

Use of microcosms to determine persistence of *Escherichia coli* in recreational coastal water and sediment and validation with *in situ* measurements

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ABSTRACT

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Aims: To determine the persistence of the faecal indicator organism *Escherichia coli* in recreational coastal water and sediment using laboratory-based microcosms and validation with *in situ* measurements.

Methods and Results: Intact sediment cores were taken from three distinct coastal sites. Overlying estuarine water was inoculated with known concentrations of *E. coli* and decay rates from both overlying water and sediment were determined following enumeration by the membrane filtration method at fixed time intervals over a 28-day period. It was demonstrated that *E. coli* may persist in coastal sediment for >28 days when incubated at 10°C. *Escherichia coli* survival was found to have an inverse relationship with temperature in both water and sediment. In general the decay rate for *E. coli* was greater in water than in sediment. Small particle size and high organic carbon content were found to enhance *E. coli* survival in coastal sediments in the microcosms.

Conclusions: Results of this microcosm study demonstrated the more prolonged survival of *E. coli* in coastal sediments compared with overlying water, which may imply an increased risk of exposure because of the possible resuspension of pathogenic micro-organisms during natural turbulence or human recreational activity.

Significance and Impact of the Study: A more accurate estimate of exposure risk has been described which may subsequently be used in a quantitative microbial risk assessment for recreational coastal waters.

Keywords: *Escherichia coli*, microcosm, persistence, recreational water, sediment, survival.

INTRODUCTION

Organisms released into the aquatic environment are exposed to numerous factors which may cause stress including temperature change, salinity, nutrient deficiencies, predation and sunlight (Davies *et al.* 1995; Özkanca and Flint 1997; Thomas *et al.* 1999). Studies have indicated that greater numbers of micro-organisms are attached to suspended particles and sediment than those free in surface waters under both environmental and laboratory conditions (Goulder 1977; Shiaris *et al.* 1987; Fish and Pettibone 1995;

Crump and Baross 1996). In coastal waters this may indicate a potential increase in the risk of infection to human beings because of the resuspension of potentially pathogenic micro-organisms from the surface sediment layer during recreational activities.

It has been demonstrated that exposure to coastal water subject to faecal contamination during recreation may increase the risk of disease, in particular gastroenteritis, but also nonenteric diseases caused by respiratory, eye, ear and skin infections (Fleisher *et al.* 1993, 1996; Kay *et al.* 1994). The organism *Escherichia coli* is commonly used as a faecal indicator organism. It is assumed that *E. coli* behaves in a similar manner to other bacteria of faecal origin when released into the environment. Known inadequacies exist for the use of *E. coli* as an indicator organism, but its widespread

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use continues in the absence of any viable alternatives (Hood and Ness 1982; Koh *et al.* 1994; Ferguson *et al.* 1996).

A study by Flint (1987) demonstrated that *E. coli* incubated at 25°C in autoclaved filtered river water survived for >260 days without any loss of culturability. In contrast the number of *E. coli* inoculated into nonfiltered river water had reduced by 2 log (t_{99}) from the original count in 2.5 days, indicating predation may contribute greatly to the disappearance of *E. coli* under *in situ* conditions (Flint 1987). In addition to predation, the decay of *E. coli* in seawater exposed to sunlight has been determined to be greater than that in freshwater (Davies and Evison 1991; Sinton *et al.* 1999).

Microcosms of varying complexity have been used in numerous studies to investigate the survival of micro-organisms in both water and sediment (Gerba and McLeod 1976; Wagner-Döbler *et al.* 1992; Bordalo 1993; Davies *et al.* 1995; Brenner *et al.* 1999; Thomas *et al.* 1999). Unlike *in situ* experiments, the use of microcosms allows for investigation of the response of micro-organisms to specific environmental conditions in isolation. In this study, a laboratory-based microcosm experiment was undertaken utilizing intact sediment cores taken from three coastal areas, each with distinct sediment characteristics. The effect of temperature on the decay rate of *E. coli* was determined in both overlying water and sediment from these microcosms.

In an effort to validate results observed from the laboratory-based microcosm experiment, the persistence of faecal coliforms in both the water column and sediment at a recreational coastal site following a high rainfall event (and subsequent high bacterial load) was investigated. Henley Beach is situated at the outlet of the Torrens River in metropolitan Adelaide and is used substantially for recreation during the summer months (at the time the study was undertaken). This research aims to more accurately describe environmental exposure in the first stage of a health risk assessment for recreational coastal waters.

MATERIALS AND METHODS

Microcosm design and sampling

For each test condition, six intact sediment cores were collected from Henley Beach, Onkaparinga River (estuary/T) and Port Adelaide River in metropolitan Adelaide (South Australia) (Fig. 1). Particle size analysis was performed on the sediments by the pipette method of Sheldrick and Wang (1993). The percentage of organic carbon present in the sediment was determined by the titrimetric dichromate redox method (Tiessen and Moir 1993).

Prior to sampling, all microcosm equipment was treated using sodium hypochlorite and rinsed with sterile water to remove any micro-organisms which may have been present.

Perspex columns (70 mm diameter, 310 mm length) were inserted into sediment and overlying water at respective sites to a depth of *ca* 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. In addition to these, six columns were filled only with overlying water to determine the decay rate of *E. coli* in the water column alone.

In the laboratory, the microcosms were placed in a water bath and maintained at a constant temperature for the duration of the experimental period (10, 20 or 30°C). Overlying water was removed and replaced with 500 ml of water from the Onkaparinga River estuary to maintain a constant total dissolved solid (TDS) concentration between different sediment types. This removed the potential influence of changes of salinity in overlying water caused by tidal and environmental changes at the different sites. The mean TDS concentrations were $27\,766 \pm 805$, $28\,663 \pm 1889$ and $23\,450 \pm 929$ mg l⁻¹ for the experiments run at 10, 20 and 30°C, respectively. Water pH (HACH, Loveland, CO, USA) and conductivity (Hanna Instruments, Keysborough, Victoria, Australia) were measured with hand held meters when microcosms were sampled.

Inoculation of microcosms

A stock bacterial suspension was prepared by inoculating *E. coli* (ATCC 25922) into 10 ml of nutrient broth and incubating overnight at 37°C. *Escherichia coli* ATCC 25922 was used to allow comparison with other published survival studies. Cells were harvested by centrifugation at 2500 g for 10 min. The pelleted sample was resuspended in 1 ml of 0.1 mol l⁻¹ phosphate-buffered saline (PBS; pH 7.2), washed by centrifugation at 8000 g in a microcentrifuge, followed by resuspension in 1.5 ml PBS. All stock *E. coli* suspensions were stored at 4°C until use. Each column was inoculated by adding 50 µl of stock *E. coli* suspension to overlying water giving a final concentration of *ca* 1×10^7 colony forming units (CFU) 100 ml⁻¹. A set of columns were not inoculated with *E. coli* to act as a negative control. Columns were sparged with air at a rate which was determined not to disturb the upper layer of sediment.

Determination of *E. coli* survival

Both sediment and water from the columns were analysed on days 0 (1 h after inoculation), 1, 2, 7, 14 and 28. A new column was used for each sampling date. Because of a rapid decay of *E. coli* when incubated at 30°C, columns incubated at this

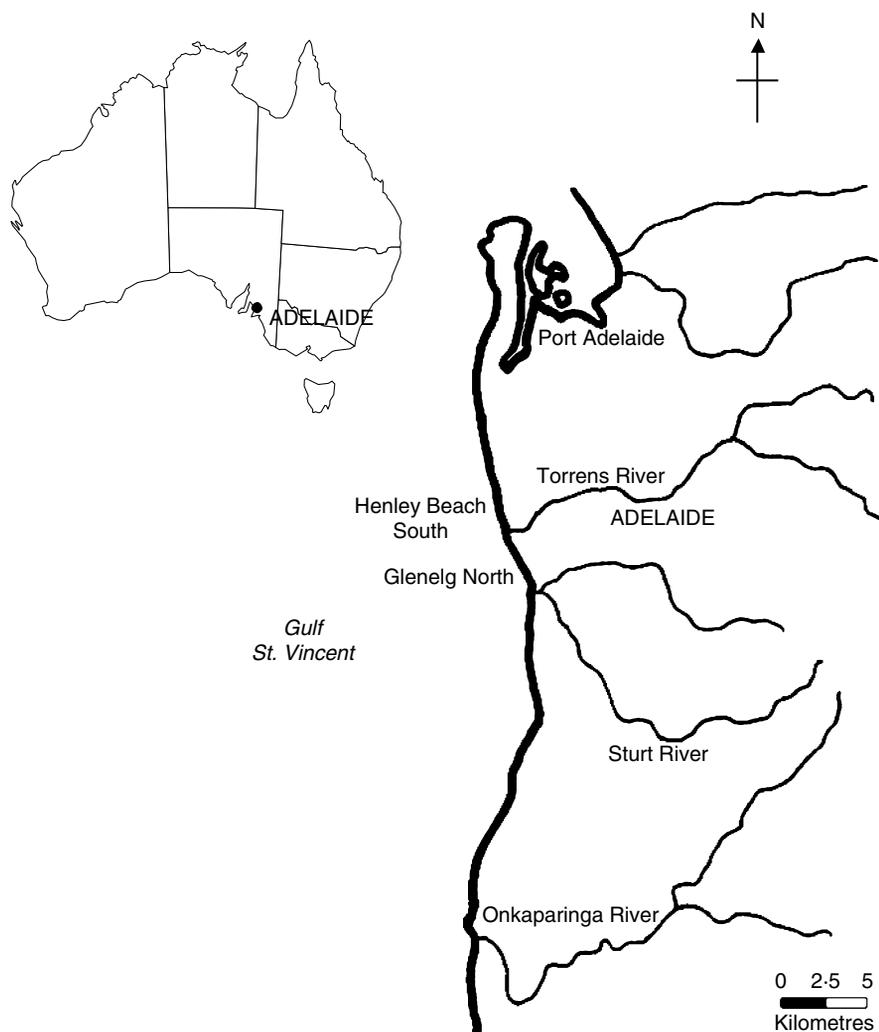


Fig. 1 Adelaide metropolitan coastline, South Australia

temperature were analysed on days 0, 1, 2, 4 and 7 only. The number of *E. coli* present in the water was determined by membrane filtration and incubation on lauryl sulphate agar plates (Oxoid Australia, West Heidelberg, Victoria, Australia) as per Australian Standard AS4276-7, (1995). Results were expressed as number of CFU 100 ml⁻¹.

Sediment samples were obtained by first removing the remaining overlying water. The column was then destructively sampled by placing on a coring device that extruded the sediment at controlled intervals. The top 10 mm of sediment was removed and placed into a sterile beaker. Of this sediment, triplicate samples of 25 g were placed into 75 ml of sterile 0.1% peptone water (Oxoid) and sonicated in a sonication bath for 10 min, stirred and sonicated a further 10 min to separate the bacteria from sediment particles. This method has previously been found to be an effective method to separate micro-organisms from sediment particles (Craig *et al.* 2002). Faecal coliforms were enumerated from the supernatant using

the method previously described. The results for *E. coli* concentration in sediment were expressed as number of CFU 100 g⁻¹ (dry weight) sediment.

To determine the possible loss of *E. coli* from the water column because of adhesion to biofilm on the surface of the column wall, biofilm was sampled by scraping a marked 20 mm × 20 mm area on the inside of columns containing only overlying water with a sterile cotton swab on days 0, 1, 2, 7, 14 and 28. The swab was mixed vigorously in 9 ml of 0.1% peptone water (Oxoid) and *E. coli* were enumerated by the method previously described.

The decay rate constant (k) was calculated as the slope of the line when $\log_{10} (N_t N_0^{-1})$ 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).

In situ samples

In February 2000, triplicate intact sediment cores and grab samples of overlying water (1 l) were taken from Henley Beach South, ca 400 m north of the Torrens River outlet, each day following the rain event for a period of 10 days. The natural current along the Adelaide metropolitan coastline is northward, which can result in large areas of the beach being affected by the contaminated plume emanating from the Torrens River outlet. Faecal coliforms from the surface sediment layer and overlying water were enumerated using the same methods as for the microcosm experiment. Water conductivity (Hanna Instruments), dissolved oxygen (WTW, Weilheim, Germany) and temperature were measured during sampling using hand-held meters at times of sampling.

Statistical analysis

All analyses were undertaken in triplicate and expressed as the mean \pm standard deviation. Slopes of the lines from linear regression (decay rates) were compared by analysis of covariance using Graphpad Prism (version 3.0; Graphpad Software Inc., San Diego, CA, USA). One-way ANOVA was used to determine differences between faecal coliform concentrations in the overlying water compared with the surface sediment layer for *in situ* measurements using SPSS (version 10.0.5; 1999, SPSS Inc., Chicago, IL, USA). Significance for all analyses was expressed at $P \leq 0.05$.

RESULTS

Laboratory-based microcosm study

With respect to sediment, sites were chosen to investigate the effect of sediment type on survival. Sediment from Henley Beach consisted mainly of sand, with very little silt and clay, and low percentage of organic carbon (Table 1). In comparison, Port Adelaide sediment contained much higher proportions of silt, clay and organic carbon. Sediment from the Onkaparinga River could be described as intermediate. At the Port Adelaide River the sediment was distinctly

Table 1 Particle size analysis and organic carbon content of surface sediment layers (≤ 20 mm)

Site	% Sand	% Silt	% Clay	% Organic C
Henley Beach (sand)	98.47	0.08	1.41	0.05
Onkaparinga (intermediate)	95.48	1.26	2.91	0.35
Port Adelaide (clay)	83.05	4.24	10.33	2.38

Particles sizes for sand 2–0.2 mm; silt 0.2 mm to 2 μ m; clay <2 μ m. All results normalized to 100%.

stratified, with the top 2-cm layer comprising anaerobic horizon and below that a layer consisting of a mixture of sand and silt/clay. Only the surface layer (≤ 10 mm) of each column was examined as this would provide the main source of exposure to micro-organisms in any recreational activity.

Monitoring of overlying water demonstrated that conductivity and pH remained stable over the sampling period of 28 days. The adhesion, or inclusion, of *E. coli* into biofilm attached to the surface of the column was not significant (results not shown). The disappearance of micro-organisms observed from the water column could therefore be explained as occurring either by inactivation or partitioning into the surface sediment layer and not simply incorporation into biofilm.

In control microcosms (not inoculated with *E. coli*) only very small numbers of faecal coliforms (or <1 CFU 100 ml⁻¹) were detected in overlying water and sediment suggesting that the presence of indigenous faecal coliforms did not significantly influence results. The survival curves of *E. coli* in both sediment and water at 10, 20 and 30°C are illustrated in Figs 2, 3 and 4, respectively. The initial concentration of *E. coli* in the water column was relatively constant between columns, however, the concentration of *E. coli* enumerated on day 0 from the surface sediment layer slightly varied between sediment types. As the surface sediment layer was analysed 1 h following inoculation, this difference may reflect the intrinsic differences between sediment types, which may lead to variation in settling velocities and/or association with sediment particles. Persistence of *E. coli* was greatly reduced when samples were incubated at 20 and 30°C, respectively, compared with 10°C, while no significant variations were found for Henley Beach sediment at 20°C with respect to 10°C. Even when incubated at 10°C, there was a relatively rapid decline in the

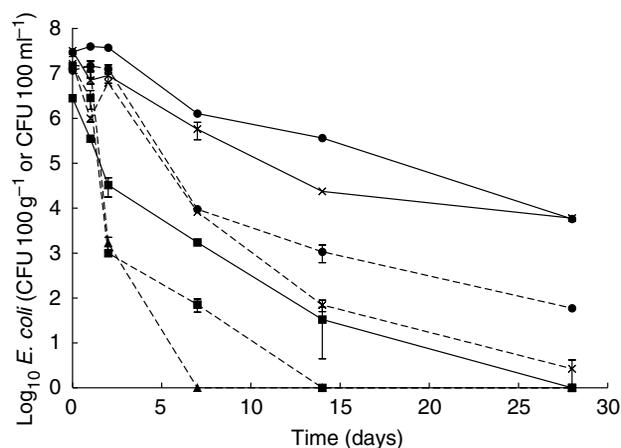


Fig. 2 Survival of *Escherichia coli* incubated at 10°C in sediment (—) and water (---) from Henley Beach (■), Onkaparinga (×), Port Adelaide (●) and column containing water only (▲); (mean \pm S.D.; $n = 3$)

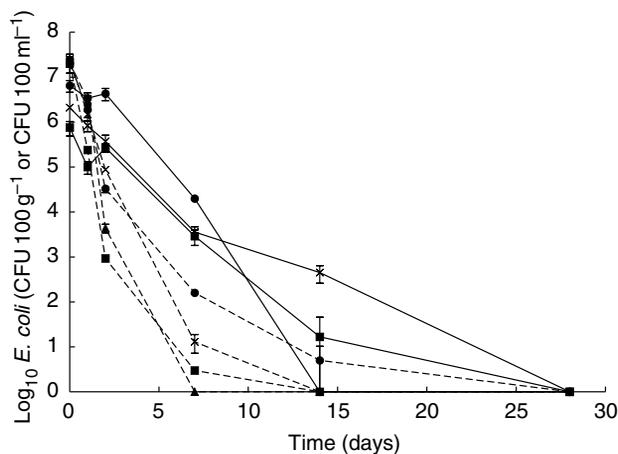


Fig. 3 Survival of *Escherichia coli* incubated at 20°C in sediment (—) and water (---) from Henley Beach (■), Onkaparinga (×), Port Adelaide (●) and column containing water only (▲); (mean ± S.D.; $n = 3$)

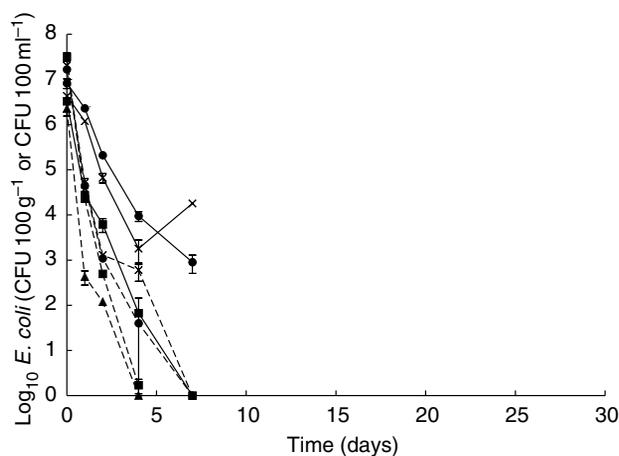


Fig. 4 Survival of *Escherichia coli* incubated at 30°C in sediment (—) and water (---) from Henley Beach (■), Onkaparinga (×), Port Adelaide (●) and column containing water only (▲); (mean ± S.D.; $n = 3$)

concentration of *E. coli* in the overlying water. This was most pronounced in the microcosm containing water only, with no *E. coli* being detected after 7 days. However, in Onkaparinga and Port Adelaide sediments $>5 \times 10^3$ CFU 100 g^{-1} were enumerated after 28 days incubation at 10°C (Fig. 2).

In general, *E. coli* decay (t_{90} , Table 2) plotted as $\log_{10}(N_t \cdot N_0^{-1})$ against time were significantly linear ($P < 0.05$). This was based on the r^2 value of the decay rate plot, and the number of data points (number of samples taken until no *E. coli* detected). At all temperatures, survival was greater (lower decay rates) in sediment compared with that in water (Table 2). The greatest decay was observed in the column containing water only incubated at 30°C ($t_{90} = 0.47$ days). In general, sediment from Henley Beach (mainly sand) was significantly less conducive to the survival of *E. coli* compared with Onkaparinga and Port Adelaide sediment (higher silt, clay and organic carbon content) at all temperatures (Figs 2–4).

In situ measurements

The average monthly rainfall total for Adelaide in February is <20 mm (Australian Bureau of Meteorology). A significant rainfall event occurred in metropolitan Adelaide on 20 February 2000 with a total of 37.8 mm recorded on one day (Fig. 6). In the previous 26 days a total of 1 mm of rainfall was recorded and therefore this event could be described as a 'first flush'. This resulted in a large flow of water from the Torrens River outlet at Henley Beach South, as shown by the increase in TDSs concentration measured during the sampling period (Fig. 5). Temperature was also recorded during this period (Fig. 5), to enable comparison with microcosm experiments. During this period of high rainfall, a large amount of debris, including faecal material originating from the agistment of horses near the Torrens River was deposited onto Henley Beach.

The baseline faecal coliform concentration in overlying water and sediment at Henley Beach prior to the high flow of the Torrens River was relatively low (Fig. 6). The

Table 2 Rate of decay (t_{90} ; days) for *Escherichia coli* in water and sediment

	Water						Sediment					
	10°C		20°C		30°C		10°C		20°C		30°C	
	t_{90}	r^2										
Henley Beach	2.13	0.80*	1.12	0.86	0.57	0.95*	3.13	0.93*	3.13	0.98*	0.90	0.95*
Onkaparinga	4.17	0.90*	1.92	0.89*	1.10	0.89*	7.69	0.89*	4.55	0.97*	1.15	0.99*
Port Adelaide	4.76	0.86*	2.22	0.89*	1.05	0.89*	7.14	0.97*	2.04	0.98*	1.72	0.96*
Water only	0.96	0.88	0.97	0.94*	0.47	0.84						

* $P \leq 0.05$.

Fig. 5 Total dissolved solids concentration (○) and temperature (■) of water at Henley Beach South during the sampling period

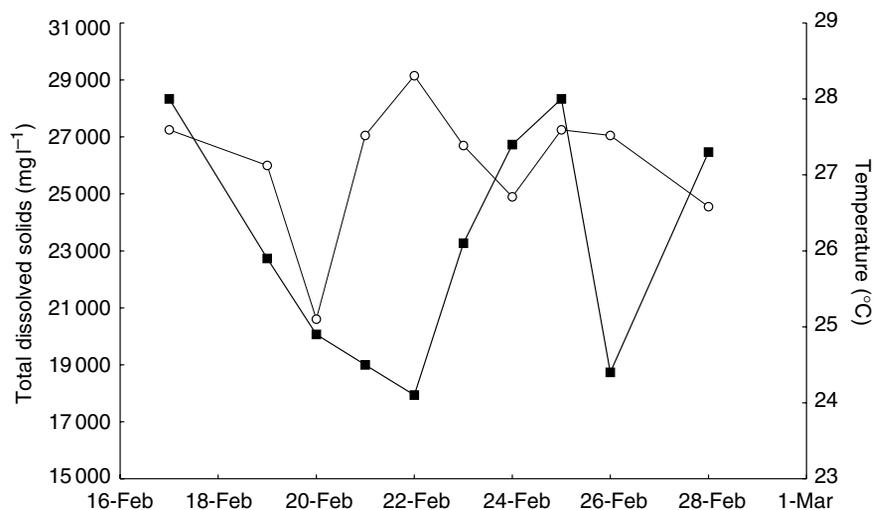
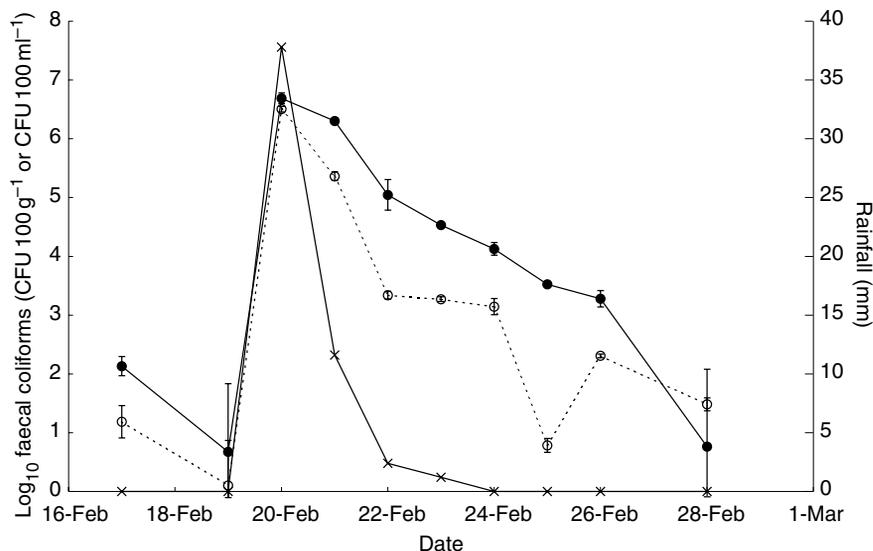


Fig. 6 Concentration of confirmed faecal coliforms in sediment (—●—) and water (---○---) samples taken from Henley Beach (mean \pm S.D.; $n = 3$) and rainfall (—×—) recorded during the sampling period (source: Bureau of Meteorology, Adelaide Airport)



baseline concentration of faecal coliforms in the sediment were, however, greater than in the water column (143 ± 57 CFU 100 g⁻¹ and 17 ± 11 CFU 100 ml⁻¹, respectively). On 20 February, when the high rainfall occurred, there was a dramatic increase in the concentration of faecal coliforms in sediment and water, with $>1 \times 10^6$ CFU 100 g⁻¹ or $>1 \times 10^6$ CFU 100 ml⁻¹ being detected in both matrices, which was similar to the initial concentration used for the microcosm study. Two days following the initial peak flow of the Torrens River, the concentration of faecal coliforms in the water at Henley Beach had decreased to 2.2×10^3 CFU 100 ml⁻¹. In contrast the concentration of faecal coliforms in sediment remained at 1.2×10^5 CFU 100 g⁻¹. The rate of decay (t_{90}) for faecal coliforms was determined to be greater in overlying water

compared with sediment at Henley Beach during the sampling period (1.28 and 1.67 t_{90} per day, respectively).

Comparison between laboratory and *in situ* results

Modelling the microcosm decay rate (t_{90}) against temperature using an exponential relationship,

$$t_{90} = \alpha e^{-\beta T}, \quad (1)$$

where T is temperature (°C), resulted in values for α and β in overlying water of 4.09 and 0.07, respectively ($r^2=1.00$; $n = 3$). For microcosm sediment, α was 5.10 and β was 0.04 ($r^2=0.64$; $n = 3$). Using this exponential model, the estimated t_{90} for *in situ* samples of overlying water and sediment at Henley Beach following the high rainfall event, was 0.75

and 1.79. The modelled estimate for *E. coli* decay rate in overlying water was somewhat lower than the measured value (measured t_{90} of 1.28 compared with modelled value of 0.75), whereas, the modelled estimate for sediment was much closer to the actual measured decay rate (measured t_{90} of 1.67 compared with modelled value of 1.79).

DISCUSSION

Over the 28 days microcosm sampling period, overlying water TDS and pH remained relatively stable. Conditions of sediment and overlying water in microcosms of similar design have previously been shown to closely resemble that of *in situ* conditions over this period of time (Wagner-Döbler *et al.* 1992). In a 12-month *in situ* study investigating the microbiological quality of the same recreational coastal sites as those used in this current study, water conductivity was measured to be $24\,735 \pm 5876$, $25\,543 \pm 4334$ and $20\,245 \pm 5875$ mg l⁻¹ for Henley Beach, Onkaparinga River and Port Adelaide, respectively (Craig *et al.* 2002). Therefore, taking into account the natural variation of water TDS concentration caused by factors such as rainfall and tidal movement, the use of Onkaparinga River water for all microcosms provided a similar TDS as that observed *in situ*.

In general, in these nonsterile microcosms, the number of *E. coli* detected in both overlying water and sediment declined more rapidly with increased temperature. This agrees with other studies, demonstrating prolonged survival of *E. coli* in nonsterile water at lower temperatures (Flint 1987; Rhodes and Kator 1988; Özkanca and Flint 1997). Significantly, from a public health viewpoint, the survival of enteropathogenic *E. coli* in freshwater has also been identified to be greater at lower temperatures (Terzieva and McFeters 1991).

Results indicated that survival was influenced by sediment characteristics, which may reflect intrinsic differences between the sediment types. The greatest rate of decay occurred in Henley Beach sediment which consisted of large particle size and low organic carbon content (statistically significantly at 10 and 30°C; $P < 0.05$). There has been limited investigation into the effect of sediment type on the survival of *E. coli* (or other pathogenic micro-organisms). Of the studies undertaken, many have used sterile sediment and water (Brenner *et al.* 1999; Thomas *et al.* 1999). Other than having the effect of eluting nutrients from sediment, making them available to micro-organisms (Gerba and McLeod 1976), the use of sterile sediment and water removes the added pressure on survival by competition with, and predation by, natural organisms. For this study, the persistence of *E. coli* was determined using intact nonsterile sediment cores, therefore retaining the effect of natural flora. The authors are aware that exposure to

sunlight has been demonstrated to reduce survival of *E. coli* in coastal waters (Davies and Evison 1991; Sinton *et al.* 1999). However, we believe that high turbidity because of increased suspended solids associated with stormwater events may significantly reduce penetration of sunlight exposure through the water column and thus the significance of this parameter to this particular study. The inaccuracy of the modelled vs measured values for die off in the water column, could be partially explained by this phenomenon, while in sediment, which would not be subject to influence of u.v. light, measured and modelled values were very well correlated.

In a study of indicator and pathogenic micro-organism survival in freshwater sediments at 20°C, Burton *et al.* (1987) identified greater survival of *E. coli* in sediments containing high proportions of clay and nutrients compared with sandy sediments (>98% sand and low nutrient levels). Results from the current study confirm, in agreement with Gerba and McLeod (1976), that nutrient availability (measured by organic carbon) may have a significant effect on microbial survival. Under most experimental conditions (all except incubation at 20°C), *E. coli* survival was lowest in Henley Beach sediment (lowest organic carbon content) compared with sediment from Onkaparinga River and Port Adelaide (higher organic carbon content, respectively), particularly at 10°C.

The effect of relatively high rainfall over a short period of time which occurred in February 2000 provided a unique opportunity to study the persistence of faecal coliforms at Henley Beach in both overlying water and sediment, thus allowing a unique comparison with the microcosm studies which had been carried out using samples from the same location.

Prior to the high flow of the Torrens River, the concentration of faecal coliforms in both water and sediment were below the guideline value for recreational waters of <150 faecal coliforms 100 ml⁻¹ (NHMRC 1990). Following the high rainfall there was a dramatic increase in faecal coliform concentration, that at this time, reached similar values both in water and sediment. Two days after this peak, the concentration of faecal indicator organisms was *ca* 100 times greater in the sediment compared with the water. Water at Henley Beach met recreational water quality guidelines for primary contact recreation 5 days after the initial high bacterial load, but concentrations in the sediment did not meet guidelines for recreational water until 7 days after the rain event. At a marine site subjected to sewage contamination, Shiaris *et al.* (1987) demonstrated the concentration of indicator bacteria two to four orders of magnitude higher in marine sediments compared with overlying water. These results suggest that indicator organisms released into the coastal environment can accumulate in sediment, leading to increased persistence.

Faecal material from horses, agisted along the banks of the Torrens River outlet, is likely to have contributed significantly to this high concentration of faecal coliforms. The risks posed to human health from pathogens potentially present in this faecal material need to be determined to more accurately estimate infection risk following recreational contact with this water source.

Results from the microcosm experiment showed a similar decrease in decay of *E. coli* in sediment from Henley Beach compared with overlying water. Considering the average water temperature at Henley Beach during the sampling period was $26.1 \pm 1.6^\circ\text{C}$, the decay rates observed in overlying water was lower than that observed in the laboratory microcosm experiment. However, for the sediment, estimated decay rates using the microcosm model closely represented that observed under *in situ* conditions. In a study by Wait and Sobsey (2001) decay rates observed for *E. coli* in water were greater under *in situ* conditions (using diffusion chambers) compared with laboratory conditions. However, it should be identified that unlike the microcosm experiment undertaken in the current study, the natural environment is not static. Factors such as turbulence and tidal movement, exposure to sunlight as well as the addition of faecal coliforms during the sampling period will influence decay rates.

Results of this *in situ* study confirmed that of the laboratory-based study, illustrating the extended persistence of faecal coliforms in coastal sediments compared with water. After the initial peak, the concentration of faecal coliforms and *E. coli* in the overlying water decreased dramatically in a relatively short period of time. However, the concentration of faecal coliforms in the sediment remained above those guidelines set for recreational water for a period of 6 days following the peak.

This study allowed for the validation of laboratory-based microcosm studies in the estimation of *in situ* decay rates of faecal coliforms in both coastal water and sediment. The presence of *E. coli* in coastal sediments (particularly in sediments consisting of small particle size and high organic carbon) may indicate an increased risk of infection because of the possible resuspension of other pathogenic microorganisms during natural turbulence or human activity. Studies are currently being undertaken to estimate this likely risk, as well as to determine the survival of other pathogenic microorganisms under the same conditions. Results of this study could be used to better manage risk at specific recreational coastal sites, depending on sediment type and average water temperatures.

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