AN EVALUATION OF TRANSIENT BACTERIAL POPULATION IN A POLLUTED BATHING SITE IN PORTO ALEGRE – BRAZIL

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ABSTRACT

Water is of vital importance to all living beings, and problems with the quality of fresh water have become more serious due to pollution that watercourses suffer. Most severe consequences include the economic losses and spreading of water-borne diseases. This study was designed to assess water bacterial quality of a bathing site in the city of Porto Alegre, state of Rio Grande do Sul, Southern Brazil, through coliform counts and identifying the bacterial population there present. Monthly sample collections took place from July, 1999 to August, 2000. Samples were spreaded onto plates with tryptic soy agar (TSA) and selective media eosin-methyl blue agar (EMB), xylose-lysin desoxycholate agar (XLD), xylose-lysin tergitol$_4$ agar (XLT$_4$), and Salmonella-Shigella agar (S-S). The isolated colonies were identified by biochemical tests. Results showed a prevalence of genus *Bacillus* (45.3%), followed by genera *Enterobacter* (17.8%) and *Escherichia* (10.7%). High coliform counts were observed, indicating that the water of the bathing site received considerable load of fecal pollution. Thus on this condition the site was unsuitable for bathing. These results are related to analyses done before the construction of a sewage treatment plant at that region. Nowadays, a new evaluation of microbial population should be considered, to see if there have been changes as expected.

Key words: surface waters, bacteria, total coliform counts, *Escherichia coli.*

RESUMO

Uma avaliação da população bacteriana temporária num lugar de banho poluído em Porto Alegre – Brasil

A água é de importância vital para todos os seres vivos, e problemas com a qualidade da água têm tornado-se mais sérios devido à poluição que os cursos de água sofrem. As conseqüências mais severas incluem perdas econômicas e difusão de doenças transmitidas pela água. Este estudo foi desenhado para testar a qualidade da água de um local de banho na cidade de Porto Alegre/RS, região sul do Brasil, através da contagem de coliformes e identificação da população bacteriana lá presente. Coletas mensais foram efetuadas de Julho de 1999 a Agosto de 2000. As amostras foram espalhadas sobre placas com TSA e meio EMB, XLD, XLT$_4$ e S-S. As colônias isoladas foram identificadas com testes bioquímicos. Os resultados mostraram a prevalência do gênero *Bacillus* (45,3%), seguidos pelos gêneros *Enterobacter* (17,8%) e *Escherichia* (10,7%). Alta contagem de coliformes foi observada, indicando que a água do local de banho recebeu considerável carga de poluição fecal. Desta forma nestas condições o local foi considerado inadequado para banho. Estes resultados estão relacionados às análises realizadas antes da construção da planta de tratamento de esgoto na região. Atualmente, uma nova avaliação da população microbiana deveria ser considerada, para verificar se houve as mudanças esperadas.

Palavras-chave: água de superfície, bactérias, total de colifórmios, *Escherichia coli.*

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INTRODUCTION

Water is essential for the survival of all living organisms, though its accessibility is extremely low: of the 1,370 million km³ of all the water existing in the planet, only 98,000 km³ are suitable for human consumption (MOTA, 1997). This problem becomes more evident when the quality of these waters comes under investigation, because population growth, associated with disorderly technological development have led to use water without the appropriate responsibility, thus speeding up the contamination of sources (IMPERIANO, 1998). Several and severe are the consequences of misuse of waters. When contaminated with pathogenic microorganisms and radioactive or chemical compounds, the expected consequences range from economic losses, ecological imbalance of the environment, landscape degeneration, to impacts in quality of life standards and outbreaks of disease (MOTA, 1997).

Studies carried out by PRÜSS (1998) and FLESISCHER et al. (1998) revealed the occurrence of several diseases like gastroenteritis, skin, eye, throat, ear and respiratory infections happened when a given community has got the habit to swim in contaminated waters. From 1999 to 2000, 59 disease outbreaks in United States were attributed to recreational water exposure, and 61% of these outbreaks were of gastroenteritis (ALM et al., 2003). Yet, the identification of pathogenic microorganisms in water is difficult, inasmuch as they are short-lived and present in low numbers. For this reason, the microbiologic assessment of waters is based on the detection of microorganisms that may be regarded as indicators of pollution. This group of microorganisms comprises especially bacteria of fecal origin, including total coliforms (TC), *Escherichia coli*, and enterococci (ENT) (EVANSON and AMBROSE, 2006).

The U.S. Environmental Protection Agency (EPA) recommended that *E. coli* is a better indicator of fecal pollution than fecal coliform for purposes of evaluating ambient fresh water quality. The presence of *E. coli* in lake water indicates that the water was contaminated by fecal material of humans or other warm-blooded animals, and indicates the potential for the presence of pathogenic organisms (AN et al., 2002).

In Brazil the standards for fresh and brackish water bathing sites have been designed by CONAMA (*Conselho Nacional do Meio Ambiente*). According to them waters are satisfactory for bathing when carry at most 1×10^3 fecal coliforms and 8×10^2 of *Escherichia coli* or 100 *Enterococcus* per 100 mL of water. Waters are not suitable for bathing fecal coliformes are higher than 2.5×10^3, 2×10^3 of *Escherichia coli* and with 400 *Enterococcus* (CONAMA, 2006). The standards of *E. coli* together with the analysis of bacterial diversity in the waters were considered in this study to examine water bacterial quality of Belém Novo bathing site in Porto Alegre, RS, Southern Brazil.

MATERIALS AND METHODS

Sample collection

Surface water samples from Belém Novo bathing site were collected monthly among July 1999 to August 2000. Samples of 100 mL were carried out at one point in the bathing site (Figure 1) by the technicians of the Municipal Water and Sewage Department (DMAE, Porto Alegre, RS, Brazil), using suction pumps. The samples were collected at 15 cm of depth and were kept under refrigeration upon arrival to the laboratories.

Isolation of bacterial populations

Raw water samples were collected between July 1999 to March 2000 and were inoculated, in triplicate, onto tryptic soy agar (TSA) medium and incubated at 37 °C for 24 to 48 h. Samples collected between April to August 2000 were inoculated in TSA growth medium and in selective media eosin-methyl blue agar – EMB, xylose-lysin desoxycholate agar – XLD, xylose-lysine tergitol$_4$ agar – XLT$_4$, and Salmonella-Shigella agar – S-S. The samples seeded onto S-S and XLT$_4$ were pre-enriched in tetrathionate broth for 16 to 18 h at 42°C. The plates were incubated at 25ºC for 24 to 48 h. After growth had been confirmed, colonies were randomly selected. A map from a Petri plate was drawn and squares of 1 cm² were cut off from the map (OLIVEIRA et al., 2006, SCHIMIDT et al., 2003, STRAUCH, 1988). The colonies inside these squares were picked up and seeded onto brain-heart infusion (BHI) and incubated at 37° C for 24 h. After growth de samples were seeded onto BHI slants, which served for stocking the isolates for subsequent bacterial identification.

Bacteria identification

Identification of the bacteria present in all samples was carried out using classic microbiological techniques starting with the Gram staining followed

by biochemical tests as: methyl red, Voges Proskauer, TSI – triple sugar iron, motility, indol and H_2S production, carbohydrate fermentation, oxidation/ fermentation, descarboxylase (arginine, ornithine, and lysine), production of gelatinase, phenylalanine-desaminase, oxidase, catalase. The establishment of the genera and species present in the samples was carried out as described in the literature (HOLT et al., 1994; MACFADDIN, 2000).

Coliforms determination

In order to establish the total coliform counts and the *E. coli* counts of the samples, the Multiple-Tube Fermentation Technique were used (APHA, 1998). The determination of coliforms and *E. coli* were done in the laboratories of the DMAE – Porto Alegre.

Statistical analysis

The microbial diversity was established among each sampling and between the different sampling periods. ANOVA ($p = 0.05$) was used for the variance analysis.

RESULTS

In this study, 382 bacteria were isolated and identified, out of which 297 were classified as species and the others 85 isolates were only possible to classify on level of genus. Among the identified isolates, 212 were Gram positive bacteria and 170 were Gram negative. A predominance of the genus *Bacillus* (45.5%) was observed, followed by 17.8% for *Enterobacter* genus and 10.7% for *Escherichia.*

Bacillus stearothermophilus (15.5%), *B. brevis* and *B. circulans* (8.02% each), and *B. pasteuri* (7.07%) were the most commonly species observed in the genus *Bacillus* (Table 1). For the other genera of Gram positive bacteria, *Staphylococcus* was the most frequent one (Table 2).

The Gram negative isolates are listed on Table 3. *Enterobacter* (40%) was the most common genus among this group. However when we consider the species representation *Escherichia coli* (24.11%) was the most frequent followed by *Enterobacter aglomerans* (23.53%). Most of the other genera showed a very low frequency of isolates.

The data for bacterial diversity throughout the four seasons of the year were assessed using ANOVA variance analysis. On the whole, no significant difference was found for the overall diversity data ($p < 0.05$) of all bacterial species (data not shown). The same observation was obtained when the analysis was done considering only the Gram-positive group (Table 4). Yet for the Gram-negative group there was a significant bacterial diversity ($p < 0.05$) for two collection periods in the Summer/Autumn – 2000 ($p = 0.04$) and in the Spring/Winter 1999 ($p = 0.03$) (Table 5).

Total coliform counts were high in most of samples (above 7.4×10^3). Only in two collections, febuary/2000 and may/2000, the numbers of *E. coli* counts per 100 mL of water were satisfactory for bathing according to CONAMA legislation (2006) (Table 6).

DISCUSSION

The bacterial diversity in the Belém Novo bathing site was considerably large. During the period of this study, 382 isolates were classified in 16 genera and 46 different species. Generally, the bacteria detected in aquatic environment are, as specified by the AMERICAN WATER WORKS ASSOCIATION (1995), divided in three categories: (i) bacteria indigenous to water, generally non pathogenic and comprising several *Pseudomonas, Serratia,* and *Chromobacterium* species; (ii) bacteria native to ground, brought to the surface waters in times of floods and heavy rains, whose most common constituent species are the Gram-positive, within them *Bacillus* genus and (iii) bacteria whose origin lies in the intestinal tract of man and homothermal animals or in sewage, there being considered as indicators of fecal pollution, a category in which *Escherichia coli*, *Proteus*, *Enterobacter* and *Enterococcus* are the most common representatives. All the genera just mentioned, had been present in the environment assessed in this study, even though there was a prevalence of bacteria common in the ground, here portrayed eminently by the *Bacillus* genus. The existence of these bacteria, in the aquatic environment shows their ability to survive in places to which they are not native. According to FLINT (1987) and LIM and FLINT (1989), the survival of a given microorganism in such a circumstance depends on the ability to withstand physical, chemical and biological conditions that differ from those of its natural habitat. It is relevant to say that in this study the bacteria indigenous to the aquatic environment may have had their growth impaired by the incubation temperature of 37°C which was mostly used in this work. According to YASSUDA (1969), saprophytic bacteria

grow under temperature ranging from 20 to 25ºC – the temperature ordinarily measured in the kind of environment assessed in this study.

One aspect that probably contributed to the bacterial diversity detected in Belém Novo bathing site was the magnitude of climatic changes that the place undergoes. During autumn and winter – seasons that present higher rainfalls – bacterial diversity and the number of microorganisms increased considerably, chiefly for the *Bacillus*, *Enterobacter*, and *Escherichia* genera (Tables 1,3). As the species belonging to the *Bacillus* genus presented increased counts during winter and autumn, it is possible to hypothesize that these microorganisms must have been carried out from the native ground they inhabited into the waters of the bathing site by force of the heavy rainfalls (Table 1). When ANOVA variance analysis was used to assess the set of data obtained for Gram-positive bacteria, no statistically significant diversity difference was obtained.

The increased counts for *Escherichia coli* and for the bacteria belonging to the *Enterobacter* genus during the rainy seasons had also been observed by SOLO-GABRIELE et al. (2000), EGWARI and ABOABA (2002), and CROWTHER et al. (2001), who respectively analyzed waters from the underground, from a river in Nigeria, and from the sea in UK. SOLO-GABRIELE et al. (2000) also ascertained that the high *Escherichia coli* counts in the aquatic environment are influenced by predation and soil humidity. AN et al. (2002) studying *E. coli* and total coliforms in Texoma Lake, Oklahoma, observed a significant difference in *E. coli* density during the seasons of the year. They observed that in summer there was a decrease in *E. coli*, and that there was a positive relationship of *E. coli* densities in lake water with rainfall period. Also microbial survival and rate of decay seems to depend on the ecological conditions of the lake in this period. They also hypothesize that temperature may be the most important single environmental factors, including other factors such as protozoan predation, solar radiation, algae growth (Flint, 1987; Sinclair et al., 1993). CHANDRAN and HATHA (2005) describe that *E. coli* and *Salmonella* Typhimurium were inactivated by solar light, and this was due to the accumulation of exogenous and endogenous peroxidases produced by the system of catalase or respiratory cycle. Out of the Gram-negative bacteria identified in the Belém Novo bathing site, the *Enterobacter* and *Escherichia g*enera prevailed upon others, mainly in autumn and winter collections which might be related to the high coliform counts found, with period of rains and smaller temperature. As in the work of AN et al. (2002) and CHANDRA and HATHA (2005) the sun light in the summer might be the most important inactivating factor on the survival of fecal indicator bacteria *E. coli and* other bacteria present in Belém Novo bathing site.

The values observed for Gram-negative bacteria, in particular those of the *Enterobactereaceae* family, might have been higher, if at the beginning of the experimental procedures of this study, the selective media such as S-S, XLD, XLT4, and EMB had been employed. The use of selective media is quite common in order to favor the growth of Gram-negative bacteria when we are working with environmental samples. Some genera such as *Proteus*, which accounted for approximately 10% of the Gram-negative micro-organisms identified in this study, were essentially identified via non-selective media. The same happened for *Klebsiella* and *Citrobacter* – an observation that imparts strength to the importance of using selective media with different indicator systems when assessing bacterial diversity in a given environment.

The choices of a certain bio-indicator to estimate how safe recreational waters are, is still a large controversial issue. According to MARTINS et al. (1995), the best indicator to examine biological safety of bathing sites is subject to controversy. On one hand, some researchers point to bacteria that establish fecal pollution as being the best indicators (AN et al., 2002). On the other hand, microorganisms belonging to the normal flora of skin, nose, and mouth are sometimes seen as the most effective indicators for pollution (MARTINS et al., 1995). Even those scientists who consider fecal indicators as the best choice in fact maintain different opinions, as some regard *E. coli* a more specific fecal pollution indicator than fecal coliforms. According to BAYAMUKAMA et al. (2000) and MCLELLAN et al. (2001), fecal coliforms may originate from sources other than fecal, and may yet thrive in the environment when and where carbohydrate concentrations are high. Regarding *E. coli,* its ability to replicate in the environment is weak, which enables the microorganism to become a good pollution indicator (BAYAMUKAMA et al., 2000).

The present study allowed observing that both fecal and total coliform counts are efficient pollution indicators since these counts have lay above the permitted levels. Accordingly, ALM et al. (2003) proposed that bathing sites with more than 126 *E. coli*

and 33 enterococos in 100 mL of water for a period superior then 30 days represent health risks. This was no surprise, owing to the fact that the waters from which samples were taken received raw sewage, without any previous treatment. The *E. coli* counts were high, but the values found for *Staphylococcus* were low (Table 2). *Staphylococcus* belongs to the normal flora inhabiting the human skin, nose, and mouth. Thus the low values found may be due to the fact that the bathing site was closed for holiday-makers during sample collections of this study. This is in accordance with PRÜSS (1998), who stated that *Staphylococcus* species are directly related with the recreational use of waters, and the detection of those microorganisms may be acknowledged to no other reason than the cross-reaction between bathers.

The results obtained in this study point to the bathing site of Belém Novo as being inappropriate to bathing for presenting high *E. coli*, fecal and total coliform counts. Yet, in the light of the health hazard that polluted waters pose upon the population, the city government of Porto Alegre has opened the Sewage Treatment Plant of Belém Novo, which is treating wastewater before disposal onto that bathing site. Still, more research has to take place to assess the present situation.

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TABLE 1 – *Bacillus* isolates identified in the water samples Belém Novo bathing site, Porto Alegre, RS, Brazil. July 1999 to August 2000 (n = 173).

Species	Winter	Spring	Summer	Autumn	Winter	Total
Bacillus sp.	8	18	12	5	4	47
B. stearothermophilus	16	7	0	8	2	33
B. brevis	5	0	1	6	5	17
B. circulans	2	0	5	8	2	17
B. coagulans	4	1	1	3	1	10
B. pasteuri	3	1	1	9	1	15
B. licheniformis	4	0	1	1	3	9
B. acidocaudarius	4	1	1	1	0	7
B. pumilus	1	0	3	0	1	5
B. megaterium	0	0	1	3	0	4
B. macerans	1	0	0	1	0	2
B. alvei	2	0	0	0	0	2
B. larvae	1	0	0	0	0	1
B. firmus	1	0	0	0	0	1
B. subtilis	0	0	1	0	0	1
B. polymyxa	1	0	0	0	0	1
B. mycoides	0	0	0	0	1	1
Total	53	28	27	45	20	173

TABLE 2 – Other Gram-positive bacteria identified in the water samples of the bathing site Belém Novo, Porto Alegre, RS, Brazil. July 1999 to August 2000 (n = 42).

Species	Winter	Spring	Summer	Autumn	Winter	Total
Listeria sp.	2	0	0	2	0	4
L. murray	0	0	3	1	0	4
L. denitrificans	0	0	0	0	2	2
L. grayi	0	0	0	0	1	1
Corynebacterium mycetoides	0	0	1	1	0	2
C. paurometabolum	1	0	1	1	0	3
C. minitissimum	0	0	0	0	1	1
C. pseudotuberculosis	0	0	0	1	0	1
Micrococcus varians	0	0	0	1	1	2
Staphylococcus xylosus	1	0	3	0	3	7
S. haemolyticus	1	0	3	0	0	4
S. caseolyticus	4	0	0	0	0	4
S. sacharolyticus	1	0	0	0	1	2
S. lentus	1	0	0	1	0	2
S. warnerii	0	0	0	0	2	2
S. epidermidis	0	0	1	0	0	1
Total	11	0	12	8	11	42

TABLE 3 – Gram-negative species identified in the water samples Belém Novo of the bathing site, Porto Alegre, RS, Brazil. July 1999 to August 2000 (n = 170)

+	Winter	Spring	Summer	Autumn	Winter	Total
Escherichia coli	1	0	7	20	13	41
Hafnia alvei	2	1	0	9	4	16
Enterobacter sp.	2	1	1	14	2	20
E. aglomerans	2	1	8	25	4	40
E. aerogenes	0	1	0	1	3	5
E. cloacae	0	0	0	0	3	3
Aeromonas sp.	1	0	0	0	0	1
Yersinia sp.	1	0	0	0	0	1
Y. enterocolitica	1	0	0	1	0	2
Acinetobacter sp.	1	1	0	1	1	4
A. calcoacetitus	1	0	0	2	0	3
Proteus sp.	0	0	0	6	2	8
P. mirabilis	0	0	3	5	1	9
P. rettgeri	0	0	1	0	0	1
P. morganni	0	0	0	0	1	1
Klebsiella pneumoniae	0	0	0	4	1	5
Citrobacter freundii	0	0	0	0	3	3
Serratia lichefaciensis	0	0	0	1	0	1
S. rubidae	3	0	0	1	1	5
Edwardsiella tarda	0	0	0	1	0	1
Total	15	5	20	91	39	170

TABLE 4 – Variance ANOVA analyzes for the Gram-positive bacteria identified at Belém Novo bathing site ($p < 0.05$).

	F	p- Value
Winter/Spring 1999	0.98	0.33
Spring 1999/Summer 2000	0.12	0.73
Summer 2000/Autumn 2000	0.73	0.36
Autumn/Winter -2000	1.74	0.19
Winter 1999/Winter 2000	2.86	0.09
Summer 2000/Winter 1999	1.57	0.21
Summer/Winter 2000	0.10	0.74
Spring 1999/Autumn 2000	0.63	0.43
Winter 2000/Spring 1999	0.02	0.89
Winter 1999/Autumn 2000	0.07	0.79

TABLE 5 – Variance ANOVA analyzes for the Gram-negative bacteria identified at Belém Novo bathing site ($p < 0.05$)*.

	F	p-Value
Winter/Spring 1999	4.87	0.03*
Spring 1999/Summer 2000	1.98	0.16
Summer 2000/Autumn 2000	4.44	0.04*
Autumn/Winter 2000	2.25	0.14
Winter 1999/Winter 2000	1.30	0.26
Summer 2000/Winter 1999	0.14	0.70
Summer/Winter 2000	0.71	0.40
Spring 1999/Autumn 2000	3.47	0.06
Winter 2000/Spring 1999	2.94	0.09
Winter 1999/Autumn 2000	2.65	0.11

TABLE 6 – Counts of total coliform and *Escherichia coli* detected in the water samples of Belém Novo bathing site Porto Alegre, RS, Brazil. August 1999/August 2000.

Month of collection	Total Coliform MPN/100 mL	*Escherichia coli*- MPN/100 mL
August 1999	7.4×10^3	1.0×10^3
September 1999	2.8×10^4	4.9×10^3
October 1999	6.4×10^4	2.0×10^3
November 1999	2.9×10^4	1.0×10^3
December 1999	1.4×10^5	4.1×10^3
February 2000	7.7×10^3	$*1.4 \times 10^2$
March 2000	3.9×10^4	1.4×10^4
April 2000	1.2×10^4	1.7×10^3
May 2000	9.6×10^3	$*7.7 \times 10^2$
June 2000	5.2×10^4	4.9×10^3
July 2000	2.3×10^4	3.9×10^3
August 2000	2.4×10^4	3.3×10^3

* Collections in which the numbers of *E. coli* counts were satisfactory for bathing according to CONAMA legislation.

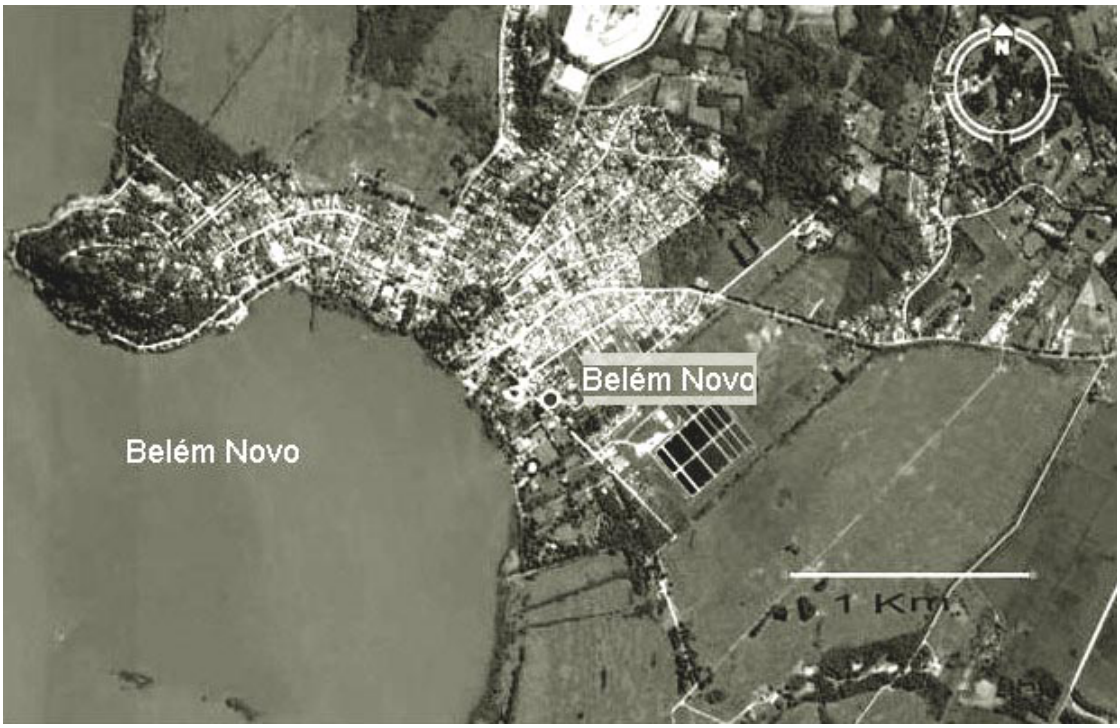

Fig. 1. Guaíba Lake. Left margin, Belém Novo bathing site. BN (?) collection point at 50 m from the margin.