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Distribution of viable aerobic heterotrophic bacteria, total number of microorganisms, *α-, β-, γ-* and *δ-proteobacteria* in the eastern coastal zone of Havana and in oceanic waters southwest of Cuba

Distribución de bacterias heterótrofas aerobias viables, número total de microorganismos y de α-, β-, γ- y δ-proteobacterias en un sector costero al E de La Habana y en la aguas oceánicas al SW de Cuba

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ABSTRACT

It was determined the distribution of viable heterotrophic bacteria, Phylum *proteobacteria* and the total number of microorganisms in a coastal sector east of Havana and in oceanic waters southwest of Cuba. Samplings were carried out in September 2005 and May 2006. In general, the concentration of microorganisms was higher in the coastal zone; although in the *SW* of the Exclusive Economic Zone (EEZ), high values were found mainly in stations closest to the shelf, where there is an exchange between nutrient enriched waters from the Gulf of Batabano and the adjacent oligotrophic oceanic waters, and in areas characterized by complex water mass dynamics. In most stations, *proteobacteria* were the highest contributors to the total number of microorganisms. It was observed that the total average concentration of *proteobacteria* was 1.5 times higher in the coastal area, in relation to that in oceanic waters southwest of Cuba, where the α-*proteobacteria* predominated; while the other subclasses were represented by lower percentages.

RESUMEN

Se determinó la distribución de bacterias heterótrofas viables, de las diferentes clases de *proteobacterias* y el número total de microorganismos en un sector de la zona litoral al *E* de La Habana y en las aguas oceánicas al SW de Cuba. Los muestreos se realizaron en septiembre del 2005 y en mayo 2006. En general, la concentración de microorganismos fue mayor en la zona costera; aunque en la Zona Económica Exclusiva (ZEE) al SW se encontraron valores altos, fundamentalmente, en las estaciones más cercanas a la plataforma, donde se produce un intercambio entre las aguas enriquecidas en nutrientes del Golfo de Batabanó y las oceánicas oligotróficas adyacentes, y en zonas que se caracterizan por presentar una dinámica compleja de las masas de agua. En la mayoría de las estaciones la contribución de las *proteobacterias* al número total de microorganismos fue elevada, y se observó que la concentración total promedio de *proteobacterias* fue 1,5 veces superior en la zona costera con relación a la encontrada en las aguas oceánicas al SW de Cuba, donde predominaron las α-*proteobacterias*, mientras que las otras subclases estuvieron representadas en menor porcentaje.

Keywords: Heterotrophic bacteria, *proteobacteria*, coastal waters, oceanic waters, Cuba.
***Palabras clave**: Bacterias heterótrofas, proteobacterias, aguas costeras, aguas oceánicas, Cuba.*

INTRODUCTION

The knowledge on the structure of microbial community, including diversity and total and relative abundance, and their relation to environmental factors is of great importance to understand the role of bacteria in biogeochemical cycles and to detect predictable patterns, since this would allow for more effective management of marine ecosystems (Fenchel, 2008).

Bacterioplankton has a significant role as a major contributor in biogeochemical processes of estuarine and marine environments, and is a key component in microbial food chains (Schauer *et al*., 2003; Peressutti *et al*., 2010). In recent years, the concept of the classical food chain has changed to a more complex alternative model called microbial loop, based on the ability of heterotrophic bacteria for decomposing and recycling organic matter to make it available to other plankton components and, indirectly, also to other higher trophic levels in the marine ecosystem. In the oceans, microorganisms are responsible for 98% of primary production and are involved in all stages of matter degradation, with a key role on the fate of contaminants in highly anthropized ecosystems (Sogin *et al*., 2006).

The Phylum *Proteobacteria* is the largest and most diverse in the Bacteria domain. As a group, these organisms show metabolic diversity and represent the majority of known gram-negative bacteria of medical, industrial, and agricultural significance. It constitutes a group from the evolutionary, geological and environmental points of view, and it is divided into Classes alpha-, beta-, gamma-, delta- and epsilon- *proteobacteria* based on rRNA sequences (Garrity, 2005).

In Cuba, no investigation had been conducted so far on the distribution of *proteobacteria* groups in marine ecosystems and their contribution to the total number of microorganisms. The objective of this study was to determine the distribution pattern of α-, β-, γ- and δ-*proteobacteria*, of viable heterotrophic bacteria and total number of microorganisms during the summer period in a coastal area east of Havana and in oceanic waters southwest of Cuba, as well as their relation to some abiotic factors.

MATERIALS AND METHODS

Description of the study areas

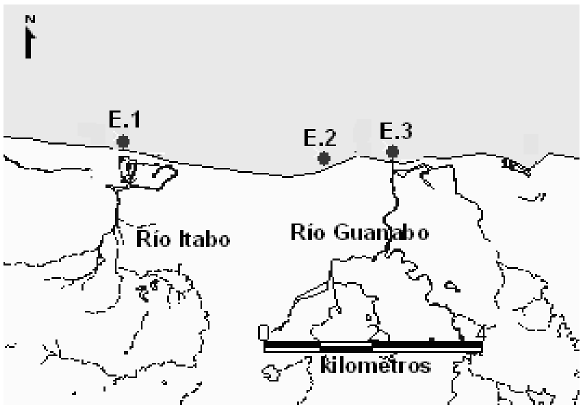
Playas del Este are located on the northeast coast of Cuba's capital, and consist of a series of calcareous sand beaches with a total length of 12.6 km and 30 m wide averagely. This tourist resort covers a coastal area of 2690 ha that limits on the north with the Gulf of Mexico, on the south with Justiz-Campo Florido Valley, on the east with Rincon de Guanabo, and on the west with Bacuranao River. This coastal area is a place where there are natural beaches, river coves, archaeological remains, coral formations and unique vegetation of high endemic value, among other attractions of interest (Acevedo, García & Fernández, 2008).

The oceanic waters south of Cuba cover an extensive maritime region that extends from San Antonio Cape to Maisi Point, and from the island shelf edge and the line formed by Jagua, Bucanero, Silvertown and Paz del Norte banks to the legal limit of southern oceanic waters (Fernández *et al*., 1990).

The southwest region of Cuban Exclusive Economic Zone (EEZ), in summer time, has a mean temperature of 29.2 °C (Gutiérrez *et al*., 2001). Water dynamics is characterized by convergence, divergence and frontal zones, as well as the contribution of waters enriched with organic matter and nutrients from the Gulfs of Batabano and Ana Maria (Arriaza *et al*., 2008).

Samplings

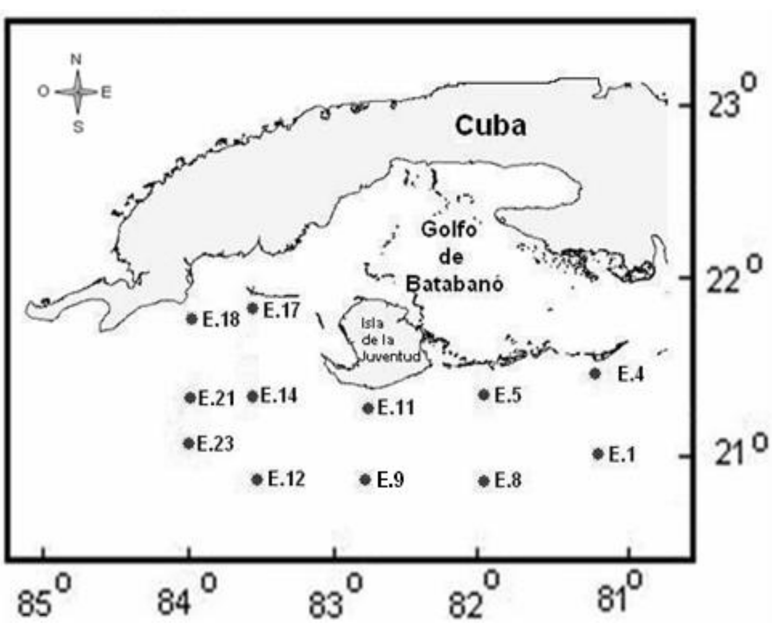
Water samplings were carried out in September 2005 and May 2006, at 15 stations: three in the coastal area east of Havana, and 12 in the Exclusive Economic Zone southwest of Cuba (Figures 1 and 2).



Stations	Latitude	Longitude
E.1	23^{0}10´22.9´´	82^{0}09´48´´
E.2	23^{0}10´14.6´´	82^{0}08´23´´
E.3	23^{0}10´24.0´´	82^{0}07´24´´

Figure 1. Location of sampling stations in the coastal area east of Havana, Cuba.

Figura 1. Localización de las estaciones de muestreo en la zona costera al este de la Habana, Cuba.



Stations	Latitude	Longitude
E.1	21^{0} 04´00´´	81^{0} 31´5´´
E.4	21^{0} 32´0´´	81^{0} 33´00´´
E.5	21^{0} 24´3´´	82^{0} 13´2´´
E.8	20^{0} 56´0´´	82^{0} 13´1´´
E.9	20^{0} 56´0´´	83^{0} 56´ 6´´
E.11	21^{0} 19´20´´	82^{0} 55´1´´
E.12	20^{0} 56´5´´	83^{0} 35´1´´
E.14	21^{0} 23´08´´	83^{0} 36´0´´
E.17	21^{0} 53´5´´	83^{0} 36´ 1´´
E.18	21^{0} 53´5´´	83^{0} 35´ 0´´
E.21	21^{0} 23´2´´	83^{0} 59´ 6´´
E.23	21^{0} 08´0´´	83^{0} 59´5´´

Figure 2. Location of sampling stations in oceanic waters southwest of Cuba.

Figura 2. Localización de las estaciones de muestreo en las aguas oceánicas al SW de Cuba.

Water samples were taken from the subsurface layer, in the coastal zone by means of scuba diving, using 250-mL sterile bottles, and in oceanic waters with 1.5-L Nansen oceanographic bottles.

Bacteriological analysis

For determining the viable aerobic heterotrophic bacteria (VHB) concentration, water samples were cultured by triplicate using spread plate technique in Petri dishes containing marine agar 2216 E medium (Miravet *et al*., 2009). Water samples were diluted up to 10^{-4} (coastal zone) or to 10^{-2} (oceanic waters) with sterile seawater. Petri dishes were incubated at 30° C ± 2 for 72 h and total count of colony forming units (CFUs) was carried out for 3 consecutive days.

After analysis, water samples were preserved by adding 4 % paraformaldehyde (Merck KGaA, Germany). Bottles were stored in the dark until analysis.

The total number of microorganisms was determined by counting total cells stained with 4', 6-diamidino-2-phenylindole (DAPI) (Porter & Feig, 1980), using epifluorescence microscopy (Hobbie, Daley & Jasper, 1977). In each sample, no less than 300 cells were counted to ensure an adequate sample size.

In situ hybridization *of α-, β-, γ-,* and *δ-proteobacteria* groups by using specific probes were determined according to Pernthaler *et al*., 2001. Before hybridization, 0.5 mL of the samples were pre-fixed with 4% paraformaldehyde and filtered using polycarbonate membrane of 0.22 micron porosity and diameter of 25 mm.

The Classes *alphaproteobacteria* (*α*), *betaproteobacteria* (*β*), *gammaproteobacteria* (*γ*) and *deltaproteobacteria* (*δ*) were determined using probes specific to each group. Four oligonucleotide probes labeled with Cy3 (Cyanine 3) and two competitor unlabeled oligonucleotide sequences for classes *α-, β- , γ- and δ-proteobacteria* were used (Table 1).

Table 1. Probes used for counting *proteobacteria* classes using Fluorescent *In Situ* Hybridization (FISH) method.

Tabla 1. Sondas utilizadas para el conteo de las clases de proteobacterias empleando el método de hibridización flourescente in situ (FISH).

Probe	Specificity (subclases)	Probe sequence (5´- 3´)	Market site (position of rRNA) [b]	% FA [c]	References
ALF1b	Alphaproteobacteria	CGT TCG YTC TGA GCC AG [a]	16S (19-35)	30	Manz *et al.*, 1992
BET42a	Betaproteobacteria	GCC TTC CCA CTT CGT TT	23S (1027-1043)	30	Manz *et al.*, 1992
GAM42a	Gammaproteobacteria	GCC TTC CCA CAT CGT TT	23S (1027-1043)	30	Manz *et al.*, 1992
DELTA	Deltaproteobacteria	CGG CGT CGC TGC GTC AGG	16S (385-402)	20	Amann *et al.*, 1995

Where:
a Y indicates C or T.
b Position to *Escherichia coli* (Brosius *et al.*, 1981).
c Percentage of formamide (FA) in the hybridization solution.

Data of abiotic parameters were taken from the Database of the Oceanography Department, under the Cuban Institute of Oceanology.

Statistical analyzes were performed using Origin 7.0 software (Microcal Corp.) and Statistica version 8. Thematic maps were prepared with MAPINFO version 6.5 software.

RESULTS AND DISCUSSION

In the coastal area east of Havana, it was found that the concentration of viable aerobic heterotrophic bacteria (VHB) ranged between 642 and 3910 CFU.mL^{-1} (Table 2). The highest concentration was found in E.3 when compared with the other two stations. This result could be explained due to the location of E3 station close to Guanabo River, a source of organic matter supply of domestic and industrial origin to the adjacent coastal area. Organic matter enrichment in the coastal zone from the river promotes the growth of microorganisms, particularly heterotrophic bacteria. Meanwhile, in ocean waters southwest of Cuba, VHB concentration ranged between 25 and 746 CFU, with an average of 302 ± 33.2 CFU. mL^{-1} (Table 3). In oceanic waters, the highest values were obtained mainly at stations closer to the SW shelf, where there is an exchange between enriched waters from the Gulf of Batabano and the adjacent ocean (Arriaza *et al*., 2008).

Table 2. Viable aerobic heterotrophic bacteria concentration, total number of microorganisms and *α-, β-, γ-, and δ- proteobacteria* in coastal stations northwest of Havana, Cuba.

Tabla 2. Concentración de bacterias heterótrofas aerobias viables, número total de microorganismos y α-, β-, γ-, y δ- proteobacteria en estaciones costeras al NO de La Habana, Cuba.

Sampling station	Viable heterotrophic bacteria (CFU.mL^{-1}) Mean ± SE	Total number of microorganisms X 10^4 (cells.mL^{-1})	*Proteobacteria* X 10^4 (cells.mL^{-1})			
			α	β	γ	δ
E.1	700 ± 35.6	53.1 ± 1.5	4.7	9.1	2.5	4.0
E.2	2400 ± 55.6	52.0 ± 2.0	5.4	1.8	1.8	1.9
E.3	3500 ± 310.9	132.0 ± 6.9	5.5	2.9	5.8	1.8
Zone mean ± SE*	2200 ± 418.8	79.0 ± 13.4	5.2 ± 0.4	4.6 ± 3.9	4.0 ± 2.1	3.0 ± 1.0

*SE: mean standard error.

Table 3. Concentrations of viable aerobic heterotrophic bacteria, total number of microorganisms, *α-, β-, γ-* and *δ- proteobacteria* in oceanic waters southwest of Cuba.

Tabla 3. Concentración de bacterias heterótrofas aerobias viables, número total de microorganismos y α-, β-, γ-, y δ- proteobacteria en las aguas oceánicas al SW de Cuba.

Sampling station	Viable heterotrophic bacteria (CFU.mL^{-1}) Mean ± SE	Total number of microorganisms X 10^4 (cells.mL^{-1})	*Proteobacteria* X 10^4 (cells.mL^{-1})			
			α	β	γ	δ
E.1	466 ± 32.4	11.0 ± 1.0	0.8	2.3	0.7	1.5
E.4	190 ± 12.9	9.5 ± 5.0	2.0	1.0	1.3	0.9
E.5	70 ± 9.3	11.2 ± 1.0	3.2	1.7	1.2	1.3
E.8	30 ± 2.9	20.0 ± 2.0	1.1	0.4	0.8	0.6
E.9	146 ± 16.2	6.4 ± 0.6	1.3	0.4	0.3	0.3
E.11	513 ± 33.6	7.0 ± 0.4	0.6	0.6	0.6	0.8
E.12	296 ± 29.7	34.5 ± 4.0	10.0	7.2	8.0	6.9
E.14	200 ± 12.9	21.0 ± 2.0	6.0	7.0	1.5	0.8
E.17	700 ± 26.3	8.0 ± 1.0	0. 6	0.5	0.6	0.8
E.18	500 ± 31.3	7.0 ± 0.4	0. 7	0.8	0.6	0.5
E.21	220 ± 14.1	14.0 ± 0.6	3.0	4.0	0.5	0.8
E.23	290 ± 13.6	15.0 ± 0.7	6.0	3.0	2.0	0.6
Zone mean ± SE	302 ± 33.2	14.0 ± 0.01	3.3 ± 0.8	2.4 ± 0.7	1.5 ± 0.6	1.3 ± 0.5

By comparing the results with those reported by Delgado *et al.* (2009) for the west coast of Havana, it was observed that in summer VHB concentrations in water were similar to those obtained for the coastal area east of Havana and the São Sebastião Channel, Brazil (2.6 x 10^1 to 3.4 x 10^3 CFU. mL^{-1}) (Almeida, 2009), and higher than those found for the Gulf of Batabano, southwest Cuba (2.8 x 10^2 CFU. mL^{-1}) (Miravet, 2003). However, they were lower than those reported by Lugioyo *et al.* (2010) for the waters of Playas del Este beaches in the summers of 2007 and 2008, which oscillated between 1500 and 6500 CFU. mL^{-1}. These authors reported that BOD_5 values were high (3.1 to 9.7 mg. mL^{-1}), thus showing the availability of organic matter in the ecosystem. On the other hand, in the analyzed period the temperature ranged between 28.7 and 32 °C, favoring the VHB growth and duplication.

In September 2005, VHB concentration in the southwest of the Exclusive Economic Zone (EEZ) was lower than that reported by Lugioyo (2003) for Cuban northern EEZ (3.5 x 10^1 - 12 x 10^4 CFU. mL^{-1}).

In May 2006, the total number of microorganisms in the coastal area east of Havana ranged between 4.8 x 10^5 cells.mL^{-1} and 1.44 x 10^6 cells.mL^{-1} (Table 2). In addition, the highest value of microorganisms was found at E.3 station located near Guanabo River.

Nevertheless, in oceanic waters southwest of Cuba, in September 2005, it was found that the total number of microorganisms oscillated between 5.6 x 10^4 cells.mL^{-1} and 42.7 x 10^4 cells.mL^{-1} (Table 3). The highest values of this variable were found in E.12 and E.14; however, these stations are far from the shelf. This apparently contradictory result is explained by the complex dynamics of water masses that characterizes the area, with predominance of interrelated cyclonic and anticyclonic eddies, which is maintained over time and forms a large anticyclonic circulation (Victoria & Penié, 1998; Arriaza *et al*., 2008). This water dynamics appears almost permanently in the southwestern zone (Arriaza *et al*., 2008) and enhances energy input into the system, favoring the vertical mixing that fertilizes the photic layer. Besides, the contributions from neighboring shelves confirm the significantly higher values of nutrient concentrations found in the southwest region, facilitating the development of epipelagic plankton communities as a whole (phytoplankton, bacteria and microzooplankton), despite the oligotrophic condition of Cuban oceanic waters (Lugioyo, 2003).

The total number of microorganisms was similar to those reported for the Northern coast of Germany, where the highest concentration was around 10^6 cells.mL^{-1} (Eilers *et al*., 2000). It was also comparable to those in Santa Monica Bay, California, that varied between 10^5 and 10^6 cells.mL^{-1} (Karner & Fuhrman, 1997). But it was lower than the values reported for the northern part of Cuban Exclusive Economic Zone (Lugioyo, 2003) and the São Sebastião Channel, Brazil (6.3 x 10^6 to 2.5 x 10^7 cells.mL^{-1}) (Almeida, 2009).

The mean total number of microorganisms was five times higher in the coastal area of Havana, in relation to the southwest region of Cuban ocean waters. It is logical to expect this result if we consider that in coastal areas the concentration of organic matter – of both native and allochthonous origin – is greater than in oceanic waters, which are impoverished and, particularly those adjacent to Cuba, are classified as oligotrophic according to their trophic status (Lugioyo, 2003).

In the coastal area east of Havana, salinity values varied from 26.8 to 33.7 UPS, water temperature ranged between 28.7 and 32.0 ^{0}C, NH_4 concentrations were between 2.43 and 3.12 µmol.L^{-1}, and total phosphorus oscillated between 0.69 and 1.41 µmol.L^{-1}. Moreover, the high values of Biochemical Oxygen Demand (BOD_5) (between 3.54 and 4.29 mg.L^{-1}) and DOC (2.19 - 2.93 mg.L^{-1}) show the organic load received by this coastal area.

Similar behavior was observed in different classes of proteobacteria, where the mean total concentration was higher in the coastal area in relation to that found in oceanic waters southwest of Cuba (Tables 2 and 3).

In the coastal area east of Havana, the mean concentration of each *proteobacteria* class with respect to the total number of cells was α 6.6%, β 5.8%, γ 5.0% and δ 4.0%; while in the southwest of the EEZ it was 23.6%, 17.1%, 10.7% and 9.3% respectively.

Oliveira *et al.* (2006) report that in the Marine Aquaculture Station in Rio Grande do Sul, Brazil, the concentrations of α-, β- and γ-*proteobacteria* groups were 2.1 x 10^7 cell.mL^{-1}, 7.8 x 10^5 cel.mL^{-1} and 6.4 x 10^5 cel.mL^{-1}, respectively; which were up to two orders higher than those found in Cuban waters.

However, if the analysis is performed by station in each study area, it is evident that every sampling point has its specificities, primarily related to the geographic location and physical-chemical characteristics of marine waters (Tables 2 and 3, Figures 1 and 2).

Regarding the distribution of the different *proteobacteria* classes with respect to the total number of microorganisms in the stations of Havana coastal area, any class of *proteobacteria* was dominant in any of the stations. In the station E.1, β-*proteobacteria* predominated with 17.1%; in E.2, α-*proteobacteria* with 10.4%, and in E.3, γ-*proteobacteria* with 4.4%, followed by α-*proteobacteria* with 4.2%, compared to the total count of *proteobacteria* (Table 2). The analysis by station of the behavior of each class with respect to the total *proteobacteria* concentration showed a similar pattern (Figure 3 A).

In oceanic waters, α-*proteobacteria* (43%) predominated in 50% of the sampled stations, followed by β-*proteobacteria* (33%); while the other classes were represented to a lesser extent (Table 3, Figure 3B).

As observed (Figure 4A and 4B), in most studied stations the *proteobacteria* were the largest contributors to the total of microorganisms. In oceanic waters southwest of Cuba, in 83% of the studied stations the frequency of *proteobacteria* was higher than 35 % of the total of microorganisms; in E.12, E.14 and E.23 it was even above 70 %. However, of the sampled sites in Havana coastal area only in one of them (E.1) the *proteobacteria* were relatively high (40%); while in E.2 and E.3 they were 21% and 12.1%, respectively.

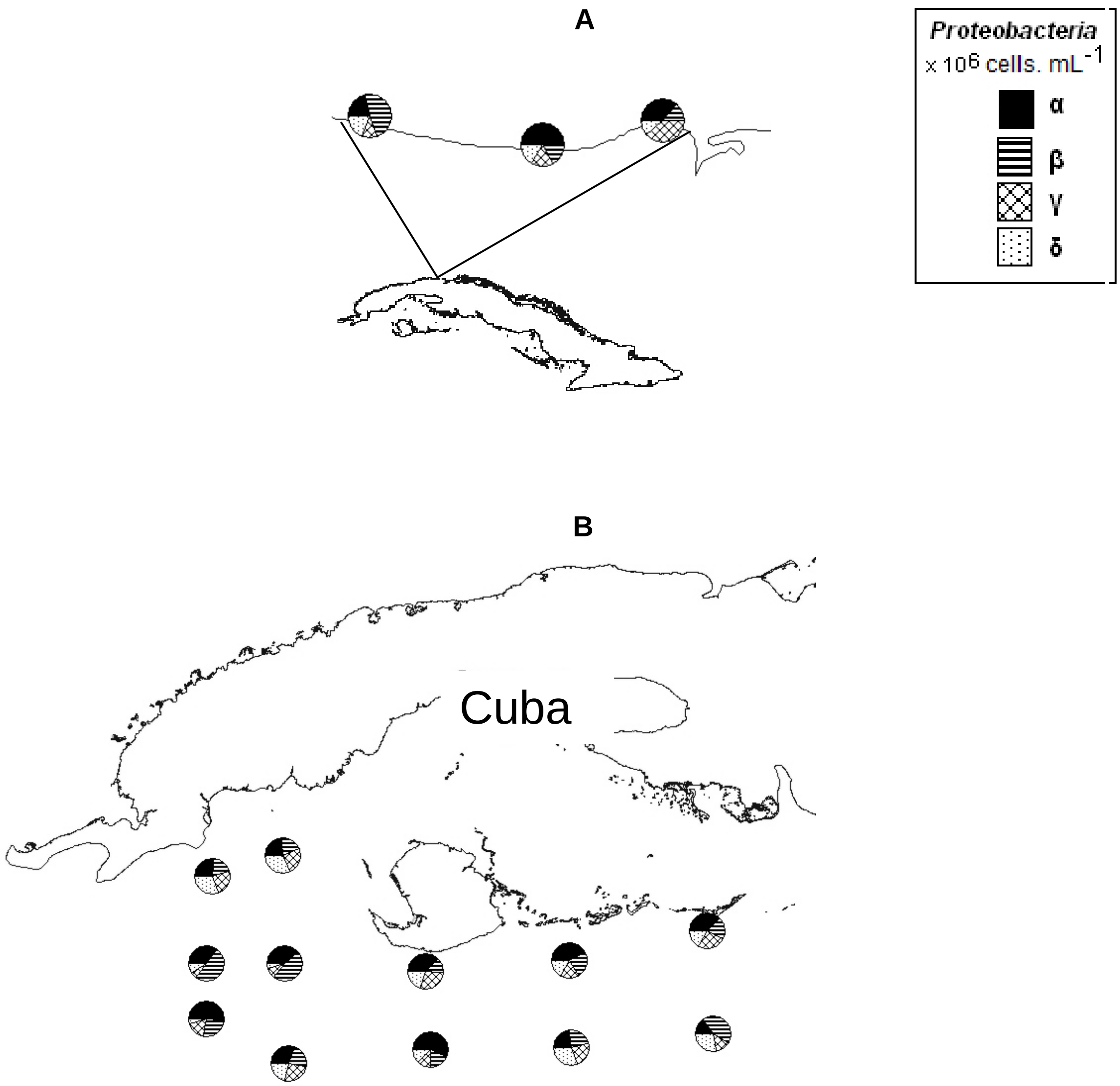


Figure 3. Relative contribution of different classes of *proteobacteria* in stations located in a coastal area east of Havana (A) and in oceanic waters southwest of Cuba (B), in summer.

Figura 3. Contribución relativa de las diferentes clases de proteobacterias en estaciones localizadas en una zona costera al NE de La Habana (A) y en las aguas oceánicas al SW de Cuba (B), en verano.

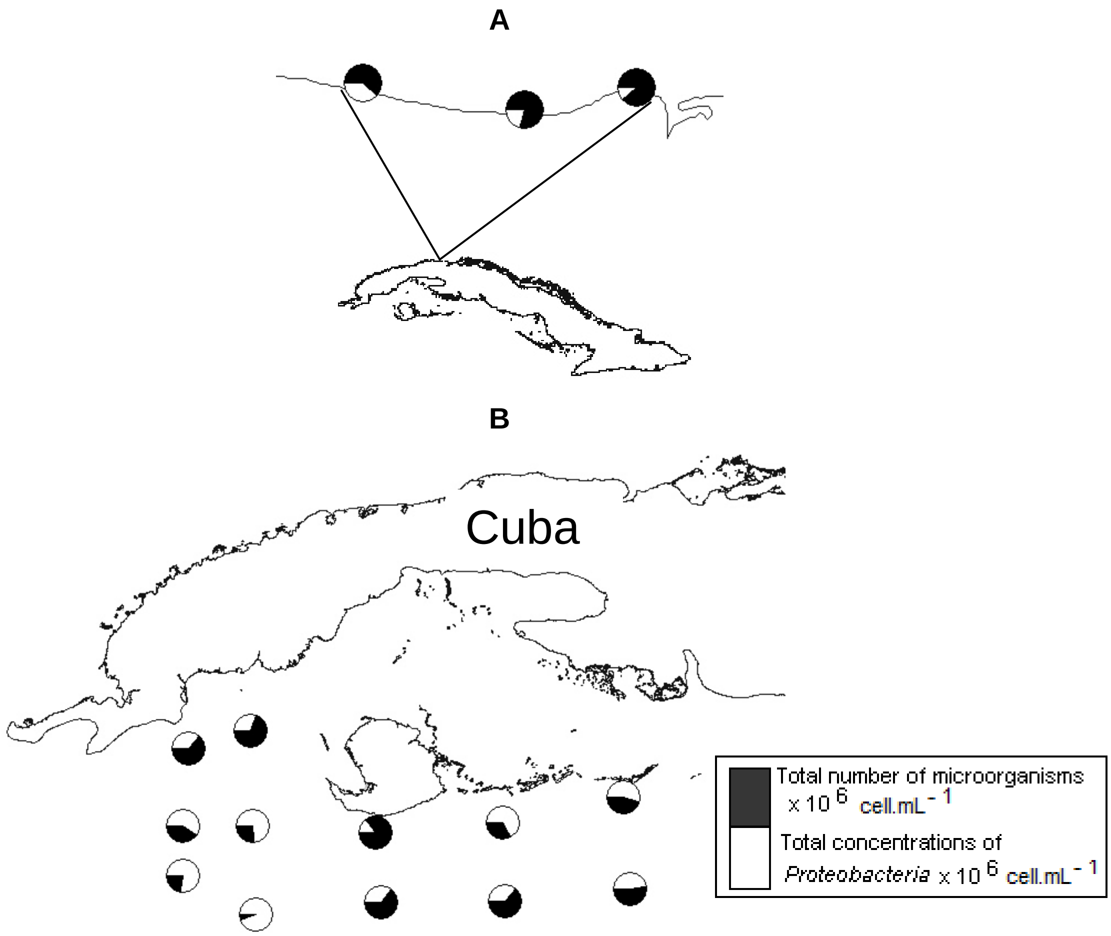


Figure 4. Relation between the total number of microorganisms and the total concentration of *proteobacteria* at stations located in the coastal area east of Havana (A) and in ocean waters southwest of Cuba (B).

Figura 4. Relación entre el número total de microorganismos y la concentración total de proteobacterias en estaciones localizadas en una zona costera al NE de La Habana (A) y en las aguas oceánicas al SW de Cuba (B).

Almeida (2009) showed in the São Sebastião Channel, Brazil, the predominance of β-*proteobacteria* which accounted for 10.2% of total cells, followed by α-*proteobacteria* and γ-*proteobacteria*, with 9.5% and 8.1 % respectively. These percentages are lower than those found for Cuban waters.

Similar proportions between cells hybridized with fluorescent probes and total cell numbers were obtained in seawater in Germany, where the predominant group was α-*proteobacteria* (27%) (Sekar *et al*., 2004). Likewise, in the coastal zone of the Mediterranean Sea it was found that α-*proteobacteria* was the dominant class with 29%; however, only 8% corresponded to γ-*proteobacteria* (Alonso-Sáez *et al*., 2007).

The fact that the δ-*proteobacteria* subclass is in low frequencies could be related to the inclusion in this group of sulfate-reducing bacteria, which are characteristic of anoxic environments, thus being uncommon in marine waters (Parkes *et al*., 1989; Takii *et al*., 2008).

It is unquestionable that abiotic factors such as temperature, dissolved oxygen concentration, and the availability of organic matter and nutrients may influence the concentration of different groups of bacteria in marine ecosystems (Cottrell & Kirchman, 2000; Heidelberg, Heidelberg & Colwell, 2002).

With regard to the nature of organic matter, Cottrell & Kirchman (2000) reported that the consumption of organic matter varies between phylogenetic groups and even that α-*proteobacteria* is the highest consumer of amino acids, but also the lowest consumer of protein.

In general, in the surface layer of oceanic waters southwest of Cuba it was found that *proteobacteria* (considering all classes assessed) represent 72% of all microorganisms. These results indicate that this area is generally dominated by Gram-negative bacteria, which is consistent with the reports by Giovannoni & Rappe (2000), and Perez-Nieto (2001) for oceanic ecosystems.

However, it is not possible to reach a generalization from the results obtained for Havana coastal area so far, since the proportion of total *proteobacteria* was only 20% compared to the total number of microorganisms. It is possible that in coastal areas the predominant group is Gram-positive bacteria with low G – C content, as these are areas that receive a strong influence from land and different sources, such as rivers, that provide organic matter in a quasi-permanent manner, which could promote the growth and replication of genera such as *Clostridium*, *Bacillus*, *Lactobacullus*, and *Staphylococcus* (Guerrero, 2001).

CONCLUSIONS

1. In the coastal area east of Havana, the concentrations of viable aerobic heterotrophic bacteria, total number of microorganisms and *proteobacteria* were higher if compared to oceanic waters southwest of Cuba.
2. In general, in the oceanic waters southwest of Cuba, the contribution of *proteobacteria* (considering the four assessed classes) to the total number of microorganisms was high; but in the coastal area east of Havana it was not observed the same behavior.
3. In oceanic waters southwest of Cuba, *α-proteobacteria* predominated, followed by *β-proteobacteria*. While a different contribution of every *proteobacteria* class at each station was obtained in the coastal area east of Havana.

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