ENUMERATION OF SOMATIC COLIPHAGES IN SEWAGE POLLUTED WATER SAMPLES IN ALEXANDRIA.

By

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ABSTRACT

Seasonal enumeration of somatic coliphages (SC), total coliforms (TC), faecal coliforms (FC) and faecal streptococci (FS) has been performed in different wastewater polluted water source points at and near the eastern treatment plant in Alexandria. Somatic coliphages were detected in all collected samples. A highly significant correlation was found between counts of SC and either TC or FC but not FS in all seasons tested. Predictive curves that could allow the assessment of SC counts from TC, FC or FS counts and vice versa have been constructed. Different hydrochemical parameters of the water samples were measured. Stepwise multiple regression analysis was used to rank various parameters based on their effect on counts of somatic coliphages.

INTRODUCTION

Pathogenic bacteria, viruses and parasites in faecally contaminated water constitute potential health hazards to persons using such waters (Kabler, 1959). These microorganisms are discharged into natural waters from sewage and their presence denotes faecal pollution and may constitute a risk of transmission of waterborne diseases to the human population. More than 140 serotypes of viruses are transmitted via wastewater. Hepatitis A virus, caliciviruses, adenoviruses, rotaviruses and enteroviruses have the greatest effect on public health (Anderson and Strenstrom, 1987; Yao, 1989; Bosch et al., 1991).

Bacteriophages could play a valuable role as indicators of enteric viruses because their structure, composition, morphology and size closely resemble that of enteric viruses (Grabow et al., 1984). The importance of coliphages as indicators of faecal contamination in water was first recognized by Guelin (1948, 1950).

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 estimates in comparison to traditional indicators were performed in different countries such as Kuwait (Qureshi *et al.*, 1988); Finland and Nicaragua (Mustonen and Heinonen-Tanski, 1994), Al-Bahrain (Qureshi and Qureshi, 1989); France (Gantzer *et al.*, 1998); Saudi Arabia (Fattouh and Al-Kahtani, 2002), U.S.A. (Griffin *et al.*, 2000) and others.

Seasonal enumeration of somatic coliphages (SC) in wastewater, brackish water (Marriout Lake) and fresh water samples near the eastern sewage treatment plant in Alexandria was 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 physiochemical parameters of the water samples were also determined. The correlation between estimates of somatic coliphages and all other parameters will be discussed.

MATERIALS AND METHODS

Water samples:

The eastern sewage treatment plant has a capacity of 530,000 m^3 / day. It receives wastewater from most of the eastern district of Alexandria through four main pumping stations. Following primary treatment, wastewater flows through El Kalaa drain and finally to Marriout lake. The Eastern Treatment water source points included this study were: The Eastern Treatment Plant Influent (E.T.P.I), Eastern Treatment Plant Effluent (E.T.P.E.), El Kalaa Drain (K.D.), Marriout Lake (M.L.) and Fresh Water Canal (F.W.C.).

Seasonal estimation of the four indicators: somatic coliphages (SC), total coliforms (TC), faecal coliforms (FC) and faecal streptococci (FS) was carried out from the winter season of 2001 to the winter season of 2002.

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 taking them. After collection, the samples were sent to the laboratory, and examined within two to three hours 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 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 onto Petri dishes containing TYG-base agar, allowed to solidify and incubated at 37°C. The plaques were counted following 16 hours incubation (Mustonen and Heinonen-Tanski, 1994).

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 onto the surface of LES-endo agar and incubated at 37°C ± 0.5 for 24 h ± 2h.

Faecal coliforms (FC)

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

Faecal Streptococci (FS)

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

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 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 (APHA, 1995). In the laboratory, 100 ml of sample were titrated against 0.02 N sodium thiosulphate solution. Dissolved oxygen content was calculated in mg O_2/l. The percentage saturation of dissolved oxygen was calculated using the tables of the National Institute of Oceanography of Great Britain and UNESCO.

Total phosphorus and total nitrogen (TP and 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 significant difference (LSD). A difference between treatment mean of $p \leq 0.05$ was regarded as significant. Correlation coefficient was applied to asses 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 (Neter *et al.,* 1996).

RESULTS

Somatic coliphages and the bacterial indicators,: total coliforms, faecal coliforms and faecal streptococci were enumerated in water samples from different source points near the eastern treatment plant, Alexandria. Log average counts of all indicators during 5 different seasons starting winter 2001 and up to winter 2002 are shown in Fig. (1). Analysis of variance using 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. The counts however, for SC were significantly lower at other source points: El Kalaa drain, Marriout lake and the fresh water canal. Counts within those latter source points showed no further significant reduction of SC counts with increasing distance away from the eastern treatment plant.

The same observation was seen for TC counts of winter, summer and autumn 2001. It was interesting to note that in case of FC estimates there always was a significant reduction in count of inffluent and effluent samples in all seasons tested. The 3 other source points outside the treatment plant had FC counts that were not significantly different as shown Fig. 1.

Estimates of FS showed more variation as shown in Fig. 1. In winter and summer 2001, no significant difference in counts between influent and effluent samples and no significant difference in counts in the 3 points outside the treatment plant. In spring 2001, the effluent sample, El Kalaa drain and Marriout lake samples were not significantly different, yet the fresh water canal sample was significantly lower.
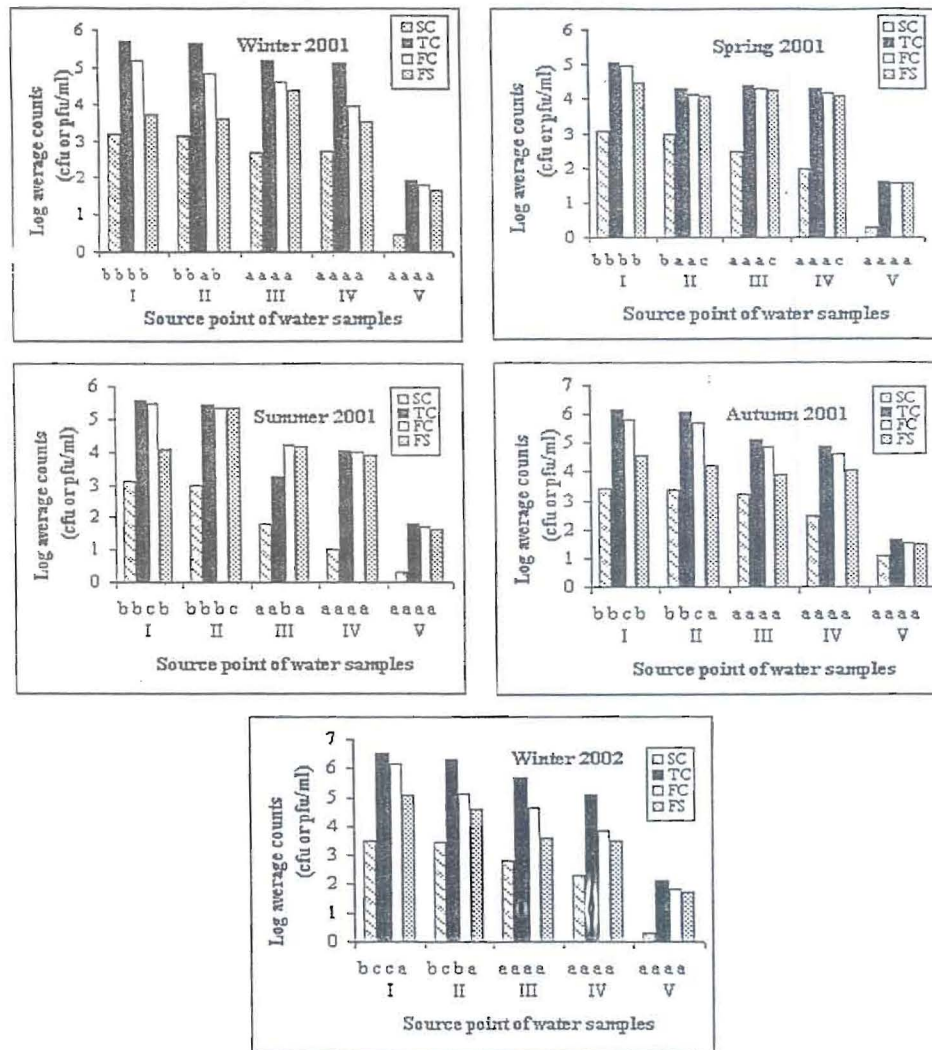
Fig. 1. Log average counts of the indicators: somatic coliphages (SC), total coliforms (TC), faecal coliforms (FC), and faecal streptococci (FS) in different seasons at the Eastern Treatment Plant (E.T.P.). Source point of water samples are: I: E.T.P. influent, II: E.T.P.
Effluent, III: El Kalaa Drain, IV: Marriout Lake, V: Fresh water canal. Different letters (a, b, c,) indicate a significant difference in counts (p< 0.05) at different source points of water samples according to one way ANOVA test.

Autumn 2001 samples of influent was significantly different from all other source points. Finally, winter 2002 samples of all source points were not significantly different according to one way ANOVA test. Analysis of variance of data for each indicator

showed that counts of the 4 indicators were significantly higher during the colder seasons of autumn 2001 and winter 2002.

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

Table 1 : Simple correlation coefficient between the four indicators in different seasons at the Eastern Treatment Plant (E.T.P.).

[a] Simple Correlation Coefficient	Winter 2001	Spring 2001	Summer 2001	Autumn 2001	Winter 2002
Total coliforms	0.998**	0.749*	0.999**	0.601*	0.638*
Faecal coliforms	0.867**	0.736*	0.889**	0.881**	0.602*
Faecal streptococci	0.464	0.710*	0.853**	0.771*	0.864**

If a relationship between coliphages as model organisms is to be of practical use, it is important that there is a good correlation between them and the bacterial indicators for individual types of water samples and also that the relationship between the organisms is consistent for different types of water samples. Least square linear regression analysis was applied to predict the variation in the number of SC, TC, FC, and FS. The regression equations describing the relationship between the number of somatic coliphages and that of the other indicators in different types of water samples are given in Fig. 2 I, II, and III.

Levels of the hydrochemical parameters in and near the eastern treatment plant water source points during various seasons are outlined in Table (2). 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 over all 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:

$Y = 234.38 + 0.90\ TC + 0.43\ BOD + 0.27\ TP - 0.44\ FC - 0.18\ salinity + 0.16\ PO_4 + 0.10\ NO_3$.

Table (2): Levels of the hydrochemical parameters in the Eastern Treatment Plant water source points (E. T. P.) during various seasons.

Stations	Temp. °C	pH	Salinity ‰	DO (mg/l)	BOD (mg/l)	H2S (mg/l)	NH4 (mg/l)	NO2 (mg/l)	NO3 (mg/l)	TN (mg/l)	PO4 (mg/l)	TP (mg/l)
Winter 2001 I	17.2	7.00	0.92	ND	408	11.69	750.00	0.21	18.81	795.67	9.49	24.48
II	17.6	7.02	0.98	ND	285	0.86	718.00	0.46	19.09	800.80	9.27	17.28
III	18.0	7.10	1.02	ND	212	16.54	650.00	0.06	4.96	691.20	7.21	24.48
IV	18.2	7.03	1.06	ND	125	14.67	649.00	0.14	8.81	677.86	10.40	24.00
V	18.9	7.41	0.24	13.28	2.42	ND	12.00	3.04	19.39	91.02	0.13	0.22
Correlation Coef.	-0.95**	-0.78*	0.56	-0.67*	0.94**	1.45**	0.79*	-0.59	0.37	0.79*	0.69*	0.53
Spring 2001 I	25.2	8.20	0.52	ND	430.00	5.47	931.00	0.38	0.68	1072.87	11.81	88.12
II	26.0	8.20	0.66	ND	292.40	5.56	899.00	0.40	0.75	995.89	56.71	94.82
III	25.6	8.40	1.20	ND	126.40	17.47	995.86	0.33	0.63	1015.00	48.91	75.79
IV	26.2	8.50	2.80	ND	46.60	14.00	649.00	0.22	2.00	662.30	48.24	72.31
V	26.6	8.10	0.28	6.25	3.24	ND	12.00	7.31	8.95	77.11	10.01	16.10
Correlation Coef	-0.72*	-0.33	-0.42	-0.53	0.99**	-0.22	0.61*	-0.52	-0.61*	0.73*	-0.02	0.74*
Summer 2001 I	31.0	7.00	0.45	ND	317.00	12.90	575.50	0.25	0.46	792.59	20.23	36.05
II	31.9	7.05	0.59	ND	222.20	0.25	526.00	0.18	0.26	805.93	22.22	42.61
III	32.5	7.15	1.06	ND	182.00	15.80	493.50	0.17	2.09	816.46	23.83	29.08
IV	32.6	7.06	2.37	ND	112.00	0.15	508.00	0.13	0.81	820.31	19.83	26.71
V	32.8	7.52	0.29	5.10	2.19	ND	1.18	1.94	21.59	241.78	2.88	3.62
Correlation Coef	-0.95**	-0.59*	-0.47	-0.42	0.84**	0.21	0.52	-0.38	-0.47	0.39	0.37	0.70*
Autumn 2001 I	18.6	8.20	0.61	ND	338.00	1.34	770.00	0.54	9.81	796.41	12.48	46.4
II	18.9	8.10	0.77	ND	243.20	0.80	758.80	0.14	5.31	796.41	14.21	42.9
III	18.9	8.10	1.25	ND	111.60	16.54	673.30	0.05	2.31	793.59	14.86	44.3
IV	19.2	7.60	1.01	ND	67.40	14.20	558.80	0.01	0.05	788.20	12.96	39.5
V	19.4	7.80	0.27	9.97	6.41	ND	5.02	4.43	9.42	723.52	1.44	1.66
Correlation Coef	-0.96**	0.91**	0.61*	-0.65*	0.94**	-0.21	0.83**	-0.61*	0.25	0.73*	0.65*	0.74*
Winter 2002 I	21.0	8.60	1.01	0.60	396.80	ND	1200	0.19	1.50	1384.46	22.19	25.1
II	21.0	8.50	1.23	4.02	274.60	ND	1100	0.21	2.70	1363.93	20.47	22.6
III	22.0	8.50	1.15	ND	192.20	6.00	820	0.63	2.98	980.47	16.46	27.4
IV	20.0	8.60	1.13	ND	66.30	0.63	820	0.77	1.10	1039.50	24.12	27.9
V	22.0	8.10	0.28	6.02	2.74	ND	15	8.29	32.35	193.53	1.82	2.5
Correlation Coef	-0.13	0.49	0.49	-0.08	0.94**	-0.31	0.78*	-0.57	-0.51	0.79*	0.51	0.4

[a] Water source points at Eastern Treatment Plant (E.T.P.): I: E.T.P. Influent, II: E.T.P. Effluent, III: El Kalaa Drain, IV: Marriout Lake, V: Fresh Water Canal.

[b] Simple correlation coefficient between somatic coliphage counts and the hydrochemical parameters of water samples in Eastern Treatment Plant.

Insignificant = < 0.6, * = Significant (0.6-0.8), ** = Highly significant,> 0.8, - = Indirect Correlation.

DISCUSSION

Members of the coliform bacteria groups are measured to determine whether the water has been contaminated by faecal material (APHA, 1999). Faecal material also contains viruses that can cause disease in humans (Marino *et al.*, 1995; Lee *et al.*, 1997). Viruses are more resistant to chlorine and other disinfectants used in water treatment than are coliforms (Berg *et al.*, 1978). Coliform numbers do not serve as adequate indicators of enteric viruses contamination water (Stetler, 1984; Havelaar, 1994; Marino *et al.*, 1995; Gantzer *et al.*, 1998). Estimation of somatic coliphages, in addition to the traditional indicators, total coliforms, faecal coliforms and faecal streptococci has been thus performed in different types of sewage polluted water. Raw sewage, treated sewage, brackish water and fresh water samples were used in this study. Results indicated that somatic coliphages could be detected and recovered in all types of sewage-polluted water.

The highest estimates of somatic coliphages were 30×10^2, 27×10^2 and 5×10^2 pfu/ml from influent, effluent sewage samples and brackish water samples respectively at E.T.P. in winter season 2002. Only 12 pfu/ml were detected in the fresh water canal samples during autumn 2001. Seasonal variations in estimates of somatic coliphages, however, indicate a general trend of higher phage concentrations during the cooler months in comparison to the warmer months. This is in agreement with studies reported by Mahdy (1979), who reported that viruses discharged in water environment survived longer than bacteria and that low temperature and high degree of domestic pollution favored virus survival. Qureshi and Qureshi (1989), reported higher concentrations of coliphages to be observed during January and February in Bahrain, with average daytime temperatures of 20 °C. However, other investigators reported as high estimates as 7.5×10^5 pfu/ml in winter (Qureshi *et al.*, 1988). Furuse *et al.* (1981) have also reported somewhat higher phage densities in summer 10^6 to 10^8 pfu/ml than in winter 10^3 to 10^5 pfu/ml.

Results in this study, also indicate that there was no significant decrease in coliphage estimates in influent and effluent samples. This could suggest that the treatment procedures employed in the station may not be adequate enough to decrease somatic coliphage loads. Such high number of coliphages could indicate the presence of human enteric viruses in the water. A correlation between the presence of coliphages and enteric viruses has already been proposed (Pretorious, 1962; Kott *et al.* 1974, Gerba *et al.*, 1978; Gantzer *et al.*, 1998 and others).

Though the counts of the four indicators in samples taken at different source points other than the sewage influent and effluent decreased with the increase of distance away from sewage source, yet statistical analysis of data by one-way ANOVA test did not indicate that there was a significant decrease. This observation could be explained for El Kalaa drain, Marriout lake and the fresh water canal by the fact that such water bodies could be receiving discharges of unidentified amounts from agricultural,

domestic and industrial wastewater drainage from Alexandria city. This shows that the E.T.P. station is not the sole source of sewage pollution of such source points and that other uncontrolled sources contribute to the indicators count. Our results in this aspect thus do not coincide with other similar results (Ng *et al.*,1993; Paul *et al.*, 1997; Saleem *et al.*, 2000), which indicated the decrease in counts of indicators away from the sewage pollution source. The presence of coliphages in the fresh water canal is not surprising as incidence of coliphages in the River Nile were recorded (El Abagy *et al.*,1988; El Abagy, 2001). There were few exceptions to this generalization especially for the faecal streptococci samples of Spring 2001. Variable survival rates of the faecal streptococcus group species are known. *Streptococcus bovis, S. faecalis* and *S. faecium* tend to survive longer (Edwards *et al.*, 1997).

To establish the validity of coliphages as indicators of faecal pollution, these viruses should first correlate with their bacterial hosts in the aquatic media. Results of this study show that somatic coliphages have significant or highly significant correlation with both TC and FC in all types of water samples collected at various seasons. The relationship between somatic coliphages and FS was variable, however, they are not hosts of somatic coliphages. This is in agreement with may other reports (Goyal *et al.*,1980; Borrego *et al.*, 1987; Qureshi and Qureshi, 1989; Havelaar *et al.*,1993). This study allowed the development of regression equations illustrating the relationship between somatic coliphages and the other three bacterial indicators. Using such equations, it was possible to construct a graphical presentation of the relationships between the indicators. This should allow the prediction of somatic coliphages, as models of viruses from counts of the bacterial indicators and vice versa in all types of water source points tested in this study. Similar prediction curves were constructed for F-RNA phages (Havelaar *et al.*,1993) and somatic coliphages (El Rakchy, 1989).

Measurement of some physicochemical water parameters in association with estimates of the indicators was performed. This was done in order to determine the parameters that affect most the survival and recovery of the indicator organisms in various types of water samples. Biological oxygen demand (BOD) values ranged between 2.4 and 408 mg/l and in most cases correlate positively with the four indicators. Mitwally and Hossam El-Deen (1996) reported that the BOD range was 140 to 210 mg/l in El Kalaa drain. However, El Sharkawy (1978) stated that the BOD was 57 mg/l. The increase in BOD indicates an increase in the oxygen consumption needed for organic matter decomposition, which in turn refers to an increase in the microbial population growth due to the increase in sewage pollution over the years.

Simple correlation coefficient values between somatic coliphage counts and the various water parameters included in the study were variable and ranged from non-significant to highly significant. Other factors in the environment besides the parameters measured might also be influencing the viral and bacterial survival in various water samples such as microbial antagonism, antimicrobial products, heavy metals in sewage or industrial discharges, organic matter, detergents, hydrocarbons and

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