Journal of Industrial Microbiology & Biotechnology (2000) 24, 127–131

www.nature.com/jim

The passage and propagation of fecal indicator phages in birds

DM Ricca and JJ Cooney

Environmental, Coastal and Ocean Sciences Program, University of Massachusetts Boston, Boston, MA 02125-3393, USA

The presence of F-specific phages in the diet of birds influenced the presence of these fecal indicators in their feces. F-specific phage concentrations in the feces of Canada geese and pigeons, which are normally low, increased greatly the same day coliphage MS2 was added to their diets. F-specific phage concentrations decreased to the original low levels a week after the phage-spiked feed was removed. Geese kept in pens that were cleaned regularly to reduce fecal-oral contamination had significantly lower somatic coliphage concentrations in their feces than wild geese had in their feces. Somatic coliphage concentrations in feces of feral pigeons were typically low with an occasional fecal sample having high numbers of either one of the two types of phages seen in this population of birds. Sometimes many birds had high numbers of only one type of phage in their feces. This lasted only a day and was probably due to fecal contamination of the feeding pans by the pigeons. The degree to which birds are a source of phage indicators of fecal pollution can change in a short period of time. Thus the presence of contaminated feeding sites should be considered before ruling out animals as a possible source of fecal indicators. F-specific phages may be useful tracers for modeling viral transmission and tracking feeding habits in birds. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 127–131.

Keywords: viral tracers; fecal indicator sources; viral transmission; coliphages; phage MS2; birds; pigeons; Canada geese

Introduction

Microbial indicators that are commonly used to determine water quality, including fecal coliforms and other bacterial indicators, do not always correlate with human pathogens [20]. Many indicators suggest fecal pollution but do not provide any information regarding the source of the pollution. Some progress has been made in finding indicators that are source-specific. For instance, *Bacteroides fragilis* bacteriophages appear to be specific to human feces [19]. Different serotypes of F-specific phages have been grouped into those that are found mainly in human wastewater and those mainly from animal feces [9,11].

Although animals have frequently been tested to see if they are sources of fecal pollution, conditions that could alter their status as a source have not been studied. We found F-specific phages in the feces of herring gulls (*Larus argentatus*) but not in the feces of geese (*Branta canadensis*) or pigeons (*Columba livia*) [13]. These gulls may have had F-specific phages in their feces, in part, because their diet included fish and invertebrates from Boston Harbor which received partially-treated sewage. Marine animals bioaccumulate phages [10,12,18]. We found F-specific phages in Boston Harbor water [14], therefore it is possible that exposure to these phages in the diet of these birds contributed to F-specific phages in their feces. We added F-specific phage-spiked feed to the diet of geese and pigeons to see if these birds were inherently unable to harbor F-specific phages or were not a source of these phages due to a simple lack of exposure to them. Somatic coliphage concentrations were monitored in the feces of these pigeons and geese to shed light on causes of high fecal phage numbers.

Most studies of phages or other microbial indicators in animal feces examined animals kept in captivity (eg zoo animals, farm animals or domesticated pets) [5,7,9,11], although some wild populations were tested [4,6,13]. If diet affects the presence and/or numbers of fecal indicators, characterizing animal sources of fecal indicators according to species is insufficient. We examined wild and captive geese to see if effects of captivity influenced phage numbers in their feces. We chose to test the effects of captivity on the presence of phages in feces of geese because the feces of wild geese are known to foul fresh water bodies yet the availability of domestic geese may tempt researchers to test their feces instead.

Materials and methods

Microorganisms and media

Microorganisms and media used were the same as previously described [13]. Somatic coliphages were tested on *Escherichia coli* C, and *Salmonella typhimurium* WG49 was used as host for F-specific coliphages [8]. Positive controls for plaque assays for somatic coliphages and F-specific phages were phage ϕX174 and phage MS2 respectively. Tryptone-glucose-yeast extract (TGYE) agar and broth were used for culturing all host bacteria and for all plaque assays. *S. typhimurium* is resistant to kanamycin and nalidixic acid so these antibiotics were added to media used to assay F-specific phages in order to reduce background bacteria [8].

Sample collection and analysis

Sampling and phage enumeration procedures were as previously described [13]. Fecal samples were collected daily. Goose droppings (5–10 g) were collected by sterile tooth-

Correspondence: JJ Cooney, Environmental, Coastal and Ocean Sciences Program, University of Massachusetts Boston, Boston, MA 02125-3393, USA. E-mail: jjc@capecod.net
Received 6 July 1999; accepted 7 November 1999

pick from a golf course in eastern Massachusetts for wild geese and from a pen holding five captive geese at the Franklin Park Zoo in Boston, MA. Pigeon feces (0.2–1 g) were collected by sterile toothpick from nesting areas under highway bridges. All fecal samples were added to TGYE broth in centrifuge tubes, agitated to break up the droppings and brought to the lab on ice. Samples were then centrifuged ($6000 \times g$ for 10 min at 4°C). Dilutions of the supernatant phases (in phosphate-buffered saline) were added along with host bacteria to soft agar and poured over bottom agar according to standard double agar overlay methods [2]. Grab samples of sewage were taken from the influent at a sewage treatment facility, brought to the lab on ice, and tested for phages using the double-agar overlay method. Plates were incubated overnight at 37°C then plaques were counted and recorded according to their morphology. The high variability among fecal samples required us to test large numbers of samples. Each fecal sample required double-agar overlays of several dilutions. Therefore we used a method we developed to pour 12 plates at a time [15].

Phage-spiked bird feces

Fecal samples were tested to see if phage numbers in feces change over time when samples are brought to the lab. The following tests were done on pigeon and on goose feces. Twelve to fourteen fecal samples were collected aseptically and mixed in a sterile plastic centrifuge tube. Approximately 0.5 g of each composite fecal sample was removed at various times, mixed in TGYE broth, centrifuged and the supernatant was tested for F-specific and somatic coliphages. Ten-fold serial dilutions of triplicate samples of each supernatant were spotted onto double-agar overlay plates prepared with host bacteria only. Plates were then incubated overnight at 37°C. Cleared spots indicated the presence of phage(s). The concentration of phages in the original supernatant samples were then determined using most-probable number calculations [1].

Fecal samples were tested for background concentrations of F-specific and somatic coliphages before the samples were spiked with stock solutions of a somatic coliphage (ϕX174) or the F-specific phage MS2. Phage concentrations were then determined in the fecal samples at 0 h, 1 h, 6 h and 24 h after the samples were spiked with the phages. The spiked fecal samples were kept in the lab in plastic centrifuge tubes at room temperature between sample times.

F-Specific phages fed to geese and pigeons

Initial phage counts were made on fecal samples from five Canada geese confined in a pen at the zoo and on fecal samples from a population of 20–40 wild pigeons nesting under highway bridges in order to verify low phage concentrations in their feces. The geese were fed a mixture of cracked corn and food pellets daily. This was occasionally supplemented with lettuce or other vegetable matter. The pigeons were provided with a bird seed mix for several days before, and several days after the day spiked feed was added to their diet. Each morning the food pans were cleaned and refilled with feed. The birds ate intermittently from the feed pans over the course of a day. Feed, mixed with a lysate of phage MS2 to give approximately 10^7 pfu per gram of feed, was fed to the geese and pigeons for one day. Fecal samples were collected once on each of the days indicated in Figure 2. F-specific phage numbers in feces were monitored and changes in phage numbers were observed over time. Somatic coliphage numbers and plaque morphologies of phages from each fecal sample were also recorded.

Somatic coliphages in wild and captive geese

Somatic coliphage counts of fecal samples from free-ranging, wild Canada geese were compared to counts from the five geese confined at the zoo. The wild geese ate grass at the golf course and the captive geese were given a diet of cracked corn and food pellets, occasionally supplemented with lettuce. The pen and food pan of the confined geese were cleaned daily.

Results and discussion

Phage-spiked bird feces

Somatic coliphage and F-specific coliphage concentrations were negligible in the goose fecal samples and in the pigeon feces prior to spiking the feces with phages. No significant differences were seen in F-specific phage or somatic coliphage concentrations in either goose or pigeon feces for up to 24 h after the spike was added (Figure 1). Somatic coliphage concentrations and F-specific coliphage numbers were comparable in goose and in pigeon feces. This suggests that phage inactivation, viral replication or predation of viruses did not cause significant changes in phage concentrations when fecal samples were kept in the lab.

Birds fed F-specific phages

F-specific coliphage counts in the feces of the geese and pigeons were low prior to giving them phage-spiked food. All five geese at the zoo showed F-specific phages in their feces when coliphage MS2 was added to their diet (Figure 2). Phage concentration decreased about 100-fold to low, background levels in a week. These geese did not maintain high concentrations of phages internally since phages were purged from their systems.

All of the wild pigeons sampled showed F-specific

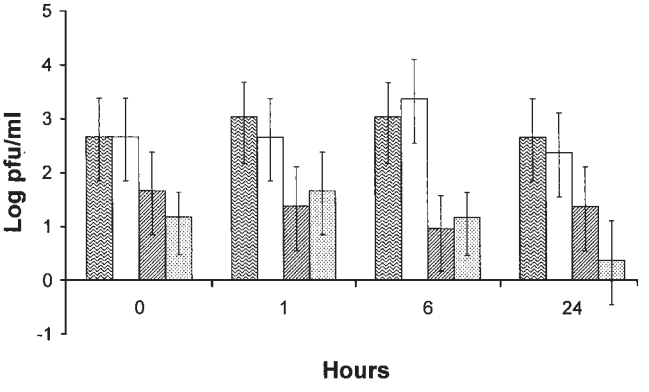

Figure 1 Somatic and F-specific coliphage numbers in phage-spiked pigeon and goose feces over 24 h. Phage ϕX174-spiked feces from pigeons (wavy) and geese (clear). Phage MS2-spiked feces from pigeons (diagonal) and geese (stippled). Error bars are 95% confidence intervals.

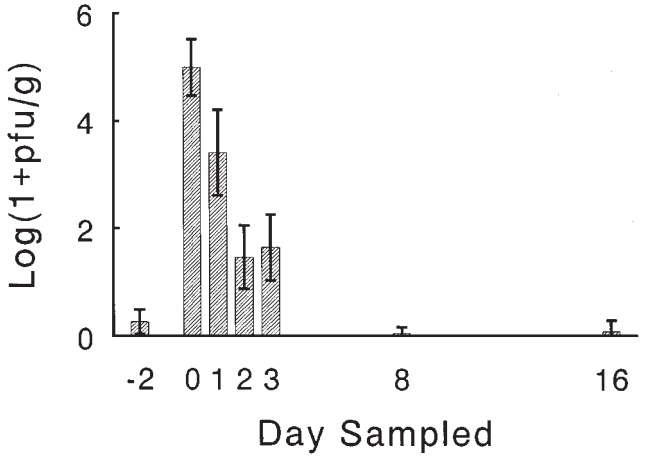

Figure 2 F-specific phages in the droppings of confined geese. Phage-spiked feed was fed to confined geese. Spiked feed was given to the birds on day zero. Error bars are ± two standard errors.

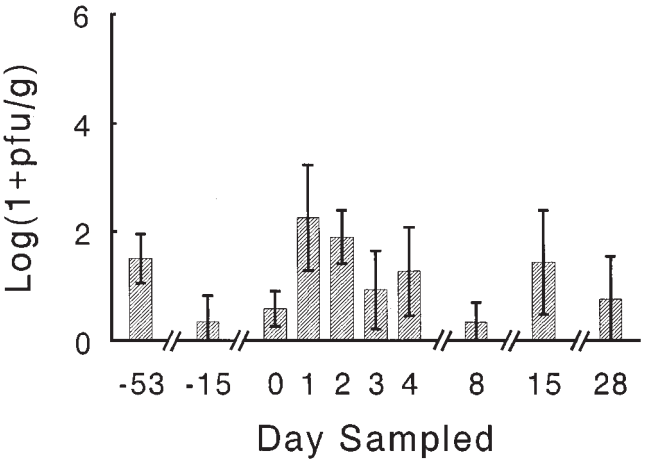

Figure 4 Somatic coliphages in droppings from wild pigeons. Error bars are ± two standard errors.

phages in their feces when MS2 phage-spiked feed was included in their diet (Figure 3). After phage-spiked food was removed, high numbers decreased over a week, as was seen in the geese, but counts leveled off at higher levels. This reflected the greater chronic presence of the phage in the pigeons than was seen in the five captive geese. Such chronic presence of F-specific phages, although low, was higher in pigeons than in geese, presumably since the pigeons lived in an environment allowing more fecal-oral contamination. Chronic levels of F-specific phages in pigeon feces resembled phage counts from somatic coliphages in that plaque counts varied greatly from no plaques to about 10^4–10^5 pfu g^{-1} (Figure 4). These results show that some, but low numbers of, F-specific phages were present in the feces of pigeons and geese. High numbers of F-specific phages in the feces of these birds depend, at least in part, on a phage-contaminated food source. Birds reverted to nominal phage concentrations soon after their diet was changed from a contaminated source.

Phage MS2 is a member of FRNA-phage serotype I. This serotype is found in animal feces and presumably replicates in animals. Phages of this serotype are regarded as indicators of animal sources of fecal pollution [9,11]. Therefore the rapid purging of phage MS2 observed was not expected. The inability of a population of birds to maintain indicator phages impacts the reliability of these phages as indicators of animal fecal pollution. The ability to maintain MS2 may be dependent on the presence of suitable host bacteria or on a proper environment in the birds to allow phage replication on those hosts.

All birds showed F-specific phages in their feces soon after coliphage MS2 was included in their diet yet phage numbers in the birds dropped off when they were no longer fed phage-contaminated food. These results suggest a strong tie between food source contamination and expression of phages in bird feces since replication in the birds did not obscure phage expression due to feeding. Coliphages may be useful tracers of bird feeding habits. Bacteriophages have been useful as tracers of the flow of water [17].

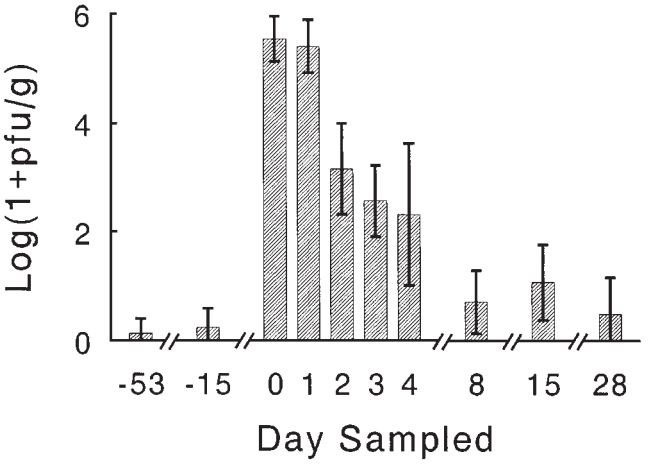

Figure 3 F-specific phages in pigeon droppings. Phage-spiked feed was fed to wild pigeons on day zero. Error bars are ± two standard errors.

Effect of captivity on somatic coliphage numbers in geese

Wild geese had high sample-to-sample variability in somatic coliphage concentrations, with phage numbers up to 10^8 pfu g^{-1} of feces (Figure 5). However, on some days (days labeled b and d in Figure 5), all droppings from geese had low somatic coliphage numbers. The captive geese had consistently low somatic coliphage counts in their droppings on all days tested.

Geese can vary greatly in the degree to which they are a source of indicator phages. The addition of lettuce to the diet of the captive geese had no effect on somatic coliphage concentration. Furthermore, low somatic coliphage concentrations in the feces of wild geese on some days but not others implies a mechanism regulating phage concentrations in these birds that is not strictly dependent on the type of feed. The opportunity for fecal-oral cycling of phages was reduced for the five confined geese since their pen and food pan were cleaned daily. Variations in the wild geese populations' feces may be due to eating from more or less contaminated sites from day to day. Differences in the capacity for fecal-oral contamination is a factor contributing to differences in indicator counts in animals

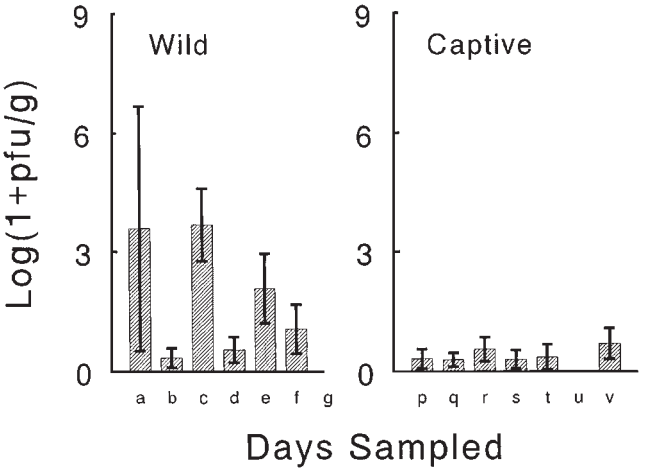

Figure 5 Somatic coliphage concentrations in feces from wild and captive geese. Days sampled are labeled with lower-case letters. Error bars are ± two standard errors.

[4–6,11,13]. Differences in phage concentrations in geese may also be influenced by season since feces from captive geese were collected in the winter but feces from wild geese were collected in the summer.

Geese are not self-contained producers of phages but are dependent on favorable conditions. Perhaps, in this case, recontamination of their food by their own feces was required to maintain phages in their feces. Therefore it may be imprudent to extrapolate indicator phage counts from captive animals to predict indicator phage numbers in wild animals.

Somatic coliphages in pigeons

Based on plaque morphology, we observed only two types of somatic coliphage plaques in the feces of the population of wild pigeons: phages that produced tiny clear plaques and phages that made large clear plaques. Peaks at 0.5 and 6 mm diameter plaques for a typical day's set of pigeon samples (Figure 6) reflects the distribution of the two classes of plaque morphotypes seen in these pigeons. Sewage, on the other hand, showed a variety of plaque morphotypes with a single peak at 1-mm diameter plaques. Plaques from sewage samples showed other signs of variability such as plaques that were faint or haloed. Furthermore, counts of phages in sewage remained fairly constant (about 10^3 pfu ml^{-1}) whereas, on a typical day (eg Jan 25, Table 1), most fecal samples from pigeons had very low numbers of somatic coliphages. The chronic, low somatic coliphage numbers in the pigeon feces were punctuated by an occasional sample with high numbers of plaques of one or the other morphotype. The results presented in Table 1 for January 25 were representative of results observed on seven other occasions ranging from November 1998 to February 1999.

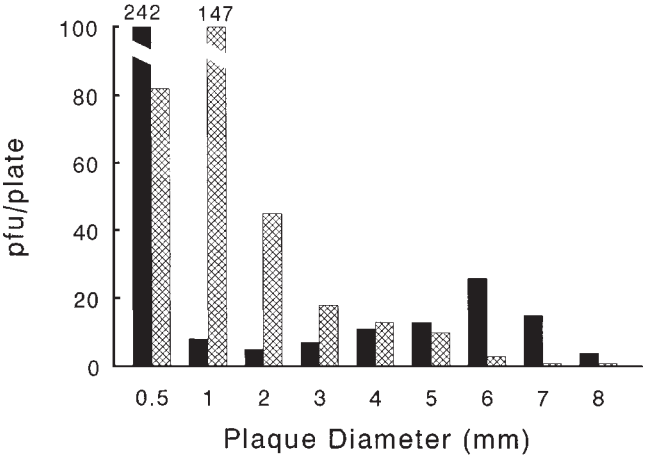

Figure 6 Plaque size distribution of somatic coliphages from pigeon feces (solid bars) and sewage (cross-hatched bars). Distributions are for a representative day's samples.

Somatic coliphage plaque size distributions among pigeon fecal samples for January 23 and January 24 were different from the usual pattern of a few samples with high numbers of one type of plaque among many samples with very few plaques. On Jan 23 (Table 1), seven of twelve samples had large numbers of phages that produced tiny plaques followed, the next day (Jan 24), by a similar number of samples with large numbers of phages that produced large plaques. The duration of these 'epidemics' of each plaque morphotype lasted no more than a day. By contrast, sewage had a much more uniform sample-by-sample and day-by-day somatic coliphage concentration as well as the consistent presence of a variety of plaque morphotypes (data not shown).

This population of pigeons maintains at least two types of phages. Expression of phages in these birds was characterized by bursts of one type of phage in the feces of a few birds among the majority of birds which deposited a background of low numbers of large-plaque and small-plaque phages. Such bursts of phages lasted no more than a day. Thus pigeons can vary greatly in the degree to which they are a source of indicator phages. Only the phages that produced large or small clear plaques were seen in pigeon feces over the time range sampled. The absence of diverse plaque morphotypes in that time suggests that these birds

Table 1 Somatic coliphage plaque size distribution in the feces of wild pigeons

Plaque diameter (mm)								
Jan 23			Jan 24			Jan 25		
0.5	1	>2	0.5	1	>2	0.5	1	>2
6.8	6.8	0	22	13	580	0	0	0
0	0	28	0	5.1	5.1	11	5.3	150
1200	5.9	0	8.2	0	8.2	0	0	4.5
83000	190	0	8.2	0	0	0	0	0
1300	71	0	17	5.8	220	9700	0	0
0	0	0	0	0	33	0	0	20
670	0	47	0	0	170	0	0	0
1100	0	0	12	3.9	130	19	6.5	6.5
850	0	0	0	0	3.3	33	0	0
0	0	0	0	0	49	0	0	0
3300	0	0	22	25	1400	0	0	0
0	0	0	210	101	880	0	0	0

Size distributions listed for January 23, 24 and 25 corresponded to days 1, 2 and 3 in Figures 3 and 4. Fecal samples were picked randomly each day, therefore samples for rows across days are not from the same birds. Values are pfu g^{-1}.

were isolated from new sources of phages and that there was minimal spread of viruses between different populations of pigeons in this region. Coliphages may be useful models of the propagation of viruses in populations of birds [3,16].

The increases in coliphage concentrations of one type of phage in the feces of pigeons seen on January 23 and 24 were not due to changes in the type of food alone. Such 'epidemics' did not appear on all days when the pigeons were fed bird seed. For example, the feces of pigeons not fed bird seed had somatic coliphage distributions that resembled distributions from the feces of pigeons that were fed bird seed for several days. Also, the increases of phages in the feces of pigeons were of only one plaque morphotype at a time even though, since the birds' diet was unchanged, there should have been increases of both plaque morphotypes at the same time.

Sometimes the pigeons would leave feces in the feed pans. One of the rare fecal samples with high numbers of either large-plaque-forming or small-plaque-forming phages among the feed could cause the infection of many birds. Presumably, the 'epidemics' of each type of phage seen on January 23 and 24 (Table 1) were caused by birds eating feces-contaminated seed. This implies sporadic fecal-oral contamination for the maintenance of phages in these birds.

Fecal samples taken from wild pigeons, both in summer [13] and winter, showed high variability in somatic coliphage numbers. Summer pigeon fecal samples had higher somatic coliphage concentrations and a greater proportion of samples with somatic coliphages than fecal samples taken in winter (data not shown).

Conclusions

These data show that pigeons and geese that are not normally a source of some phages can be a source if their food is contaminated with the phages. The phage concentrations revert to nominal levels when the contaminated food source is removed. Since the presence of phages in the diet of birds can influence whether the animals are a source of these indicators, any study concerned with sources of fecal pollution must consider the feeding habits of the animal source. Since birds can rapidly change the degree to which they express phages in their feces, the presence of high numbers of coliphages in the feces of birds may be an indication of nearby fecal-contaminated areas where the birds feed.

Acknowledgements

We are grateful to Fred Beall, Curator for Birds and Reptiles and to other employees at the Franklin Park Zoo, Boston, MA. We are also grateful to Bill Hedlund, Braintree Director of Parks and Recreation and employees at the Braintree Municipal Golf Course. This work was supported, in part, by a NATO grant (Envir. LG 950387).

References

1 APHA. 1965. Standard Methods for the Examination of Water and Wastewater. pp 608, American Public Health Association, New York.
2 Adams MH. 1959. Bacteriophages. Interscience Publishers, New York.
3 Brussow H, O Nakagomi, N Minamoto and W Eichhorn. 1992. Rotavirus 993/83, isolated from calf faeces, closely resembles an avian rotavirus. J Gen Virol 73: 1873–1875.
4 Calci KR, W Burkhardt, WD Watkins and SR Rippey. 1998. Occurrence of male-specific bacteriophage in feral and domestic animal wastes, human feces, and human-associated wastewaters. Appl Environ Microbiol 64: 5027–5029.
5 Dhillon TS, EKS Dhillon, HC Chau, WK Li and AHC Tsang. 1976. Studies on bacteriophage distribution: virulent and temperate bacteriophage content of mammalian feces. Appl Environ Microbiol 32: 68–74.
6 Grabow WOK, TE Neubrech, CS Holtzhausen and J Jofre. 1995. *Bacteroides fragilis* and *Escherichia coli* bacteriophages: excretion by humans and animals. Water Sci Technol 31: 223–230.
7 Havelaar AH, K Furuse and WM Hogeboom. 1986. Bacteriophages and indicator bacteria in human and animal faeces. J Appl Bacteriol 60: 255–262.
8 Havelaar AH and WM Hogeboom. 1984. A method for the enumeration of male-specific bacteriophages in sewage. J Appl Bacteriol 56: 439–447.
9 Havelaar AH, WM Pot-Hogeboom, K Furuse, R Pot and MP Hormann. 1990. F-specific RNA bacteriophages and sensitive host strains in faeces and wastewater of human and animal origin. J Appl Bacteriol 69: 30–37.
10 Lucena F, J Lasobras, D McIntosh, M Forcadell and J Jofre. 1994. Effect of distance from the polluting focus on relative concentrations of *Bacteroides fragilis* phages and coliphages in mussels. Appl Environ Microbiol 60: 2272–2277.
11 Osawa S, K Furuse and I Watanabe. 1981. Distribution of ribonucleic acid coliphages in animals. Appl Environ Microbiol 41: 164–168.
12 Paul JH, JB Rose, SC Jiang, CA Kellogg and L Dickson. 1993. Distribution of viral abundance in the reef environment of Key Largo, Florida. Appl Environ Microbiol 59: 718–724.
13 Ricca DM and JJ Cooney 1998. Coliphages and indicator bacteria in birds around Boston Harbor. J Ind Microbiol Biotechnol 21: 28–30.
14 Ricca DM and JJ Cooney. 1999. Coliphages and indicator bacteria in Boston Harbor, MA. Environ Toxicol 14: 404–408.
15 Ricca DM and JJ Cooney. 1999. Screening environmental samples for source-specific bacteriophage hosts using a method for the simultaneous pouring of 12 Petri plates. J Ind Microbiol Biotechnol 24: 124–126.
16 Rohwedder A, KI Schutz, N Minamoto and H Brussow. 1995. Sequence analysis of pigeon, turkey, and chicken rotavirus VP8* identifies rotavirus 993/83, isolated from calf feces, as a pigeon rotavirus. Virology 210: 231–235.
17 Sinton LW and SB. Ching. 1987. An evaluation of two bacteriophages as sewage tracers. Water, Air and Soil Pollut 35: 347–356.
18 Sobsey MD and L Jaykus. 1991. Human enteric viruses and depuration of bivalve mollusks. In: Molluscan Shellfish Depuration (Otwell WS, GE Rodrick, RE Martin, eds), pp 71–114, CRC Press, Boca Raton.
19 Tartera C and J Jofre. 1987. Bacteriophages active against *Bacteroides fragilis* in sewage-polluted waters. Appl Environ Microbiol 53: 1632–1637.
20 Toranzos GA, CP Gerba and H Hanssen. 1988. Enteric viruses and coliphages in Latin America. Toxicity Assessment 3: 491–510.