Comparison of decay rates of faecal indicator organisms in recreational coastal water and sediment

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Abstract
A laboratory based microcosm study utilising intact non-sterile sediment cores was undertaken to determine the survival of the faecal indicator organisms *Escherichia coli*, *Enterococcus faecium* and somatic coliphage in both recreational coastal water and sediment. Overlying water was inoculated with the test organisms and incubated at 10°C, 20°C or 30°C. *E. coli*, enterococcus and coliphage were enumerated from the water column and sediment by the membrane filtration method, Enterolert (IDEXX Laboratories) and the double-agar overlay methods respectively on days 0, 1, 2, 7, 14 and 28 following inoculation. It was demonstrated that for all organisms, greater decay (k; d^{-1}) occurred in the water column compared to sediment. Sediment characteristics were found to influence decay, with lowest decay rates observed in sediment consisting of high organic carbon content and small particle size. Decay of *E. coli* was significantly greater in both the water column and sediment compared with enterococcus and coliphage under all conditions. Decay of enterococcus was found to closely resemble that of coliphage decay. Survival of all organisms was inversely related to temperature, with greatest decay at 30°C. However, increased temperature had a less significant impact on survival of enterococcus and coliphage compared with *E. coli*. The importance of this study for estimating risk from recreational exposure is great if some pathogenic microorganisms behave similarly to the organisms tested in this study. In particular if survival rates of pathogens are similar to enterococcus and coliphage, then their ability to accumulate in coastal sediment may lead to an increased risk of exposure if these organisms are resuspended into the water column due to natural turbulence or human recreational activity.

Keywords
coliphage; enterococcus; *Escherichia coli;* indicator organisms; microcosm; recreational water; sediment

INTRODUCTION

Bathing in recreational coastal waters subject to high levels of faecal contamination is known to increase the risk of disease, in particular gastroenteritis, but also non-enteric diseases caused by respiratory, eye, ear and skin infections (Kay *et al*., 1994; Fleisher *et al*., 1996). In Australia, the level of faecal contamination in recreational coastal waters is estimated by the enumeration of faecal coliforms and *Escherichia coli* from the water column (ANZECC, 1992). These indicator organisms were traditionally selected as they were believed to behave similarly to pathogenic organisms of faecal origin when released into the environment. The ability of faecal coliforms to act as indicators of enteric viral and protozoan pathogens has, however, been questioned (Hood and Ness, 1982; Koh *et al*., 1994; Ferguson *et al*., 1996).

Organisms released into the coastal environment are subjected to numerous stressors such as temperature change, salinity, nutrient deficiencies, sunlight and predation (Davies *et al*., 1995; Mezrioui *et al*., 1995; Özkanca and Flint, 1997; Thomas *et al*., 1999). Studies have demonstrated that organisms associated with suspended particles and sediment contribute greater numbers than in the water column under many in-situ conditions (Davies *et al*., 1995; Goulder, 1977; Obiri-Danso

and Jones, 2000; Shiaris *et al.*, 1987). Pathogenic microorganisms associated with sediment particles have the possibility of being resuspended back into the water column due to natural turbulence or human recreational activity (Irvine & Pettibone, 1993; Obiri-Danso & Jones, 2000).

To determine the capability of *E. coli* to act as an accurate estimate of the risk of other organisms of faecal origin being present in recreational coastal water, a laboratory based microcosm experiment utilising intact sediment cores was undertaken. The decay rate of *E. coli* in both overlying water and the surface sediment layer was determined and the influence of sediment characteristics and temperature were investigated. These decay rates were then compared with those observed for the other potential faecal indicator organisms, enterococcus and somatic coliphage, under the same conditions. This research attempts to more accurately environmental exposure in the first stage of a health risk assessment for recreational coastal waters.

METHODOLOGY

Sediment samples
Intact sediment cores were taken at low tide from Henley Beach South, Onkaparinga River and the Port Adelaide River along the greater metropolitan Adelaide coastline, South Australia. These sites were chosen to represent differences in sediment characteristics in addition to all being sites of known recreational contact. Perspex columns (70 mm diameter, 310 mm length) were inserted into sediment and overlying water at respective sites to a depth of approximately 100 mm. The top of the column was capped with a rubber bung to aid the removal of the core from the sediment. The sediment core was kept in place by inserting a combination of neoprene (5 mm thick) and closed-cell foam (20 mm thick) bungs into the bottom of the core. This prevented the movement of both sediment and water from the column.

Sediment was characterised by particle size analysis (percentage sand, silt and clay) using the pipette method (Sheldrick & Wang, 1993). The percent of organic carbon present in the sediment was determined by the dichromate method (Tiessen & Moir, 1993).

Microcosm design
Intact sediment cores were placed in a water bath held at a constant temperature (10°C, 20°C or 30°C). Overlying water was removed and replaced with 500 mL of water from one site to reduce the effect of differences between total dissolved solids (TDS) concentration and volume between cores. Overlying water pH and redox potential (HACH Ltd) and conductivity (Hanna) were measured with hand held meters when microcosms were sampled. The redox potential of the top 1 cm of sediment was also measured at time of sampling.

Preparation of indicator organism cultures
Stock bacterial suspensions of *E. coli* (ATCC 25922) and *Enterococcus faecium* (ATTC 19434) were prepared by inoculation into 10 mL nutrient broth (Oxoid) and incubated overnight at 37°C. Cells were harvested by centrifugation at 2,500 g for 10 minutes. The pelleted samples were resuspended in 1 ml of 0.1 M phosphate buffered saline (PBS) at pH 7.2, washed by centrifugation at 8,000 g, followed by resuspension in 1.5 ml PBS.

For preparation of coliphage suspension, host *E. coli* (FCC 84) cells were inoculated into 100 ml nutrient broth (Oxoid) and incubated overnight at 37°C. Raw primary-treated sewage (100 mL) was added to this suspension and incubated a further 24 h at 37°C to increase the number of viral particles. Coliphage were isolated by a double-agar overlay method. Briefly, 4 mL of sample, or an

appropriate dilution, was added to 4 mL molten nutrient agar (maintained at 50°C in a water bath) and 250 μL of host *E. coli* suspension, mixed and poured onto a petri dish containing a base layer of nutrient agar. To obtain a single coliphage suspension, a plaque isolated from the raw sewage was sub-cultured into a fresh 100 mL suspension of host *E. coli* in nutrient broth and incubated at 37°C for 24 h. To remove host bacterial cells, the suspension was centrifuged at 8,000 g for 3 min and the supernatant (containing coliphage) was filtered through a 0.2 μm syringe filter (Satoris) to further remove any bacterial cells present and collected in a sterile container. All stock suspensions were maintained at 4°C until use.

Determination of indicator organism survival
Microcosms were inoculated by adding 500 μL of stock coliphage suspension and 50 μL of stock *Enterococcus faecium* suspension into the overlying water. To prevent the possible interference between *E. coli* and coliphage survival, separate columns were inoculated with 50 μL of stock *E. coli* suspension. In addition to sediment and overlying water, columns containing only water were inoculated.

Indicator organisms were enumerated from both overlying water and sediment on days 0 (1 h after inoculation), 1, 2, 7, 14 and 28 following inoculation. Sediment samples were prepared by removing the top 10 mm of sediment from the intact core, of which 25 g was placed into 75 mL of 0.1% peptone in a sterile beaker. Sediment was sonicated in a sonication bath for 10 min, stirred and sonicated a further 10 min to separate bacteria from sediment particles. This method has been determined to be very effective for separating microorganisms from sediment particles (Craig *et al*. 1998).

E. coli in sediment and water samples were enumerated by membrane filtration (GN-6, Gellman) and incubation on membrane lauryl sulphate agar (Oxoid) (Australian Standard AS 4276.7, 1995). Plates were incubated at 30°C for four hours, followed by 44°C for 18 h. Enterococcus were enumerated from overlying water and sediment using the Enterolert (IDEXX Laboratories) method and quantified using 97-well Quanti-trays. The double-agar overlay method, as described above, was used to isolate coliphage from both overlying water and sediment.

Potential loss of indicator organisms in the water column through adhesion to biofilm on the surface of the microcosm walls was determined. Biofilm was isolated by scraping a marked 20 mm × 20 mm area on the inside surface of the microcosm column using a sterile cotton swab. The swab was mixed vigorously in 9 mL of 0.1% peptone water (Oxoid) and *E. coli*, enterococcus and coliphage were enumerated by the methods previously described.

The decay rate constant (k) was calculated as the slope of the line when $\log_{10} (N_t/N_0)$ was regressed against time, where N_t is the number of bacteria at time t and N_0 is the number of bacteria at time 0 (Davies and Evison, 1991). The decay rate constants could then be used to calculate T_{90} values, which are the times required for a 1-$\log_{10}$ reduction in organism concentration (Pesaro *et al*., 1995).

Statistical Analysis
Statistical analysis was undertaken using one-way ANOVA and significance expressed at $P \leq 0.05$ (SPSS version 10.0.5, 1999). All results were expressed as the mean ± standard deviation of three determinations.

RESULTS AND DICUSSION

Sediment characterisation, as determined by particle size analysis and organic carbon content, identified distinct differences between sediments from the three sites investigated (Table 1). Sediment from Henley Beach consisted mainly of sand, with very low proportions of silt, clay and organic carbon. Sediment from Port Adelaide, however, contained much greater proportions of silt, clay and organic carbon. Onkaparinga sediment could be considered as intermediate. The influence of sediment characteristics on survival of indicator organisms could therefore be investigated.

Table 1. Particle size analysis. Particles sizes for sand 2 – 0.2 mm; silt 0.2 mm – 2 μm; clay < 2 μm. All results normalised to 100%.

SITE	%SAND	%SILT	%CLAY	%ORGANIC C
Henley Beach	98.47	0.08	1.41	0.05
Onkaparinga	95.48	1.26	2.91	0.35
Port Adelaide	83.05	4.24	10.33	2.38

Examples of the decay of the three indicator species investigated in both overlying water and sediment (Figure 1 a, b and c) show clearly the differences in survival both within sediment types and between sediment and water. When decay rates were plotted (eg. Fig 1d) they largely followed first-order kinetics as illustrated by r^2 values in Tables 2 – 4. Thus, results were expressed as decay rate constants (k), with more negative decay rates indicating a more rapid decline in the number of organisms enumerated.

The incorporation of *E. coli*, enterococcus and coliphage into biofilm attached to the surface of the column was not significant (results not shown). The disappearance of organisms observed from the water column could therefore be explained as either inactivation or partitioning into the surface sediment layer and not simply incorporation into biofilm.

The decay of *E. coli* in both the water column and sediment was greater compared to enterococcus and coliphage at all temperatures (represented by more negative decay rates). This was illustrated in the survival curves, with enterococcus and coliphage persisting in the water column for longer periods of time compared with *E. coli* (Figs 1 a - c). When directly comparing results with other studies, experimental differences must be recognised. This study utilised intact, non-sterile sediment cores which include added pressures on organism survival through competition with natural fauna, presence of predators and association with sediment nutrients and particles. It has been suggested that autoclaving sediment may increase the transfer of nutrients from the sediment into the water column, therefore influencing survival (Gerba and McLeod, 1976). The decay rates of *E. coli*, and other pathogenic microorganisms, have been determined to be greater in unfiltered (non-sterile) water compared with filtered water (Flint, 1987; Rhodes and Kator, 1988). The increased survival of enterococcus compared with *E. coli* in seawater (using in-situ diffusion chambers) has previously been reported by Vasconcelos and Swartz, (1976). A study by Sinton *et al*. (1999) identified a greater survival of somatic coliphage in seawater compared with *E. coli*. The increased decay rate of *E. coli* in both overlying water and sediment compared with enterococcus and coliphage is of concern, raising concerns about its ability to be used as an indicator of the presence of other pathogenic organisms of faecal origin in the water column.

An inverse relationship was identified between temperature and indicator organism survival in both overlying water and sediment. Greatest decay occurred in the water column when incubated at 30°C (Tables 2 – 4). Temperature was found to have a more significant impact on *E. coli* survival compared with enterococcus and coliphage. In particular, increased temperature had a relatively

insignificant impact on survival of coliphage in sediment. These results compare with other studies demonstrating prolonged survival of indicator organisms at lower temperatures (Flint, 1987; Rhodes and Kator, 1988, Sinton *et al*., 1999; Özkanca and Flint, 1997; Wait and Sobsey, 2001). In a study by Özkanca and Flint (1997) it was demonstrated *E. coli* incubated at 37°C displayed greater signs of stress, as determined by decreased electron transport system and respiratory enzyme activity and reduced cell size, compared with organisms incubated at lower temperatures. This may partially explain the increased decay rates observed at higher temperatures observed in this current study.
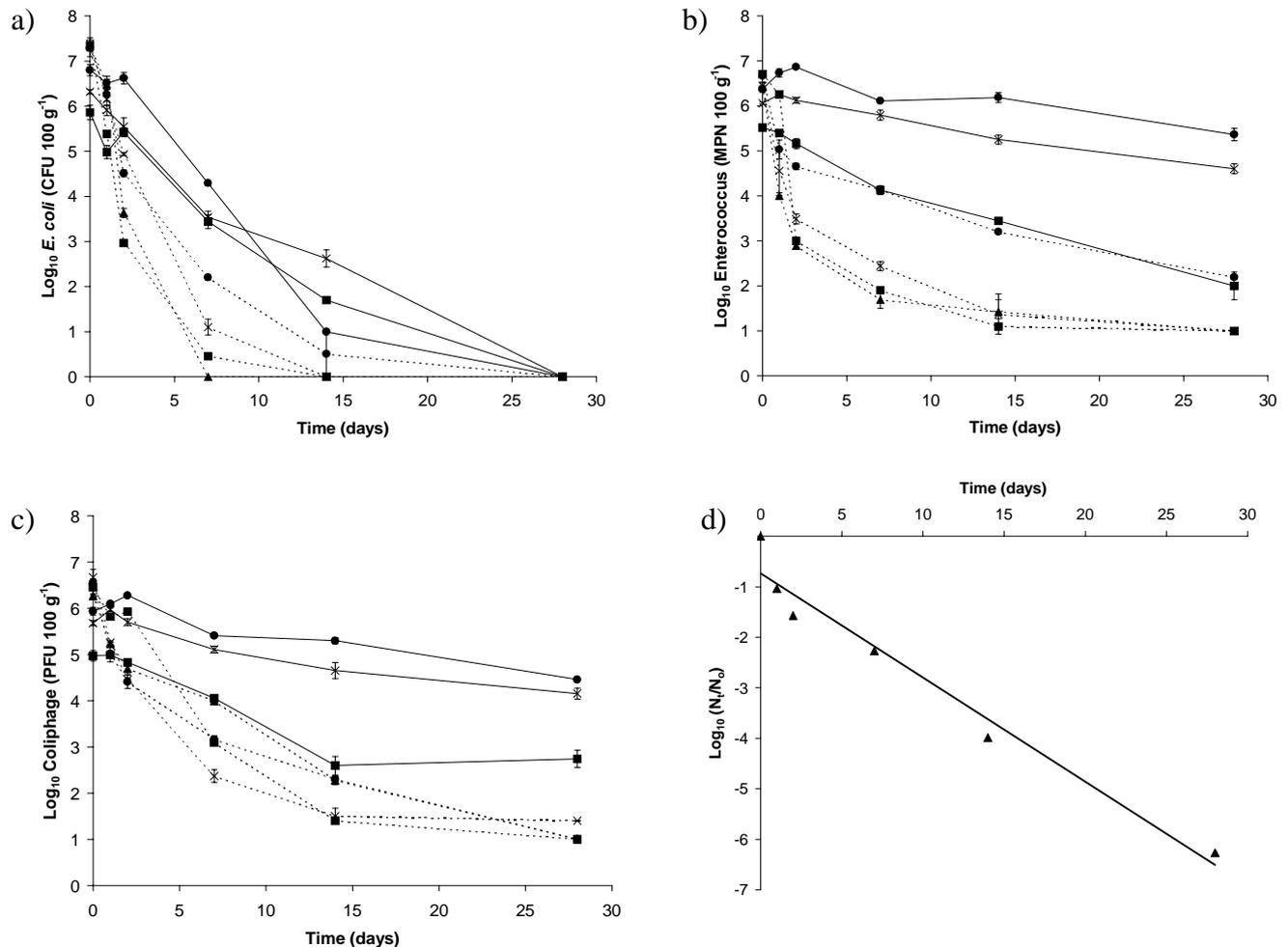
Figure 1 Survival curves for *E. coli* (a), enterococcus (b) and coliphage (c), in sediment (—) and water (----) from Henley Beach (■), Onkaparinga (×), Pt. Adelaide (•) and control column containing water only (▲) all incubated at 20°C. Figure 1 (d) an exponential decay plot used to calculate decay rate constants (coliphage decay in a microcosm containing water only incubated at 20°C).

Under all experimental conditions, greater decay rates were observed in overlying water compared to sediment. Enterococcus and coliphage persisted in sediment for more than 28 days at all temperatures. Decay rates observed for both coliphage and enterococcus in sediment remained relatively constant irrespective of temperature. The decay rates for these organisms in the water column, however, increased at higher temperatures. At Pt Adelaide, coliphage in sediment incubated at 30°C decreased from an initial concentration of 4.9×10^5 PFU 100 g^{-1} to 7.0×10^3 PFU 100 g^{-1} after 28 d. In contrast the concentration in the overlying water had decreased from 5.8×10^5 PFU 100 g^{-1} to 42 PFU 100 mL^{-1} after 7 d.

Table 2. Decay rate constants (k; d^{-1}) for *E. coli* in water and sediment; *P <0.05.

| | Water | | | | | | Sediment | | | | | |
| | 10 °C | | 20 °C | | 30 °C | | 10 °C | | 20 °C | | 30 °C | |
	k	r^2	k	r^2	k	r^2	k	r^2	k	r^2	k	r^2
Henley Beach	-0.47	0.80*	-0.89	0.86	-1.74	0.95*	-0.32	0.93*	-0.32	0.98*	-1.11	0.95*
Onkaparinga	-0.24	0.90*	-0.52	0.89*	-0.91	0.89*	-0.13	0.89*	-0.22	0.97*	-0.87	0.99*
Port Adelaide	-0.21	0.86*	-0.45	0.89*	-0.95	0.89*	-0.14	0.97*	-0.49	0.98*	-0.58	0.96*
Water only	-1.04	0.88	-1.03	0.94*	-2.13	0.84						

Table 3. Decay rate constants (k; d^{-1}) for enterococcus in water and sediment; *P <0.05.

| | Water | | | | | | Sediment | | | | | |
| | 10ºC | | 20ºC | | 30ºC | | 10ºC | | 20ºC | | 30ºC | |
	k	r^2	k	r^2	k	r^2	k	r^2	k	r^2	k	r^2
Henley Beach	-0.36	0.93*	-0.66	0.74	-0.80	0.95*	-0.08	0.92*	-0.12	0.97*	-0.16	0.95*
Onkaparinga	-0.20	0.85*	-0.30	0.78*	-2.40	0.94*	-0.04	0.75	-0.06	0.91*	-0.09	0.74*
Port Adelaide	-0.14	0.72*	-0.13	0.81*	-0.13	0.54	-0.04	0.74	-0.06	0.90*	-0.10	0.95*
Water only	-0.35	0.90*	-0.54	0.71	-0.73	0.91*						

Table 4. Decay rate constants (k; d^{-1}) for coliphage in water and sediment; *P <0.05.

| | Water | | | | | | Sediment | | | | | |
| | 10ºC | | 20ºC | | 30ºC | | 10ºC | | 20ºC | | 30ºC | |
	k	r^2	k	r^2	k	r^2	k	r^2	k	r^2	k	r^2
Henley Beach	-0.19	0.89*	-0.40	0.98*	-0.60	0.95*	-0.06	0.73	-0.18	1.00*	-0.08	0.83
Onkaparinga	-0.16	0.93*	-0.34	0.87*	-0.50	0.82	-0.04	0.92*	-0.06	0.91*	-0.04	0.52
Port Adelaide	-0.12	0.75*	-0.20	0.91*	-0.55	0.90	-0.04	0.92*	-0.06	0.90*	-0.06	0.92*
Water only	-0.17	0.97*	-0.21	0.96*	-0.58	0.99*						

Sediment characteristics were found to influence survival of *E. coli*, enterococcus and coliphage. In general, of the columns containing sediment, greatest decay occurred in sediment from Henley Beach, which consisted mainly of sand (large particle size). Small particle size and high organic carbon content were found to be more conducive to microbial survival. Greatest decay overall was observed in the column containing water only. A study by Howell *et al*. (1996) also identified a significantly greater decay of both *E. coli* and enterococcus in sediment consisting of large particle size, compared with sediments consisting of greater proportions of silt and clay. A weak correlation between small particle size and decreased bacterial decay has also been reported for freshwater sediments (Burton *et al*., 1987). The greatest decay observed in the column containing water only suggests nutrient availability may be a very influential factor in determining microbial survival under the conditions tested.

CONCLUSION

Results of this study demonstrated a reduced decay rate (increased survival) of faecal indicator organisms in coastal sediment compared with overlying water. Small particle size and high organic carbon content were found to be more conducive to survival. The ability of *E. coli* to persist in the coastal environment was significantly less than enterococcus and coliphage, suggesting limited effectiveness for its use as an indicator of all faecal contamination. The fact that enterococcus survival more closely resembled that of coliphage survival suggests that it may provide a better estimate of exposure to pathogenic microorganisms of faecal origin, especially viruses.

This research questions the usefulness of setting guideline faecal indicator organism concentrations using the water column alone to estimating health risk. If pathogenic microorganisms behave similarly to the organisms tested in this study, in particular to enterococcus and coliphage, then their ability to accumulate in coastal sediment may lead to an increased risk of exposure if these organisms are resuspended into the water column due to natural turbulence or human recreational activity. Further studies are required, however, to determine the decay of pathogenic microorganisms of faecal origin and estimate their likely rate of resuspension from sediment into the water column.

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