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DISTRIBUTION OF SOME DOMINANT BACTERIA IN ALEXANDRIA EASTERN HARBOUR THAT CAN BE USED AS MARINE CONTAMINANT INDICATORS

By

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ABSTRACT

The population distribution of the fecal coliform bacteria (Escherichia coli, Streptococcus sp., Staphylococcus aureus, Staph. epidermidis and Enterobacter aerogenes) in the Eastern Harbour of Alexandria (EHA) as well as the most abundant bacterial group (Bacillus spp.) during 2000-2001 were studied. Bacterial counts were determined and statistically analyzed in order to verify the correlation between these isolates, and to establish which isolate can be used as a bio-indicator for microbial pollution. The results indicated that the most contaminated seasons were winter (159 cfu /ml) and autumn (155 cfu /ml), while the most contaminated site was location (II) (171 cfu/ml). The mean counts of the tested bacteria showed high significant differences ($P<0.01$) except for Enterobacter aerogenes, Bacillus circulans and B. megaterium, they were insignificantly different (39, 38 and 43 cfu /ml, respectively). The most abundant bacterial species in all locations and seasons were found to be Bacillus lentus with a mean-count 341 cfu /ml followed by Staphylococcus epidermidis with a mean-count 208 cfu /ml. Therefore, they can be used rather than the classical indicators for detecting the microbial contamination in the aquatic bodies suffering from similar environmental conditions.

INTRODUCTION.

Many investigators selected the coliform group to assess the contamination in the marine environment (Mancini, 1978 and Lara *et al.*, 1991). While, others studied the factors affecting the survival of such classical bacterial indicators and how to eliminate or reduce them from the contaminated area (Fujioka *et al.*, 1981; McCambridge and McMeeckin, 1981; Lara *et al.*, 1991; Baron and Bourbigot, 1996 and Rose *et al.*, 1996), disregarding the other bacterial species that may play an important role in such marine environments and can be used as alternative indicators for pollution.

Eastern Harbour of Alexandria (EHA) is a shallow semicircular basin, covering an area of about 2.8 km^2 with a water volume of $1.5x107m^3$. The harbour is also sheltered from the sea by a middle breakwater leaving two openings where the exchange of seawater between the harbour and the Mediterranean sea takes place, the western inlet (El-Boughaz), and the eastern inlet (El-Silsila) (Lotfy and Badr, 1999).

Sewage wastes from the northern side of Alexandria were discharged through some outlets into the coastal line of the Mediterranean sea including EHA which received about $1.5 - 6.3 \times 10^4$ $m^3.d^{-1}$ of the domestic sewage (Said and Maiyza, 1987 and Saad, *et al.*, 1987). Recently, only one outlet was left in EHA for rain discharge. It was found that from the 15th October 2000 to 15th April 2001 about $1.47x10^5$ m^3 of rain water were discharged (personal communication). However, the only outlet that discharge domestic sewage at this area is located behind the Kayiat Bey castle, which discharges about $1.48x10^5$ $m^3.d^{-1}$. Various investigations were carried out concerning oceanography, hydrography, pollution and the discharge of wastewater inside this harbour regardless the microbial communities (El-Deek *et al.*, 1990 and Abd El-Moati *et al.*, 1991).

Therefore, this study aimed to investigate the seasonal distribution of some abundant bacterial species present in different locations outside and in the EHA during the period 2000-2001, with special interest to the members that can be used as a new indicator for bacterial pollution.

MATERIALS AND METHODS

Sampling process

Three sub-surface samples (50cm below the sea level) were seasonally collected from seven locations (I-VII) outside and in the EHA basin on 15th of May (spring-2000), 15th of August (summer-2000), 15th of November (autumn-2000) and 15th of February (winter-2001). The seawater samples (500ml /each) were collected in sterile glass blue-capped bottles using special sampler and kept cool after sampling in an ice-box until they were transferred to the laboratory (Fig. 1).

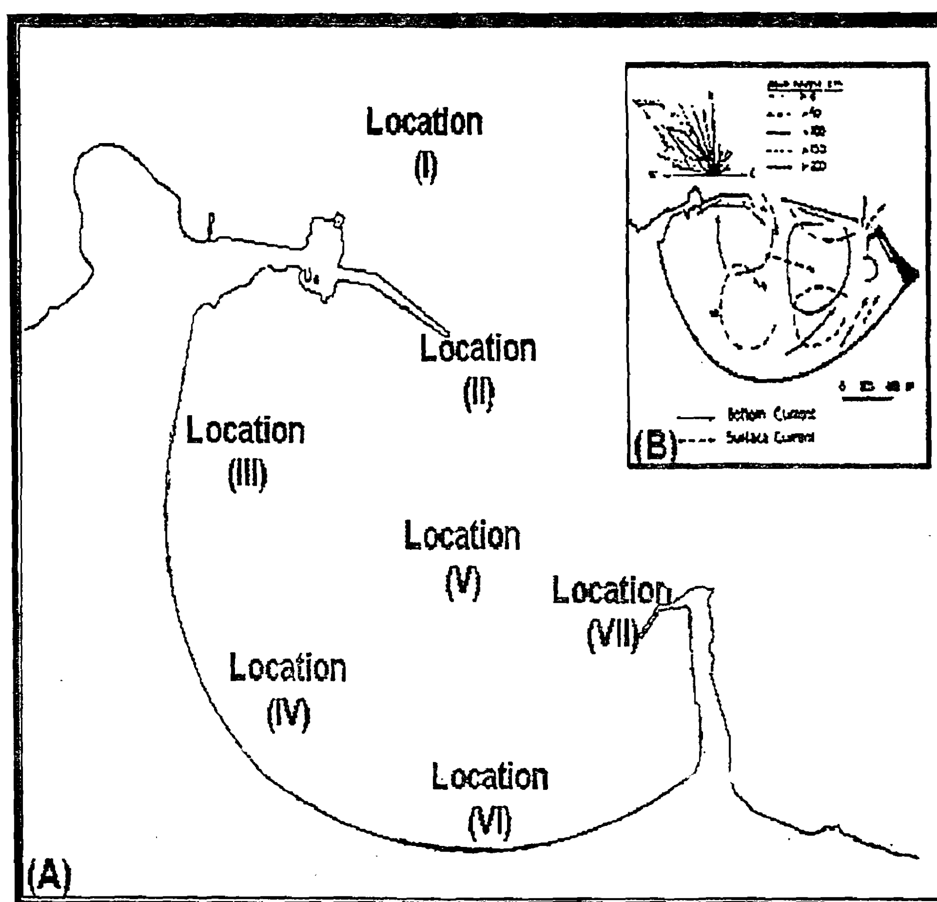

Fig. 1: Eastern harbor of Alexandria (EHA) showing the sampling locations (A), and the current pattern inside (EHA) (B).

Isolation of the common bacterial species

Seawater samples were serially diluted down to 10^{-5} using sterile saline solution (0.8% Na Cl). One hundred µl was spread onto the surface of sterile nutrient agar medium containing peptone (5g), beef extract (3g) and agar (20g) per one liter of sea water. The seeded plates were left for 10 min, then they were inverted and incubated at 30°C for 24h. Enumeration was carried out in plates containing 30-300 cfu /plate. Isolation of bacterial strains was based on colony characteristics and microscopic examination of bacterial cells. After grouping similar isolates, the common isolates were *Bacillus* members. These were identified by biochemical methods using API - 50CH specific identification kits for *Bacillus sp.* and API -20E specific identification kits for additional confirmatory tests needed for complete identification of the *Bacillus sp.*

Isolation of E. coli

Three replicates of 1, 10 and 100ml seawater samples were filtered through standard membrane filters with pore size 0.45mm, then transferred onto a selective Difco- m FC agar medium, 52g/l were suspended in distilled water, heated to boiling, then supplemented with 10ml of 1% Rosolic acid in 0.2N Na OH (no autoclaving). The seeded plates were incubated for 24h at 44.5°C, then blue colonies were enumerated. The confirmation and identification were carried out using API- 20E specific identification kits for *Enterobacteriaceae.*

Isolation of Staphylococcus *species*

Staphylococcus species were isolated from seawater samples using a selective Difco- Mannitol salt agar medium, where 111g/l were dissolved in distilled water, heated to boiling then autoclaved for 15 min. Two types of colored colonies (yellow and red) appeared after 24h incubation at 37°C. The obtained colonies were examined for pigment production using another selective medium, Difco -*Staphylococcus* medium 110, where 149g/l were completely dissolved in distilled water, heated to boiling then autoclaved for 10 min. After 48h incubation at 37°C the tested yellow colonies showed positive pigment production indicating the presence of *Staph. aureus* while, the red colonies showed negative pigment production indicating the presence of *Staph. epidermidis*. The final identification was carried out using API -STAPH. specific identification kits for *Staphylococcus* species.

Isolation of Streptococcus *species*

The detection of *Streptococcus* species in seawater samples was carried out according to APHA (1995). A selective medium (Difco-KF *Streptococcus* agar medium) was used, 76.4g/l were suspended in distilled water, heated to boiling (no autoclaving). Then 1% of TTC solution (triphenyl-tetrazolium chloride) was added per

100ml sterile medium used. The inoculated plates were incubated for 48h at 37°C. Red or pink colonies appeared indicating the presence of *Streptococcus* species. Difco- bile esculin azide agar medium (57 g/l were completely dissolved, heated to boiling then autoclaved for 15 min). This selective medium was used to detect the isolation of group D streptococci which hydrolyze esculin producing a dark brown color. The seeded plates were incubated for 24h at 37°C and a positive hydrolysis of the esculin was obtained. In addition, API-20 STREP specific identification kits for *Streptococcus* species were used for final identification which indicate the presence of *Streptococcus faecalis*.

Isolation of other fecal coli form bacteria

Three replicates of 1,10 and 100ml seawater samples were filtered using 0.45mm membrane filters then transferred aseptically to plates containing Difco-SS agar medium, 60g/l were suspended in distilled water, heated to boiling (no autoclave), then dispensed into sterile petri dishes (5 cm in diameter). After 24h incubation at 37°C the appeared colonies were pinkish-cream indicating the isolation of *Enterobacter aerogenes*.. The conformation and identification of these colonies were carried out using API-20E specific identification kits for *Enterobacteriaceae*,

Statistical analysis

The statistical analysis of the data was carried out in a factorial experimental design and was completely randomized according to Steel and Torrie, 1980. The analysis of variance, F test, LSD, and factorial combinations were estimated using ANOVA program and MSTAT-C software package ver.1.2 1994.

RESULTS

The classical fecal bacterial indicators (*Escherichia coli, Streptococcus sp., Staphylococcus aureus, Staph. epidermidis* and *Enterobacter aerogenes)*, in addition to the most abundant bacterial species found in EHA basin (*Bacillus* members), were isolated from 84 subsurface seawater samples collected from seven locations at EHA basin during 2000-2001. Results of bacterial counts were statistically analyzed in relation to the collection seasons (Table 1) and locations (Table 2). The highest counts were recorded in winter followed by autumn with mean counts of 177 and 155 cfu /ml, respectively, while, the lowest mean bacterial count was recorded in summer (74 cfu /ml). The sampling locations showed also high significant differences ($P<0.01$) in bacterial counts, where location-II at the basin-inlet was the most contaminated one followed by location-I outside the basin with mean counts of 171 and 164 cfu /ml, respectively. Meanwhile, location-III was the least contaminated location with a mean-count of 66 cfu /ml (Table 2).

Table 1: The statistical analysis of the mean bacterial counts (cfu/ml) in four seasons during 2000-2001.

Organisms \ Seasons	Spring	Summer	Autumn	Winter	Mean
E. coli	244*	10	84	265	151[d]
Staphylococcus auerus	46	71	188	19	81[f]
Staphylococcus epidermidis	189	261	183	200	208[b]
Streptococcus faecalis	51	20	92	525	172[c]
Enterobacter aerogenes	28	19	18	92	39[g]
Bacillus lentus	287	229	576	272	341[a]
Bacillus pumilus	9	29	83	32	38[g]
Bacillus megaterium	20	44	233	81	95[e]
Bacillus circulans	9	24	67	72	43[g]
Bacillus cereus	39	28	29	30	32[h]
Mean	92[b]	74[c]	155[a]	159[a]	

* Each value is a mean of three replica then approximated to a whole number.

@denotes means followed by the same letter are not significantly defferent at L.S.D $_{0.01}$ level of probability, but the different letters are significantly different at the same level of probability.

The presence of the bacterial community within the EHA basin showed a certain trend following the surface water-current in the basin except location-V. It was noticed that, the bacterial counts increased from location-III to location-VII with mean counts of 66, 90, 128 and 138 cfu /ml, respectively. The mean count at location-V (83 cfu /ml) was almost the median of mean-counts in locations (III), (IV), and (VI), which can be attributed to the surface inter-current between those locations at the basin center.

In general, variations between the mean bacterial counts showed that *B. lentus* recorded the highest mean (341 cfu /ml) followed by *Staphylococcus epidermidis* , *Streptococcus sp.* and *E. coli* with mean counts of 208 , 172 and 151 cfu /ml, respectively, (Table 1&2).

Table 2: The statistical analysis of the mean bacterial counts (cfu/ml) at seven locations in EHA during 2000-2001.

Organisms \ Locations	I	II	III	IV	V	VI	VII	Mean
E. coli	73*	223	81	78	120	199	280	151^{d}
Staphylococcus auerus	153	105	26	160	46	48	28	81^{f}
Staphylococcus epidermidis	325	258	152	145	92	208	278	208^{b}
Streptococcus faecalis	475	406	48	23	56	86	110	172^{c}
Enterobacter aerogenes	43	47	41	25	52	40	27	39^{g}
Bacillus lentus	378	377	209	255	321	551	296	341^{a}
Bacillus pumilus	44	37	6	81	49	39	11	38^{g}
Bacillus megaterium	134	205	13	22	40	55	196	95^{e}
Bacillus circulans	2	36	2	83	49	48	80	43^{g}
Bacillus cereus	15	20	79	28	5	6	69	32^{h}
Mean	164^{b}	171^{a}	66^{g}	90^{e}	83^{f}	128^{d}	138^{c}	

@denotes means followed by the same letter are not significantly defferent at L.S.D $_{0.01}$ level of probability, but the different letters are significantly different at the same level of probability.

The variation in counts for each bacterial isolate during the period from spring 2000 to winter 2001 was considerably pronounced. The highest count for *E. coli* (780 cfu /ml) was determined in winter at location-VII. It was noticed that, this bacterium is widely represented in spring, where it followed certain trend within EHA locations (Figure 2-A). It showed a low count of 120 cfu /ml at location-I with a gradual count increase within the basin starting at the western inlet (location-II) with a count of 180 cfu/ml, to location-VI (400 cfu /ml) then location-VII (380 cfu /ml), following the surface current in EHA which is illustrated in Figure 1-B.

Meanwhile, in case of *Staphylococcus* species (*Staph. aureus* and *Staph. epidermidis*), EHA locations were more contaminated with *Staph. epidermidis,* especially in summer and spring, where the highest counts were 420 and 300 cfu /ml, respectively, which were determined at location (I) and (II), respectively, (Figure 2-B). Moreover, in spring this bacterium showed an ascending trend contrary to that of the *E. coli* inside the EHA basin. Meanwhile, the maximum counts for *Staph. aureus* were only obtained in autumn at locations (I) and (IV) with a count of 480 and 520 cfu /ml, respectively, (Fig. 2-C).

Generally, the distribution of *Streptococcus sp*. showed a maximum bacterial count in winter compared with the other seasons but only at locations (I) and (II) with a count of 1.8×10^3 and 1.2×10^3 cfu /ml, respectively. However, this species was detected at all locations in summer with counts ranging between 25 to 100 cfu /ml, (Figure 2-D). Monitoring the count of *Enterobacter aerogenes* showed that it is more represented in winter at all locations with count ranging from 40 to 120 cfu /ml (Figure 2-E).

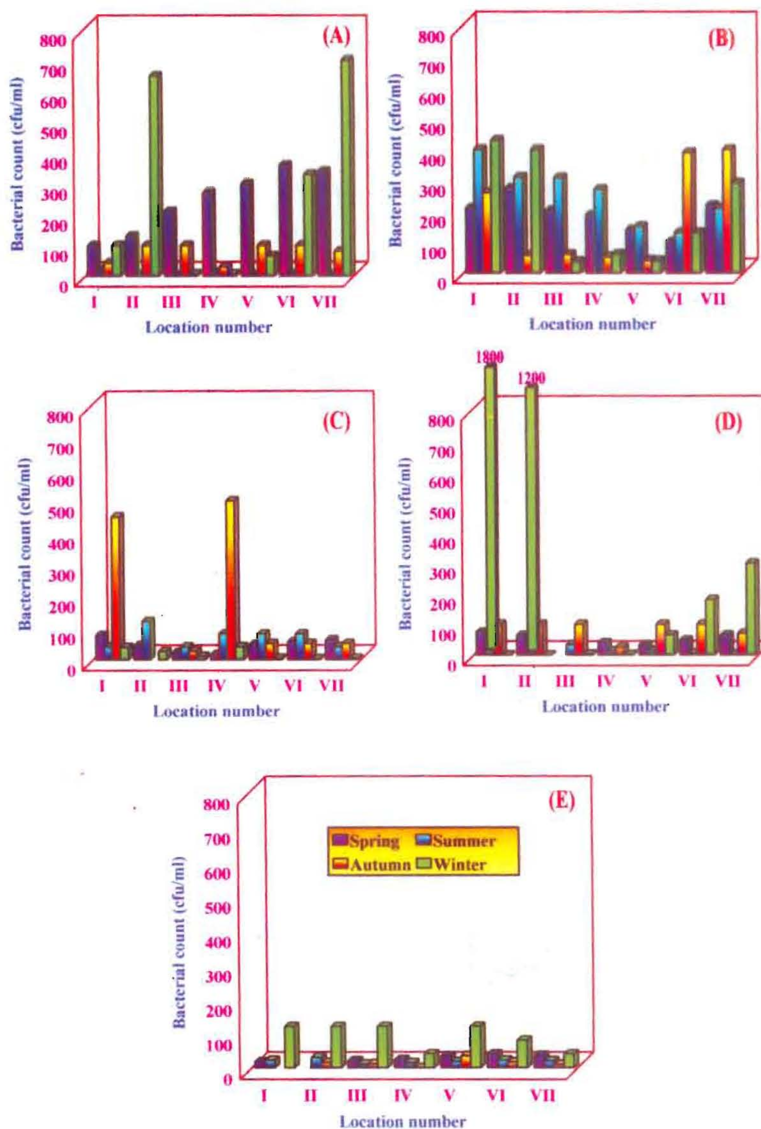

Fig. 2: Monitoring the bacterial count ofE._coli (A), Staph. epidermidis (B), Staph. auerus (C), Streptococcus faecalis (D) and Enterobacter aerogenes (E) during 2000-2001 in seven locations of EHA.

The monitoring of Bacillus group in the sampling locations (Fig. 3, A-D) showed that the most detectable species within this group is B. lentus during the period of collection at all locations (Fig. 3-A). B. pumilus was also detected in all locations, but with lower counts compared to B. lentus. It was regularly detected in winter with a high count of 540 cfu/ml at location-I, and with least count of 100 cfu /ml at locations (III) and (IV), Figure (3-B). B. circulans was detected inside EHA at locations (II-VII) especially in autumn, with a mean count of 83 cfu /ml, (Fig. 3-C & Table 1). On the other hand, B. megaterium and B. cereus showed scattered distributions along the tested locations (Fig. 3-D and E).

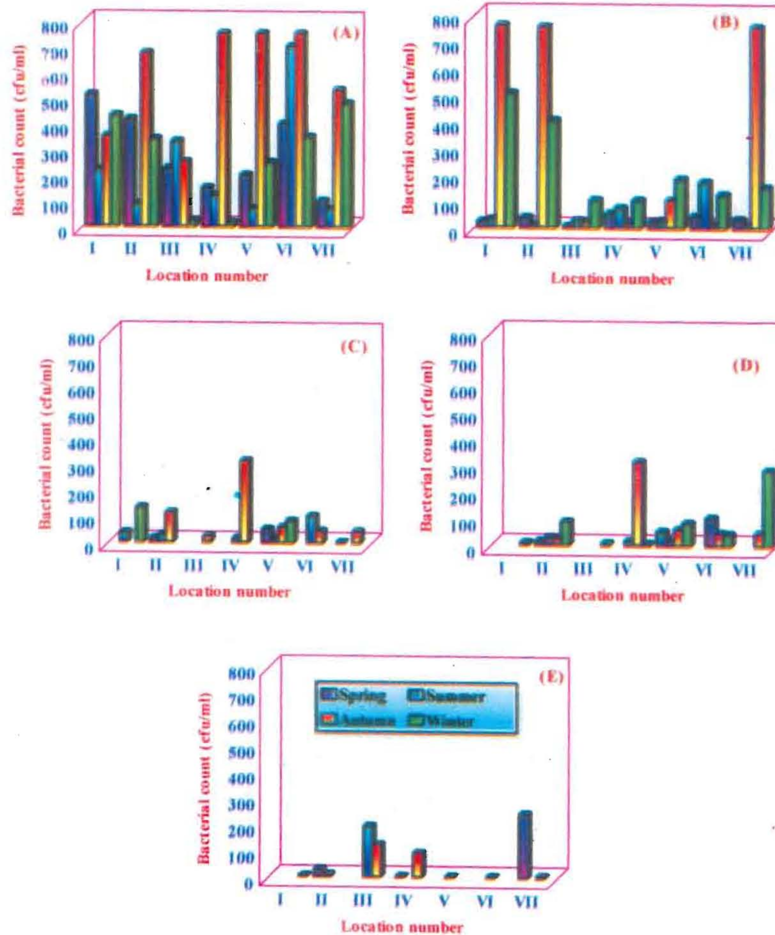

Fig. 3: Monitoring the bacterial count of Bacillus Lentus (A),B. pumilus (B), B. circulans (C),B. megaterium (D) and B. cereus (E) during 2000-2001 in seven locations of EHA.

DISCUSSION

A wide variety of enteric microbial pathogens found in wastewater may cause diseases such as dysentery, typhoid and gastroenteritis, which showed to be transmitted primarily through untreated sewage (Bitton, 1994 and Rose *et al.*, 1996). Therefore, assessment of such contamination in EHA was a must to detect and to determine the type and the level of contamination.

However, reassessment of the model currently used for predicting the bacteriological quality of aquatic systems subject to fecal contamination was needed, because the models used in the assessment of aquatic contamination did not follow a consistent trend especially in environments contaminated with wide varieties of bacteria.

The results obtained in this study showed great variations in the ecological distribution of the coliform group during the collection period and it may be inadequate for assessing the exact microbial contamination occurring in EHA. This is in agreement with the results obtained by Havelaar (1991), who indicated that the coliform system is an inadequate measurement for contamination. It was also observed that there are several factors responsible for the mortality rate and the rapid disappearance of fecal bacteria in the aquatic ecosystems, (including salinity, temperature, duration of light and the presence of predators) (Aubert *et al.*, 1981).

In addition, grazing by mono-flagellates has been identified as a major factor in depleting the bacterial community (Pace, 1988). Lara *et al.* (1991) have also reported that, protozoan grazing of *E. coli*, *Salmonella* sp., and *Streptococcus* sp. was the most important factor for bacterial mortality in seawater samples, and the biochemical differences of these organisms did not result in grazing specificity. It was also reported that bacterio-phages were very abundant in the aquatic environments and play a significant role in bacterial mortality (Bergh *et al.*, 1989; Proctor and Grines, 1990). Moreover, Simek *et al.* in 2001, observed that viral concentrations and frequencies of infected cells were highly significant correlated with grazing rates, suggesting that protistan grazing may stimulate viral activity. Furthermore, Fricker and Fricker (1996) mentioned that, the expense needed for these bacterial determinations may be wasted if the presumptive results are not confirmed. Therefore, it may be useful to use other bacterial indicators that have a wide presentation and have been established in marine environments suffering from contamination instead of the classical and depleting fecal bacteria.

Lotfy and Badr in (1999) noticed that, the water exchange between the harbour and the open sea takes place in a dominant direction where the coastal current enters the harbour through El-Boughaz and exit through El-Silsila following an anti-clockwise

current, except in winter when the current runs in a clockwise direction. In general, the current regime is also affected by refracted and diffracted waves inside the harbour, which may explain the variation noticed in the bacterial counts between the sampling locations. It was observed that a gradual accumulation of bacteria followed the surface current within the basin to El- Silsila opening (Table 2).

On the other hand, the seasonal variations in count for the fecal indicators and the *Bacillus* group showed that winter and autumn were the most contaminated seasons. This may be correct or reasonable since the only outlet left in EHA showed to be workable in the period from 15th October to 15th April. These results are in agreement with the observations of Moran *et al.*, 2001, who concluded that the productivity of heterotrophic bacteria may be enhanced when transferred to dark condition, like in case of changing from summer to autumn or from autumn to winter.

Moreover, it may be noticed that the variations in the temperature values (~15 °C) along the collection period may indirectly affect the bacterial community (data not showed), where the predators prefer the warm environment as in spring and summer encouraging predator growth flourishing rather than the low temperatures as in autumn and winter.

This study may present an out-view about the ecological distribution of the abundant bacterial species in EHA. Also, it may be concluded that the use of one or two bacterial indicators for detecting the fecal contamination in the aquatic environments all over the year is not realistic. It may be useful to use *E. coli* (in spring) or *Streptococcus faecalis* (in summer), where they showed to be widely spread in all locations, while *Staphylococcus epidermidis* may be used for detecting the fecal contamination in both summer and spring. These selected bacteria showed to be more abundant and presentable as compared to the other fecal bacteria tested under such environmental conditions.

On the other hand, *Bacillus lentus* showed to be the most detectable species in such environments in comparison with the other bacterial isolates all over the collection period (2000-2001), and at all studied locations (I-VII), followed by *Bacillus pumilus* which was also detected at all locations but in lower counts. Moreover, this study showed that there was a relationship between the presence of *B. lentus* and the bacterial contamination in EHA, where it was abundant at the studied locations all over the year with a highest mean-count compared to the other species. Therefore, *B. lentus* may be used as a new indicator for monitoring the bacterial contamination in the marine environments suffering from such conditions. Further investigations are recommended to prove the reality of this conclusion in order to avoid the effect of many depleting factors discussed before that affect the presence of the classical fecal indicators used for detecting the bacterial contamination in water bodies.

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