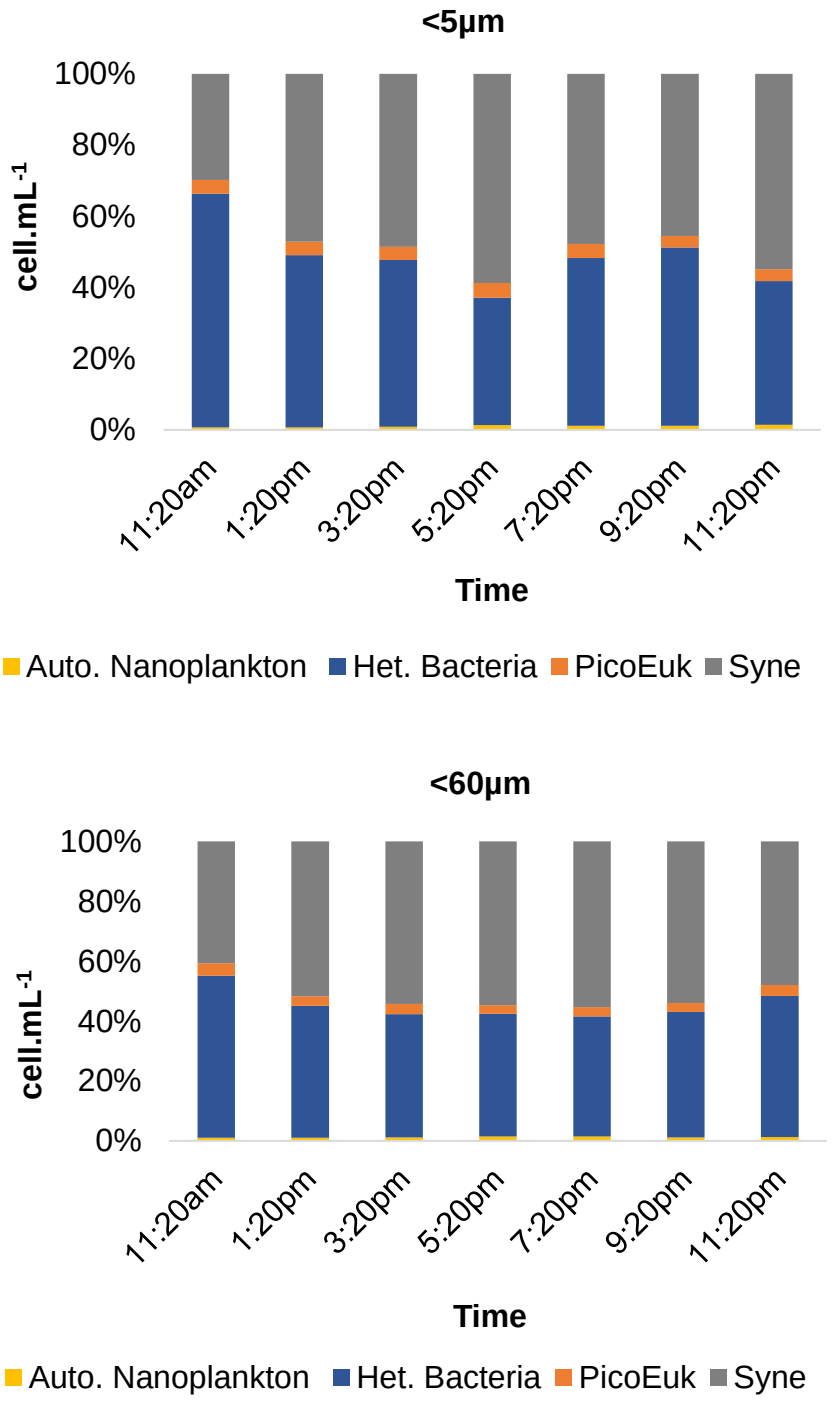

Figure 7: Variations of the planktonic cells inside the<5µm and <60µm microcosms represented in cell per mL over the 12h of the experiment with sampling every 2 hours (from T0- 11h20am to T12- 11h20pm).

The relative importance of *Synechococcus* carbon biomass dominated in both microcosms. Moreover, their concentrations in the <60µm filtered water were higher than in <5µm ranging from 53.24 µgC.L-1 per day (11:20am) to 79.99 µgC.L-1 per day (9:20pm). On the last sampling there was an inversion of concentrations (Figure 8). On average, heterotrophic bacteria contributions of carbon biomass remained similar in both microcosms from 5.72 - 5.56 µgC.L-1 per day to 4.79 - 4.64 µgC.L^{-1}. Picoeukaryotes in the <5µm and <60 µm microcosms ranged respectively from 49.37 - 55.92µgC.L-1 per day to 50.02 – 45.80µgC.L-1 per day (Figure 8).

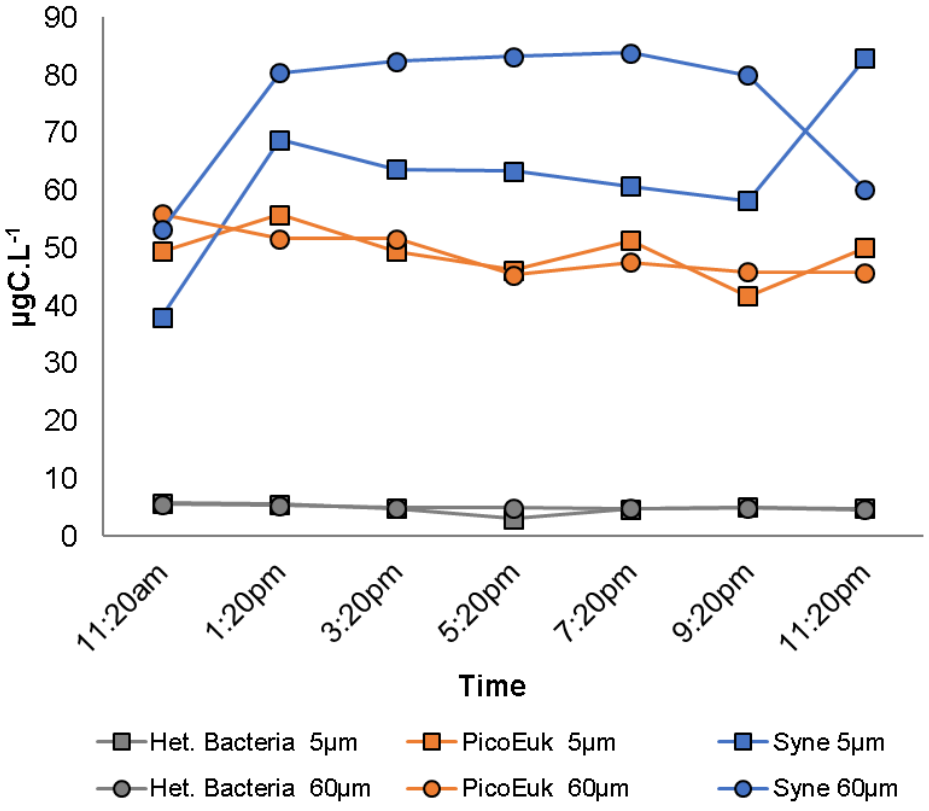

Figure 8: Biomass of carbon µgC.L-1 per day of all the organism analyzed over the 12h of the experiment sampled every 2 hours (from T0- 11h20am to T12- 11h20pm)

The mean temperature was 24°C. The average of nutrients concentrations was analyzed in all the dilutions on Exp^{Uba1} that ranged from 0.18µM - 0.22µM to 0.06µM - 0.15µM for nitrite it remained similar in all the dilutions besides the 50% that showed a decrease (Table 2). Nitrate concentrations were from 1.06µM - 1.60µM to 0.78µM - 2.31µM this last was considered an abnormal high value (Table 2). Phosphate concentrations varied from 0.19µM – 0.30µM to 0.03µM – 0.21µM (Table 2). Analyses of the nutrients on Exp^{Uba2} were conducted only for 100% unfiltered water. Then, the nitrite concentrations in Exp^{Uba2} diminished at the end of the incubation from 0.08 and 0.01 µM (T0) to 0.01 and 0.003 µM (Tf) (Table 2). Nitrate concentrations for Exp^{Uba2a} decreased from 0.65 µM to 0.20 µM. The Exp^{Uba2b} started with low concentration of nitrate 0.05 µM, but increased to 0.30 µM (Table 2). Phosphate concentrations for Exp^{Uba2} were from 0.30 µM and 0.37 µM to 0.22µM and 0.21 µM evidencing a small reduction (Table 2).

Table 2: Nutrients concentration (µM) in Ubatuba's experiments

Experiment	Dilution	Nitrite		Nitrate		Phosphate	
		T0	Tf	T0	Tf	T0	Tf
Uba1	25%	0.21	0.15	1.41	2.31	0.25	0.14
	50%	0.21	0.06	1.60	0.78	0.30	0.04
	75%	0.22	0.14	1.06	1.22	0.24	0.03
	100%	0.18	0.15	1.41	1.32	0.19	0.21
Uba2a	100%	0.08	0.01	0.65	0.20	0.3	0.22
Uba2b	100%	0.01	0.003	0.05	0.30	0.37	0.21

*µM

Chlorophyll-*a* concentrations analyses were only conducted in Exp^{Uba2}, On average Exp^{Uba2a} started the incubation with 2.67µg.L^{-1} and at the end it was increased to 3.26µg.L^{-1}. On the other hand, the average of chlorophyll-*a* concentration in the beginning of Exp^{Uba2b} was 2.08 µg.L^{-1} but at the end decreased to 1.56 µg.L^{-1}. Through inverted microscope analyses the dinoflagellate *Prorocentrum* was the most abundant in Ubatuba's experiment (Figure 9).

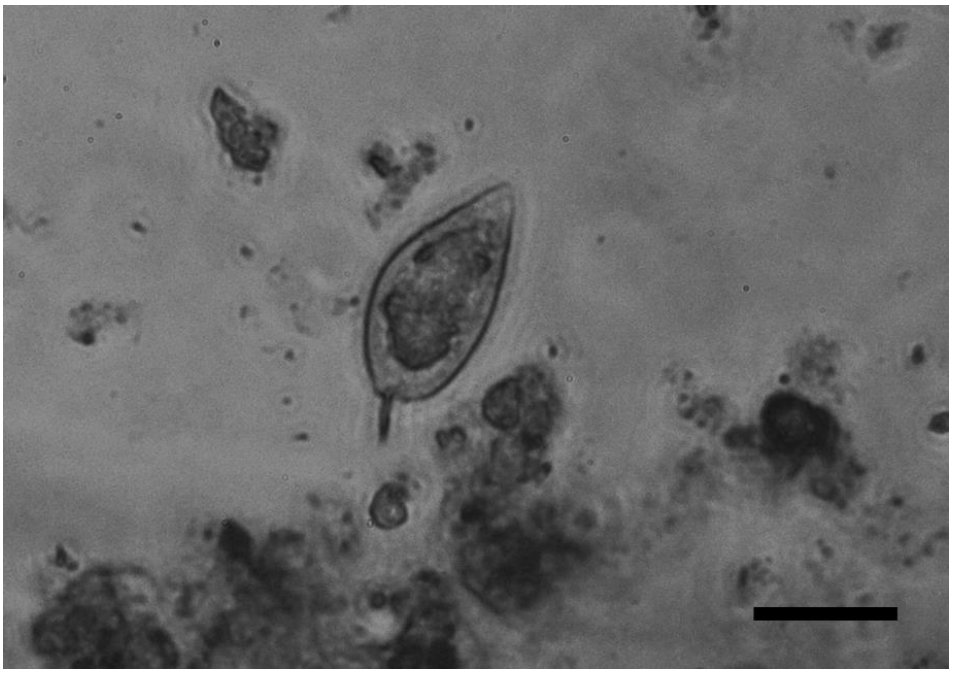

Figure 9: Dinoflagellate *Prorocentrum* most abundant organism found in Ubatuba's experiment. Scale10µm

GPP in Exp^{Uba2a} was 8.95 $mgCm^{-3}h^{-1}$ and the RESP measured was 0.22 $mgCm^{-3}h^{-1}$ then the NCP rate was 8.73 $mgCm^{-3}h^{-1}$. Overall respiration of the organisms was supported by the GPP. For Exp^{Uba2b} the GPP concentration was 7.31$mgCm^{-3}h^{-1}$ in the beginning of the incubation, but RESP was bigger than the production so NCP presented a negative value -6.61 $mgCm^{-3}h^{-1}$. Comparing concentrations of chlorophyll-*a* and NCP rates of both Exp^{Uba} the relative importance of photosynthetic organism on the second experiment was smaller.

Table 3: Gross Primary Production (GPP), Respiration (RESP) and Net Community Production (NCP) in all three experiments conducted in Ubatuba.

Experiment	GPP	RESP	NCP
Uba1	19.32	10.88	8.44
Uba2a	8.95	0.22	8.73
Uba2b	7.31	13.92	-6.61
	$mgCm^{-3}h^{-1}$		

Growth and grazing rates are summarized in Table 4. Exp^{Uba1} was conducted for 18h and then its values were converted to a 24h experiment in

order to have daily rates. *Synechococcus* growth rate (μ) was 0.049 day^{-1} with Ki from 0.069 to 0.085 day^{-1} in the 25% and 100% unfiltered seawater respectively and mortality rate (m) 0.011 day^{-1}, for this group the dilution did not interfered significantly in *Synechococcus* growth. The growth rate of Picoeukaryotes depicted a positive slope along the dilution (μ =0.128) and mortality rate were not possible to be measured. Heterotrophic bacteria growth was 0.078 day^{-1} and mortality 0.068 day^{-1} (R^2=0.80). Nanoeukaryotes growth rate was 0.105 day^{-1} which were the highest among the autotrophic organisms in Exp^{Uba1}. Their mortality rate was 0.055 day^{-1} (R^2=0.72) (Figure 10; Table 4).

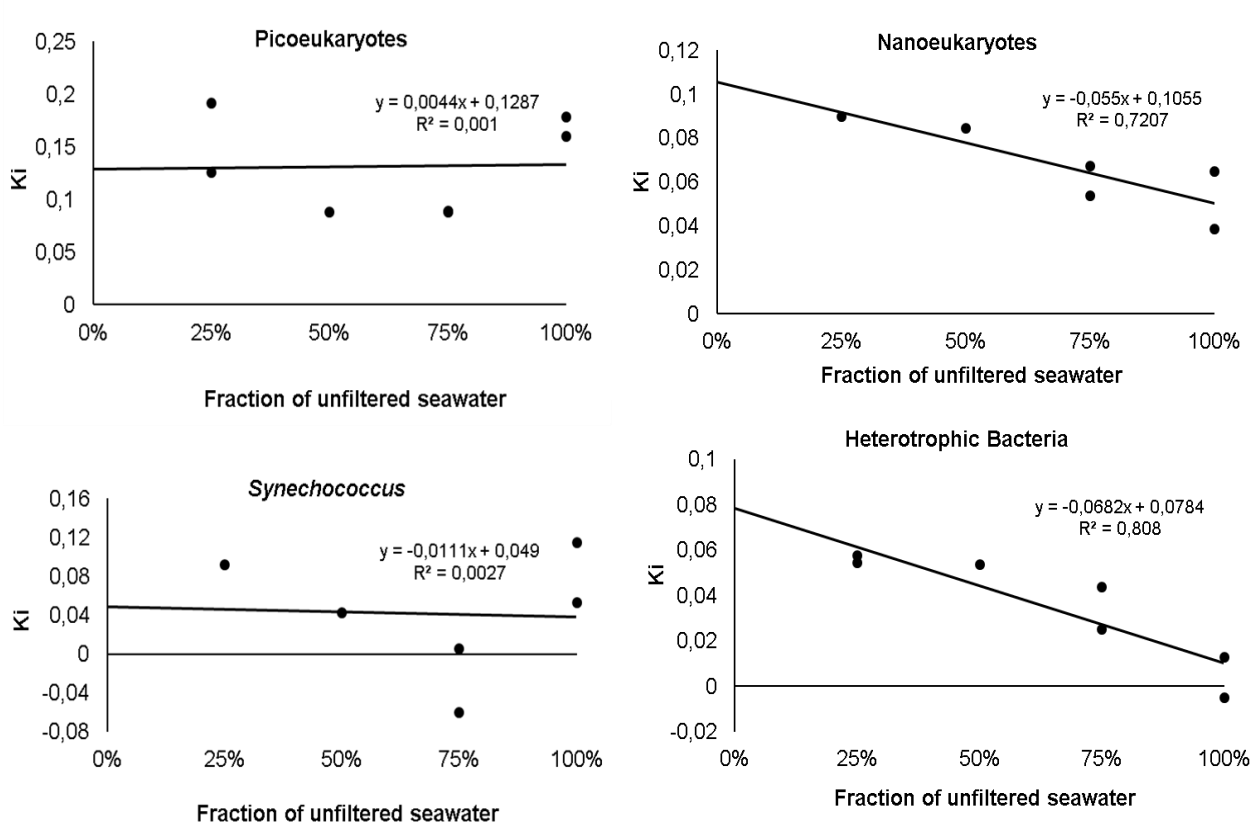

Figure 10: Results of Exp^{Uba1} dilution experiment to estimate growth and mortality rates for all biological groups identified. Regression analyses between the fraction of unfiltered water and apparent growth (Ki).

In Exp^{Uba2a} the growth rate of nanoeukaryotes were the highest amoung the autotrophic groups (μ =0.036 day^{-1}) and their mortality was (m) 0.023 day^{-1}. *Synechococcus* growth decreased and after 50% unfiltered water it was observed

a negative Ki from -0.018 to -0.042 day^{-1} with mortality rate above the growth (μ =0.014 day^{-1}; m =0.017 day^{-1}) (R^2=0.65). The same occurred in picoeukaryotes growth (μ =0.024 day^{-1}) with negative Ki of -0.0047 day^{-1} in 75% and -0.003 day^{-1} in 100% unfiltered water and mortality rate (m) of 0.027 day^{-1} (R^2=0.77). Heterotrophic bacteria showed a low growth and mortality rate, respectively 0.008 day^{-1} (μ) and 0.002 day^{-1} (m), but the results were not statistically significant (Figure 11; Table 4).

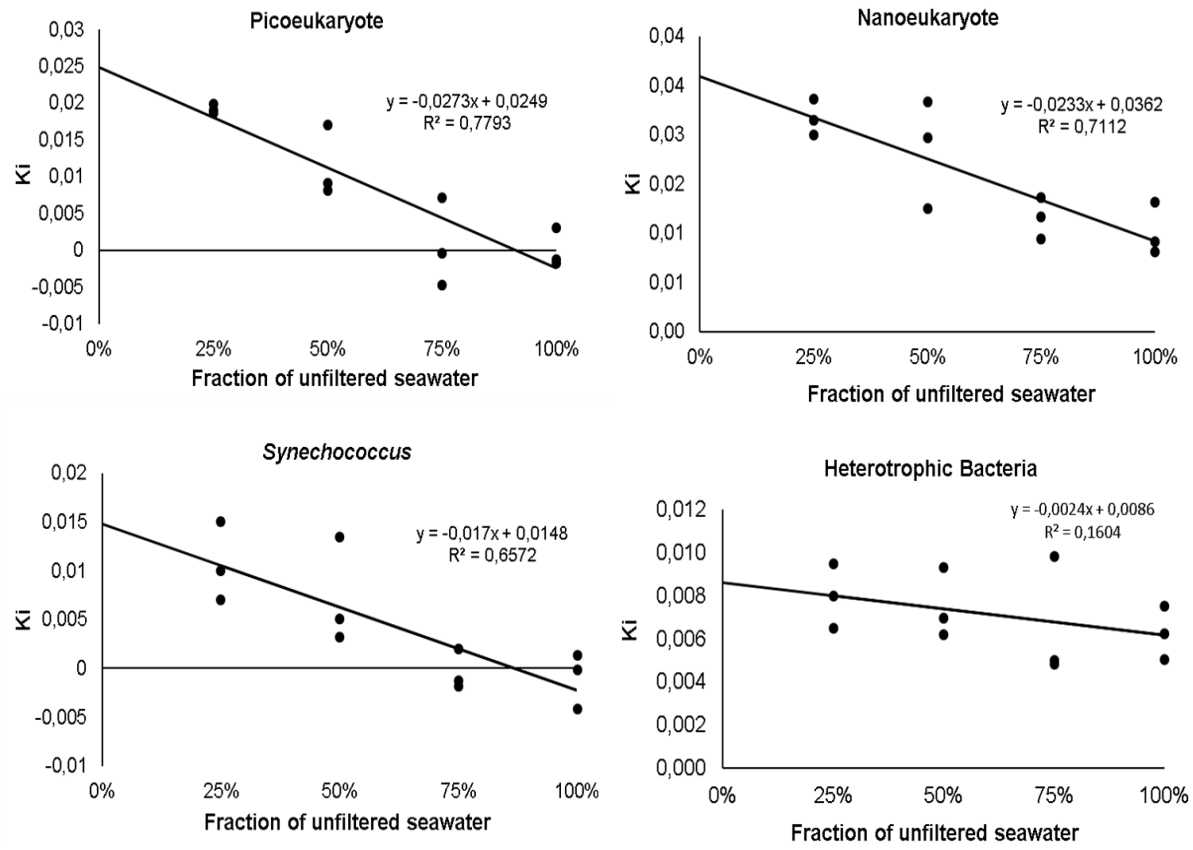

Figure 11: Results of Exp^{Uba2a} dilution experiment to estimate growth and mortality rates for all biological groups identified. Regression analyses between the fraction of unfiltered water and apparent growth (Ki).

In Exp^{Uba2b} nanoeukaryotes mortality rate was 0.022 day^{-1} (m) which was similar to the rate observed in Exp^{Uba2a}, although growth rate of 0.017 day^{-1} (μ) was lower than in Exp^{Uba2a}. *Synechococcus* growth rate (μ =0.002 day^{-1}) was negative and mortality rate (m) was similar to the other two experiments 0.015

day^{-1} (R^2=0.60). Picoeukaryotes growth was the highest among the autotrophs in this experiment (μ =0.052day^{-1}) and mortality rate (m) was 0.037 day^{-1} being the most grazed group of this experiment (R^2=0.89). Heterotrophic bacteria growth rate (μ) was 0.029 day^{-1} and mortality rate (m) 0.002 day^{-1} similar to Exp^{Uba2a} (Figure 12; Table 4).

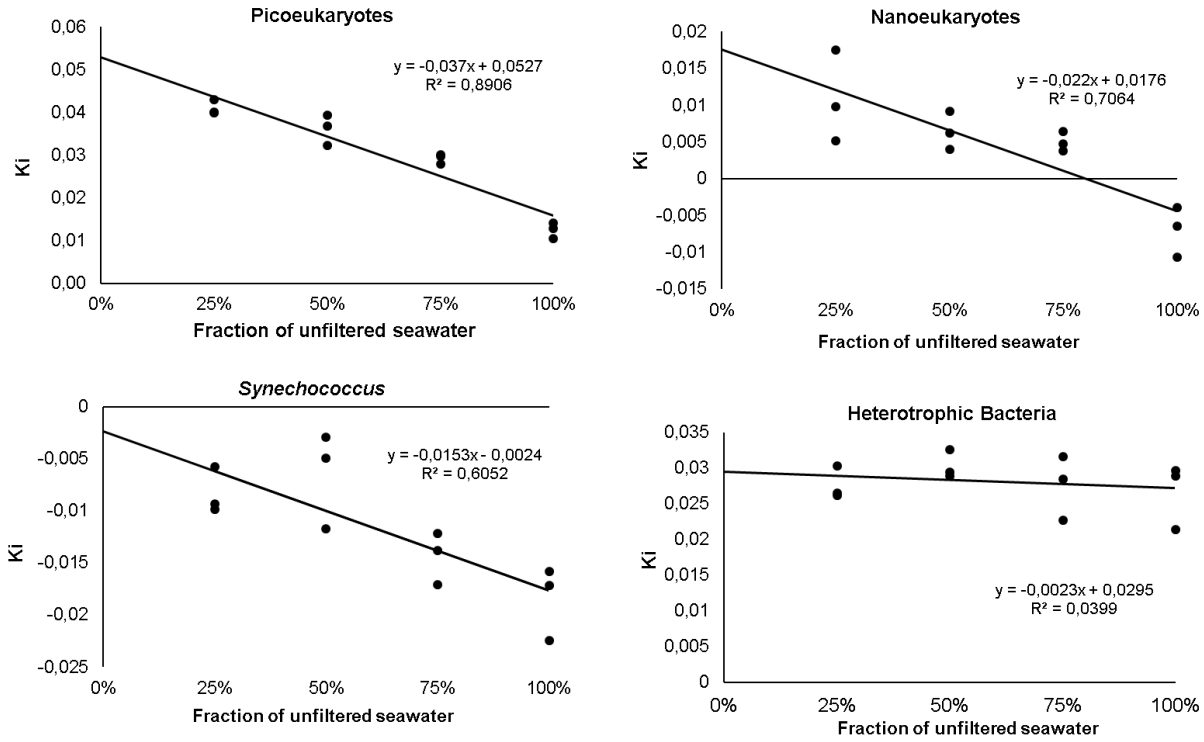

Figure 12: Results of Exp^{Uba2b} dilution experiment to estimate growth and mortality rates for all biological groups identified. Regression analyses between the fraction of unfiltered water and apparent growth (Ki).

Table 4: Summary of growth (μ) and grazing (m) rates per day quantified in all the experiments in Ubatuba.

	Growth rate (μ)	Grazing rate (m)	R^2
	Picoeukaryotes		
Uba1	0.128	*0.004	0.001
Uba2a	0.024	0.027	0.77
Uba2b	0.052	0.037	0.89
	Synechococcus		
Uba1	0.049	0.011	0.002
Uba2a	0.014	0.017	0.65
Uba2b	**0.002	0.015	0.60
	Nanoeukaryotes		
Uba1	0.105	0.055	0.72
Uba2a	0.036	0.023	0.71
Uba2b	0.017	0.022	0.70
	Het. Bacteria		
Uba1	0.078	0.068	0.80
Uba2a	0.008	0.002	0.16
Uba2b	0.029	0.002	0.03

* Positive Slope and no grazing quantified

** Negative growth rate

It was possible to estimate the growth and grazing of the organisms due to a linear regression model that analyzed a hypothetical dilution close to 0% unfiltered water in which consumption did not occur. Conversion factor for biomass of carbon of nanoeukaryotes were not represented since there is no conversion factor for this group (Table 5). In Exp^{Uba1} only heterotrophic bacteria could be analyzed due to statistical significance (R^2=0.80; Table 4).

Heterotrophic bacteria grazing was 53.85 μgC.L-1 per day in Exp^{Uba1} and 97.61 μgC.L-1 per day of final biomass (Table 5). In Exp^{Uba2a} heterotrophic bacteria grazing was 2.53 μgC.L-1 per day and final biomass was 44.98 $\mu gC.L^{-1}$. The autotrophic biomass of carbon grazed in Exp^{Uba2a} was 2.26 μgC.L-1 per day for *Synechococcus* and 35.97 $\mu gC.L^{-1}$ for picoeukaryotes (Table 5). In Exp^{Uba2b}

heterotrophic bacteria final biomass was 61.41 µgC.L-1 per day and only 3.00 µgC.L-1 per day was consumed. *Synechococcus* in Exp^{Uba2b} grazed biomass was 1.14 µgC.L-1 per day of 3.90 µgC.L-1 per day final growth. Picoeukaryotes in Exp^{Uba2b} grazed biomass was 50.46 µgC.L-1 per day of 92.73 µgC.L-1 per day final growth (Table 5).

Table 5: Final biomass, net biomass and grazing in µgC.L-1 per day estimated trough linear regression. Plotted only 100% unfiltered water.

PicoCells	Final biomass	Net biomass	Grazing	R^2
		Uba1		
HetBact	97.61	43.76	53.85	0.85
		Uba2a		
HetBact	44.98	42.45	2.53	0.15
Synecho	6.86	4.60	2.26	0.63
Peuk	76.67	40.69	35.97	0.79
		Uba2b		
HetBact	61.41	58.40	3.00	0.03
Synecho	3.90	2.76	1.14	0.58
Peuk	92.73	42.28	50.46	0.92

*Final biomass without grazing activity µgC.L^{-1}

**Net biomass with grazing activity

6. DISCUSSION

Results of the pilot experiment conducted in Mamanguá revealed a dynamic balance between grazing losses and gains due to cell growth because the numbers of cells did not change throughout the experiment. This is what occurs in a natural system where the cell number of these pico-sized organisms does not change considerably. The abiotic parameters remained constant throughout the whole incubation. Low concentrations of the nutrients corroborated with AIDAR *et al.* (1993) who characterized the southeast of Brazil as a meso-oligotrophic ecosystem. The dynamics of these microbial community were in

equilibrium between growth and loss due to grazing as in “steady state” production systems (CUSHING, 1989).

Even though the abiotic parameters were the same, the results are not always consistent from one day to the other because we can deal with different communities. Therefore, variations of microbial processes in these experiments were not caused by the abiotic conditions such as nutrients concentrations, light and temperature because the incubations were under similar conditions. Indeed, associated just with microbial processes.

S*ynechococcus* represented approximately 40% of the total amount of organisms in number of cells throughout the experiment, and so did heterotrophic bacteria. *Synechococcus* high density in the microcosms experiment is in accordance with the dominance of pico-sized cells dominance along the coast of São Paulo state where these organisms have the ability of growing at low nutrient conditions at these trophic levels (AIDAR *et al.* 1993).

There was an inversion of biomass of carbon between *Synechococcus* in 60μm and 5μm filtered microcosms in Exp^{MMG} on the end of the incubation. This may be the result of cells division since this group usually divides at night (DOLAN *et al*. 1999). Moreover, within the 60μm filtered microcosms there were more grazers and hence it was occurring more grazing pressure.

Exp^{MMG} was not enough to test the hypothesis that heterotrophic bacteria are the most relevant pico-sized cells to the carbon flux, and therefore, grazing rates could not be evaluated appropriately. On the other hand, dilution technique applied in $Exps^{Uba}$ allowed a better assessment of grazing and growth rate of the picoplankton which were necessary to determine flux of carbon at these low trophic levels. The abiotic parameters in both experiments in Ubatuba were similar and in accordance with AIDAR *et al.* (1993). Therefore, cell densities in both experiments were mostly due to microbial processes and not due to environmental parameters. Also, there were consistency between results confirming that there were no methodological problems.

In Exp^{Uba1} picoeukaryotes growth rate did not change along the dilutions and therefore mortality rate was not quantified. It is showed a positive slope in the graph that according to CALBET and SAIZ (2013) are frequent when carnivorous

predators are heavily predating herbivorous grazers. For instance, the presence of trophic cascades during incubations may be the reason that the linearity of the relationship between dilution levels and picoeukaryotes could not be seen. Then, *Synechococcus* and nanoeukaryotes growth rates were also high and their mortality rates could be quantified. However only nanoeukaryotes showed a statistically significant data. Heterotrophic bacteria biomass was elevated and so did the respiration rate of the community. According to their mortality rate there was an increasing grazing pressure. It was grazed more than half of the heterotrophic bacteria production (Table 5) that was supposed to diminish the respiration level, yet the opposite occurred evidencing that not only bacteria but also their grazers respiration lead to a positive NCP. Also, may be due to *Prorocentrum*'s mixotrophic activity Sometimes, mixotrophy can control the natural population dynamics of their preys including heterotrophic bacteria.

In Exp^{Uba2a} GPP was lower than in Exp^{Uba1}, growth rates of all the autotrophs were low and so did heterotrophic bacteria. However, there was 10x less heterotrophic bacteria than in Exp^{Uba1}, their biomass of carbon was not higher than picoeukaryotes' biomass and for this reason total respiration decreased and the ecosystem became more autotrophic. The autotrophic biomass was dominated by nanoeukaryotes, the only group with growth rate above mortality. Both *Synechococcus* and picoeukaryotes growth were below their consumption and yet chlorophyll-*a* increased from 2.67 $\mu g.L^{-1}$ to 3.26 $\mu g.L^{-1}$ evidencing that all these autotrophic organisms were normally photosynthesizing. According to DUARTE (2013) GPP increases proportionately with chlorophyll-*a* concentration as observed here leading to a positive NCP that in the end was similar to the rate quantified in Exp^{Uba1}.

In the Exp^{Uba2b} the autotrophic groups were being actively grazed as in Exp^{Uba2a}. However, the growth rate of *Synechococcus* did not balance their grazing pressure. The same role is true in the case of Nanoeukaryotes because their mortality rate was above their growth meaning the group could also not keep the balance between gain and loss. Differently from Exp^{Uba2a} autotrophic biomass was dominated by picoeukaryotes as the only autotrophic group actively growing above their mortality. However, chlorophyll-*a* in these pico-sized cells are not as representative as in nanoeukaryotes (RII *et al.* 2016) then chlorophyll-*a*

concentration decreased from 2.08 μg.L^{-1} to 1.56 μg.L^{-1}. Heterotrophic bacteria mortality rates in Exp^{Uba2a} and Exp^{Uba2b} were similar, but growth rate was higher in Exp^{Uba2b} than in Exp^{Uba2}. Therefore, respiration increased and overpassed GPP leading to negative NCP.

Higher photosynthetic rates in Exp^{Uba1} and Exp^{Uba2a} and low respiration rates lead to a positive NCP. In Exp^{Uba2a} respiration was higher therefore NCP was negative. Production and grazing vary daily, affecting the balance between gains and losses. According to WILLIANS *et al.* (2013) oligotrophic ecosystems exhibit alternated bursts of autotrophy that can be potentially transferred and be rapidly consumed in the local pelagic system. But usually respiration exceeds photosynthesis in these low production areas. The coastal waters off Ubatuba depicted meso-oligotrophic characteristics with absence of significant input of nutrients (AIDAR *et al.* 1993). Nevertheless, lateral advection of different water masses can change the taxonomic compositions of its low trophic levels.

Heterotrophic bacteria and picoeukaryotes were the most important biomass of carbon in the experiments here conducted corroborating with AGAWIN *et al.* (2000) that also found higher biomass of picophytoplankton in less productive waters. DUARTE *et al*. (2013) described that communities of oligotrophic oceans are considered heterotrophic because of the bacterial carbon consumed that tends to exceed primary production. It was observed that grazing rate of heterotrophic bacteria in Exp^{Uba1} was higher than all the others experiments and even though NCP in this experiment was positive. The contribution of biomass of corroborated with BERGO *et al.* (2017) and GÉRIKAS *et al.* (2016) (Table 6)

Table 6: picofitoplankton and bacterioplankton contribution of carbon biomass. Comparison among this current study with other reseachers.

Contribution of biomass of carbon *				
	Current study	Bergo *et al.* 2017	Gérikas *et al.* 2016	Linacre *et al.* 2015
	SBC	SBB	SBB	SGM
Synecho	6.86	9.06	15.44	1.67
	3.90			
PEuk	76.67	20.92	16.35	3.09
	92.73			
HetBact	53.85	67.60	59.65	11.22
	2.53			
	3.00			

SBC= Southern Brazilian Coast SBB= South Brazilian Bight SGM= Southern Gulf of Mexico

*µgC.L^{-1}

7. CONCLUSION

In contrast to what is usually expected in oligotrophic ecosystems dominated by the heterotrophism that prevail in the microbial loop (sensu AZAM, 1983), the results of my experiments showed the flux of Carbon from both heterotrophic and autotrophic bacteria to grazers of the second trophic level was not as important as the flux of Carbon from picoeukarytes to grazers. One of the reasons why this result came out of the experiments may rely on the preferential removal of autotrophic cells by mixotrophic dinoflagellates. The dominant grazer in the microscosm experiments was *Prorocentrum* sp. which is in fact a well-known mixotrophic behavior induced by nutrients starvation (JOHNSON, 2014). Nutrients, mainly Nitrogen, is in fact limiting autotrophic cells in the coastal zone off Ubatuba (AIDAR *et al.*, 1993). Anyway, the results here obtained may not conclude that the exceptionally higher grazing rates on autotrophic cells than on heterotrophic bacteria might be done by mixotrophism. It is premature to consider pico-eukaryotes as the main source of carbon to grazers on a regular basis, since the experimental results may represent exceptions along the annual carbon flow dynamics in the study area. Nevertheless, it is important to consider the major role of pico- and nanoeukariotes in the carbon flow at these meso-oligotrophic systems. More experiments such as these must be conducted in order to have a better assessment of the origin and fate of organic carbon at low trophic levels in the meso-oligotrophic coastal systems of the southeastern Brazil and overall at any western boundary coastal subtropical ecosystem similar to my study area.

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