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Full Length Research Paper

Factors influencing multi-drug resistant and plasmid DNA harbouring *Aeromonas hydrophila* isolated from Lake Manzala, Egypt

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Lake Manzala is considered one of the most important coastal Lakes in Egypt. This study revealed the highly polluted condition of water and fish samples taken from three of the more important sites (Kapoty, Bashtier and Mataryia areas), representative to human activity and different ecosystems in the Lake water environment. Total suspended solids (TSS), ammonia and nitrates, of the samples taken showed figures exceeding the national and international standards. The samples from the study yielded high levels of bacterial content, which were found through plate counts and by using different growth media such as agar and endo agar. Total viable bacteria (TVB) reached more than 10^4 cfu/ml in water samples and 10^5 cfu/g in fish samples, particularly in Kapoty and Mataryia areas. Faecal coliform counts reached 10^2 cfu/ml in water samples and 10^3 cfu/g in fish samples. Statistical analysis revealed a positive correlation between *Aeromonas* spp. counts and some minerals, such as chlorides, calcium and magnesium ($r = 0.9$, 0.8, and 0.6 respectively; $p < 0.001$), which revealed the interactions and adaptability of such organisms to environmental factors. A total of 88 isolates from *Aeromonas* differential agar were identified using the API 20E system; 17% of these isolates were *Aeromnas hydrophila*, and most of these strains were resistant to three or more antibiotics including pencillinG, ampicillin and chloramphenicol. Isolation of plasmid DNA from these strains of *A. hydrophila*, showed that most harboured plasmids ranging from less than 2.2 - 4 Kb_P. Such findings could explain the adaptability and multi-drug resistance of such bacterial pathogens, which may have an impact on the course of infection and active causal agents of diarrhoeal diseases in this important coastal area of Egypt on the Mediterranean Sea.

Key words: *Aeromonas hydrophila*, multi-drug resistance, plasmid DNA, Lake Manzala.

INTRODUCTION

Lake Manzala is considered one of the most important coastal Lakes in Egypt. It is situated in the northeastern part of the country, on the Delta of the Nile River bordering of the Mediterranean Sea. It receives a high load of different polluted waste water effluents including sewage water, industrial and agricultural waste from many drains, such as Bahr El-Bakar, Ramsis, El-Sirw and Hadous. The effluents from these drainages negatively affect the Lake environment to the eutrophic level as well as human health among communities around the Lake.

Aeromonas spp. particularly *Aeromonas hydrophila* and *Aeromonas sobria* are widely distributed in fresh, brackish and saline water environments, as well as in lakes and sewage water; they have also been isolated from drinking water and food, such as meat, fish, shellfish, raw milk and vegetables (Araujo et al., 1990; Gonzalez et al., 2002; Hanninen et al., 1997; Kirov et al., 1990; Montesw

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Figure 1. Map of Lake Manzala, showing different sampling sites and sources of pollution.

1990; Montesw et al.,1999; Ortega et al.,1996; Ringo and Vadstein, 1998).

MATERIALS AND METHODS

Sampling location

Lake Manzala is a coastal shallow lake, which consists of 30 basins with depths ranging from 0.7 - 1.5 m and the navigation canal is about 3 m deep (Maclaren et al., 1980). It is situated east of the Nile's Delta between the Damitta branch of the Nile River and the Suez Canal. The Mediterranean Sea is immediately north of the narrow coast, which separates the two water bodies. Lake Manzala did not exist before the 6th century - before then it was an area of cultivated land with fertile soil until a tectonic plate movement caused an earthquake that created the lake (Maclaren et al., 1980). Lake Manzala is quite different today, the construction of canals and drains, such as Bahr El-Bakar, Sirw, Ramsis and Hadous drains have created eutrophic conditions and low salinities in portions of the lake. The area closest to the drain outlets is characterized by brackish water in the south and west; and by saline water in the northeast area near the sea outlets (El-Gamil outlet). The sampling localities were selected close to the most important communities around the Lake and to the areas where the discharge of different types of pollutants occur, which negatively affect the Lake's condition and human health in the area (Figure 1).

Samples were taken from the end of the junction canal, which connects the Suez Canal with Lake Manzala, and the source of pollution in this area is most likely from the Port-Said city. Effluents such as sewage water and different types of industrial waste from many factories are disposed of in the Lake. This site is close to EL-Kapoty village which is a fishermen village in Port-Said and where raw sewage is disposed directly into the lake water, and has been designated as the Kapoty site. The Bashtier sampling site is considered a mid-point between the El-Kapoty and the Mataryia areas; it receives water currents from different directions resulting in high water levels. The depth reaches three meters and is part of the navigation canal. The area has many islets which are inhabited by people who work in fishing and raise animals. The Mataryia area is considered as the fresh-water part of the Manzala Lake; however, it receives high amounts of different types of pollutants. Untreated sewage from the El Dakahlyia governorate disposed of six million m^3/ day of industrial and agricultural waste from the El-Siwr, Hadous, Ramsis and Bahr El-Bakar drains. The drains empty into the El Genka reservoir, a part of the lake that is characterized by vast vegetation composed of reeds and other aquatic plants like the water hyacinth. This area is particularly important for fishing, especially the fishing of *Tilapia* spp.

Sampling methods

One litre water samples was taken from all three sites (Kapoty, Bashtier and Mataryia) on Lake Manzala in clean sterile glass bottles and transported on ice from the lake to the laboratory within six hours for direct examination (Tucker et al., 1990). Fish samples were collected from the Bashtier and Mataryia sites utilizing gill nets and samples were immediately packed in sterile polyethylene cases

and preserved on ice. All collected samples were transported and prepared for immediate bacteriological analysis.

Preparation of samples

Water samples were directly diluted with 0.8% saline distilled water for bacterial counting using the dilution and pour plate methods. The fish were dissected and 1 g of the intestinal contents was aseptically stripped out with sterile forceps. Samples of gill lamellae and intestinal contents (1 g) were aseptically resuspended in 100 ml of 0.1% w/v chilled peptone water (pH 7.0), homogenized and poured with different dilutions into Petri dishes (Zaky, 1995).

Physicochemical characterization of water samples

The temperatures of water samples were determined by using an ordinary mercuric thermometer. The pH values were determined using a combination of pH electrode (Eil series 1180) and pH meter (Eill, Model 7030) calibrated against pH 4, 7, and 9 buffers. The salinity of the water was determined using the refractometer model Leica No. 10419. The dissolved oxygen of water was determined using the Oxygen meter Model YSI 58. Chemical analysis of the water for determination of Total Suspended Solids (TSS), ammonia, nitrates, calcium, magnesium and chlorides was done using the recommended standard methods of water analysis (APHA, 1995).

Microbiological analysis

Counts of total viable bacteria were evaluated using nutrient agar, which contains 5 gm of peptone; 5 gm of sodium chloride; 3 gm of beef extract and 15 gm of agar per liter of distilled water used. Incubation was at 37°C for 24 h. Counts of faecal coliforms were conducted with Endo agar medium composed of 10 gm of lactose, 10 gm of peptone, 3.5 gm of dipotassium hydrogen phosphate, 2.5 gm of sodium sulphite, 0.5 gm basic fuchsin, and 15 gm of agar per 1 L of distilled water used. Incubation was at 44°C for 24 h. Counts of *Aeromonas* spp. were determined utilizing *Aeromonas* differential agar media, which contains 15 gm of peptone from casein, 1.6 gm sodium sulphite, 0.25 gm of basic fuchsin, 7.75 gm of disodium hydrogen phosphate, and 15 gm of agar, per 1 L of distilled water. The pH was 7.5, and it was incubated at 37°C for 24 h. Typical colonies of *Aeromonas* spp. were taken from the selective media and identified according to the recommended API 20 E system (bioMerieux) for identification of the family Enterobacteriaceae.

Resistance to antimicrobial agents

The resistance to antimicrobial agents was determined by the MIC method. The bacterial strains of *A. hydrophila* were grown overnight in nutrient broth, then 1 ml of the broth was injected into a Petri dish and nutrient agar media (50°C) was poured on top. Different antibiotic discs (Oxoid) were inserted into the plates before complete solidification of the media and incubated at 37°C for 24 h. Results were obtained by observing and measuring the clear zones around each antibiotic disc. The absence of clear zones was an indication of the resistance of the bacteria to the antibiotics. The antibiotics used were chloramphenicol 30 mcg, ampicillin 10 mcg, penicillin G 10 mcg, streptomycin 10 mcg and gentamycin 10 mcg (Zaky, 1995).

Isolation of plasmid DNA

The alkaline lysis method was used in mini preparations as follows: 5 ml of medium was inoculated with a serial colony and incubated at 37°C overnight with vigorous shaking; 1.5 ml of the culture was poured into an Eppendorf tube and centrifuged. The remainder of the overnight culture was stored at 4°C. The solution was poured off and the last drop was removed with a Pasteur pipette. The pellet was resuspended by vortexing in 100 µl of an ice-cold solution consisting of 50 mM glucose, 10 mM EDTA, 25 mM tris-HCL (pH.8.0), 4 ml / ml lysozyme, and stored for 5 min at 37°C in a water bath. A 200 µl of freshly prepared solution of 0.2 M NaOH, 1% SDS was added and the contents were mixed by inverting the tube gently**,** two or three times and then stored on ice for 5 min. A volume of 150 µl of an ice cold solution of potassium acetate (pH 4.8) was added and prepared as follows: 11.5 ml of glacial acetic acid and 28.4 ml of water were added to 60 ml of 5 M potassium acetate, the resulting solution was mixed 10 times and stored on ice for 5 min, and the supernatant was transferred to a fresh tube. Two volumes of ethanol were added at room temperature and mixed gently by hand and left at room temperature for 2 min; the solution was then centrifuged for 30 min in an Eppendorf centrifuge at room temperature. The supernatant was removed and the tube was left in an inverted position on a paper towel to allow all the fluid to drain; 10 - 50 ml of TE (pH 8.0) containing DNase free pancreatic RNase (20 µg/ml) was added and vortexed briefly, then 20 µl of the solution was removed to a new Eppendorf tube, and 3 µg of loading buffer was added and then injected into the gel. The remainder of the preparation was stored at -20°C (Maniatis et al., 1984).

Statistical analyses

The correlation matrix is the relationship between a numbers of data sets that are scaled to be independent of the unit of measurement. It is used to determine whether data sets move together; that is whether large values of one set are associated with large values of the other (positive correlation); whether small values of one set are associated with large values of the other (negative correlation); or whether the values of the sets are unrelated (Fowler and Cohen, 1996).

RESULTS

Physical and chemical properties were estimated for all water samples collected from the Kapoty, Bashtier and Mataryia sites seasonally from autumn to summer. Most samples taken from these sites exceed the limits of pollution standards, such as TSS, ammonia and nitrate, respective to their different levels of salinity (Table 1).

Bacteriological counts were higher in the summer months; particularly in the water samples of most sites, with seasonal variation in counts. Total viable bacterial counts (Figure 2) reached a mean value of 20 and 112 cfu.ml^{-1}. Faecal coliform counts (Figure 3) reached a mean value of 776 cfu.ml^{-1} and *Aeromonas* spp. counts (Figure 4) reached a mean value of 206 cfu.ml^{-1}. There were obvious variations of total viable bacteria counts (Figure 5); faecal coliforms (Figure 6) and *Aeromonas* spp. (Figure 7) in different anatomical parts of fish from different sampling sites. The highest mean value counts (102756.3 cfu.g^{-1}) of total viable bacteria were recorded in the intestine of fish taken from the Mataryia area (Table 2).

The correlation matrix of the environmental data and *Aeromonas* spp. counts has shown variation of correlations

Table 1. Mean values of seasonal variations of different physico-chemical parameter in different sites (Mean ± standard deviation and range).

Parameter	Kapoty	Mataryia	Bashtier
Temp (℃)	21.7 ± 1 (21-23)	21.5 ± 1 (21 -23)	21 ± 1 (20 – 22)
pH	9.2 ± 1 (8.2 – 10.2)	8.2 ± 1.3 (6.9 -9.9)	8.2 ± 1 (7.2 -9.2)
Salinity	16 ± 1 (15-17)	4.3 ± 1 (3.3 – 5.3)	8.3 ± 1 (7.3 – 9.3)
D.O (mg/l)	3 ± 1 (2 – 4)	4.3 ± 1 (1.2 – 2.2)	3.4 ± 1 (2.4 -4.4)
TSS (mg/l)	127 ± 1 (126 - 128)	50 ± 10 (40 - 60)	67 ± 1 (66 - 68)
Ammonia (mg/l)	1.2 ± 0.1 (1.1-1.3)	2.0 ± 1 (1 – 3)	1.9 ± 0.1(1.8 –2)
Nitrate (mg/l)	1.6 ± 0.1 (1.5 – 1.7)	1.3 ± 0.8 (0.5 – 2)	0.1 ± 0.0 (0.11 – 0.13)
Chlorides (mg/l)	5500 ± 1000 (4500 -6500)	1300 ± 0.0 (1300 -1300)	2300 ± 533.9 (1400 – 2700)
Calcium (mg/l)	1088 ± 11 (1077 - 1099)	210 ± 10 (200 – 220)	260 ± 10 (250 – 270)
Magnesium (mg/l)	433 ± 11 (422 - 444)	73 ± 1 (72 – 74)	230 ± 10 (220 – 240)

Table 2. Mean values of seasonal variations of different bacterial species in different sites (Mean ± standard deviation and range).

	Kapoty	Mataryia	Bashtier
T.V.B	4700 ± 100(4600 -4800)	20150 ± 50 (20100 -20200)	8900 ± 4273.2 (1500 – 11500)
F.C	776 ± 1(775 - 777)	151 ± 1 (150 – 152)	59 ± 1 (58 – 60)
Aeromonas spp.	183 ± 1(182 - 184)	206 ± 1 (205 – 207)	152 ± 1 (151 – 153)

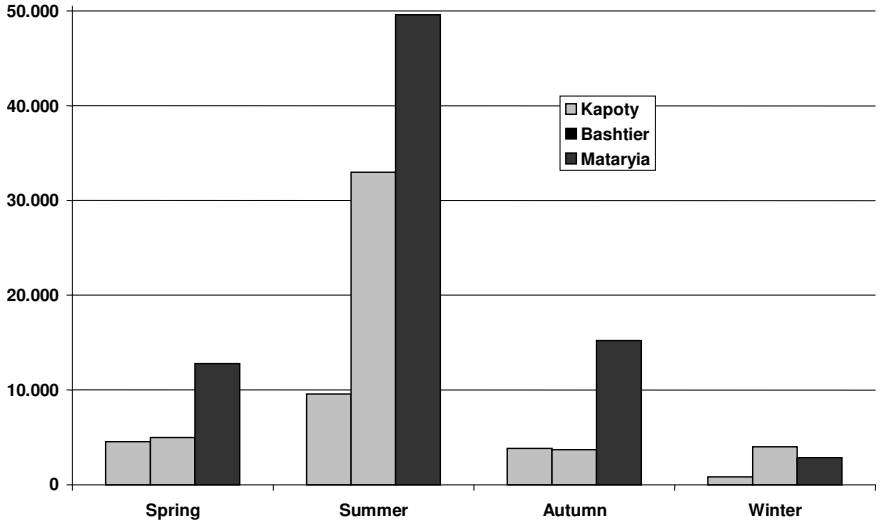

Figure 2. Total viable bacterial counts of water samples taken from different sites of Lake Manzala (cfu ml^{-1}).

correlations in different sites. The data from the Kapoty site demonstrated that there were significant strong correlations between *Aeromonas* spp. counts with ammonia, nitrate and magnesium (r = 0.96036, 0.883244 and 0.866242, respectively, p <0.001), and significant strong correlations with calcium, chlorides and salinity (r = 0.780722, 0.635003 and 0.548988, respectively, p < 0.001). The data from the Bashtier demonstrated that there were significant strong correlations between *Aeromonas* spp. counts with calcium, magnesium and salinity (r = 0.996895, 0.849515 and 0.812784, respectively, p < 0.001) and significant strong correlation

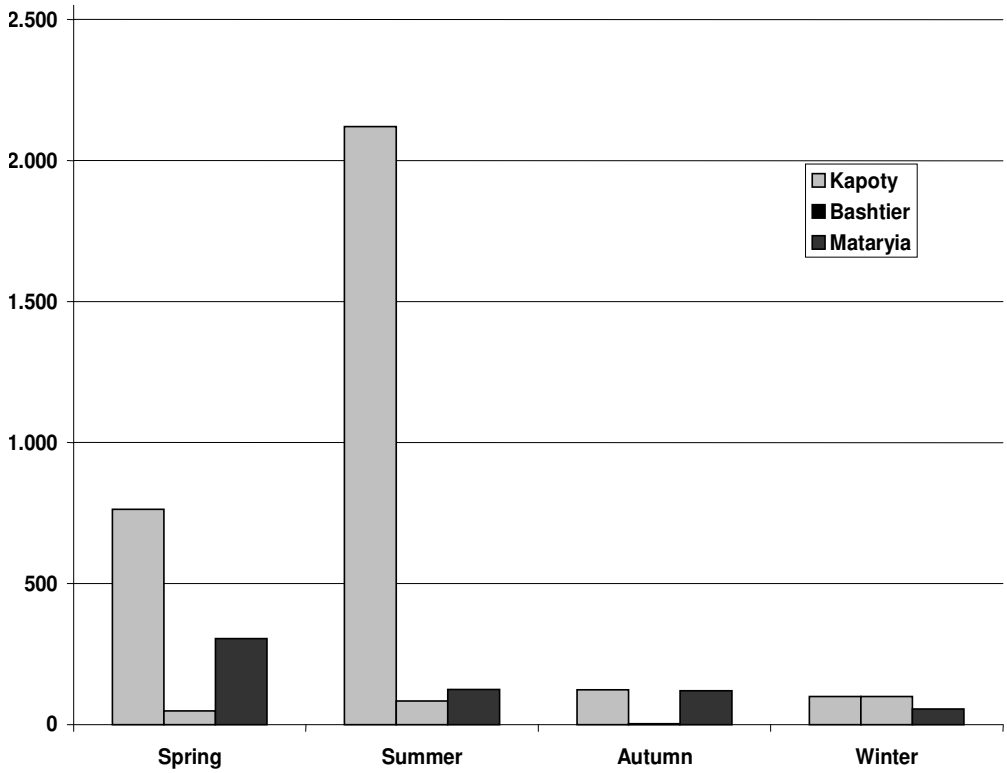

Figure 3. Counts of faecal coliforms of water samples taken from different sites of Lake Manzala (cfu ml^{-1}).

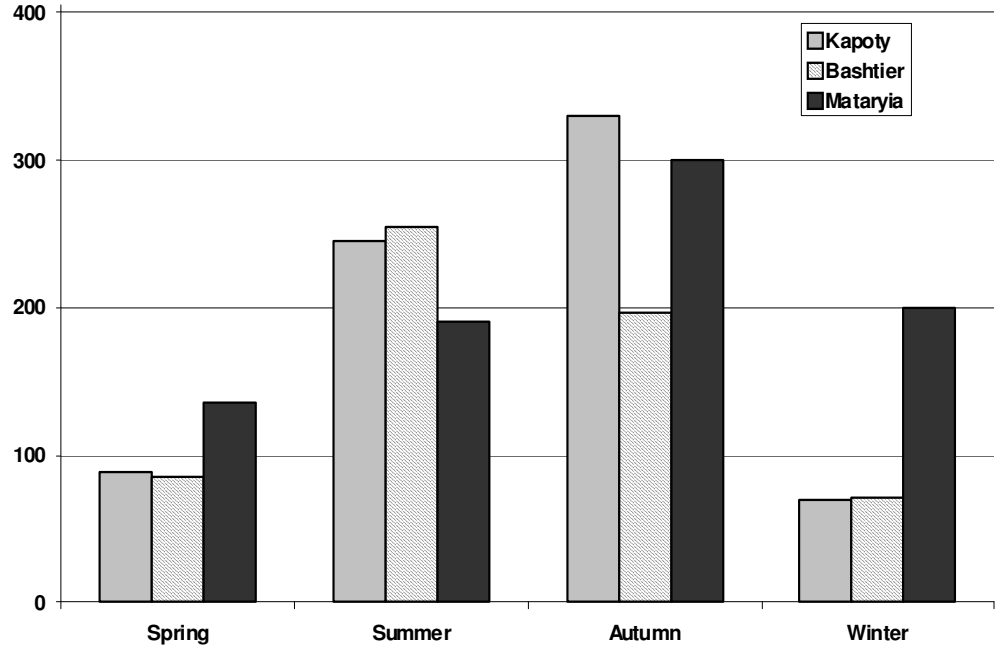

Figure 4. Counts of *Aeromonas* spp. of water samples taken from different sites of Lake Manzala (cfu ml^{-1}).

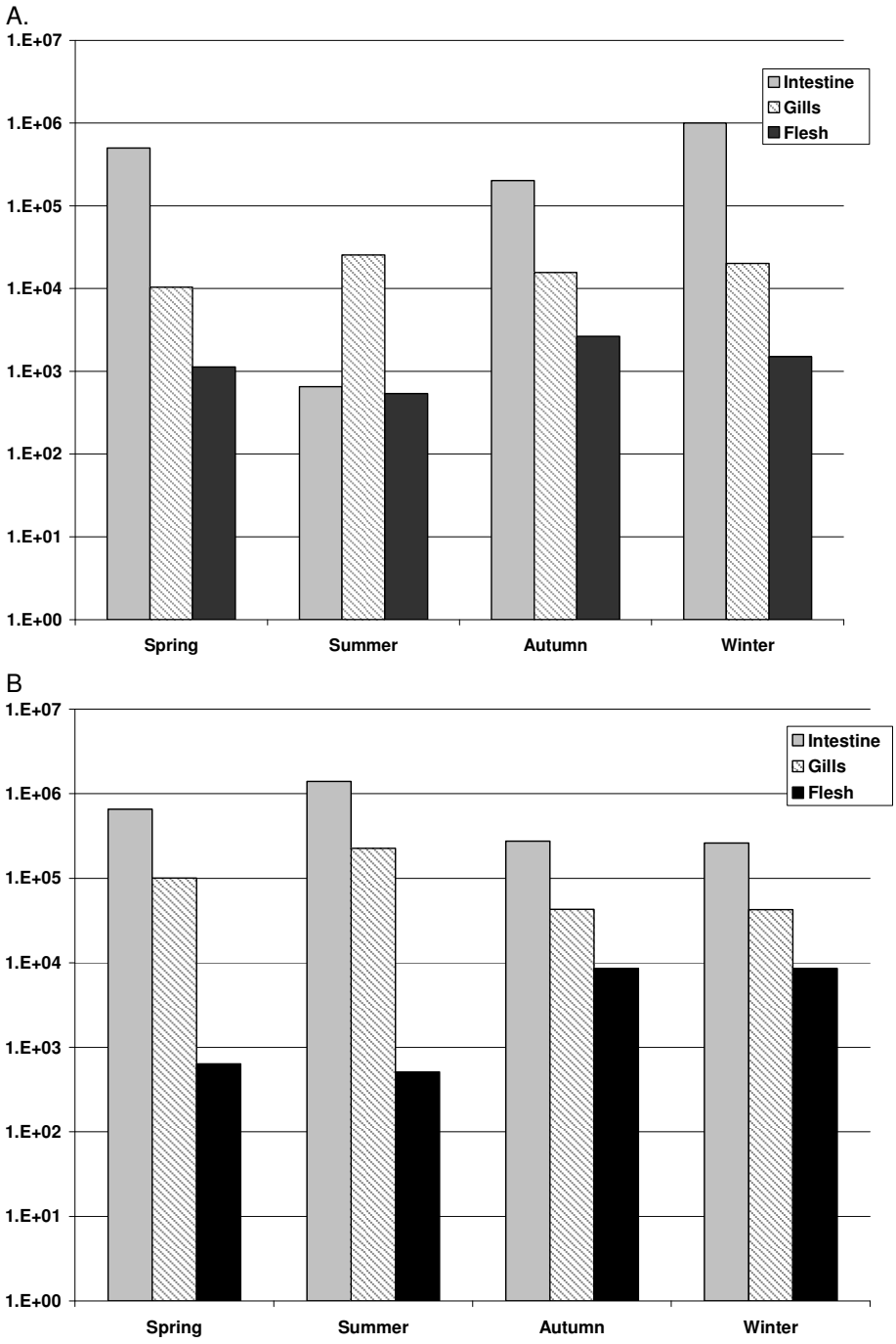

Figure 5. Counts of total viable bacteria (cfu g^{-1}) of different anatomical parts (intestine, gills and flesh) of fish taken from A) Bashtier area and B) Mataryia area of Lake Manzala (cfu g-1).

with chlorides (r = 0.795329; p < 0.001). The data from the Mataryia demonstrated that there were significant strong correlations between *Aeromonas* spp. counts with nitrate and magnesium (r = 0.912488 and 0.828159, respectively, p <0.001) and there was a significant strong correlation with salinity (r = 0.521413, p <0.001).

Antibiotic resistance of *A. hydrophila*

Antibiogram figures showed variation of the susceptibility and resistance of different strains isolated from water and fish. Some strains were only resistant to ampicillin and penicillin G, others were resistant to three or more antibiotics

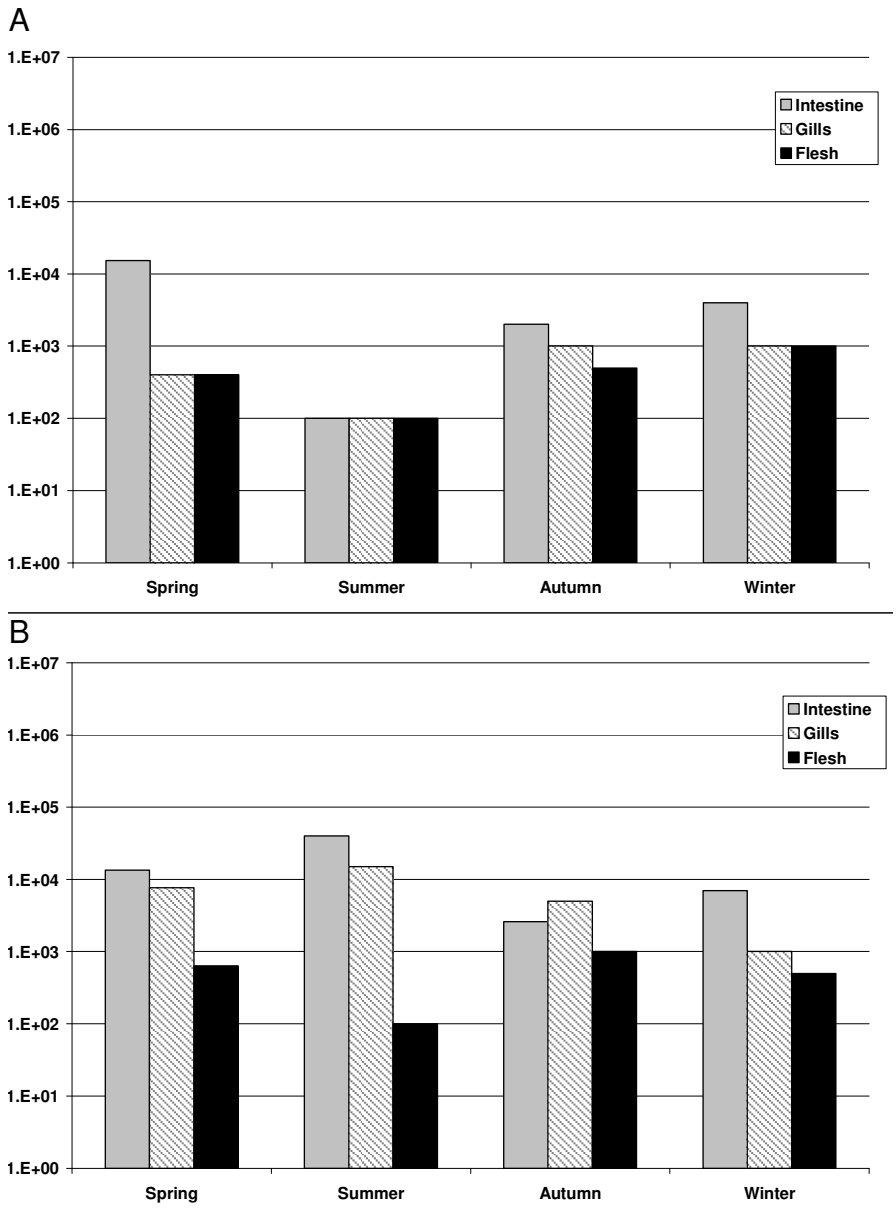

Figure 6. Counts of faecal coliforms of different anatomical parts (intestine, gills and flesh) of fish taken from A-(Bashtier area) and B- (Mataryia area) in Lake Manzala.

antibiotics and two strains isolated from fish were completely resistant to the five antibiotics used in this study (Table 3).

Plasmid DNA profiling of *A. hydrophila*

The Plasmid DNA profiling using agarose gel showed variation of the different strains isolated from water and fish in number and size with regard to λDNA digested with Hind III, where some strains that harbored plasmids less than 2 Kb_P, others had plasmids more than 2.3 Kb_p and others were equal to or more than 4.3 Kb_P (Table 4 and Figure 8).

DISCUSSION

There are many studies that have established the polluted condition of Lake Manzala, as the worsening of physicochemical and bacteriological conditions have adversely affected the lake's ecosystem. For example, during the 1921 - 1926 the lake water was marine brackish but now is brackish fresh (Awadi and Khalil, 1989)

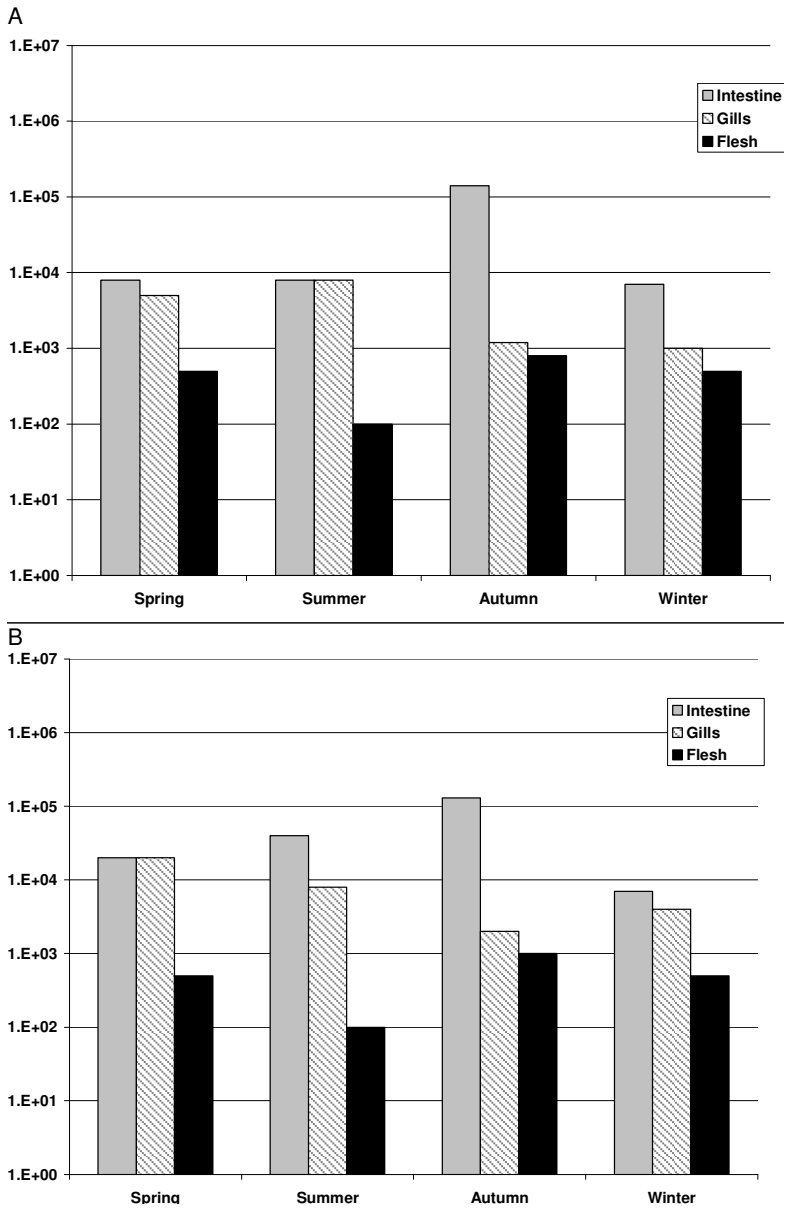

Figure 7. Counts of *Aeromonas* spp. of different anatomical parts (intestine, gills and flesh) of fish taken from A-(Bashtier area) and B- (Mataryia area) in Lake Manzala.

Table 3. Mean values of different bacterial counts (cfu.g^{-1}) in different anatomical parts of fish in tow sampling sites of Lake Manzala.

Sampling site/ anatomical parts	Total viable bacteria	Faecal coliforms	*Aeromonas* spp.
Bashtier			
Intestine	425.675	5,345	40.750
Gill	17.933	625	3.800
Flesh	1.458	500	475
Mataryia			
Intestine	646.648	15,750	49.250
Gill	102.756	7,162.5	8.500
Flesh	4.553	558.75	525

Table 4. Antibiogram of the multi-drug resistant *A. hydrophila* isolated from water and fish taken from Lake Manzala.

Strain No.	Source	Ampicillin	Penicillin G	Gentamycin	Streptomycin	Chloramphenicol
8W	Water	R	R	S	S	S
18W	Water	R	R	S	S	S
22W	Water	R	R	S	S	S
29W	Water	R	R	S	S	S
30W	Water	R	R	R	S	S
32W	Water	R	R	S	R	S
42W	Water	R	R	S	S	S
45W	Water	R	R	S	S	S
50W	Water	R	R	S	R	R
52W	Water	R	R	S	R	S
62W	Water	R	R	S	R	R
70W	Water	R	R	S	S	S
72W	Water	R	R	S	R	S
74W	Water	R	R	S	R	R
4F	Fish	R	R	S	S	S
12F	Fish	R	R	S	S	S
30F	Fish	R	R	R	R	S
43F	Fish	R	R	S	R	S
51F	Fish	R	R	R	R	R
63F	Fish	R	R	S	R	S
71F	Fish	R	R	R	R	R

R-Resistant; S- Susceptible.

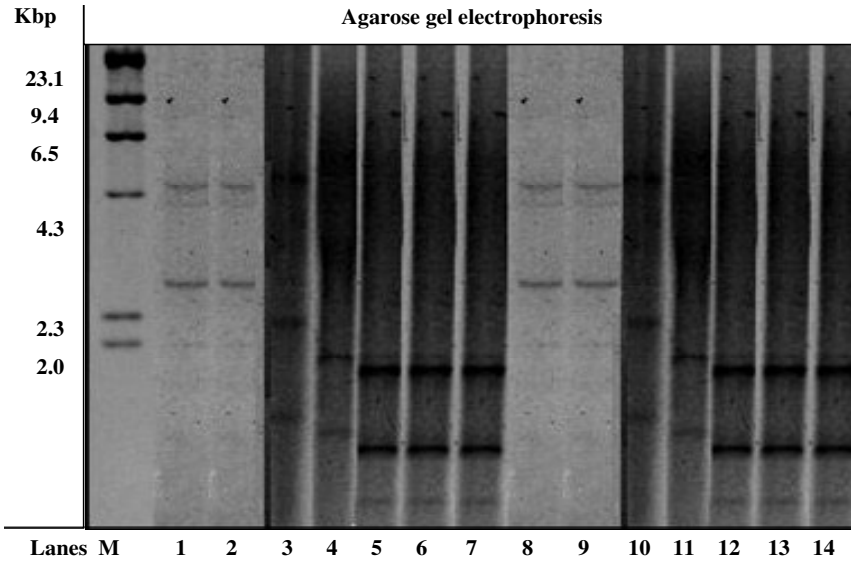

Figure 8. Plasmid profiling of multi-drug resistant *A. hydrophila* isolated from water and fish of Lake Manzala (M: λ DNA digested with Hind III, Lanes from 1 - 14 represented AH strains No. 8, 42, 62 and 70 W, 71, 63 and 30 F, 52, 72, 50 and 74 W, 51, 43 and 12 F, respectively, where W: from water and F: from fish).

1989). The effluents of fresh water from drains like Bahr El-Bakar which oppose the current from the Mediterranean Sea resulting in slow water movement to the point where the Lake is almost stagnant or at least is without currents. The heavy loads of suspended solids and organic matter have raised the bed of the lake and

have created shallow water depths. This study revealed the high densities of microbial populations particularly those of bacteria.

This study revealed high records of pollutants, like ammonia, the presence of these can be explained by the anoxic conditions created by the depletion of oxygen. In addition, high suspended particles whether of organic or mineral origin adsorb bacteria to their surfaces. The adsorption of debris particles not only provides microbes with a more favourable nutritional environment than those found in free water but also neutralizes inhibitory and toxic substances. Thus, the suspended particles have a favourable effect on bacterial growth. Faecal coliform counts, well known water pollution indicators, were performed on the different anatomical parts of the fish samples from the Kapoty and Mataryia areas. These counts were used in the study to assess and understand the level of pollution of Lake Manzala. The deterioration of the environmental conditions in Lake Manzala can be attributed to the people inhabiting the region and their role as an element of the ecosystem. The complaints and ever increasing recorded cases of diarrhea, gastroenteritis, kidney failure and other diseases may be partially explained by the aforementioned lake condition. Not only the Lake Manzala water samples but also the fish samples were found to have very high pathogenic bacteria contents (Uerbia et al., 2003; Zaky, 1995).

Aeromonas spp. is always found in faecal polluted water such as waste water ponds, fresh and brackish eutrophic estuaries and lagoons in other parts of the world, as well as in the water, sediment, soil and fish of Lake Manzala's water environment (Chao et al., 2003; Marcel et al., 2002; Sumpono et al**.**, 2003). The statistical analysis of previous studies revealed a strong correlation between counts of *Aeromonas* spp. and faecal coliforms in Lake Manzala, a brackish water environment. Thus *Aeromonas* spp. can be a powerful tool for the assessment of the microbial pollution in brackish water environments (Zaky, 2005; Zaky et al., 2005). In other studies *Aeromonas* spp. dominated other species, both in water and fish samples taken from Lake Manzala, where they represented 44% of the total; this result is in conformity with other studies which described the Mediterranean region as an ideal environment for the proliferation of *Aeromonas* spp., and with the established correlation between the distribution patterns of *Aeronomas* spp. and level of faecal pollution in waters, particularly of the lakes in this area (Araujo et al.,1990; Ben-Dan and Stone,1991).

In this study a strong positive correlation between counts of *Aeromonas* spp. and minerals such as calcium and magnesium was found (r-0.996895 and 0.849515, respectively) as well as with salinity and chlorides (r-0.812784 and 0.795329, respectively). Such positive correlations indicate the importance of the brackish water environment in physiological and genetic terms as well as in the behaviour of the aforementioned microorganism. The manipulation of the growth media (SS Agar, TCBS and Endo Agar) and its effect on the growth of *Aeromonas* spp. attests to the ability of the species to adapt to different environments. Aeromonas spp. shares similar characteristics with the members of the Enterobacteriaceae family more specifically with the *Salmonella* and *Shigella* spp. (Delaat, 1984). The haemolysins produced by *A. hydrophila* and *A, sobria* (Hirono et al., 1992; Tucker et al., 1990) are virulence-linked factors. *A. hydrophila* is a well-known fish pathogen that causes a serious disease in fish and ultimately leads to death; the ability of these species to live on fish maximizes the health risks they pose to humans (Popoff, 1984; NitetoandEllis, 1986). *A. hydrophila* is probably partially responsible for the decrease in the number of fish caught in Lake Manzala because of its toxic activity and as a common species in the Lake Manzala environment (Gurwith et al., 1978; Zaky, 1995; Mansour et al., 2005). *A. hydrophila* and *A. sobria* are opportunistic pathogens that have cytotoxic effects. These pathogenic bacteria thrive in polluted water environments like those found in many developing countries. Their roles as human pathogenic agents causing diarrhoeal and other gastrointestinal diseases have been repeatedly established in studies (Ashbolt, 2004; Dumontet et al., 2003; Pianietti et al., 2004).

In this study, *A. hydrophila* showed a resistance pattern to different antibiotics, particularly ampicillin and penicillin. The resistance is an indication of the presence of β-lactamases, common in bacterial pathogens in polluted water environments in this area, and common in the Egyptian environment. These environments are rich in nutrients like calcium, magnesium and chlorides, and there is a strong correlation between the existence of these nutrients and *Aeromonas* spp. counts in brackish water environments (Zaky, 2006, unpublished). The presence of these minerals stretches bacterial cell walls facilitating the transfer of genetic material, particularly the transfer of antibiotic resistance plasmids.

The different strains of *A. hydrophila* found in the Lake; particularly strains No. 50 W which harbours four plasmids, two of them more than 2 and 4.3 Kbp and is resistant to four antibiotics; and strain No. 52F which is resistant to five antibiotics, and harbours three plasmids less than 2 Kbp, could explain the effect that the water environment has on genetic transfer among bacteria, which could be congruent with environmental changes and factors that enable such processes. The antibiotic resistance found in the fish tissue samples may be due to conditions controlled by the physiological processes and the immune systems of the fish. These conditions may cause the loss of some plasmids or enhance the incorporation of these with chromosomal DNA. The stabilization of drug-resistance is essential for such bacteria to express virulent characteristics (Heesemann et al., 1993; Rakin et al., 1999). The morbidity of diarrhoeal and gastrointestinal diseases has increased in

the populations residing along the coastal areas of Egypt where the staple is seafood. The increase of such illnesses is presumably due to the prevalence of toxic *Aeromonas* spp. The importance of studying the mode of infection of pathogenic *A. hydrophila,* the epidemiology of diarrhoeal and gastrointestinal diseases along with the accurate clinical diagnosis of diarrhoeal cases in this area is evident.

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