Operational Paper

Recovery of somatic coliphages in wastewater and seawater samples in relation to bacterial indicator organisms and water hydrochemical parameters in Kaiet Bay station, Alexandria

Faiza A. Fattouh, Mohamed A. El Shenawy and Sahar W. Hassan

ABSTRACT

Somatic coliphages were enumerated seasonally in addition to the traditional bacterial indicators total coliforms, faecal coliforms and faecal streptococci at different source points at Kaiet Bay wastewater treatment station in Alexandria. There was a highly significant correlation between counts of somatic coliphages, total coliforms and faecal coliforms but not faecal streptococci in all samples tested. Significant reduction in counts was shown in pipe outfall water samples; this is hypothesized to be due to dilution. Coliphages persisted in all seawater samples collected. Different hydrochemical parameters of the water samples were measured which showed variable correlation with coliphage counts. Biochemical oxygen demand, ammonium, total nitrogen, reactive phosphorus and total phosphorus showed highly significant direct correlation with somatic coliphages counts for all water source points in all seasons tested. Stepwise multiple regression analysis was performed to rank various parameters based on their effect on counts of somatic coliphages.

Key words | hydrochemical parameters, indicators, somatic coliphages, wastewater

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INTRODUCTION

Somatic coliphages are a heterogeneous group of bacteriophages of different morphology. They occur frequently in human and animal faeces and wastewaters. They are known to infect host cells via receptor molecules in the cell wall. Somatic coliphages may multiply in the environment and are readily inactivated by water treatment processes with the exception of a few types. The persistence of somatic coliphages in the environment is similar to viruses, but their behaviour in water treatment processes could be variable (Wenstel *et al.* 1982; Grabow *et al.* 1984; Borrego *et al.* 1990; Payment & Franco 1993; Mustonen & Heinonen-Tanski 1994).

Many studies have been performed over the years to evaluate the use of coliphages as indicators of the virological quality of water. Evidence by Grabow *et al.* (1984) indicated that coliphages meet the basic requirements of an indicator in sewage-polluted water in combination with the standard plate count of coliform bacteria. Coliphages used as indicators of enteric viruses in activated sludge showed that phages giving rise to plaques greater than 3 mm in diameter were positively related to enteric viruses in secondary effluent (Funderberg & Sorber 1985).

Enumeration of coliphages in comparison with traditional bacterial indicators has been performed in different countries, such as Kuwait (Qureshi *et al.* 1988), Finland and Nicaragua (Mustonen & Heinonen-Tanski 1994), Bahrain (Qureshi & Qureshi 1989), France (Gantzer *et al.* 1998), Saudi Arabia (Fattouh & Kahtani 2002), the USA (Griffin *et al.* 2000) and others. The correlation between coliphages and coliform indicators has been reported in such studies.

Seasonal enumeration of somatic coliphages (SC) in wastewater and seawater samples at Kaiet Bay station was

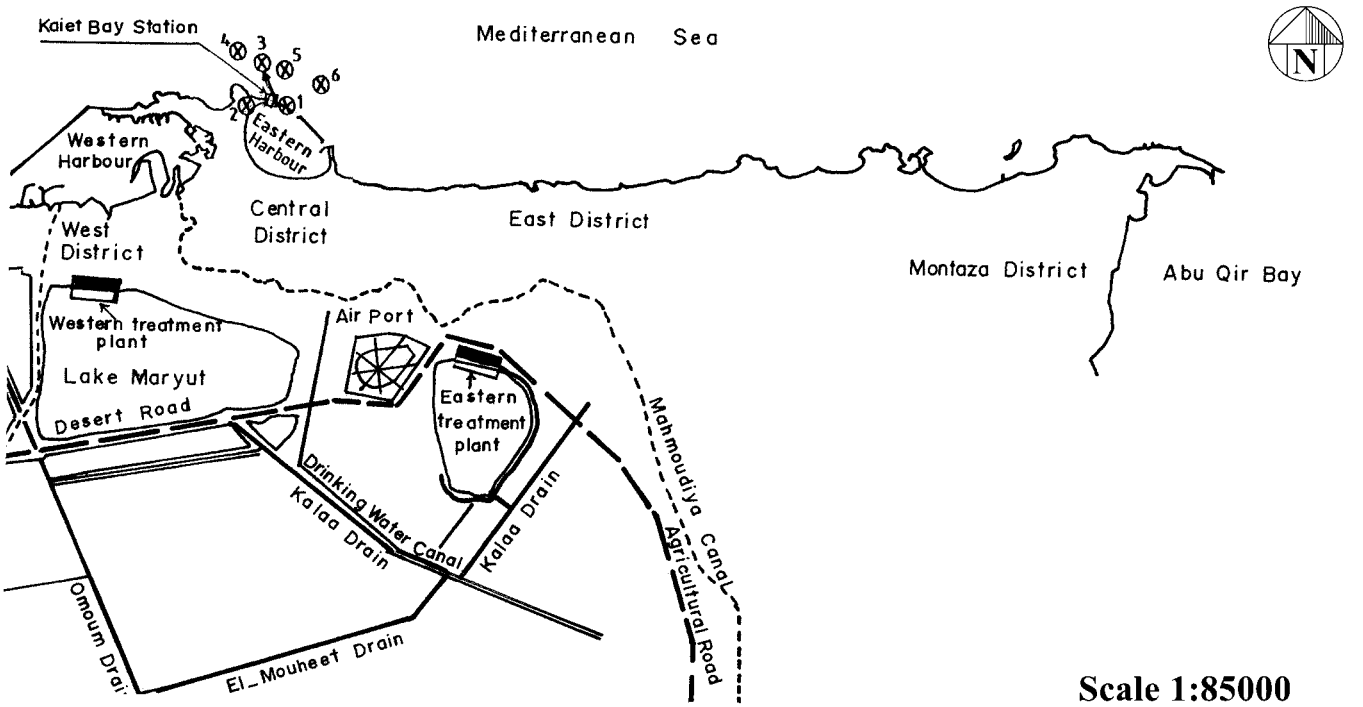

Figure 1 | Location of Kaiet Bay Wastewater Treatment Station in Alexandria and the source points of water samples: 1, Kaiet Bay Station influent; 2, Kaiet Bay Station effluent; 3, pipe outfall into the sea; 4, west of pipe outfall; 5, east of pipe outfall; 6, wind direction point.

carried out from the winter season of 2001 to the winter season of 2002. The indicator organisms, total coliforms (TC), faecal coliforms (FC) and faecal streptococci (FS), were estimated in the same water samples. Several physicochemical parameters of the water samples were also determined. The aim of this work is to investigate the use of coliphages as indicators of faecal pollution in wastewater and seawater samples. Assessment of the degree of correlation between coliphages, bacterial indicators and various hydrochemical parameters of water samples will be attempted. All parameters included in the study will be ranked according to their effect on the counts of the somatic coliphages.

MATERIALS AND METHODS

Water samples

Kaiet Bay station is a lateral sewage treatment station in Alexandria with a capacity of 72,000 m^3 day^{-1}. It receives sewage from the middle part of Alexandria city. Following primary treatment, the effluent is disposed of by means of an outfall into the sea. This outfall is 750 m long, 16 m deep and is made of steel with a diameter of 1.26 m. Seasonal estimation of the four indicators, somatic coliphages, total coliforms, faecal coliforms and faecal streptococci, was carried out from the winter season of 2001 to the winter season of 2002. Estimates of the indicators and measurements of all parameters in the water samples were performed every 2 weeks. The source points of the water samples tested in this study were: Kaiet Bay Station influent (KBSI), Kaiet Bay Station effluent (KBSE), pipe outfall into the seawater (PO), west of the pipe outfall (WPO), east of the pipe outfall (EPO) and wind direction point (WDP), as shown in Figure 1. Water samples from the last three source points were taken within a circle of 10 m diameter around the pipe outfall.

Collection of water samples

The bacteriological sampling guidelines of the International Organization for Standardization ISO

5667/1 (1980) and ISO 5667/2 (1990) were used for water samples collection. Sterile glass sampling bottles with wide-mouthed openings and screw cap-closures with capacity of 250 ml were used for collecting water samples. Special stainless steel sampling rods were used. Samples were collected about 25–35 cm below water surface. The bottles were kept unopened until the moment of collection; samples for the physicochemical analysis were fixed in position immediately after collection. After collection, the samples were sent to the laboratory, and examined within 2 to 3 h of collection.

Enumeration of somatic coliphages in water samples

The double-agar-layer technique (DAL) described by Adams (1959) was used for phage detection and enumeration. Bacterial growth and background flora in the water sample were eliminated by pre-filtration through a low protein binding filter (Millipore, 0.45 μm pore size). One ml of the water sample or appropriate dilutions of the sample and 0.2 ml of exponentially growing host culture (*E. coli* ATCC 13706) were added to 3 ml of liquified soft agar. The mixture was poured on to Petri dishes containing TYG-base agar, allowed to solidify and incubated at 37°C. The plaques were counted following a 16-h incubation.

Bacteriological analysis

The bacteriological analysis was carried out according to ISO 9308/1 (1990) using the filtration technique. Different dilutions of the water sample were filtered using a sterile glass filtration unit and a vacuum pump at a pressure of 65 kPa.

Total coliforms (TC)

For the detection of total coliforms, the membranes were placed on to the surface of LES-endo agar, incubated at 37°C ± 0.5 for 24 h ± 2 h.

Faecal coliforms (FC)

For the detection of thermotolerant coliforms (*E. coli*), the same dilutions of samples were filtered and the membranes were placed on to the surface of m-FC agar. Incubation was done at 44°C ± 0.5 for 24 h ± 2 h.

Faecal streptococci (FS)

For the detection of faecal streptococci, different dilutions of water sample were used for filtration. The membranes were placed on to the surface of m-enterococcus agar, incubated at 37°C ± 0.5 for 48 h ± 4 h.

Physicochemical analysis of water samples

Temperature measurements

Water temperature was measured at the time of sampling, using an ordinary thermometer.

Hydrogen ion concentration

The pH value of water samples was determined at the time of sampling, using a digital portable pH-meter. All readings were made up to 0.01 pH units.

Dissolved oxygen (DO)

Determination of dissolved oxygen was carried out according to the classical Winkler method (Standard Methods 1995). In the laboratory, 100 ml of sample was titrated against 0.02 N sodium thiosulphate solution. Dissolved oxygen content was calculated in mg O_2 l^{-1}. The percentage saturation of dissolved oxygen was calculated using the tables of the National Institute of Oceanography of Great Britain and UNESCO.

Biochemical oxygen demand (BOD)

The BOD determination was carried out according to Standard Methods (1995). The values of dissolved oxygen (DO) were expressed in mg O_2 l^{-1}. BOD is computed from the difference between initial and final DO.

$$\text{BOD (mg l}^{-1}) = \text{D1} - \text{D2}$$

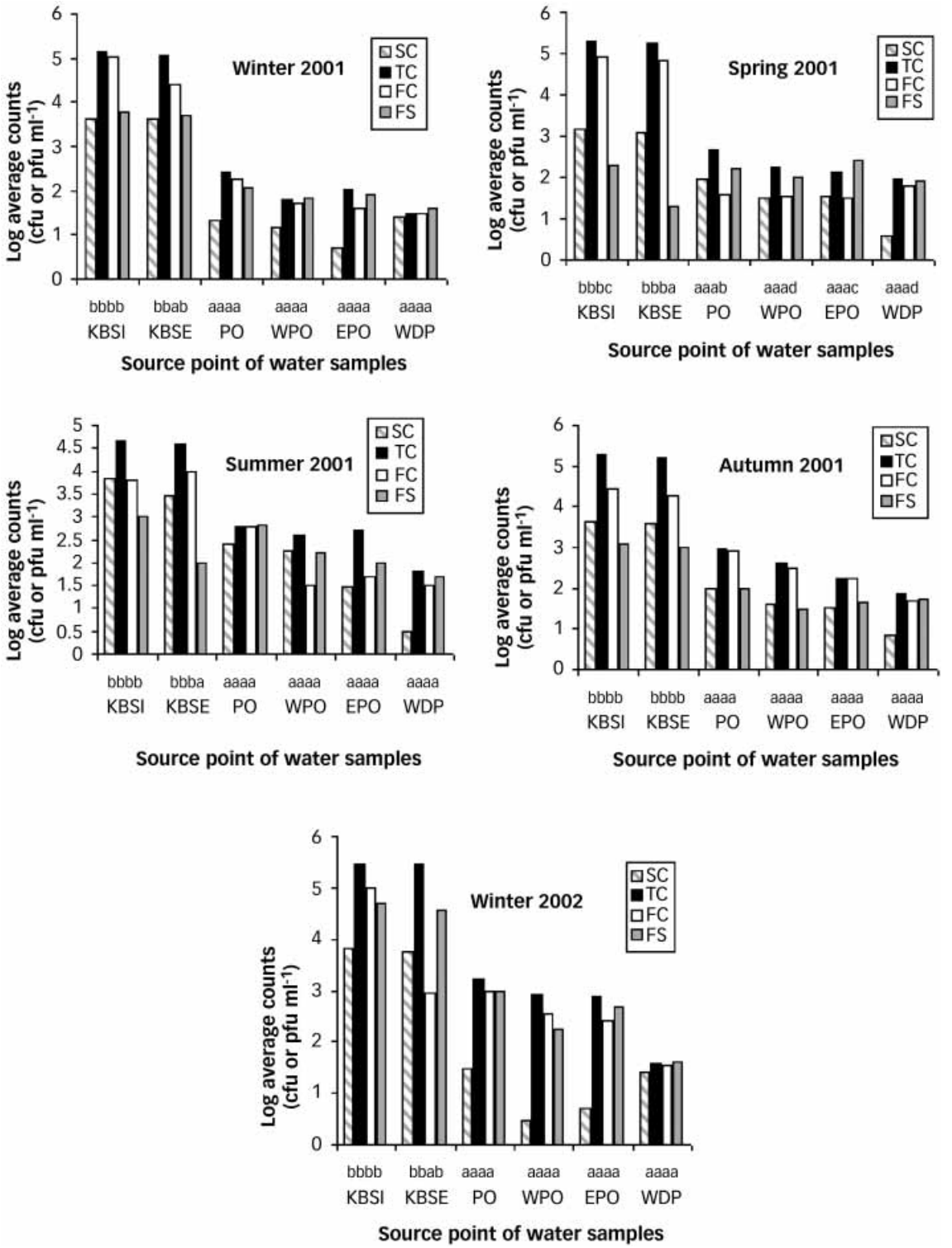

Figure 2 | Log average counts of the indicators: somatic coliphages (SC), total coliforms (TC), faecal coliforms (FC) and faecal streptococci (FS) in different seasons at Kaiet Bay Station. Source point of water samples are: Kaiet Bay Station influent (KBSI), Kaiet Bay Station effluent (KBSE), pipe outfall (PO), west of pipe outfall (WPO), east of pipe outfall (EPO) and wind direction point (WDP). Different letters (a, b, c, d) indicate a significant difference in counts ($p<0.05$) at different source points of water samples according to one-way ANOVA test.

Table 1 | Simple correlation coefficient between the four indicators in different seasons at Kaiet Bay Station (KBS)

Simple correlation coefficient[a]	Winter 2001	Spring 2001	Summer 2001	Autumn 2001	Winter 2002
Total coliforms	0.999**	0.999**	0.992**	0.996**	0.998**
Faecal coliforms	0.823**	0.999**	0.880**	0.984**	0.711*
Faecal streptococci	0.998**	− 0.157	0.348	0.997**	0.999**

[a]Simple correlation coefficient between somatic coliphages and bacterial indicators at $p<0.05$ using one way ANOVA test.
Insignificant=<0.6, *=significant (0.6–0.8), **=highly significant >0.8, −=indirect correlation.

Where: D1 (mg l^{-1}) = DO of the sample immediately after sample collection
D2 (mg l^{-1}) = DO of the sample after 5 days incubation at 20°C.

Salinity (‰)

Samples were collected in hard-glass, well-stoppered salinity bottles. Salinity was determined by measuring the electrical conductivity, using an induction salinometer. The conductivity ratio was measured to the nearest 0.0001. The salinometer was standardized with normal seawater, of chlorinity 19.377‰. The salinity values were calculated from the conductivity ratio, after making temperature compensation correction using the International Oceanographic tables of salinity published by UNESCO.

Hydrogen sulphide (H_2S)

Hydrogen sulphide concentration was measured spectrophotometrically by the methylene blue method (Cline 1969).

Nutrient salts

Water samples for nutrient analysis were kept in polyethylene bottles of 1-l capacity and 0.5% chloroform was added in order to prevent or at least minimize changes in the samples. After returning to the laboratory, 500 ml of each sample was filtered using a 0.45 μm membrane filter, then kept in well-stoppered bottles and frozen in a deep freezer at −20°C.

Nitrite (NO_2)

The method used for determination of nitrite was as described by Strickland & Parsons (1972).

Nitrate (NO_3)

The method used was that described by Strickland & Parsons (1972).

Ammonium (NH_4)

Ammonium was fixed immediately after collection of the samples. Ammonium was determined spectrophotometrically using the indophenol blue technique (IOC 1983).

Reactive phosphorus (PO_4)

Methods described for the determination of inorganic phosphate in water are based on the reaction of this ion with an acidified molybdate reagent to yield a phosphomolybdate complex, which is then reduced to a coloured blue compound (Strickland & Parsons 1972). The blue colour was measured at 880 nm.

Table 2 | Levels of the hydrochemical parameters in Kaiet Bay Station water source points and correlation with somatic coliphage counts during various seasons

Source points[a]	Temp. °C	pH	Salinity ‰	DO (mg l^{-1})	BOD (mg l^{-1})	H_2S (mg l^{-1})	NH_4 (mg l^{-1})	NO_2 (mg l^{-1})	NO_3 (mg l^{-1})	T N (mg l^{-1})	PO4 (mg l^{-1})	T P (mg l^{-1})
Winter 2001												
KBSI	17.2	7.20	1.21	ND	390.00	4.66	722.40	1.33	6.65	822.87	26.98	65.66
KBSE	17.4	7.40	0.62	ND	185.00	3.92	720.00	0.63	1.05	820.00	24.90	62.07
PO	18.2	8.30	37.10	6.85	62.00	ND	13.66	0.09	0.56	103.16	0.89	1.50
WPO	17.6	7.70	36.60	7.66	22.00	ND	9.00	0.06	0.38	67.49	0.58	1.31
EPO	18.2	8.35	37.20	6.85	16.50	ND	8.40	0.04	1.03	71.34	0.48	1.34
WDP	18.2	8.35	37.60	7.82	5.01	ND	11.20	0.05	1.20	44.70	0.38	0.96
Correlation coefficient[b]	0.84**	0.87**	− 0.99**	− 0.99**	0.91**	0.99**	0.99**	0.92**	0.69*	0.99**	0.99**	0.99**
Spring 2001												
KBSI	25.6	7.20	1.29	ND	410.00	4.92	707.00	1.33	6.65	936.83	26.98	65.66
KBSE	25.8	7.70	1.15	ND	122.00	3.91	690.00	1.30	5.90	850.00	29.50	60.98
PO	25.4	8.20	37.02	9.40	18.10	ND	9.01	0.70	10.05	26.64	1.73	2.41
WPO	25.1	8.20	37.86	8.59	15.31	ND	9.36	0.19	4.38	36.11	2.95	4.93
EPO	25.1	8.10	37.92	11.66	8.10	ND	8.44	0.18	2.68	35.52	1.51	1.98
WDP	25.2	7.90	37.80	6.80	6.48	ND	8.66	0.14	3.38	81.40	2.88	6.22
Correlation coefficient	0.89**	− 0.89**	− 0.99**	− 0.94**	0.87**	0.99**	0.99**	0.94**	0.27	0.99**	0.98**	0.99**
Summer 2001												
KBSI	32.6	7.70	0.25	ND	345.00	11.60	347.00	0.30	1.33	796.44	14.74	24.96
KBSE	32.8	7.50	0.39	ND	155.00	11.66	329.00	0.27	1.05	806.19	28.99	52.80
PO	31.2	8.20	36.70	6.40	46.00	ND	4.02	0.05	0.86	199.95	2.50	9.25
WPO	31.4	8.20	36.48	5.91	18.00	ND	5.02	0.30	1.93	83.03	1.10	1.47
EPO	31.3	8.20	36.91	6.16	3.81	ND	4.90	0.39	6.53	82.88	1.37	1.47

Table 2 | *Continued*

Source points[a]	Temp. °C	pH	Salinity ‰	DO (mg l^{-1})	BOD (mg l^{-1})	H_2S (mg l^{-1})	NH_4 (mg l^{-1})	NO_2 (mg l^{-1})	NO_3 (mg l^{-1})	T N (mg l^{-1})	PO4 (mg l^{-1})	T P (mg l^{-1})
Summer 2001 *continued*												
WDP	31.3	8.20	36.80	6.24	3.73	ND	4.66	0.08	23.64	99.90	0.36	1.74
Correlation coefficient	0.96**	− 0.92**	− 0.98**	− 0.98**	0.96**	0.98**	0.99**	0.29	− 0.44	0.98**	0.93**	0.79*
Autumn 2001												
KBSI	18.2	7.80	1.30	ND	329.00	3.08	774.00	0.43	6.93	793.84	18.94	67.86
KBSE	18.3	7.60	0.52	0.57	96.20	ND	634.00	0.33	1.31	757.94	21.19	70.14
PO	17.6	8.00	37.01	0.57	41.20	ND	16.76	0.20	0.91	201.13	0.91	0.99
WPO	17.6	8.00	36.60	7.53	14.60	ND	9.06	0.93	7.61	68.83	0.80	1.13
EPO	17.5	7.80	37.30	6.80	10.20	ND	8.90	0.61	10.84	72.08	0.67	3.67
WDP	17.4	7.60	38.14	7.06	7.60	ND	11.72	0.18	0.21	70.74	0.58	3.14
Correlation coefficient	0.98**	− 0.41	− 0.99**	− 0.74*	0.80**	0.67*	0.99**	− 0.18	− 0.08	0.87**	0.99**	0.99**
Winter 2002												
KBSI	18.2	7.30	1.41	ND	356.60	0.70	1055.3	1.62	5.63	1473.00	31.78	36.82
KBSE	18.7	7.60	0.54	ND	178.00	0.87	1140.3	0.18	1.53	1470.00	32.76	33.23
PO	18.2	8.40	37.18	8.05	22.46	ND	17.46	0.35	2.84	104.01	1.29	1.37
WPO	18.2	8.40	36.76	8.12	13.35	ND	14.50	0.26	2.68	67.79	0.82	1.02
EPO	17.9	8.40	37.58	8.27	3.20	ND	10.48	0.21	2.99	71.35	0.74	0.86
WDP	17.9	8.20	38.17	8.20	3.13	ND	1.64	0.31	3.66	49.49	0.48	0.59
Correlation coefficient	0.65*	− 0.98**	− 0.99**	− 0.99**	0.95**	0.97**	0.99**	0.65*	0.29	0.99**	0.99**	0.99**

[a]Water source points at Kaiet Bay Station (KBS): Kaiet Bay Station influent (KBSI), Kaiet Bay Station effluent (KBSE), pipe outfall (PO), west of pipe outfall (WPO), east of pipe outfall (EPO), wind direction point (WDP). ND: not detected.

[b]Simple correlation coefficient between somatic coliphage counts and the hydrochemical parameters of water samples in Kaiet Bay Station at $p<0.05$ using one-way ANOVA test.

Insignificant=<0.6, *=significant (0.6–0.8), **=highly significant=>0.8, −=indirect correlation.

Total phosphorus (TP) and total nitrogen (TN)

Determination was carried out according to the technique described by Koroleff (1977) and modified by Valderrama (1981).

Statistical analysis

Data were examined with analysis of variance using the COSTAT 2.00 software. All data were tested with least square difference (LSD). A difference between treatment mean of $p \leq 0.05$ was regarded as significant. The correlation coefficient was applied to assess the interrelationship between the different indicators. The coliphage level was further studied as a function of various factors of water quality using stepwise multiple regression analysis. Analysis was run for coliphage estimates as the dependent variable. This statistical technique is a method of achieving the best linear equation between a given set of independent variables and the dependent variable in question. In this approach the variable that explained the greatest amount of variance in the dependent variable is the independent variable that enters the model first.

RESULTS

Somatic coliphages and the bacterial indicators, total coliforms, faecal coliforms and faecal streptococci, were enumerated in water samples from different source points at Kaiet Bay Station, Alexandria. Log average counts of all indicators during five different seasons starting in winter 2001 and ending in winter 2002 were determined. Six different biweekly samples during the 3 months representing each season were analysed per seasonal average and are shown in Figure 2. Analysis of variance using the one-way ANOVA test at $p \leq 0.05$ for counts of each indicator at different source points showed that, for somatic coliphages and total coliforms, there was no significant difference between counts in influent and effluent samples as shown in Figure 2. However, all other source points (PO, WPO, EPO and WDP) showed a significant reduction in counts of SC and TC as seen in Figure 2. Faecal coliform counts showed the same trend in all seasons except winter 2001 samples where average counts of effluent samples showed a significant reduction compared with influent samples. Faecal streptococci counts also showed the same trend except in spring 2001 and summer 2001 where there was a significant difference in counts between influent and effluent samples. One-way analysis of variance of data for each indicator showed that the counts for all indicators were significantly higher during the colder seasons, winter 2001, 2002 and autumn 2001 (results not shown).

A simple correlation coefficient between counts of the four indicators in different seasons showed a highly significant correlation between somatic coliphages, total coliforms and faecal coliforms as shown in Table 1. Faecal streptococci showed a correlation in counts in colder seasons but not in spring and summer 2001 as illustrated in Table 1.

Levels of the hydrochemical parameters in Kaiet Bay Station water source points during various seasons are outlined in Table 2. A simple correlation coefficient between counts of different hydrochemical parameters varied from insignificant to highly significant as shown in Table 2. In order to describe the relationship between somatic coliphage counts and all variables involved in this study, we applied a stepwise multiple regression analysis of all data. The independent variables are presented in the order in which they were selected in the final model. These variables were significantly predictive of somatic coliphage counts, with an overall predictive power R^2 of 0.98 and standard error of 280.4. The developed equation describing the relationship between coliphage number (Y) and selected independent variables was found to be:

$$2770.35 + 0.30\ \mathrm{TN} + 0.15\ \mathrm{NO_2} - 1.03\ \mathrm{TC} + 0.02\ \mathrm{DO} + 0.35\ \mathrm{HN_4} - 0.72\ \mathrm{TP} + 0.55\ \mathrm{BOD} + 1.05\ \mathrm{PO_4} - 0.16\ \mathrm{FC} - 0.55\ \mathrm{H_2S} - 0.76\ \mathrm{salinity}.$$

DISCUSSION

Coliphages are better indicators of enteric viruses than bacteria are, because they persist in water longer than indicator bacteria (Havelaar 1993, 1994). The use of

coliphages as indicators is justified by data showing that coliphages were recovered as frequently as were enteric viruses from water samples (Gantzer *et al.* 1998). Seasonal enumeration of somatic coliphages in addition to traditional bacterial indicators in a wastewater lateral treatment plant in Kaiet Bay, Alexandria, has been performed. Although counts of all indicators decreased with distance away from the wastewater source (influent), the application of one-way analysis of variance at $p \leq 0.05$ showed that such a decrease was not always significant.

As shown in the results, reduction in counts of indicators in both influent and effluent samples were not significant. This indicates that the treatment process in such a station is not efficient for the removal of bacterial and viral loads. The only treatment process applied in this station is primary physical sedimentation. However, a significant reduction in counts was observed at the pipe outfall source point where wastewater effluent is released into the seawater 750 m off the seashore. The main cause of such a significant reduction is hypothesized to be dilution. Similar studies have reported a decrease in counts of indicators away from the sewage pollution source (Ng *et al.* 1993; Paul *et al.* 1997). The three source points east and west of the pipe outfall and samples taken in the wind direction showed no further significant reduction in counts. All these samples were taken within a 10-m diameter; the highest count of somatic coliphages in these samples was 40 pfu ml^{-1}. In the case of bacterial indicators the highest count was <300 cfu ml^{-1}. Such an estimate of coliphages as indicators of enteric viruses is relatively high as a single virus particle could be sufficient for the initiation of infection.

One-way analysis of variance of the data ($p \leq 0.05$) to show the effect of different seasons on counts of coliphages has indicated that counts in colder seasons were variable but higher than in warmer seasons. This is in agreement with results by Mitchell and Chamberlin (1974), who noted that survival of coliphages varies considerably with season. Kapsuscinski & Mitchell (1983) indicated that sunlight and higher temperatures are lethal for microorganisms discharged into the water mass. A simple correlation coefficient between the four indicators showed a positive, highly significant correlation with counts of total and faecal coliforms in all types of water tested, as they are the natural hosts of such phages. Several results have reported a correlation in counts (Mustonen & Heinonen-Tanski 1994; Gantzer *et al.* 1998). The correlation between SC and FS was variable in different seasons. However, the faecal streptococci are not hosts of SC. This result also agrees with findings by Goyal *et al.* (1980) and Borrego *et al.* (1983).

The correlation between various hydrochemical parameters tested and the counts of somatic coliphages were very variable as shown from the results. However, the use of stepwise multiple regression analysis indicated that the most effective parameters that could be predictive of coliphage counts were total nitrogen, nitrite and total coliforms. TN and NO_2 are much higher in wastewater than seawater samples due to a lower microbial population in the sea. In addition to *E. coli*, total coliforms include other potential bacterial hosts of coliphages that could contribute to their survival and persistence (Borrego *et al.* 1983).

Salinity was not a limiting factor in the assessment of somatic coliphages. This is in agreement with findings by Berry & Noton (1976) who stated that coliphage viability remained unaltered in artificial seawater over a long period before inactivation was observed. Furthermore, Gerba & Schaiberger (1975) pointed out that the salinity of seawater was not toxic to enteroviruses. Sewage discharge into seawater is strongly discouraged.

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