BACTERIAL DENSITY AND COLIFORM ORGANISMS IN WATERS AND OYSTERS OF PARANAGUÁ ESTUARINE COMPLEX, PARANÁ, BRAZIL

Hedda Elisabeth KOLM [1] and Theresinha Monteiro ABSHER [2]

ABSTRACT

The objective of the present research was to evaluate quantitatively the total coliforms and *E. coli* in oysters and adjacent waters of the Paranaguá Estuarine Complex and its interrelation with biotic and abiotic factors, as well as evaluate the contamination from coliforms of oysters sold in the Paranaguá market. Water and oysters were collected from April of 1997 to February of 1998, in the Paranaguá Estuarine Complex and oysters were bought from the same three merchants in the market through-out the research. Temperature, salinity and seston of the water and total heterotrophic bacteria, bacterial biomass, total coliforms and *Escherichia coli* in the water and in the oysters were analyzed. Temperature was higher in summer, salinity and seston in the winter. Total heterotrophic bacteria and bacterial biomass in the water were heigher in Ostras River in July and January and total coliforms in July. The highest values of total coliforms in oysters were observed in Rasa Island, in January. In the water the number of *E. coli* was larger than 2,419 MPN.100 ml^{-1} in Cobras' Island in April and in Puruquara, in July. In relation to the oysters from the market of Paranaguá, the largest values of total coliforms were registered in April and December, and *E. coli* in December, both from merchant 2. The results show that oysters collected in the environment as well as the ones from the Municipal Market of Paranaguá cannot be consumed raw without previous purification and that there is a need of an urgent re-form of the legislation, including the analysis of *E. coli* in the oysters to be marketed.

Key words: Oysters, water, coliform bacteria, Paranaguá Estuarine Complex

DENSIDADE BACTERIANA E ORGANISMOS COLIFORMES EM ÁGUAS E OSTRAS DO COMPLEXO ESTUARINO DE PARANAGUÁ, PARANÁ, BRASIL

RESUMO

Foram objetivos da presente pesquisa avaliar quantitativamente os coliformes totais e *E. coli* em ostras do Complexo Estuarino de Paranaguá, sua inter-relação com fatores bióticos e abióticos, assim como avaliar a contaminação por coliformes de ostras comercializadas no Mercado Municipal de Paranaguá. Foram feitas coletas de água e ostras, entre abril de 1997 e fevereiro de 1998, e foram compradas ostras ao longo do período amostral dos mesmos três comerciantes do mercado municipal homônimo. Analisaram-se a temperatura, salinidade e seston da água, além de bactérias heterotróficas totais, biomassa bacteriana, coliformes totais e *Escherichia coli* na água e nas ostras. A temperatura foi mais elevada no verão, a salinidade e o seston no inverno. As bactérias heterotróficas totais e biomassa bacteriana na água foram mais elevadas no Rio das Ostras em julho e janeiro e coliformes totais em julho. Nas ostras, os maiores valores de coliformes totais ocorreram na Ilha Rasa em janeiro. Na água o número de *E. coli* foi maior que 2.419 NMP.100 ml^{-1} na Ilha das Cobras em abril e no Puruquara, em julho. Com relação às ostras adquiridas no mercado de Paranaguá, os maiores valores de coliformes totais foram registrados em abril e dezembro, e de *E.coli* em dezembro, ambas do comerciante 2. Os resultados mostram que, tanto as ostras coletadas no ambiente, quanto as do Mercado Municipal de Paranaguá não podem ser consumidas cruas sem prévia depuração e que há necessidade de uma reformulação urgente da legislação, incluindo a análise de *E. coli* nas ostras a serem comercializadas.

Palavras-chave: ostra; água; coliformes; Complexo Estuarino de Paranaguá

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[1, 2] *Centro de Estudos do Mar, Universidade Federal do Paraná.*
Endereço/Address: Av. Beira Mar, s/no. Caixa Postal 50.002. 83.255-000 Pontal do Sul, PR - Brasil.
e-mail: hedda@ufpr.br ; tmabsher@ufpr.br

INTRODUCTION

In the Brazilian coast, the species of oysters of higher economic interest belong to the genus *Crassostrea*, of the family Ostreidae. The species of this genus are eurihaline, adapted to the estuarine environment. They are gonocorics, the gamets are spawned in the water, where fertilisation takes place. The planktonic larvae at the end of the larval phase settle in hard substrata and metamorphose (GALTSOFF, 1964). During its life cycle, from larva to adult, they feed on phytoplankton from the surrounding seawater.

Although Brazilians are not a great oysters consumer, oysters have a good acceptance in the market, mainly in coastal areas, where they are also consumed by the local population.

Due to the high commercial value, the species of oysters have been explored indiscriminately along the last decades in the Paraná coast. Now they are found, in commercial sizes, mainly in the bays of Pinheiros (Figure 1) and of Guaratuba. The merchants of the Municipal Market of Paranaguá always say that they are original of these places or of cultivations. However it is very difficult to detect its true origin. At the Paranaguá market they are marketed in amounts that vary along the year. The largest havest happen during the summer, time of larger affluence of tourists to the coast. Still, according to the merchants above mentioned, as oysters may stay alive, out of the water for some days, the ones not sold during the day are maintained, during the night, in the nearby waters of Paranaguá city, to return to the market the following day.

Due to the efficient filtration mechanism, oysters are able to accumulate, from the adjacent seawater, big amounts of microorganisms and, consequently, to store a bacterial flora excepcionally rich (KINNE, 1983). Thus, in spite of being free of bacterial diseases, the mollusks can act as carriers of human pathogenic microorganisms (BRISOU, 1974; METCALF *et al*., 1979; RIPPEY, 1994; VILLALOBOS and ELQUEZABAL, 2000; SILVA *et al*., 2003; CLAYTON, 2006; PEREIRA *et al*. 2006r). Consequently these organisms accumulate, among others, big amounts of coliform bacteria, when maintained in polluted waters (BURKHADT and CALCII, 2000). Such fact is even worse when we consider that those organisms are consumed raw. As the system of sewers treatment of Paranaguá city is deficient, in the cities of Antonina and Guaraqueçaba is nonexistent, and appropriate registrations of contamination of the estuary from domestic efluentes are lacking, the waters in which the oysters are maintained, until sold, should be contaminated. Neither registration of contamination, for total and fecal coliform bacteria (and/or *Escherichia coli*), of the areas of origin of those bivalves is known.

The objective of the present research was to evaluate quantitatively the total coliforms and *E. coli* in oysters and adjacent waters of the Paranaguá Estuarine Complex and its interrelation with biotic and abiotic factors, as well as evaluate the contamination from coliforms of oysters sold in the Paranaguá market.

STUDY AREA

The Paranaguá Estuarine Complex (25º16'34"S; 48º17'42"W) is formed, to west, by the Bays of Paranaguá and Antonina, and to the north, by the bays of Laranjeiras, Guaraqueçaba and Pinheiros and the inlets of Itaqui and Benito. The entrance of the estuary is flanked by Mel and Galheta islands (Figure 1).

Along the whole estuarine complex sandbanks and islands of medium and small size can be found. The whole border of the estuary, as well as the rivers and tidal-creeks are marginated by mangroves constituted by *Rhizophora mangle*, *Laguncularia racemosa* and *Avicennia schaueriana*. In the more sheltered areas of the estuary banks of *Spartina alterniflora* occurs. According to REBELLO and BRANDINI (1990) these floristic formations favors the enrichment of the area by organic detritus.

Along the whole estuary oysters of the genus *Crassostrea* can be found fastened in the intertidal region of rocky coasts and/or in the trunk and roots of the mangrove trees. In the 1990-decade the firsts experimental studies of oyster's cultivation for commercialization were made at Rasa Island of Guaraqueçaba. Nowdays small oyster cultivation is spread in the bays.

The most important city of the area is Paranaguá, with approximately 148.000 inhabitants (sensus of 2006). Great part of the city is located in the continent, however there is an occupation of low-income population in Valadares Island. In this same city is located the third largest port of Brazil. Besides Paranaguá the cities of Antonina and Guaraqueçaba can still be mentioned, with approximately 21,000 and 9,000 inhabitants respectively (sensus of 2006). Until 1998 none of the referred cities had systems of sewer

treatment and all the served waters were thrown in rivers and tidal creeks or directly in the sea.

Along the whole margin of the estuary and in several islands, smaller towns and sparse inhabitants still exists. As the freatic sheet, of all these areas, is very superficial, thus hindering the construction of aseptic tanks and drains, most of these sewers are also thrown in the estuary.

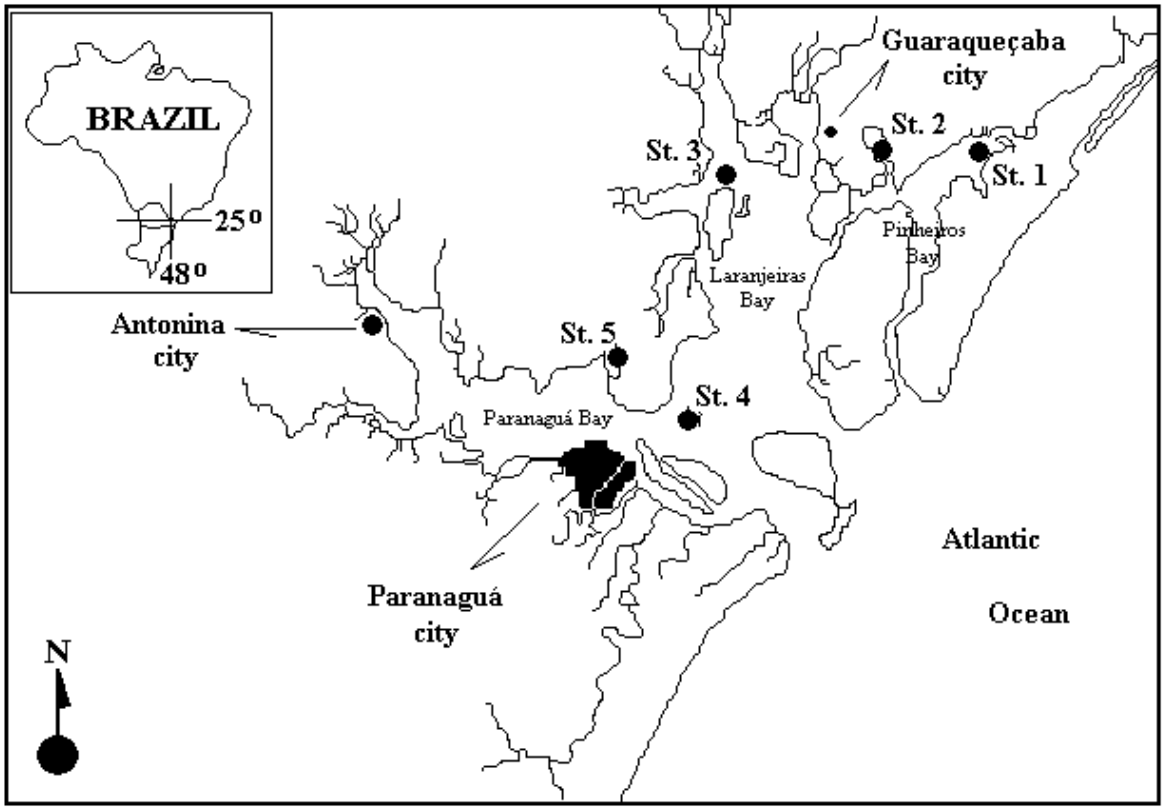

Figure 1. Map of the Paranaguá Estuarine Complex with location of the following collection points: 1 - Pinheirinho Island; 2 - Puruquara; 3 - Rasa Island; 4 - Cobra Island; 5 - Oyster River

MATERIAL AND METHODS

Between April of 1997 and February of 1998 collections of water (n=2) and oysters (n=2) in the following five stations of the Paranaguá Estuarine Complex were made: 1 - Pinheirinho Island - Pinheiros Bay (25º21´17.61"S – 48º13´54.50"W); 2 - Puruquara - Pinheiros Bay (25º19´45.13"S – 48º15´36.03"W); 3 - Rasa Island of Guaraqueçaba - experimental oyster cultivativation - Laranjeiras Bay (25º19' 48.95"S – 48º23'59.34"W); 4 -Cobras Island - Laranjeiras Bay (25º29' 13.82"S – 48º25'55.03"W) and 5 - Oysters River - Paranaguá Bay (25º28'25.70"S – 48º30'01.74"W) (Figure 1). All sampling were made during syzygy low water. At the same time, temperature (standard thermometer) and salinity (Atago refractometer) of the water were measured. Water samples collected for the other analyses were properly conditioned and brought in ice to the Laboratory of Marine Microbiology of the Centro de Estudos do Mar/UFPR. Simultaneously oysters were collected, conditioned in plastic bags and brought to the laboratory.

Three merchants (1, 2, 3) from Paranaguá market were inicialy selected and sampled during the whole period of the study. Oysters (n=2) of different sizes and positions inside the commercial boxes were bought and conditioned in plastic bags and brought to the laboratory for the analysis

Seston of the water was analyzed by filtration with GF/C filters. In the stations and months that were not possible to obtain the seston values (Pinheirinho in 01/14/1998, Cobras and Ostras in 02/19/1998) the average values of the other months were used in the statistical analysis.

The pluviosity data was obtained from the Laboratory of Marine Physics of the Centro de

Estudos do Mar/UFPR. For the present research, the sum of the values of the six days previous to day of sampling added to the value of the day of the collection was used.

For the quantification of total heterotrophic bacteria, 15 ml of water samples were preserved "in loco" to a final concentration of approximately 5% of formaldehide. Water aliquots between 0.5 and 1.0 ml were stained with acridine orange and the bacteria counted in the epifluorescence microscope, according to the methodology described by PARSONS *et al*. (1984). For the calculation of the bacterial biomass, the biovolume of the bacteria were determined from approximate geometric figures, and the conversion factor of 0.4 pgC.μm^{-3} was used (BJØRNSEN and KUPARINEN, 1991).

For the analyses of total coliforms and *Escherichia coli* the technique of the cromogenic substratum Colilert of the Idexx Laboratories Inc. (Most Probable Numbers)was used. For the analysis, the water coming from the field was not diluted. The oysters (native and acquired in the market) were very well scrubed, rinsed with distilled water, opened axenicaly and a total of 50 grams of meat and intervalvar liquid were diluted with 450 ml of distilled water (dilution of 1:10) and beaten in a blender for 10 minutes. The amount of oysters needed to comprise 50 grams varied depending on the size of the oysters. For the quantitative verification of the total coliforms and *E. coli* additional dilutions of 1:100 and 1:1000 with distilled water were made. Of each sample coming from the field (or of the market) two replicates were made. In the graphic representation the coliformes values for gram of humid weight were ploted.

Analysis of Variance (ANOVA) and Principal Component Analysis (PCA) were used for the statistical evaluation of the results. For PCA the data was logarithm transformed.

RESULTS

The results of the abiotic values of the surface water from the five stations are presented in Figure 2.

The largest temperature values, with a maximum of 32°C in Rasa Island of Guaraqueçaba, were registered in the summer (December of 1997 to January of 1998). The smallest temperatures were observed in the winter, with a minimum of 21°C in July of 1997 in Pinheirinho and in the Cobras islands (Figures 2A). Inverse relationship was observed in relation to the salinity. Its higher values, with a maximum of 31 in Cobras Island in July of 1997, were observed in the winter and the smallest ones, with a minimum of 0 in Ostra River in February of 1998, in the summer (Figure 2B). In the same way, more elevated values of seston were registered in the months of autumn, winter and spring and the smallest ones in the summer. The maxima and minima of 65.13 $mg.l^{-1}$ and 1.18 $mg.l^{-1}$ were registered in Puruquara (Figure 2C). The smallest pluviosity values were registered in July of 1997 (1.6 mm in seven days), and the highest ones (141.9 mm in seven days) in October of 1997 (Figure 2D).

ANOVA shows that there was significant variation along the period studied in relation to the temperature ($F(5,24) = 24.64$; $p < 0.0000$) with values significantly larger in the summer (Figure 3A), to the salinity ($F(5,24) = 7.38$; $p < 0.0003$), with larger values in the autumn and in the winter (Figure 3B) and to the seston ($F(5,21) = 3.20$; $p < 0.0265$), with larger values in the winter and decreasing gradualy in the summer (Figure 3C). Along the stations significant variations of the abiotic data could be verified only in relation to salinity ($F(4,25) = 2.82$; $p < 0.0467$) with the largest values registered in Cobra Island (Figure 3D).

The results of the biotic data in the five stations, along the studied period, can be observed in Figure 4.

The lowest values of total heterotrophic bacteria (603.10^3 $cel.ml^{-1}$) and of bacterial biomassa (21.01 $\mu gC.l^{-1}$) in the water were observed in April of 1997 in Pinheirinho. The highest values of these (5.10^6 $cel.ml^{-1}$ and 169.83 $\mu gC.l^{-1}$) could be observed, respectively, in Ostras River, in July of 1997 and January of 1998 (Figures 4A and 4B).

The lowest values of total coliforms in the water (180 MPN. 100 ml^{-1}) were registered in Cobras Island, and the highest ones (6,701 MPN. 100 ml^{-1}) in Ostras River, both in July of 1997 (Figure 4C). In the oysters values of total coliforms were consistent higher than 4,838 MPN per gram and the lowest 644 MPN per gram (Figure 4D).

In the water the number of *E. coli* was highest than 2,419 MPN.100 ml^{-1} in Cobras Island, in April of 1997 and in Puruquara, in July of the same year. The lowest value (18 MPN.100 ml^{-1}) was registered in Cobras Island in December of 1997. *E. coli* of the oysters was consistent higher than 4,838 $MPN.g^{-1}$. The lowest value (4 $MPN.g^{-1}$) was registered in Cobras Island in January of 1998 (Figure 4E).

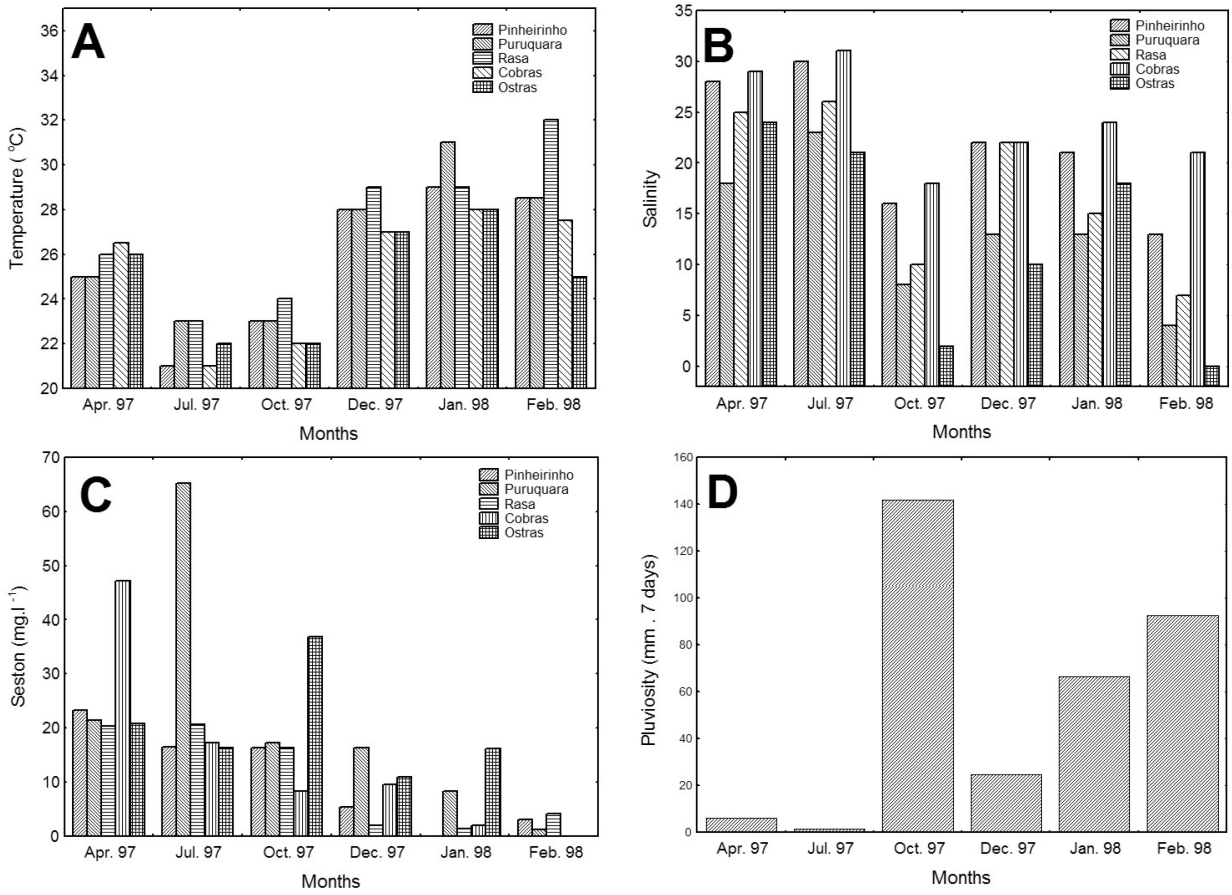

Figure 2. Absolute values of: A) Temperature; B) Salinity; C) Seston and D) Pluviosity

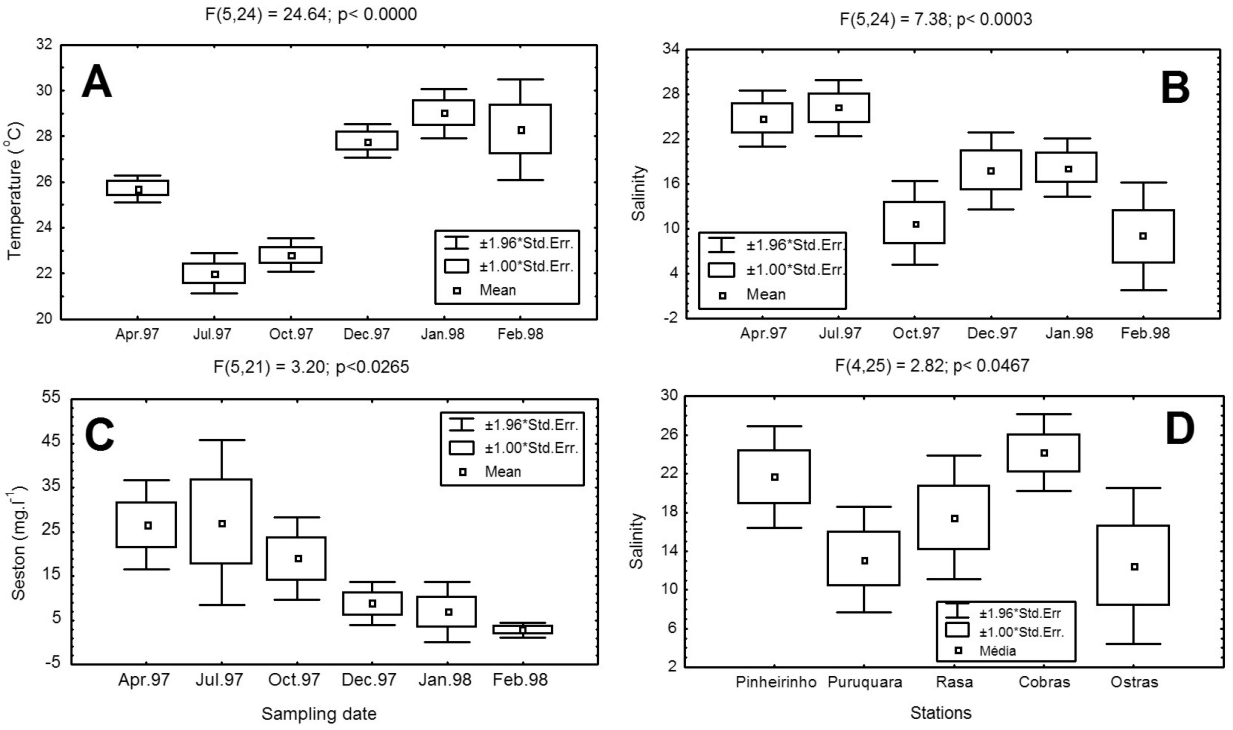

Figure 3. ANOVA results in relation to: A) Temperature; B) Salinity along the stations; C) Seston and D) Salinity in the sampling period

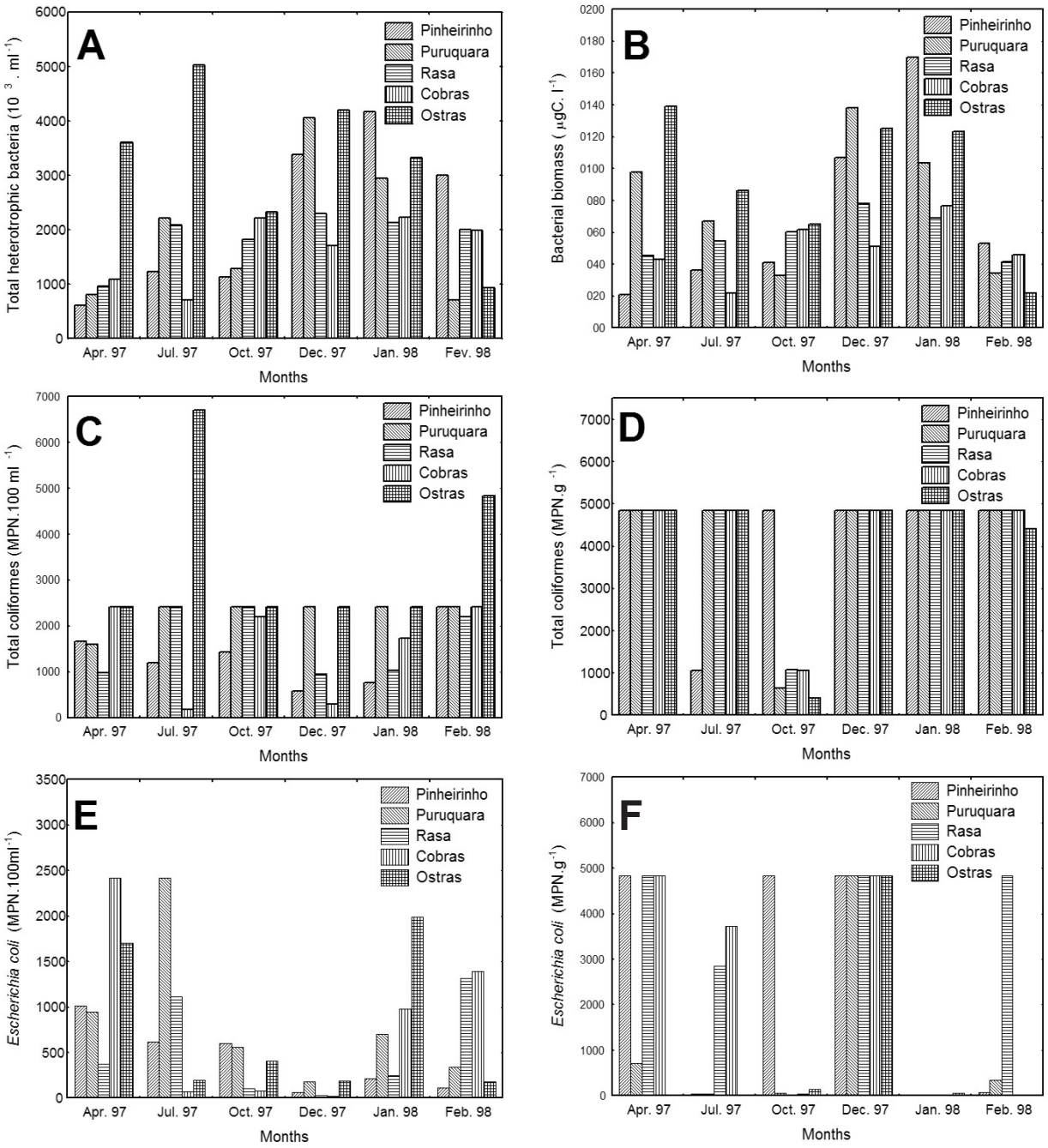

Figure 4. Absolute values, in the period and in the studied stations of: A) Total heterotrophic bacteria in the water; B) Bacterial biomass in the water; C) Total coliforms in the water; D) Total coliforms in the oysters; E) *Escherichia coli* in the water and F) *Escherichia coli* in the oysters

While the variation of the total heterotrophic bacteria in the water was not significant during the period, bacterial biomass, was significantly different ($F(5,24) = 3.78$; $p<0.0115$) with higher values in the begining of summer (Figure 5A).

The amount of total coliforms in the water presented values significantly higher ($F(4,25) = 4.13$; $p <0.0105$) in Ostras River (Figure 5B). In the other stations and along the sampled period the variation of total coliforms was not significant. There was significant variation of *E. coli* in the water.

In the oysters, the variation of total coliforms was not significant during the studied period and neither along the stations. In relation to *E. coli* the variation was significant along the studied period ($F(5,24) = 4.99$; $p <0.0029$), with higher values in the beginning of the summer (Figure 5C) and not significant among the sampling stations.

The first component of the Principal Component Analysis (Figure 6) explained 26% of the data variability and shows positive correlation among the seston, total coliforms and *E. coli* in the water, in Puruquara, in the winter, in Cobra Island in February (summer) and in Ostras River in the four seasons. The same component had positive correlation among salinity of the water, total coliforms and *E. coli* in the oysters, in the stations Pinheirinho, Rasa and Cobras in December, Rasa in January and Puruquara and Rasa in February.

The second component explained 25% of the variability and showed positive correlation with temperature, pluviosity, total heterotrophic bacteria and bacterial biomassa, in the stations Puruquara and Ostras in December, Pinheirinho, Puruquara, Cobras and Ostras in January and Pinheirinho in February, all in summer.

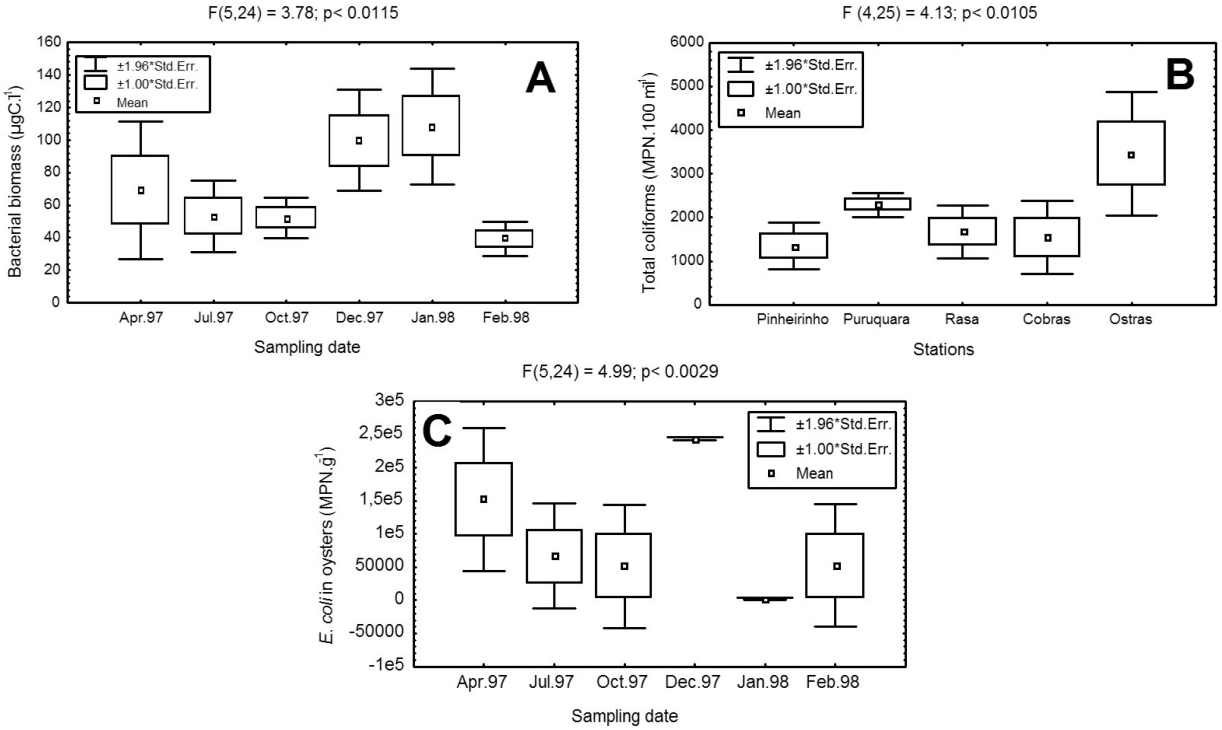

Figure 5. Results of the following variance analyses: A) Bacterial biomass in the water along the sampled period; B) Total coliforms in the water along the sampling stations and C) Fecal coliforms in the oysters along the sampling stations

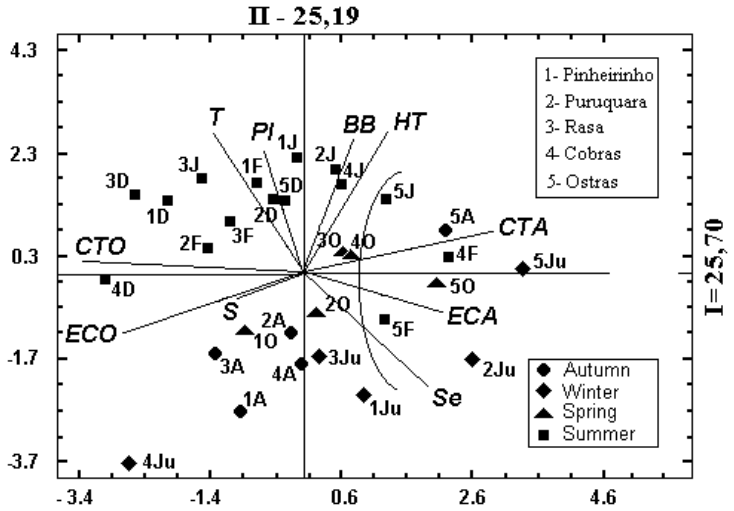

Figure 6. Results of Principal Component Analysis considering the sampling places and season

In relation to the oysters acquired in Paranaguá market, the highest value of total coliforms (>48,384 MPN.g^{-1}) was registered in April and December of 1997, and belonged to the merchant 2. The lowest value was 1,250 MPN by gram of humid weight in the merchant 3 in July of 1997 (Figure 7A).

The values of *E. coli* of these oysters were lower in April of 1997 (5 MPN.g^{-1}) and the highest in December of the same year (2,880 MPN.g^{-1}), both acquired from the merchant 2 (Figure 7B).

The Analysis of Variance showed that only the variation of *E. coli* in relation to the time of the year ($F(5,12) = 11.97$; $p < 0.0003$) was significant with higher values in December of 1997, big variability in January, and low during the other months (Figure 7C).

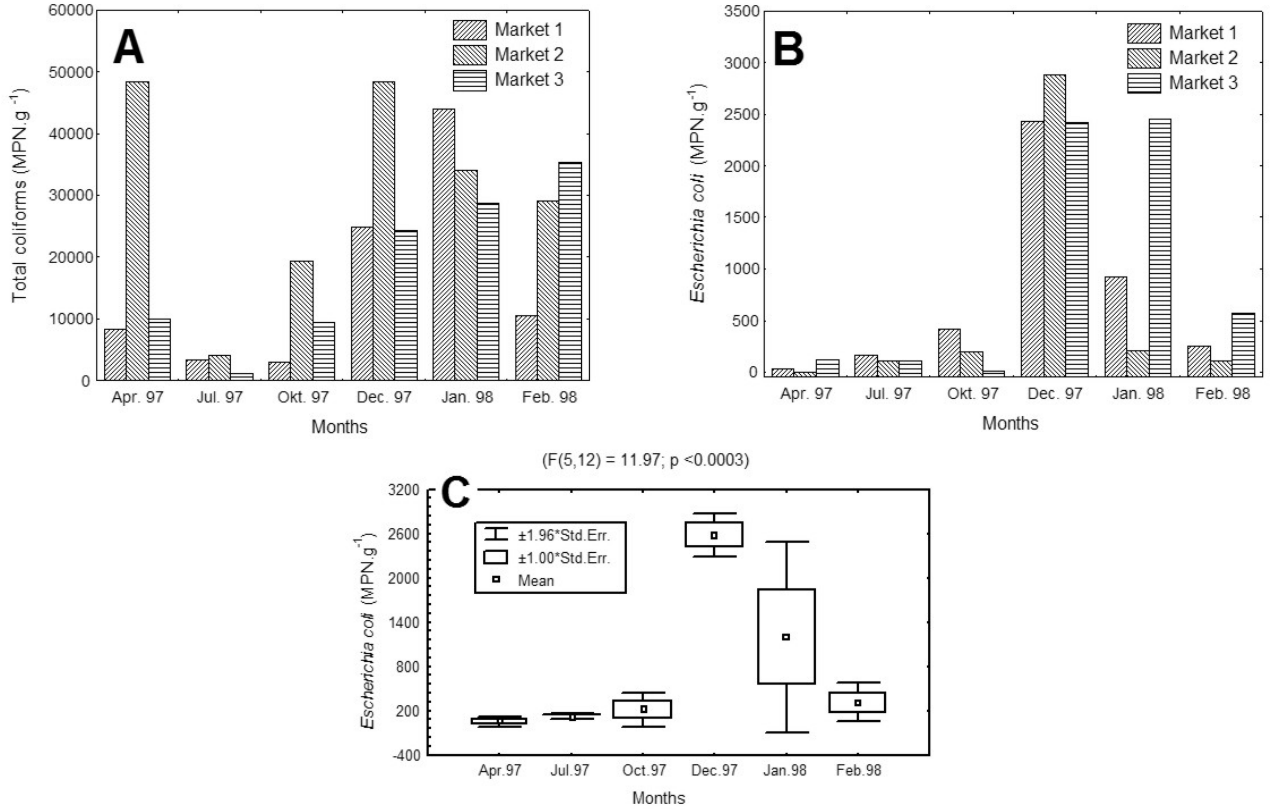

Figure 7. Graphic representation, in absolute values, of the variation of total coliformes and *E. coli* (B), and of the Analysis of Variance of *E. coli* in relation to the studied period (C)

DISCUSSION

The present research showed that the elevated values of total heterotrophic bacteria and bacterial biomass in the water occured in summer, period of higher temperature and pluviosity. Similar results were observed by SIQUEIRA and KOLM (2005) who studied the temporary variability of these microrganisms in the water column at a fixed point of the Maciel's tidal-creek, in Paranaguá Bay. There are indications that through the increase of the pluviosity organic matter is washed from the mangroves to the water column propitiating the increase of bacteria at this time of the year. However, the present research didn't show, in the water, correlation between the total heterotrophic bacteria and the coliforms.

In agreement with the Article. 18th of the Resolution number 357/05, of CONAMA (2005) the geometric average concentration of the density of fecal coliforms (termotolerants) from a minimum of 15 saline water samples collected in the same place, in which natural or intensive cultivation of organisms that are to be consumed by humans, cannot exceed 43 in 100 ml and the percentil 90% cannot exceeed 88 termotolerants coliforms in 100 mililiters. Those indexes should be maintained in a minimum of 5 samples during an annual monitoring. *E. coli* could be used in substitution of the thermotolerant coliforms but in agreement with the limits established by the competent environmental organ. Only in the waters samples analyzed from Cobras Island, in July

and October, Rasa and Cobras Islands in December the values were lower than those required by the legislation.

However direct correlation can be observed between the total coliform bacteria and *E. coli* in the water and in oysters but, in the months with higher coliforms in the oysters (summer), the values in the water were lower. Similar results were observed by MAYERLE FARIA (2002) in the studies with *Crassostrea gigas* cultivated in Babitonga Bay of Santa Catarina. This author observed higher values of *E. coli* in the water in the autumn and winter and in the oysters in the summer. As *C. gigas* is an organism from the Pacific Ocean that prefers low temperatures and high salinities, and therefore is not adapted to the local climatic conditions, the author suggested that the results could reflect the faulty physiologic conditions of the animals in the summer. However, the oysters in the present study are native of the area and therefore very well adapted to local environmental conditions. According to ABSHER (1989) they reproduce in this period. Therefore it is probable that in order to accumulate reserves for reproduction, the oysters filter more water in the summer than in the winter, thus accumulating more coliforms in this time of the year. On the other hand, according to HOOD and NESS, 1982, the survival of coliforms in marine waters depends on saline concentration, predation and competition with autochthonous microflora, heavy metals and nutrients. The present research shows that in the summer, besides temperatures and higher pluviosity also occurred higher values of salinity and total heterotrophic bacteria. The competition of these bacteria with the coliforms for the food may have contributed for the low values of coliforms found in the water. POMMEPUY *et al.* (1996) suggested that *E. coli* and others enterobacteria may survive inside the mollusks longer than when exposed to the ultraviolet rays incident on the water. As the Paranaguá Estuarine Complex is located below the Tropic of Capricorn and therefore subject to sazonality, could also explain the results of this research.

The highest values of total coliforms, *E. coli* and of seston in the water were registered in the Oyster River, located in front of the Paranaguá Port. As the sewers system of Paranaguá city is extremely defficient, and as this tidal creek is subject to great tide variations, the high coliform values should be coming from the city sewers. On the other hand low values of total coliforms and *E. coli* were observed in the oysters of this area. Due to the proximity of the urban center, the extraction of these oysters is extremely intense and individuals are of very small size and located in areas of difficult access by earth and water (due to the mud banks in the area). Thus, it is probable that the tides only occasionally, during the high spring tides, poor in coliforms, reach this animals and, due to their reduced size, have difficulty in filtering particles bigger than 45µm of the seston.

ESCOBAR NIEVES (1988) suggests that the coliform numbers detected in the water do not represent a reliable measure for the oyster quality. The results obtained in this research confirm such observation. The results show that in the beginning of December of 1997 the values of *E. coli* found in the water of the Rasa and Cobras islands were under that of the legislation but in the oysters the maximum values in all the stations were registered during this period.

Similar results obtained in oysters collected from the natural environment were observed in the Municipal Market of Paranaguá. In the summer there is a considerable population increase in Paranaguá due to tourism, thus larger amounts of oysters are commercialized than in the winter. There are also indications that in order to increase the surviving time of the animals, those not sold are maintained in waters in the proximities of the city during the night, and commercialized in the next day. As these waters are polluted, this strategy of maintenance of the mollusks can cause an increase in coliforms. Besides this, the merchants rarely know the origin of the mollusks, turning it difficult to analyze the local water from were the oyster were collected.

However the resolution RDC number 12 of the ANVISA (2001) does not refer to total coliforms, fecal and/or *E. coli* allowed in raw consumed mollusks. The Resolution number 274 of the National Environmental Council (CONAMA) refers only to patterns of swimming waters and the Resolution number 357/05 should also consider coliforms values in waters from where the bivalve mollusks will be extracted and/or cultivated. Thus we suggest that urgent alterations should be made in the Brazilian legislation that refers to the organisms studied in the present research, so guarantying to the sanitary surveillance organizations the possibility of a more rigid control of the bivalves that will be consumed raw.

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