Correlation between bacterial indicators and bacteriophages in sewage and sludge

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Abstract

The use of bacteriophages as potential indicators of faecal pollution has recently been studied. The correlation of the number of bacterial indicators and the presence of three groups of bacteriophages, namely somatic coliphages (SOMCPH), F-RNA-specific phages (FRNAPH) and phages of *Bacteroides fragilis* (BFRPH), in raw and treated wastewater and sludge is presented in this study. Raw and treated wastewater and sewage sludge samples from two wastewater treatment plants in Athens were collected on a monthly basis, over a 2-year period, and analysed for total coliforms, *Escherichia coli*, intestinal enterococci and the three groups of bacteriophages. A clear correlation between the number of bacterial indicators and the presence of bacteriophages was observed. SOMCPH may be used as additional indicators, because of their high densities and resistance to various treatment steps.

Introduction

Lack of clean water, and limited water resources in dry regions, is observed worldwide. Consequently, the reuse of treated wastewater for various activities, mainly in agriculture and aquaculture, is a rapidly developing global need (WHO, 1989).

Furthermore, the dramatic increase in sludge production as a result of extended sewerage leads to environmental policies for recycling of treated sewage sludge.

Sewage facilities reduce pathogen load, leading to a decrease in public health risks associated with exposure. Validation of the treatment processes and assurance of the microbiological quality of the effluent and the treated sludge is not easy to perform, as methods of isolation and identification of pathogens are complicated, expensive and time-consuming. Surrogate indicators (faecal coliforms, *Escherichia coli*, intestinal enterococci) are used for routine evaluation of treatment plant performance and effluent/sludge quality.

Internationally, there are no standards regulating the production and microbiological quality of reclaimed water, although WHO has developed guidelines for use, recommending monitoring of faecal coliforms and intestinal nematodes.

The utilization of treated sewage sludge in agriculture is controlled by legislation regulating the content of bacteria, parasites and heavy metals, but not viruses. European Directive 86/278 (European Union, 1986), the European Working Document on Sludge (European Union, 2000) and '40 CFR Part 503' (U.S. EPA, 1995) require acceptable procedures for the disposal and utilization of sewage sludge.

Various microorganisms exhibit different extents of inactivation after treatment processes, and the bacterial indicators do not follow the same die-off kinetics as viruses, parasitic protozoa and helminths (Stenström, 2002; Arraj *et al.*, 2005). Somatic coliphages (SOMCPH), F-RNA-specific bacteriophages (FRNAPH) and phages infecting *Bacteroides fragilis* (BFRPH) have been studied as potential indicators of water quality and/or virus content, in addition to bacterial indicators (IAWPRC, 1991). These groups of bacteriophages, also found in wastewaters, can infect bacteria of the normal flora of the human gastrointestinal tract. The methods for their detection and enumeration are simple, rapid, inexpensive and require no confirmation. An important drawback for the use of somatic coliphages as indicators is that some of them may multiply in water environments (Grabow *et al.*, 1984; Borrego *et al.*, 1990). However, according to recent studies (Muniesa *et al.*, 2003;

Muniesa & Jofre, 2004), only 3% of the environmental nonfaecal host-bacteria can support the multiplication of somatic coliphages, and the conditions that support their multiplication are rarely found in water environments. There is no indication that *F*-specific bacteriophages and phages infecting *B. fragilis* can multiply in the environment (Tartera *et al.*, 1992; Contreras-Coll *et al.*, 2002).

Many studies indicate that bacteriophages may be promising predictors of viral gastrointestinal illness and demonstrate a strong correlation between viruses and bacteriophages (Wade *et al.*, 2003). According to Havelaar *et al.* (1993) and Lee *et al.* (1997), a strong relationship exists between enteroviruses and FRNAPH in surface waters, and propose FRNAPH as indicators of health risk in recreational waters. Moce-Llivina *et al.* (2005) report that SOMCPH show a very good potential to predict the risk of viruses being present in bathing waters. Moreover, SOMCPH have been proposed as alternative faecal indicators in fresh waters in regions with tropical climate, where *E. coli* and enterococci may be less reliable as indicator organisms, because they multiply in such environments (Fujioka & Byappanahalli, 2003). FRNAPH and BFRPH have been proposed as alternative indicators of viral health risk associated with shellfish (Dore *et al.*, 2003; Muniain-Mujika *et al.*, 2003). Gantzer *et al.* (1998) report a significant correlation between SOMCPH and BFRPH and the presence of enteroviruses in wastewaters.

In previous studies (Mandilara *et al.*, 2005, 2006), correlations between bacterial indicators and bacteriophages were reported and threshold values for the presence of bacteriophages in sewage and sludge samples were determined. The aim of the present research was to study the type and grade of this correlation in raw/treated wastewater and sludge, and evaluate the use of bacteriophages as supplementary indicators to current bacterial indicators.

Materials and methods

Facilities

Samples were collected from two sewage treatment plants. Plant (A) is the main sewage treatment plant of Athens (capacity of 750 000 $m^3\,day^{-1}$) handling the sewage of the city. It includes primary treatment (sand and oil removal and primary settling). Primary sludge undergoes anaerobic mesophilic digestion for stabilization (30–35 °C for *c.* 28 days). Plant (B) treats part of the urban sewage and all the septages of Athens. Its capacity is 24 000 $m^3\,day^{-1}$ for septages and 20 000 $m^3\,day^{-1}$ for urban sewage. This plant includes primary treatment (sand and oil removal and separate primary settling for urban sewage and septages), secondary treatment (biological treatment and secondary settling) and disinfection (chlorination, residual chlorine 0.3 $\mu g\,mL^{-1}$). Primary and secondary sludge is stabilized with the mesophilic anaerobic digestion (30–35 °C for *c.* 28 days).

Sampling

Samples were collected monthly, over a 2-year period (from November 2000 to September 2002), as follows: Plant A: raw wastewater ($N = 20$) and wastewater after primary settling ($N = 20$), sludge before ($N = 20$) and after anaerobic digestion ($N = 20$). Plant B: raw urban wastewater ($N = 20$) and wastewater after primary settling ($N = 20$), raw septages ($N = 20$) and septages after primary settling ($N = 20$), wastewater after secondary treatment ($N = 20$), after disinfection ($N = 20$), and sludge before ($N = 20$) and after anaerobic digestion ($N = 20$).

The collection and transport of samples to the laboratory were carried out according to ISO 5667-2 and 5667-3.

Sewage samples

Sewage samples were homogenized by a stomacher for 2 min, and in cases of heavily polluted samples, after suspension in peptone saline at a ratio of 1 : 10. Enumeration of indicator bacteria was made using the membrane filtration method (Berg & Berman, 1989). For bacteriophage analysis, samples were decontaminated, to remove fungi and bacteria, by filtration through a 0.22-μm pore size low-protein-binding polyvinylidene fluoride membrane (Millex-GV; Millipore).

Sludge samples

Bacterial indicators (total coliforms (TC), *E. coli* and intestinal enterococci) were quantified in 10 g of sludge after suspension in peptone saline at a ratio 1 : 10 and homogenized by a stomacher for 2 min. After centrifugation at 1500 ***g*** for 15 min at 4 °C, the supernatant was used as a liquid sample for bacterial enumeration. After convenient dilutions had been made, the membrane filter assay was applied, in line with standard methods (Anonymous, 1992).

Elution of bacteriophages from sludge

Ten grams of sludge sample was suspended in 100 mL of 10% (w/v) beef extract solution and homogenized by a stomacher for 2 min. After centrifugation at 1500 ***g*** for 15 min at 4 °C, the supernatant was filtered through a 0.22-μm pore size low-protein-binding polyvinylidene fluoride membrane (Millex-GV; Millipore) to remove bacteria before phage enumeration. The filtrates were then assayed for phages (Tartera *et al.*, 1992).

Bacteriophage analysis

Escherichia coli WG5 (nalidixic acid resistant) was used to count somatic coliphages, and *B. fragilis* RYC2056 for the phages of *B. fragilis*. Bacteriophages plaquing on the host *Salmonella typhimurium* WG 49 were counted as F-total bacteriophages, and the difference between the total and the number of plaques counted on plates with 40 μg of RNase/mL into the assay medium was attributed to *F*-specific RNA bacteriophages. All phages were quantified by the double-agar-layer method (ISO 10705-1; 10705-2; 10705-4). For wastewater, five replicates of 2 mL were plated, except in the case of disinfected effluent samples, for which 10 replicates of 2 mL each were plated. For sludge, five replicates of 2 mL were plated. PFUs $100\,mL^{-1}$ and PFUs g^{-1} were calculated after 18 h of incubation. Detection limits for bacteriophages were 10 PFUs $100\,mL^{-1}$ in wastewater and 1 PFU g^{-1} in sludge samples.

Quality assurance

A first-line quality control was performed using reference materials, for bacteria and bacteriophages. Reference materials (lenticules) of *E. coli* and intestinal enterococci, provided by HPA, were used. Pure cultures of bacteriophages φX174, MS2 and B56-1, were prepared (Mooijman *et al.*, 1999) and used as reference materials for the SOMCPH, FRNAPH and BFRPH, respectively.

Statistical analysis

Statistical analyses were conducted using SPSS version 11.0. Log_{10}-transformed values were used for all computations and tests. Differences were considered significant at $P < 0.05$, as determined by the appropriate comparative test (Anon., 2001). Nonparametric statistical tests were utilized for nonnormally distributed data. Parametric tests were used for analysis of variance. The Spearman rank correlation was used to test the relationship between bacterial indicators and bacteriophages. A binary logistic regression model (SPSS 13.0) was utilized to determine whether indicator organism concentrations predicted the probability of the occurrence of bacteriophages in wastewater. The dependent variable (bacteriophages) was treated as a binary variable; that is, a score of 0 was assigned when bacteriophage was not detected and a score of 1 was assigned when bacteriophage was detected. The independent variables were continuous, and values for samples in which organisms were not detected were reported as 0. Linear regression analysis was used to estimate the coefficients of the linear equation, involving one or more independent variables (bacterial indicators/bacteriophages) that best predict the value of the dependent variable (bacteriophages/bacterial indicators) in sludge. Finally, MANOVA (Hotteling's Trace < 0.05) was used to test the effect of seasonality, October–April (winter period) and May–September (summer period), on the six measured variables.

Results and discussion

Microbial concentrations and treatment process

Concentrations of bacterial indicators and bacteriophages before and after treatments are shown in Fig. 1 in a box-plot format. TC concentrations were the highest of the microbial measurements in raw wastewater ($>10^7$ CFUs $100\,mL^{-1}$), followed by *E. coli* and intestinal enterococci ($\sim10^6$ CFUs $100\,mL^{-1}$). Among the three groups of bacteriophages, SOMCPH were the most abundant ($\sim10^5$ PFUs $100\,mL^{-1}$). Concentrations of FRNAPH ranged between 10^3 and 10^5 PFUs $100\,mL^{-1}$, and that of between BFRPH 10^3 and 10^4 PFUs $100\,mL^{-1}$.

In raw sludge, *E. coli* and intestinal enterococci concentrations ranged from 10^5 to 10^8 CFUs g^{-1} and from 10^5 to 10^7 CFUs g^{-1}, respectively. At the outlet of the anaerobic digestor, *E. coli* concentration ranged from 10^3 to 10^5 CFUs g^{-1} and intestinal enterococci from 10^3 to 10^6 CFUs g^{-1}. Among bacteriophages, SOMCPH were more abundant than the other groups and BFRPH presented slightly lower concentrations than FRNAPH. SOMCPH were detected at concentrations of 10^5 PFUs g^{-1} in untreated sludge and 10^3–10^5 PFUs g^{-1} in digested sludge. FRNAPH and BFRPH were detected at concentrations of 10^4–10^5 PFUs g^{-1} and 10^3–10^4 PFUs g^{-1}, respectively, in raw sludge. In digested sludge, FRNAPH and BFRPH presented low concentrations: 3×10^1–3.6×10^3 PFUs g^{-1} and 2×10^1–3.2×10^3 PFUs g^{-1}, respectively.

Our results agree with others, reporting that in wastewater and sludge *E. coli* and enterococci counts are similar (Gantzer *et al.*, 1998; Lasobras *et al.*, 1999; Mignotte-Cadiergues*et al.*, 2002), and that among the three groups of bacteriophages, SOMCPH are the most abundant, and BFRPH present the lowest concentrations (Jofre *et al.*, 2000).

In Table 1, the average Log_{10} reduction of the various indicators after every treatment step is presented. After primary settling, changes in concentrations of all microbiological parameters were not statistically significant ($P > 0.05$). However, after secondary treatment (biological treatment and secondary settling), a 1.54–3.15 Log_{10} reduction of all parameters was observed. Chlorination of treated effluents caused a statistically significant reduction of TC and *E. coli* ($P < 0.05$), but had no effect on enterococci, SOMCPH and FRNAPH ($P > 0.05$), as they are more resistant. BFRPH were not detectable in chlorinated wastewater. It has been reported that biological wastewater

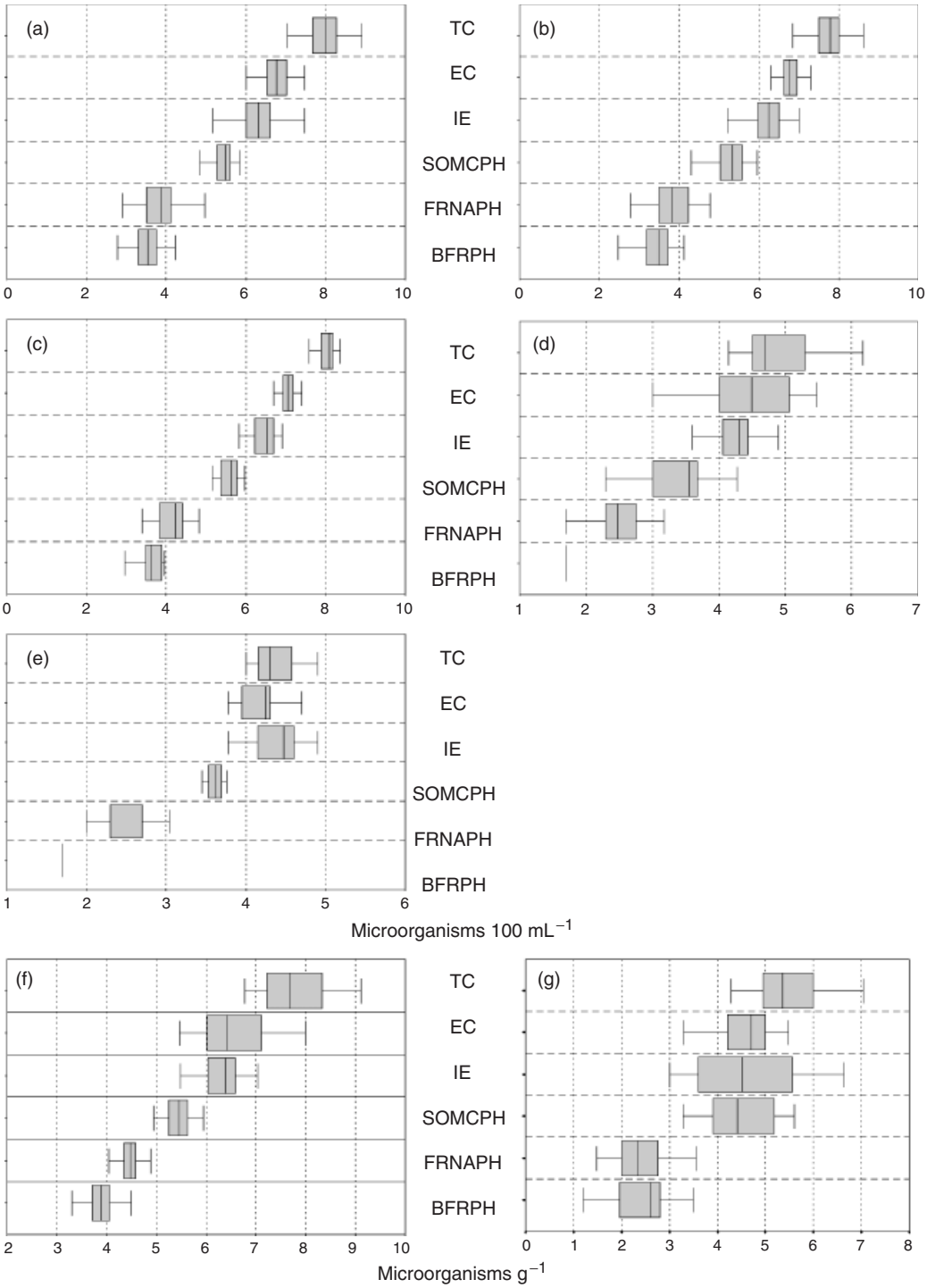

Fig. 1. Mean microorganism concentrations in: (a) raw wastewater (pooled data, Plant A and B, $N = 60$), (b) wastewater after primary settling (pooled data, Plant A & B, $N = 60$), (c) wastewater before ($N = 20$), and (d) after secondary treatment ($N = 20$), (e) chlorinated effluent ($N = 20$), (f) raw sludge (pooled data, Plant A and B, $N = 40$) and (g) sludge after anaerobic digestion (pooled data, Plant A and B, $N = 40$). Log_{10} concentrations of bacterial indicators (CFUs 100 mL^{-1} or CFUs g^{-1}) and bacteriophages (PFUs 100 mL^{-1} or PFUs g^{-1}). Boxes represent 50% of the data, the vertical lines represent the mean, lines extending from the boxes represent the 95% confidence limits. TC, total coliforms; EC, *Escherichia coli*; IE, intestinal enterococci SOMCPH, somatic coliphages; FRNAPH, F-RNA-specific bacteriophages; BFRPH, phages infecting *Bacteroides fragilis*.

Table 1. Average Log $_{10}$ reduction of microorganism concentrations after each treatment step

	Average Log_{10} reduction			
	Primary settling ($N = 60$)*	Secondary treatment ($N = 20$)	Chlorination ($N = 20$)	Anaerobic digestion of sludge ($N = 40$)*
Total coliforms	0.29	3.15	0.52	2.31
Escherichia coli	0.09	2.55	0.29	2.07
Intestinal enterococci	0.18	2.15	− 0.06	1.78
SOMCPH	0.04	2.17	− 0.20	0.98
FRNAPH	0.02	1.54	0.07	2.12
BFRPH	0.05	1.57	− 0.30	1.21

*Data from primary settling of wastewater and anaerobic digestion of sludge, pooled from Plants A & B.
SOMCPH, somatic coliphages; FRNAPH, F-RNA-specific bacteriophages; BFRPH, phages infecting *Bacteroides fragilis*.

Table 2. Percentage of samples with detectable indicator organisms and phages

	% of samples with detectable indicators and phages				
Indicators	Raw wastewater ($N = 60$)*	Wastewater after secondary treatment ($N = 20$)	Wastewater after chlorination ($N = 20$)	Raw sludge ($N = 40$)*	Sludge after anaerobic digestion ($N = 40$)*
Total coliforms	100	100	100	100	100
Escherichia coli	100	100	100	100	100
Enterococci	100	100	100	100	100
SOMCPH	100	100	95	100	100
FRNAPH	100	85	70	100	50
BFRPH	100	15	10	100	90

*Data from raw wastewater, raw sludge and sludge after anaerobic digestion, pooled from Plants A & B.
SOMCPH, somatic coliphages; FRNAPH, F-RNA-specific bacteriophages; BFRPH, phages infecting *Bacteroides fragilis*.

treatment processes have similar removal efficiency on viruses, bacteriophages and faecal bacteria (Havelaar & Nieuwstad, 1985). Chemical disinfectants, such as chlorine, inactivate faecal bacteria but have little or no effect on viruses and bacteriophages. Among bacterial indicators, enterococci present greater resistance to chlorination (Havelaar, 1987; Tartera *et al*., 1988; Mara & Cairncross, 1989).

After the anaerobic digestion of sludge, TC, *E. coli*, intestinal enterococci and FRNAPH presented a 1.78–2.31 Log_{10} ($P < 0.05$) reduction, whereas SOMCPH and BFRPH showed a 0.98 and 1.21 Log_{10} ($P < 0.05$) reduction, respectively. According to the above, SOMCPH and BFRPH are more resistant to anaerobic digestion of sludge than FRNAPH.

Our results are in agreement with previous studies. Mesophilic anaerobic digestion of sludge produces a 1–2 Log_{10} reduction of indicator bacteria, *Salmonella* sp. and enteroviruses (Berg & Berman, 1989; Soares *et al*., 1994). Lasobras *et al*. (1999) report that FRNAPH are less resistant to mesophilic anaerobic digestion than the other two groups of bacteriophages.

Percentages of pooled samples from each treatment step, containing detectable levels of microorganisms, are summarized in Table 2. All microorganisms were detected in 100% of raw wastewater and raw sludge. TC, *E. coli* and enterococci were detected in 100% of effluent after secondary treatment, chlorination of wastewater and after anaerobic digestion of sludge. SOMCPH were detected in all samples after secondary treatment, and in 95% after chlorination. FRNAPH were detected in 85% of samples after secondary treatment, and in low concentrations (200–300 PFUs 100 mL^{-1}) in 70% of samples after chlorination. BFRPH were detected in 15% and 10% of samples after secondary treatment and chlorination, respectively, in low numbers (10–100 PFUs 100 mL^{-1}). After anaerobic digestion of sludge, SOMCPH were detected in 100%, FRNAPH in 50% and BFRPH in 90% of samples.

Among the three groups of bacteriophages, SOMCPH were always the most abundant and were detected even after chlorination in high concentrations ($> 2.8\,10^3$ PFUs 100 mL^{-1}). Among all microorganisms, FRNAPH and BFRPH presented the lowest reduction after secondary treatment step, BFRPH being almost nondetectable after this treatment step. Therefore, BFRPH cannot be used to evaluate the performance of chlorination. Concerning the anaerobic digestion of sludge, SOMCPH and BFRPH

presented greater resistance than FRNAPH and bacterial indicators. Seasonal variability had no effect on the six microbiological variables and on treatment steps (MANOVA, Hotelling's Trace > 0.05)

Predictive relationships between microorganisms

Data from wastewater, and sludge also, were analysed as a pooled data set (both plants and treatment steps) to determine correlations between bacterial indicators and bacteriophages. Significant correlations between concentrations of any combination of bacterial indicators and bacteriophages were observed. Tables 3a and b present Spearman's correlation coefficients in wastewater and sludge samples, respectively. Spearman's coefficients between any bacterial indicators and bacteriophages were r_S>0.6 in wastewater, and r_S>0.75 in sludge. Spearman's correlation coefficient r_S>0.6 reveals a strong relationship between the two variables.

Logistic regression analysis was used to test the hypothesis that bacterial indicators were predictive of the presence or absence of bacteriophages in wastewater. Bacteriophage counts were converted to binary data, and the relationship between the concentration of each bacterial indicator and the presence or absence of each group of bacteriophages were assessed. Nagelkerke's R^2 ranges from 0.0 to 1.0, and denotes the strength of the association; strong associations have values closer to 1.0. Two bacterial indicator–bacteriophage combinations displayed the strongest correlations: TC's concentration and presence/absence of FRNAPH ($R^2 = 0.710$) and *E. coli* concentrations and presence/absence of BFRPH ($R^2 = 0.73$).

Multiple regression analysis was used to test the hypothesis that bacterial indicator concentrations were predictive of the concentrations of bacteriophages and vice versa. R^2 ranges from 0.0 to 1.0, and denotes the strength of the association; strong associations have values closer to 1.0. Several bacterial indicator–bacteriophage combinations displayed very strong correlation: SOMCPH and IE ($R^2 = 0.761$), FRNAPH and IE+EC ($R^2 = 0.822$), BFRPH and EC ($R^2 = 0.730$), EC and FRNAPH+BFRPH ($R^2 = 0.799$) and IE and SOMCPH+FRNAPH ($R^2 = 0.833$).

Similar studies in the literature (Contreras-Coll *et al.*, 2002) report that in marine waters, *E. coli* and bacteriophages are slightly correlated, and this correlation starts to diminish as the concentration of *E. coli* is decreased. Harwood *et al.* (2005) comment that the failure of single indicators (*E. coli*, enterococci, *Clostridium perfringens* and FRNAPH) to correlate with enteric viruses in reclaimed water suggests that public health is not adequately protected by simple monitoring schemes based on detection of a single indicator.

When effluents and digested sludge are disposed in the environment or used for agricultural purposes, pathogens may infect crops and underground waters. Bacteriophages can be very useful as model organisms for monitoring the effectiveness of treatment processes and the microbiological quality of the product. Moreover, they can be used as model/surrogates for enteric viruses as they closely meet

Table 3a. Spearman's correlation coefficients between microbiological parameters in raw and treated wastewater ($P < 0.05$)

	Total coliforms	*E. coli*	Enterococci	SOMCPH	FRNAPH	BFRPH
Total coliforms		0.806	0.639	0.663	0.627	0.702
Escherichia coli	0.806		0.645	0.696	0.635	0.758
Enterococci	0.639	0.645		0.558	0.685	0.597
SOMCPH	0.663	0.696	0.558		0.543	0.726
FRNAPH	0.627	0.635	0.685	0.543		0.683
BFRPH	0.702	0.758	0.597	0.726	0.683	

SOMCPH, somatic coliphages; FRNAPH, F-RNA-specific bacteriophages; BFRPH, phages infecting *Bacteroides fragilis*.

Table 3b. Spearman's correlation coefficients between microbiological parameters in raw and digested sludge ($P < 0.01$)

	Total coliforms	*E. coli*	Enterococci	SOMCPH	FRNAPH	BFRPH
Total coliforms		0.940	0.881	0.760	0.816	0.805
Escherichia coli	0.940		0.841	0.756	0.823	0.830
Enterococci	0.881	0.841		0.756	0.811	0.774
SOMCPH	0.760	0.756	0.756		0.773	0.662
FRNAPH	0.816	0.823	0.811	0.773		0.745
BFRPH	0.805	0.830	0.774	0.662	0.745	

SOMCPH, somatic coliphages; FRNAPH, F-RNA-specific bacteriophages; BFRPH, phages infecting *Bacteroides fragilis*.

key requirements for this function (Kott, 1981; Grabow, 1986).

According to our observations, bacteriophages are significantly correlated to bacterial indicators, and hence to faecal pollution. Considering the fact that bacteriophages present higher resistance to all treatment steps than bacterial indicators, phages seem to be a useful tool for evaluating the effect of treatment on wastewater and sludge for a wide range of microorganisms. SOMCPH seem to be the best indicators of microbiological quality and treatment performance, as they are always found in detectable concentrations in wastewater and sludge, even after chlorination, and the method for their detection is simple and rapid (results in 4 h). SOMCPH can greatly assess the validity of predictive models of treated wastewater and sludge quality.

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