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COUPLING BACTERIAL ABUNDANCE WITH PRODUCTION IN A POLLUTED TROPICAL COASTAL BAY

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Resumo

As variações das bactérias heterotróficas planctônicas em águas tropicais, em escalas de tempo de meses a anos, são pouco conhecidas. Neste estudo, a abundância de bactérias, a atividade metabólica, e parâmetros químicos foram medidos semanalmente em dois pontos estratégicos na Baía de Guanabra (Brasil), entre julho de 1998 e setembro de 1999. Os locais de coleta representam o melhor (eutrófico) e o pior (hipereutrófico) panoramas de qualidade de água na baía. No ponto eutrófico foi observado um estreito acoplamento entre densidade bacteriana ($9.44\ 10^5$ cell mL^{-1}) e atividade metabólica ($1.95\ \mu gC\ L^{-1}\ h^{-1}$), onde processos do tipo "bottom-up" controlam as populações do bacterioplâncton. O incremento na abundância bacteriana foi devido à células ativas, sendo identificada correlação das bactérias com o fitoplancton. No ponto hipertrófico, abundância e atividade bacteriana foram em geral uma ordem de magnitude superior, entretanto não se correlacionaram, indicando controle das populações bacterianas to tipo *"top-down"*. A reciclagem de fósforo e nitrogênio foi medida durante a mistura estuarina, sendo observadas correlações com as bactérias. As diferenças na qualidade de água entre os locais eutrófico e hipereutrófico, associadas à predação, salinidade e a outros fatores de inativação, podem explicar os padrões de abundância e atividade bacteriana encontrados ao longo do estuário.

Palavras-chave: syto13, citometria de fluxo, incorporação de ^{3}H-leucina, eutroficação, Baía de Guanabara.

Abstract

Little is known about fluctuation in heterotrophic planktonic bacteria in tropical waters on time-scales of months-to-years. In this study, bacterial abundance, metabolic activity, and chemical parameters were measured weekly at two strategic sites in Guanabara Bay (Brazil), from July 1998 to September 1999. The sites represent the best (eutrophic) and the worst (hypertrophic) water quality scenarios in this bay. A tight coupling between bacterial abundance ($9.44\ 10^5$ cell mL^{-1}) and bacterial production ($1.95\ \mu gC\ L^{-1}\ h^{-1}$) was observed at the eutrophic site, where bottom-up processes control bacterioplankton populations. The increment in bacterial abundance was due to active cells, and bacteria were found to be correlated to phytoplankton. At the hypertrophic site, bacterial abundance and production were generally one-fold higher. However, they were not correlated, indicating top-down control of bacterial populations. P and N recycling were measured during estuarine mixing, and correlations with bacteria were observed. Differences in water quality between eutrophic and hypertrophic sites, associated with predation, salinity and other inactivation factors, may explain the patterns of bacterial abundance and activity found along the estuary.

Key-words: syto13, flow cytometry, ^{3}H-leucine incorporation, eutrophication, Guanabara Bay.

Introduction

Heterotrophic bacteria decompose organic matter, thus making inorganic nutrients available to phytoplanktonic photosynthesis (Azam & Cho, 1987). This nutrient regeneration allows the continuity of primary production in the euphotic zone even in the absence of new inputs of enriched water. The role of heterotrophic bacteria in the dynamics of aquatic ecosystems has been better understood in the last decades due to several studies measuring both bacterial biomass and activity (Cole *et al.*, 1988; Servais, 1992; Aas *et al.*, 1996; Sommaruga *et al.*, 1997; Hoppe *et al.*, 1998).

Besides their role as decomposers, bacteria are also important in the aquatic ecosystem food web (Pomeroy, 1974). They are able to incorporate dissolved organic matter at low concentrations, converting it into particulate organic matter. Consumption of bacteria by protists, and of these by microzooplankton, creates a paralallel flux of energy to the classic food web, the "microbial loop" (Azam *et al.*, 1983). A high amount of organic matter that would not be available to the higher trophic levels, due to its small dimensions, becomes integrated to the food web by the production of bacterial biomass. This concept has greatly changed the perception of the true role of bacteria in aquatic ecosystems.

Several approaches have been introduced to measure the abundance, biomass, activity, and production of bacterioplankton (Bowden, 1977; Sorokin & Lyutsarev, 1978; Porter & Feig, 1980; Furham & Azam, 1982; Azam *et al.*, 1983; Amann *et al.*, 1995). Among these, the incorporation of ^{3}H-leucine (Kirchman *et al.*, 1985) is used to estimate bacterial production by means of their protein synthesis. Since the protein synthesis uses compounds absorbed from the environment, bacterial production rates will depend on the availability of nutrients and organic matter. Therefore, the bacterial production rates are expected to be higher in highy eutrophicated ecosystems, such as Guanabara Bay, Rio de Janeiro, Brazil.

Guanabara Bay is located in a humid sub-tropical region (43°W, 23°S), surrounded by the second largest metropolitan area in Brazil (Fig. 1). Like several other coastal regions in the world, this bay is under great antrophic pressure. It has a 4,000 km^2 drainage basin that is northernly limited by a 2,000 m high mountain range. Steep slopes favour torrential runoff during rain storms. The bay receives around 25 m^3 s^{-1} of water from rivers, diluted in its 2 10^9 m^3 and 381 km^2 area (FEEMA, 1990 and 1998). A population above 10 million people discharges 470 tons of BOD, plus 5.5 tons of garbage daily. Industrial activities produce a further 150 tons of sewage per day. Attention should be given to the largest industrial unit, the Duque de Caxias Oil Plant (Reduc).

Potential uses of this water system include, mainly, navigation, fishing and leisure. However, disorderly occupation, and both domestic and industrial

waste dilution have prevailed. Human interference has caused serious damage both in ecological and social-economical senses. As a consquence the bay, its drainage basin, and its few remaining mangrove areas are suffering from eutrophication (FEEMA, 1990; Hagler & Hagler, 1981; Pfeiffer *et al.*, 1982; Mayr *et al.*, 1989; Paranhos, 1998; Valentin *et al.*, 1999), similarly to other densely urbanised areas in the world. Despite this, Guanabara Bay has several inlets and areas with different water quality standards (Contador & Paranhos, 1996). Although this is one of the most eutrophicated coastal ecosystems in the world, it still has a surprisingly living resource potential (Mayr *et al.*, 1989).

Recent concern regarding coastal contamination, ecosystem health and long-term changes in food web structure, underscores the relevance of understanding the ecology of coastal prokaryotic assemblages (Hayward, 1996). Relatively little is known about fluctuation of heterotrophic planktonic bacteria on time scales of months-to-years, severely limiting the development of organic matter cycling models. The few studies on that subject (e.g. Murray *et al.*, 1999) were done in temperate areas, and none has covered a tropical coastal region. Our approach was to assess the bacterial abundance by the method of syto13 DNA staining and flow cytometry, and bacterial production via rates of incorporation of tritiated leucine as a proxy for bacterial protein synthesis. Our objective was to verify the coupling of bacterial abundance and production, as well as their regulating factors in two sites with very different water quality status in Guanabara Bay.

Material and methods

Sample collection

Two different sites were sampled, based on previous studies in Guanabara Bay (Mayr *et al.*, 1989; Villac *et al.*, 1991; Contador & Paranhos, 1996): (1) The outer and eutrophic Urca inlet, representing the best water quality scenario in Guanabara bay; and (2) the inner and hypertrophic Ramos beach, as a model of the worst water quality panorama (Fig. 1). A total of 46 samples has been collected weekly from July 1998 to September 1999 at each site.

Water sampling and analysis

Water samples were taken with an acid-cleaned Van Dorn bottle at 0.5 m of depth. Temperatures were determined in the field with calibrated thermometers. Sub-samples were first drained into a 300 mL BOD flask for dissolved oxygen analysis, fixed immediately with manganese and iodide solutions and kept submerged in the dark until processing in the laboratory (no longer than 1 h). Water samples were then stored in a 1 L polypropilene flask, in the dark, on ice, until taken to the laboratory (maximum 1 h) for salinity, nutrient and chlorophyll *a* analysis. Sub-samples for bacteria were collected at the surface water level with sterile flasks and stored on ice in the dark (no longer than 1 h) until further analysis.

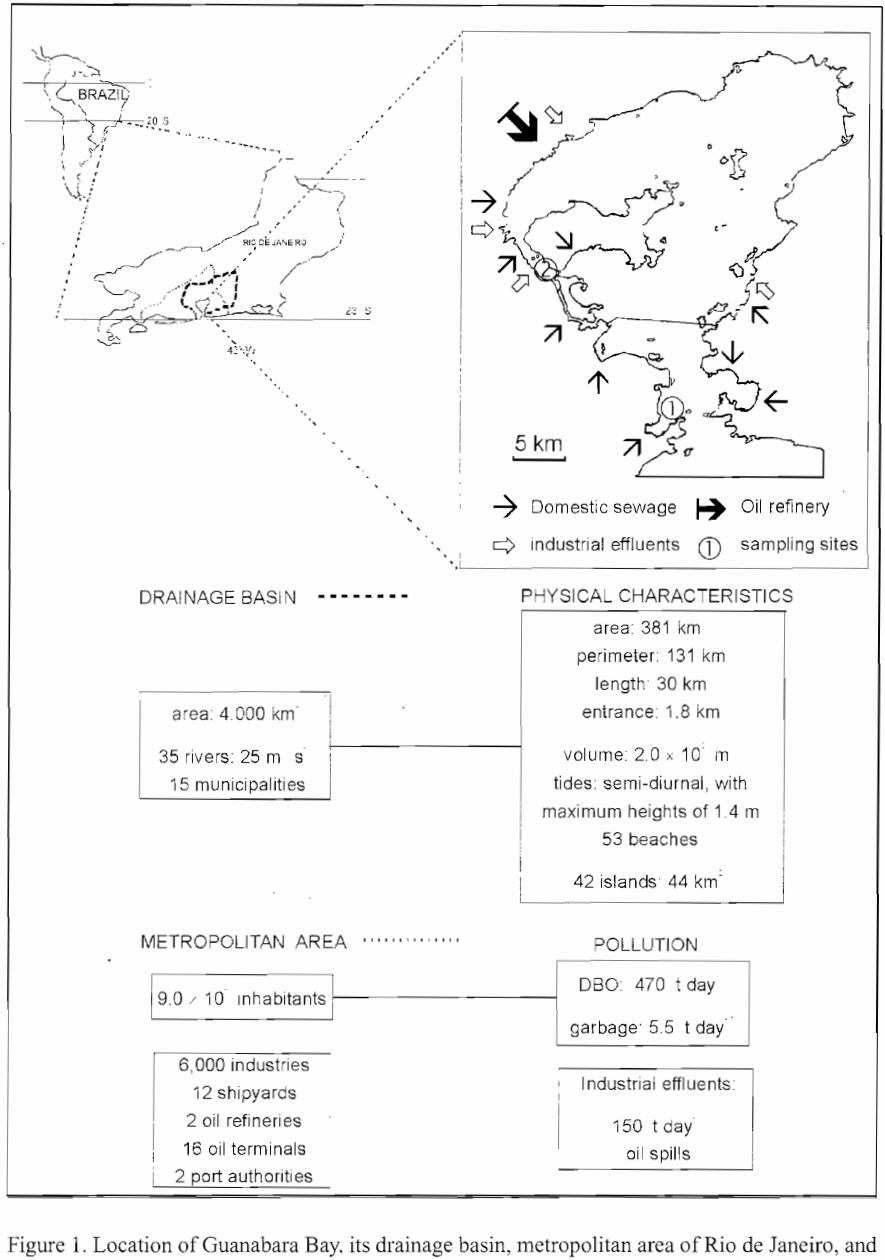

Figure 1. Location of Guanabara Bay, its drainage basin, metropolitan area of Rio de Janeiro, and the sampling sites: Outer site - Urca inlet (1) and Inner site - Ramos beach (2). Pollution data from FEEMA (1990).

Chemical analysis

For chemical analysis, triplicate samples were analyzed in the laboratory. Salinity was measured with a salinometer calibrated against Standard Sea Water and dissolved oxygen by Winkler-azide (CNEXO, 1983). Reactive orthophosphate was analysed by the molybdenium blue method (Grasshoff *et al.*, 1983). Ammoniac nitrogen ($N\text{-}NH_3 + N\text{-}NH_4^+$, hereafter referred to as ammonia) was determined by indophenol (Parsons *et al.*, 1984). Nitrite was determined by diazotation method (Grasshoff *et al.*, 1983). Nitrate was determined by reduction in a Cd-Cu column followed by diazotation (Grasshoff *et al.*, 1983). Chlorophyll *a* analysis was performed by vacuum filtration through cellulose membrane filters (Millipore® HAWP 0.45 μm), extracted with 90% acetone. Spectrophotometer readings and calculations followed Parsons *et al.* (1984), and were calibrated with pure chlorophyll *a* (Sigma C-6144). Practical Salinity Units were used (UNESCO, 1981) thereafter referred to as salinity (Symbol S). For calibration purposes, we used salinity and nutrient standards from Ocean Scientific International (http://www.oceanscientific.com).

Flow cytometric enumeration of bacterial abundance

Total bacterial cellular abundance was determined after DNA staining with fluorochrome syto13 (Molecular Probes, ref. S-7575) at 2.5 μM in samples fixed with paraformaldehyde 2% (final concentration, 0.22 μm filtered just prior to use), according to del Giorgio *et al.* (1996a), and adapted for seawater by Gasol & Morán (1999). In spite of the availability of brighter and perhaps more efficient fluorochromes for that purpose, the choice did not make a significant difference on cellular abundance determination (Lebaron *et al.*, 1998; Gasol & Morán, 1999; Gasol *et al.*, 1999).

Fifty μL of a working bead solution were added (sampled after intense sonication) to each 1.0 mL triplicate sample in a sterile cytometer plastic tube (Falcon 2052), and the content was homogenized in a vortex for 15 sec. Immediately, inside a fume hood under dim light, 40 μL of a syto13 working solution were added to each tube (final concentration 2.5 μM), and the content was homogenized in a vortex apparatus for 15 sec. The tubes were covered with a foil, and kept in the dark for 15 min until cytometer readings were taken (between 30 to 90 min). For detection, a B&D FacsCalibur flow cytometer with standard laser and optics was used: an air cooled argon ion laser emitting at 488 nm, fixed laser alignment, and fixed optical components. The sample was aspirated by a 70 μm nozzle, and sterile PBS buffer (0.22 μm filtered just prior use) was used as sheath fluid (supplied by the manufacturer). Between two successive samples, the aspiration and optical systems were cleaned with sterile Milli-Q water for 2 min. The fluorescence emitted by the samples was collected by the foward scatter (FCS), and side scatter at 90° (SSC), both with emission at 488 nm. Signals ware also collected by different photomultipliers: FL1 (530±30 nm); FL2 (585±42 nm); FL3 (> 650 nm). Counts ware made in triplicate at low mode (12 uL min^{-1}) for 30 sec, and data acquired in logarithmic mode. Data were obtained and analysed by Cell

Quest software (B&D) in Macintosh, or by WinMDI 2.5 software (Trotter, 1996) in Windows. Bacteria ware detected and cellular adundance determined by their signature in a side scatter plot (SSC, X axis, and indicative of cellular size) versus green fluorescence plot (FL1, Y axis, green fluorescence from syto13, related to DNA content), and total bacterial cellular abundance based on stained cells and fluorescent beads counts were determined by the formulae: {(bacteria ÷ beads cytometry) x beads microscope}.

For calibration of side scatter and green fluorescence signals, and as internal standard for cytometric counts and measures, fluorescent latex beads (Fluoresbrite YG carboxilate microspheres with 0.98 and 1.58 μm diameter, Polysciences) were added at a known density to each sample. From the original beads solution (~10^7 beads mL), a working solution was produced. After sonication (70 Hz, 5 min, ice bath), to avoid doubles and triplets, a 150 μL aliquot was taken and diluted with 20 mL of sterile Milli-Q water. The bead standard concentration was determined by epifluorescence microscopy, following the observations made by Lebaron *et al.* (1994) for accurate and precise counting.

^{3}H-leucine incorporation for bacterial activity measurement

Analysis followed the method of Kirchman *et al.* (1985) modified for the microcentrifuge, instead of filtration, by Smith & Azam (1992), and thus producing a minimum of radioactive waste. Following concentration-dependent incorporation experiments, 10 nM was established as the saturation concentration for Guanabara Bay waters (Gonzalez *et al.*, 2000). Triplicates of 1.7 mL of water sample were incubated with ^{3}H-leucine (Amersham TRK 510, specific activity of 171 mCi $nmol^{-1}$) at the concentrations of 10 nM in 2 mL screwcap eppendorf tubes. One tube was amended with 90 μL 100% ice-cold trichloroacetic acid (TCA) as a killed control for abiotic uptake into the macromolecular fraction. Tubes were shaken on a vortex and incubated in the dark for 30 min. Incubations were stopped by the addition of 90 μL of 100% ice-cold TCA. The tubes were again shaken on a vortex, centrifuged at 13000 rpm under 20°C (Haraeus Biofuge fresco) for 10 min and the supernatant was carefully aspired under vacuum. Pellets were washed with 1.9 mL of 5% ice-cold TCA and with 1.7 mL of 80% ice-cold ethanol, centrifuged and dried as before. A volume of 0.5 mL of liquid scintillation cocktail (1.0 g of POPOP [Sigma P-3754], 7.0 g of POP [Sigma D-4630], 1.0 L of toluene) was added and samples were counted in a Beckman LS 6000 SC liquid scintillation counter, with internal calibration and quench correction. Rates of leucine incorporation were determined by subtracting the DPM determined in the TCA-killed control, and then by converting DPM to moles of leucine incorporated per hour. Bacterial carbon production was calculated using the protein/carbon correction factor of 0,86 (Simon & Azam, 1989).

Results and Discussion

The sampling period ranged from July 1998 to September 1999, and field trips were performed on a weekly basis, with a total of 46 samples considered in this study. Significant differences were found between the oceanographic parameters recorded at the 2 locations, Compared by *t*-test, all variables, except temperature and salinity, were significantly higher at the inner site, whereas dissolved oxygen was significantly lower. These findings imply that the 2 sampling sites were substantially different and should be considered separately.

The eutrophic status of the outer site (Urca inlet), is shown by high nutrient, chlorophyll and bacterial values; however, even higher values were observed at the hypertrophic inner site (Ramos beach, see Table 1). A conservative mixing behaviour has been observed for most nutrients, as refleted by ammonia and salinity ($r^2 = -0.54$). Nitrate was positively correlated with salinity ($r^2 = 0.68$), suggesting nitrification along the estuary. This is the typical pathway from nutrient input to its removal and consumption in Guanabara Bay waters (Paranhos *et al.*, 1998). The difference in patterns observed among sampling sites is due to their characteristic pollution levels. However, most of the nitrogen and phosphorus were found in dissolved form at both sites. On a short-time scale, a great variability was observed for all water quality indicators, most of it related to the tides. The chemical patterns supported the choice of these sites as representatives of the best and the worst scenarios of Guanabara Bay. To further support the sampling location choice, a large salinity range was observed (from 17.38 to 35.33 S), indicating that most of the salinity range found in this bay (FEEMA, 1980 and 1998; Mayr *et al.*, 1989) is represented in this data set (Table 1).

Table 1. Oceanographic parameters from outer (Urca inlet) and inner (Ramos beach) sites in Guanabara Bay, obtained from 07/1998 to 09/1999, n=46: average, coefficient of variation, minimum and maximum values. # Significant differences between sampling points ($p<0.0001$).

	Outer site - Urca inlet				Inner site - Ramos beach			
	Avg	CV%	Min	Max	Avg	CV%	Min	Max
Total cellular abundance (cells mL^{-1}) #	$1.01\ 10^6$	118	$6.87\ 10^4$	$6.98\ 10^6$	$6.90\ 10^6$	70	$1.05\ 10^6$	$2.16\ 10^7$
Bacterial production ($\mu g\ C\ L^{-1}h^{-1}$) #	1.91	100	0.20	7.29	7.35	33	3.08	13.18
Temperature (°C)	23.50	10	19.00	29.00	25.30	11	21.00	31.00
Salinity (S)	33.32	4	29.98	35.12	26.29	12	17.38	30.39
Dissolved Oxygen ($mL\ L^{-1}$)#	3.10	24	0.82	4.27	1.93	91	0.00	7.26
Chlorophyll *a* ($\mu g\ L^{-1}$)#	13.33	99	0.00	58.24	118.2	83	7.26	483.5
Suspended matter ($mg\ L^{-1}$)#	21.39	38	6.20	51.60	42.51	39	12.80	87.43
Orthophosphate ($\mu M\ P\text{-}P_4^{3-}$)#	1.31	36	0.02	2.82	9.05	48	0.06	21.53
Total Phosphorus ($\mu M\ P$)#	2.56	55	0.05	7.39	13.78	39	0.22	26.42
Ammonia ($\mu M\ N\text{-}NH_3/NH_4^+$)#	8.33	65	<0.05	28.99	87.16	43	1.44	162.7
Nitrite ($\mu M\ N\text{-}NO_2^-$)	1.15	43	0.05	2.38	1.79	54	0.11	4.07
Nitrate ($\mu M\ N\text{-}NO_3^-$)#	4.09	63	<0.05	10.97	0.60	153	<0.05	3.42
Total Nitrogen ($\mu M\ N$) #	28.19	42	0.65	68.28	193.6	40	5.0	346.3
Silicate ($\mu M\ Si\text{-}SiO_2^-$)#	24.94	50	0.40	60.99	81.15	60	5.43	176.8

Bacterial abundance determined by flow cytometry and syto13 dye was consistent within sampling sites, and such bacterial data from a tropical coastal bay is reported for the first time. Large differences were observed in bacterial abundance between the sampling sites. On average, values found at the outer site ($1.01\ 10^6$ cells mL^{-1}) were significantly lower than the values found at the inner site ($6.90\ 10^6$ cells mL^{-1}) (Table 1). Despite the significant difference in cellular numbers between sampling points ($p<0,0001$), a similar pattern of variability was observed. Coupled increases and decreases in bacterial abundance were observed for both sites, in response to tidal and seasonal forcings. Bacterial numbers were always inversely correlated to the tides and directly correlated to the summer season. Tidal influence is restricted to short-scale variability, regulating bacterial number distribution along the estuary by tidal pulses. Bacterial abundance varied mostly at the outer site (100%), whereas at the inner site the variability was smaller (33%). This is related to the strongest tidal action at the outer site, and the consequent strongest estuarine mixing and water-related variability. At the inner site, smaller tidal action caused less water quality variability. Abundance peaks in summer season were found in correlation with temperature, insolation and high chlorophyll concentrations. Seasonal variability was observed by the increasing of bacterial numbers between September and April. That sort of seasonal asssessment will need a few more years to display a significant figure of bacterial variability in that tropical polluted coastal bay.

Lowest bacterial activity values were observed at the outer site. Bacterial biomass production, as measured by ^{3}H-leucine incorporation, was observed in a range from 0.20 to 7.29 $\mu gC\ L^{-1}\ h^{-1}$ (average 1.95 $\mu gC\ L^{-1}\ h^{-1}$, CV=100 %). At the hyperthrophic inner site, values were in a range of 3.08 to 13.18 $\mu gC\ L^{-1}\ h^{-1}$ (average 7.35 $\mu gC\ L^{-1}\ h^{-1}$, CV=33%). Bacterial production was significantly different between sampling sites ($p<0.00001$), as observed for cellular abundance, with the same synchronism pattern in response for tidal and seasonal forcings. Bacterial production was inversely correlated to the tides and directly correlated to the summer season.

Estuarine systems, such as Guanabara Bay, are considered changing environments, which make them a challenge to understand the factors that control bacterial populations. Physical and chemical factors that directly or indirectly affect bacterial production change greatly over relatively short distances. These large changes allow the observation of great ranges in environmental and bacterial parameters between regions that are separated by a few kilometers. Both bacterial abundance and activity were negatively correlated to salinity values ($r^2 = -0.47$ and -0.58, respectively; both $p<0,00001$), according to recent reports (Wikner & Hagström, 1999) and positively correlated nutrient values.

The abundance versus activity concept was used to assess relative importance of bottom-up (substrate limitation) and top-down (any sort of removal) processes (Billen *et al.*, 1990). Relationship between log bacterial abundance and log

leucine incorporation at outer site indicated that abundance and activity were tightly coupled (Fig. 2a, slope 0.67, $r^2 = 0.40$). According to Shiah & Ducklow (1995), slopes higher than 0.7 are indicative of strong bottom-up control processes. The cellular abundance increase observed is related to the increase in metabolic active cells. This increase of healthy cells is obviously related to the environmental conditions found at the outer site (Contador & Paranhos, 1996), where eutrophication and competition for resources are not critical. In that outer Guanabara Bay region, bacterial populations seemed to be organic-matter limited, and this demonstrated that such region can cope with organic matter supply. The linear regression between log leucine incorporation and log chlorophyll *a* concentrations indicated a relationship (slope 0.55, $r^2 = 0.29$) between bacterial activity and phytoplankton in Guanabara Bay outer region. This is further supported by the relationship between log chlorophyll *a* concentration and log bacterial abundance (slope 0.58, $r^2 = 0.34$). This might indicate the relationship between the availability of organic matter to increase in bacterial production, but the coupling of bacteria with phytoplankton as well (Hoch & Kirchman, 1993). Bacterial production could be controlled by the phytoplanktonic production despite the large allochthonous sources of dissolved organic matter (Kirchman & Hoch, 1988). We can thus suggest that bacterial populations from the outer Guanabara Bay region are controlled by nutrient limitation and phytoplankton, despite their eutrophic status (Mayr *et al.* ,1989; Contador & Paranhos, 1996, FEEMA, 1998).

An interesting pattern was observed between the two sampling sites. The average, minimum and maximum values of most variables observed further support this interpretation, as the highest values observed at the outer site were around the average observed at inner site. This is true for both bacterial abundance and activity, and also to the majority of the pollution indicators, where values were significantly different between sampling sites. This shows the representativeness of the sampling sites in displaying the extreme water quality scenarios found in Guanabara Bay.

At the hypertrophic site, bacterial abundance and production were not correlated (Fig. 2b, slope –0.09, r^2=0.004), indicating that strong top-down processes controlled the bacterial populations in inner Guanabara Bay. The linear regression between log leucine incorporation and log chlorophyll *a* concentrations indicated no relationship (slope 0.13, $r^2 = 0.09$) between bacterial activity and phytoplankton in Guanabara Bay outer region. In addition, no relationship was observed between log chlorophyll *a* concentration and log bacterial abundance (slope 0.17, $r^2 = 0.06$). Thus, phytoplankton control of bacterial populations at inner Guanabara Bay can be ruled out. The increase in bacterial abundance was not followed by an increase in metabolic active cells. In such harsh environmental conditions, several factors contribute to aquatic bacteria inactivation, as predation, salinity, nutrient and light limitation, to name a few (Rhodes & Kator, 1988; Solic & Krstulovic, 1992; Paranhos *et al.*, 1995). Predation is probably due to ranks high, as protozoplankton abundance (mostly ciliates) is great at inner site (Silva-Neto, I., Institute of Zoology,

UFRJ, pers. comm.). Thus, at the inner Guanabara Bay site, bacterial populations are not substrate-, but environmentally- limited, as a sign of deterioration in that region. The cells can even be viable, but they are not active enough to contribute to overall bacterial production. This indicates that bacterial production is probably more sensitive to environmental gradients and pressures than bacterial abundance alone. We can suggest that bacterial populations from the inner Guanabara Bay region are controlled by removal processes (top-down control), where bacterivory could be an important mechanism (del Giorgio *et al.*, 1996b), and light limitation and chemical pollution should contribute as well.

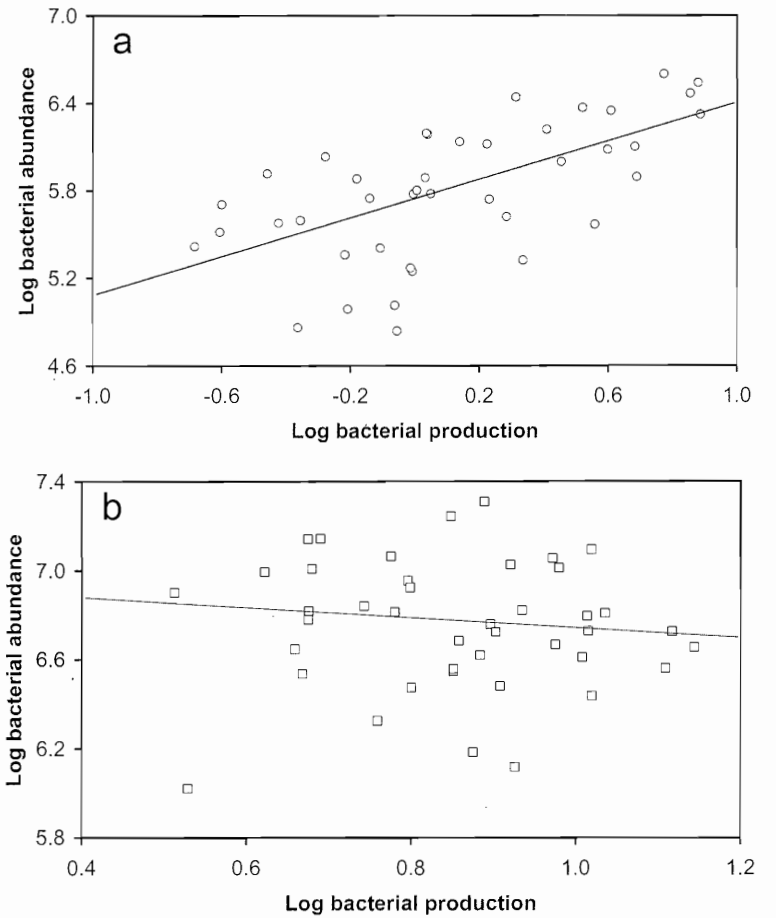

Figure 2. Log-log regression curves between total bacterial cellular abundance and bacterial production. At the outer site (a), bacterial abundance and activity were coupled ($y = 0.68x + 5.74$, $r^2 = 0.40$, $p<0.01$) whereas at the inner site (b) they were not ($y = -0.13x + 6.86$, $r^2 = 0.004$, $p<0.67$)

The coupling of bacterial abundance and activity is an important approach to understand the eutrophication in tropical estuaries submitted to pollution, where we pionnered the use of flow cytometry in aquatic microbial ecology in Brazil. The relation between bacterial variables could access what controls these populations, whether substrate limitation (bottom-up control) or removal (top-down control), and raise discussion on factors regulating the microbial biogeochemistry of tropical estuaries.

Future perspectives

In order to understand the functioning of an aquatic ecosystem, it is relevant to know the microbial component, how microbes are distributed, and what regulates their abundance and biological activity. The use of modern methods in aquatic microbial ecology is revolutionizing not only the way, but the speed with which high quality results are obtained. In such perspective, the flow cytometer approach has been receiving greater attention. Its innerent advantages, such as speed, precision, multiparameter data acquisition, to name a few, justify all the interest. Despite high installation costs, and limitations in biomass estimation, flow cytometry tends to be the reference method for the future. Based on flow cytometry detection, there are other methods and approaches we have been using to understand bacterial populations in some Brazilian water systems, such as Guanabara Bay and the central Atlantic Ocean.

The flow cytometric approach and the use of syto13 fluorochrome is a well-accepted method (see bellow). Nucleic acid differences in bacterial cytometric signatures (high nucleic acid content, related to active cells, and low nucleic acid content, related to cells with lower activity or innactive cells) and size, were not taken into account in this study. The understanding and further quantification of those fractions, accessed by the intensity of FL1 signal (green fluorescence from nucleic acid dye), were proposed as a standard procedure to determine total bacterial numbers instead of the traditional DAPI staining and microscope epifluorescence counts (Gasol *et al.*, 1999; Trousselier *et al.*, 1999; Servais *et al.*, 1999). The investigation on which bacteria are active or not, and how active they are, is being solved by the association of flow cytometry, cell sorting and activity measurement. It is possible to cytometrically identify a target bacterial population, and sort it out from the sample with a flow sorting accessory. Once a target population is sorted out, it is possible to make an activity measurement, such as ^{3}H-leucine incorporation. For more details on that refer to Servais *et al.* (1999).

To access the identity coupled with activity of target bacterial populations, one of the most successful approaches has been the Fluorescence In Situ Hybridization - FISH (Amann *et al.*, 1995), and its promising developments (Ouverney & Fuhrman, 1999). The main advantage of this method is that, in addition to cellular abundance and activity estimates, it is possible to identify groups, or even species of bacteria.

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