

Survival and Activity of *Streptococcus faecalis* and *Escherichia coli* in Petroleum-contaminated Tropical Marine Waters

Jorge W. Santo Domingo,* Francisco A. Fuentes,‡ & Terry C. Hazen§

Microbial Ecology Laboratory, Department of Biology,
University of Puerto Rico, Río Piedras, 00931 Puerto Rico

(Received 8 February 1988; accepted 30 June 1988)

ABSTRACT

The in situ survival and activity of Streptococcus faecalis and Escherichia coli were studied using membrane diffusion chambers in tropical marine waters receiving oil refinery effluents. Protein synthesis, DNA synthesis, respiration or fermentation, INT reduced per cell, and ATP per cell were used to measure physiological activity. Cell densities decreased significantly over time at both sites for both S. faecalis and E. coli; however, no significant differences in survival pattern were observed between S. faecalis and E. coli. Differences in protein synthesis between the two were only observed at a study site which was not heavily oiled. E. coli was more active in protein synthesis and respiration than S. faecalis at both oiled and unoled sites, and the percentage of the E. coli population that was respiring was significantly higher than S. faecalis fermenting cells at both sites. However, S. faecalis cells were more active in DNA synthesis and higher in ATP content than E. coli cells at both sites. Although fecal streptococci have been suggested as a better indicator of fecal contamination than fecal coliforms in marine waters, in this study both E. coli and S. faecalis survived and remained physiologically active

* Present address: Department of Crop and Soil Sciences, Michigan State University, East Lansing, Michigan 48824-1325, USA.

‡ Present address: Department of Biology, University of Puerto Rico, Humacao, 00661 Puerto Rico.

§ To whom correspondence should be addressed at: E. I. du Pont de Nemours & Company, Savannah River Laboratory, Environmental Sciences Division, Aiken, South Carolina 29808, USA.

for extended periods of time. These results suggest that the fecal streptococci group is not a better indicator of fecal contamination in tropical marine waters than the fecal coliform group, especially when that environment is high in long-chained hydrocarbons.

INTRODUCTION

Several studies have suggested that the fecal streptococci in recreational waters are good numerical indicators of human health risks, i.e. the probability of contracting gastroenteritis while swimming is directly proportional to the number of fecal streptococci in the water (Berg & Metcalf, 1978; Cabelli, 1983; Cabelli *et al.*, 1983). Fecal streptococci densities have also been reported to correlate better than densities of *E. coli* and total coliforms with the presence of enteric viruses, e.g. hepatitis A virus, Norwalk-like viruses, human rotavirus (Berg & Metcalf, 1978; Cabelli *et al.*, 1983). These viral pathogens are increasingly associated with cases of gastroenteritis where the etiological agent is unknown (Goyal, 1984). The method of detection of the bacteria has also been shown to have inherent problems. The m-enterococcus medium used for the detection of fecal streptococci is more species specific than the various media used to detect coliforms and fecal coliforms (Lavoie, 1983; Burlingame *et al.*, 1984). Thus many of the criteria used to select a good indicator organism are satisfied better by the fecal streptococci.

Fecal streptococci have also been used to differentiate between pollution of human or animal origin. Geldreich & Kenner (1969) suggest that ratios of fecal coliforms to fecal streptococci above 4.0 indicate human fecal pollution, whereas ratios below 0.7 indicate animal fecal pollution. However, studies on the fecal flora of individuals from India, Japan, and Uganda with a vegetarian diet showed higher enterococci counts than individuals from England, Scotland, and the United States with a mixed diet (Hill *et al.*, 1971). Thus, diet alone can affect the proportion of enterococci found in human fecal material. Evison & James (1975) also reported differences in survival patterns of fecal coliforms and fecal streptococci depending on the temperature. They observed that fecal streptococci survive better than fecal coliforms at low temperatures (<4°C); however, at temperatures above 20°C *E. coli* survive better because of the rapid die-off rates of the fecal streptococci. Gordon & Fliermans (1978) demonstrated that *E. coli* could survive for much longer periods of time in a lake receiving thermal effluent. Thus, environmental factors might also significantly affect the recovery and proportion of fecal coliforms and fecal streptococci in environmental samples.

Environmental factors like temperature, solar radiation, seasonal variability, and concentration of nutrients are quite different in tropical aquatic systems as compared to temperate aquatic systems. Bigger (1937) first reported the growth of coliforms in tropical waters, while in 1939 Ragavachari and Iver showed that coliforms can survive for several months in natural tropical river waters. Recent studies in Puerto Rico (Hazen *et al.*, 1987) showed that the survival of fecal coliforms increased in marine and freshwater systems possibly because of the high nutrient concentrations present. Other tropical studies have also shown that the fecal coliforms (*E. coli*) may survive better in tropical freshwater than in marine waters (Fujioka *et al.*, 1981; Fujioka & Shizumura, 1985; Hazen *et al.*, 1987). Studies in Puerto Rico have demonstrated high numbers of fecal coliforms in pristine rain forest streams where there is no apparent fecal contamination source (Carrillo *et al.*, 1985; Rivera *et al.*, 1988).

Several studies have even suggested that *E. coli* may be part of the normal aquatic flora in the tropics (Fujioka *et al.*, 1981; Fujioka & Shizumura, 1985; Hazen *et al.*, 1987). Thus, high counts of total coliforms and fecal coliforms in water supplies do not necessarily indicate recent fecal contamination. The reliability of coliforms and fecal coliforms as indicators of recent fecal contamination in tropical waters is questionable when no identifiable source of fecal contamination has been detected (Carrillo *et al.*, 1985). This has suggested to us that the fecal streptococci might be a more suitable alternative indicator of fecal contamination in tropical waters. The present study† examines the ability of *E. coli* and *S. faecalis* to survive in a tropical marine environment contaminated with petroleum.

MATERIALS AND METHODS

Study site

Yabucoa Bay is on the southeastern corner of the island of Puerto Rico. This made-made harbour was heavily oiled in 1978 with bunker C oil from a 105 m barge (NOAA, 1979). Continuous traffic of oil tankers enriches the hydrocarbon load in this area in addition to the primary treated effluents from an oil refinery. Two sites were selected for chamber studies (Fig. 1). Site A was adjacent to the harbour bridge, where the oil tankers load or unload crude oil and its derivatives. Site B was located 100 m away on the other side of the bay and was presumably much less contaminated.

† This study was part of the MS thesis of J. W. Santo Domingo at the University of Puerto Rico, Río Piedras, Puerto Rico, 1987.

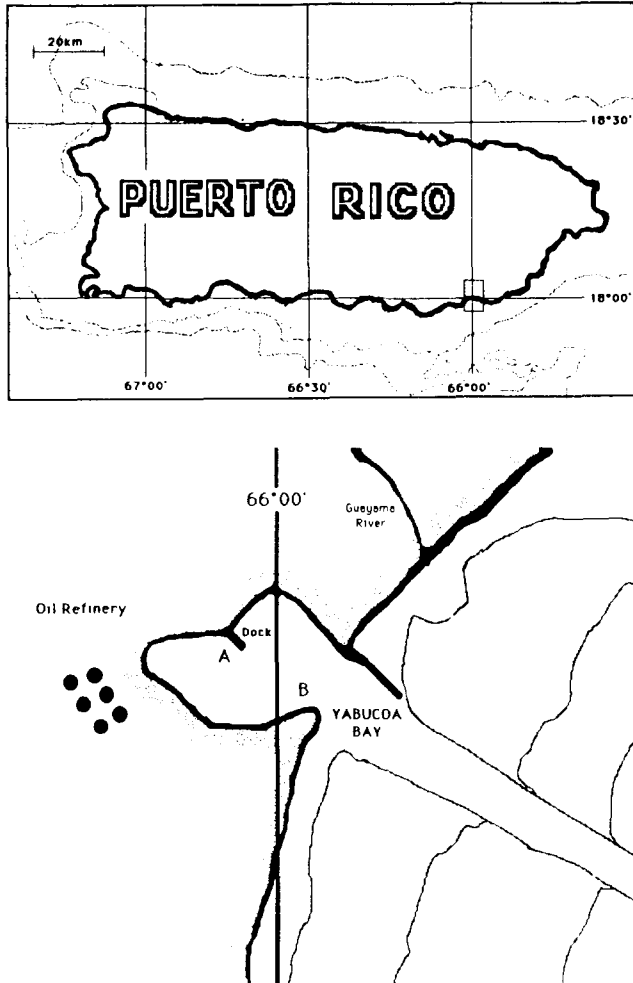


Fig. 1. Map of sampling sites in Puerto Rico.

Water analysis

Air and water temperature, pH, and conductivity were measured *in situ* with a model 4041 Hydrolab Surveyor (Hydrolab Corp., Austin, TX). Dissolved oxygen (DO) was measured using the Winkler method (APHA, 1985). Water samples were collected in dark plastic bottles and small amounts of a preservative—sulfuric acid, zinc acetate, or mercuric chloride—were added before transporting them to the laboratory where they were analyzed for nitrates plus nitrites, sulfates, and total phosphorus using *Standard Methods for Water and Wastewater Analysis* (APHA, 1985). One liter of water was

collected in an amber-coloured glass bottle and analyzed for chlorophyll *A*. Also, water samples were collected in dark plastic bottles and transported to the laboratory to test for alkalinity, ammonia, and turbidity (APHA, 1985). All samples were immediately placed on ice for transport to the laboratory.

Survival studies

Pure cultures of *E. coli* ATCC 11775 and *S. faecalis* CBSC 15-5600A were grown in nutrient broth at 37°C for 24 h. The cells were harvested by centrifuging and were washed in 0.85% sterile phosphate-buffered saline (pH 7). The final concentration was adjusted to 10⁸ cells ml⁻¹ using a model ZM Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). The final bacterial suspensions were placed in sterile membrane diffusion chambers just before immersion in the site. (For more details see Biamón & Hazen (1981), Hazen & Esch (1983), López-Torres *et al.* (1988)).

Total direct counts and cell activity

Bacterial cell densities were determined by acridine orange direct counts (AODC) and Coulter Counter counts (CC) as before (López-Torres *et al.*, 1987). The ratio of red fluorescing cells to red and green fluorescing cells under rigorous acridine orange staining was assumed to indicate cells active in protein synthesis, see López-Torres *et al.* (1988) for details. Respiring cells were determined according to Zimmermann *et al.* (1978). To determine the total INT reduced by the bacterial population, an INT-formazan alcohol extraction was performed after Jeffrey & Paul (1986). Aliquots of the same water samples, used to determine the number of respiring cells above, were filtered using 0.2- μ m-pore size, 25-mm-diameter polycarbonate membranes. The membranes were placed in clean glass containers and 1 ml of ethanol (95%) was added to extract the intracellular INT-formazan. The ethanol extracts were clarified by filtration through a Millex-GV 0.22- μ m filter, and INT-formazan content was determined by measuring the absorbance at 485 nm. INT-reduced-per-bacterial-cell was calculated using AODC counts as suggested by Kurath & Morita (1983).

Cells active in DNA synthesis were determined by microautoradiography following Tabor & Neihof (1982a), using [³H]thymidine (10 mCi/mmol) as the substrate. Water samples (1 ml) from the chambers were taken and exposed to [³H]thymidine for 2.5 h in the dark. After incubation, samples were fixed with 0.1 ml of 10% phosphate-buffered formalin. The water samples were stored on ice and transported to the laboratory for analysis. Aliquots (0.1 ml) of the water samples were filtered using 0.2- μ m-pore size, 25-mm-diameter polycarbonate membranes. The membranes were placed

facedown on a gel-matrix on microscope slides. After 24 h, membranes were removed and slides exposed for 3 days to nuclear track emulsion, NTB2 (Eastman Kodak Co., Rochester, NY). Slides were then examined by epifluorescence/transmitted brightfield microscopy. Scintillation counts were performed with aliquots of the same water samples to measure the uptake of [^3H]thymidine by the bacterial population over time. ATP concentrations were measured with the luciferin-luciferase test (Stanley & Williams, 1969) using a liquid scintillation counter model LS 7500 (Beckman Instrument Inc., Palo Alto, Calif.). ATP per bacterial cell was calculated using AODC counts as suggested by Kurath & Morita (1983).

Data analysis

Programs developed for the Apple IIe and Macintosh computers were used for statistical tests. Two-way analysis of variance was used to test for differences between sites and over time. Multiple correlation analyses were performed to establish the relationship between bacteria and water quality. Heteroscedastic data were made more homoscedastic using the appropriate transformation prior to analysis. Any statistical probability less than or equal to 0.05 was considered significant (Zar, 1984).

RESULTS AND DISCUSSION

Water quality

Representative water quality data are given in Table 1. Concentrations of total organic carbon (TOC) were greater than $4.03 \text{ mg litre}^{-1}$ at both sites (data not shown). TOC at site A was more than twice as high as site B. These high concentrations of organics are undoubtedly caused by petroleum contamination. The other water quality parameters indicate that both sites were highly productive compared to uncontaminated marine coastal areas in Puerto Rico (Valdés-Collazo *et al.*, 1987; López-Torres *et al.*, 1988). The low concentrations of nutrients (PO_4 , NO_2 and NO_3) observed suggest that this bay is limited by concentrations of these nutrients. This observation was further substantiated by microcosm studies in which phosphorus and/or nitrogen was added to water from the bay (Fuentes, F. A., 1987, Doctoral Dissertation, Department of Biology, University of Puerto Rico; unpublished). Supplemented microcosm bacterial densities increased by 3 log values in 3 days, while unsupplemented microcosm densities remained the same.

TABLE 1

Water Physicochemical Parameters by Sampling Site. All Values are Mean \pm One Standard Error ($n = 7$)

Parameter	Air temperature (°C)	Water temperature (°C)	Dissolved oxygen (°C)	pH	Alkalinity (mg liter ⁻¹ CaCO ₃)	NO ₂ + NO ₃ (mg liter ⁻¹)
Site A	25.8 \pm 1.7	29.1 \pm 0.2	6.0 \pm 0.1	7.3 \pm 0.1	100 \pm 0	0.07 \pm 0.00
Site B	25.0 \pm 1.5	28.1 \pm 0.3	6.1 \pm 0.1	7.5 \pm 0.1	97 \pm 3	0.06 \pm 0.01

Parameter	Total phosphorus (mg liter ⁻¹)	SO ₄ (\times 1000 mg liter ⁻¹)	Chl A (mg liter ⁻¹) ¹	Conductivity (\times 1000 μ mohs cm ⁻¹)	Salinity (ppt)	NH ₄ (mg liter ⁻¹)
Site A	0.01 \pm 0.00	1.84 \pm 0.11	2.61 \pm 0.38	504 \pm 2	34.5 \pm 0.4	2.34 \pm 0.09
Site B	0.00 \pm 0.00	1.96 \pm 0.03	3.07 \pm 0.53	510 \pm 2	34.2 \pm 0.1	2.25 \pm 0.10

Survival

No significant differences were found in the densities of *S. faecalis* or *E. coli* by site as determined by AODC and CC (Figs 2 and 3). However, *S. faecalis* and *E. coli* cell densities declined significantly over time for AODC ($F = 12.1$, $df = 14$ and 90 , $P < 0.001$; $F = 12.5$, $df = 14$ and 60 , $P < 0.001$; *S. faecalis* and *E. coli*, respectively) and CC ($F = 14.6$, $df = 14$ and 90 , $P < 0.001$; $F = 18.6$, $df = 14$ and 60 , $P < 0.001$; *S. faecalis* and *E. coli*, respectively). Density estimates by AODC were one order of magnitude greater than CC estimates due to the greater sensitivity of the AODC method. Both techniques showed the same trends and indicated only a 1 log decrease over the 72 h period observed. Thus *S. faecalis* and *E. coli* were surviving *in situ* in this environment. Several previous investigations have reported that high temperature and high salinity environments are stressful to enteric bacteria and some pathogens (Hanes & Fragala, 1976; Novitsky & Morita, 1976; Lessard & Sieburth, 1983; Roszak *et al.*, 1984). However, several studies (Biamón & Hazen, 1983; Lessard & Sieburth, 1983; Valdés-Collazo *et al.*, 1987; López-Torres *et al.*, 1988) have shown that enteric bacteria can overcome the bactericidal effects of seawater and temperature if the environment is high in nutrients. Yabucoa Bay has high concentrations of total organic carbon caused by petroleum contamination. This apparently increases the survivability of *E. coli* and *S. faecalis*. In addition, the gross morphological changes observed to occur in cells of several bacteria exposed to marine environments were not observed in this study (Novitsky & Morita, 1976; Xu *et al.*, 1982; Roszak *et al.*, 1984; Colwell *et al.*, 1985; López-Torres *et al.*, 1988).

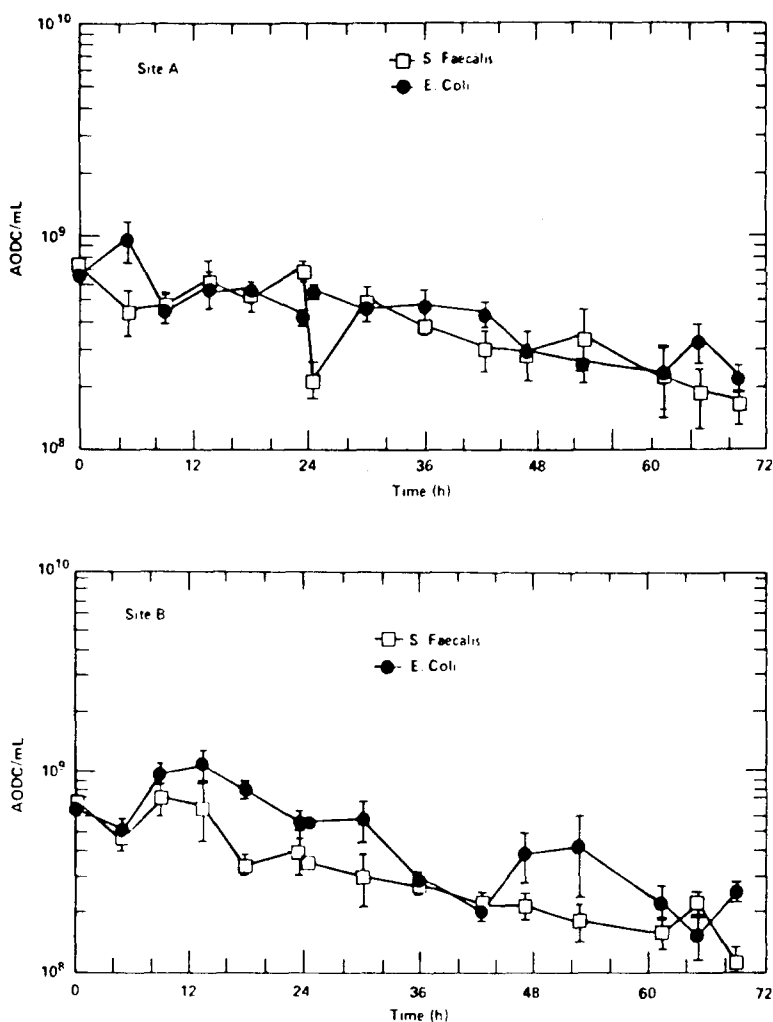


Fig. 2. Survival of *Streptococcus faecalis* and *Escherichia coli* at sites A and B of Yabucoa Bay by AODC (mean \pm one standard error, $n = 4$).

Activity

S. faecalis and *E. coli* cell activities, as determined by the percentage of cells active in protein synthesis (AODC), were significantly different over time ($F = 4.9$, $df = 14$ and 90 , $P < 0.001$ and $F = 2.5$, $df = 14$ and 60 , $P < 0.02$, respectively; Fig. 4). No evident pattern in *S. faecalis* protein synthesis was observed at site A even though the number of active cells was lower by the end of the study. High percentages of *E. coli* and *S. faecalis* cells were active in protein synthesis at both sites. Although some fluctuations were observed,

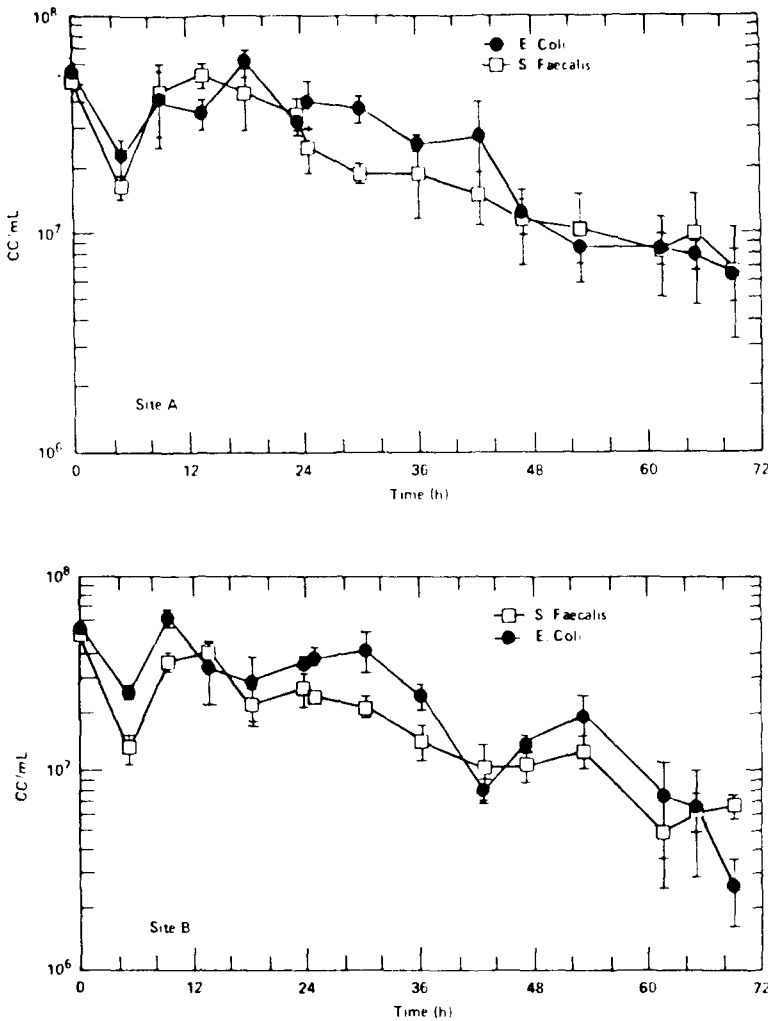


Fig. 3. Survival of *Streptococcus faecalis* and *Escherichia coli* at sites A and B of Yabucoa Bay by CC (mean \pm one standard error, $n = 4$).

the percentage of *E. coli* cells active in protein synthesis was as high at the end of the study as at the beginning. In general, *S. faecalis* and *E. coli* protein synthesis was higher at site A, where total organic carbon and total phosphorus were in higher concentrations, than at site B. This suggests that high concentrations of nutrients in marine waters can increase survival and activity.

The percentage of fermenting *S. faecalis* cells was not significantly different by site; however, it decreased significantly over time ($F = 15.0$, $df = 14$ and 90 , $P < 0.001$) at both sites (Fig. 5). The percentage of respiring

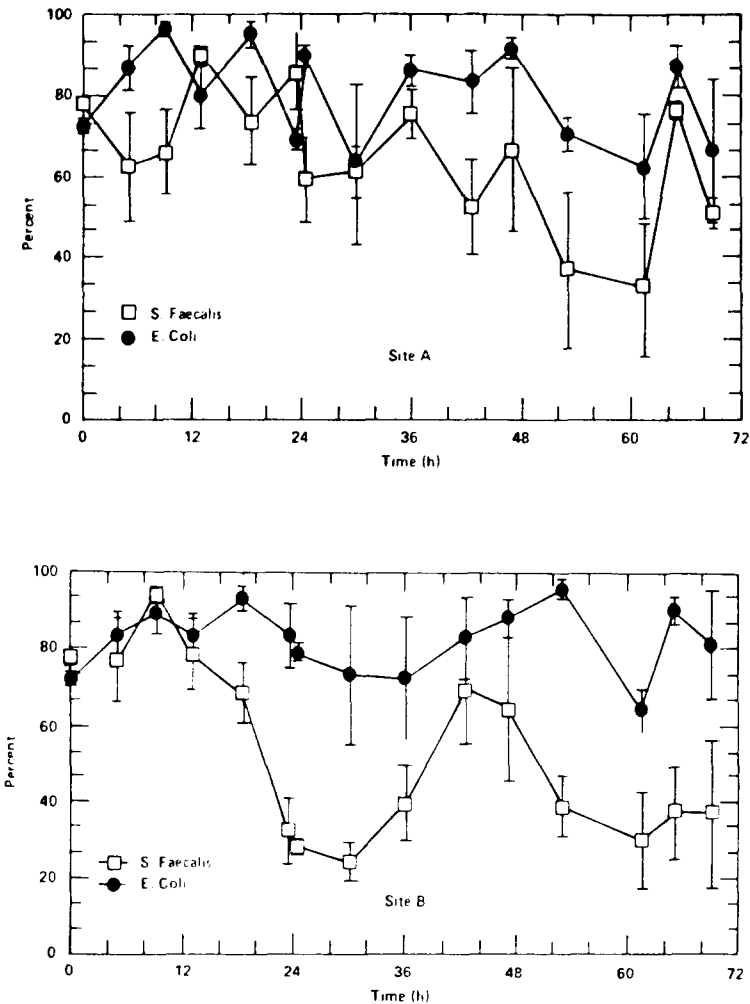


Fig. 4. Activity of *Streptococcus faecalis* and *Escherichia coli* at sites A and B of Yabucoa Bay by acridine orange staining, percentage all fluorescing cells with red fluorescence (mean \pm one standard error, $n = 4$).

E. coli cells declined significantly over time ($F = 4.6$, $df = 14$ and 60 , $P < 0.001$; Fig. 5). Differences in the interaction between site and time were also observed ($F = 4.1$, $df = 14$ and 60 , $P < 0.001$). The percentage of *E. coli* and *S. faecalis* active in respiration (*E. coli*) or fermentation (*S. faecalis*) declined slowly during the study. *S. faecalis* fermenting cells decreased more rapidly at site A, and after 3 days (69 h) only 2% of *S. faecalis* cells were fermenting. However, at site B, *S. faecalis* cells were observed to increase in activity at 24 h, although only 5% of the cells were active in fermentation at the end of the survival study. These measurements correspond to respiration

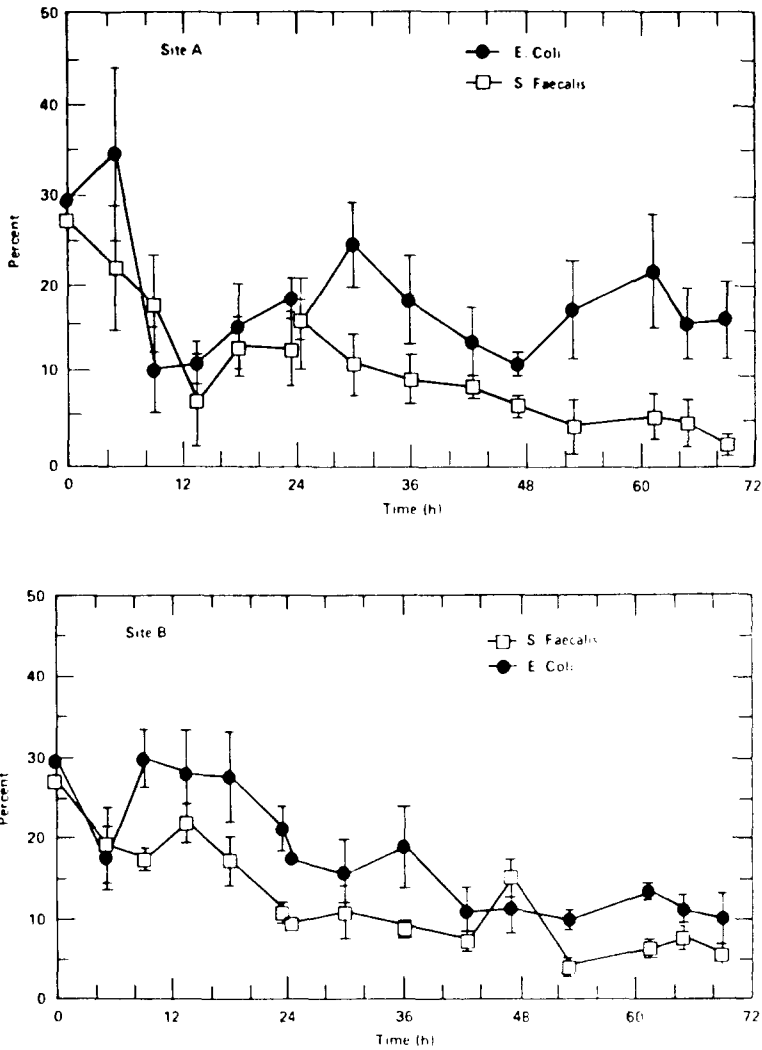


Fig. 5. Activity of *Streptococcus faecalis* and *Escherichia coli* at sites A and B of Yabucoa Bay by INT respiration, percentage of all acridine orange fluorescing cells with INT-formazan granules present (mean \pm one standard error, $n = 4$).

rates found in more oligotrophic, temperate, marine environments (Zimmermann *et al.*, 1978) and would seem to indicate that both *S. faecalis* and *E. coli* decreased in activity over time.

Two basic problems reportedly occur with the microscopic observation of INT-formazan granules: the concentration of INT reduced by some metabolically active bacterial cells may not be sufficiently high to form visible granules (Zimmermann *et al.*, 1978), and intracellular INT-formazan granules may be dissolved by immersion oil within a short period of time

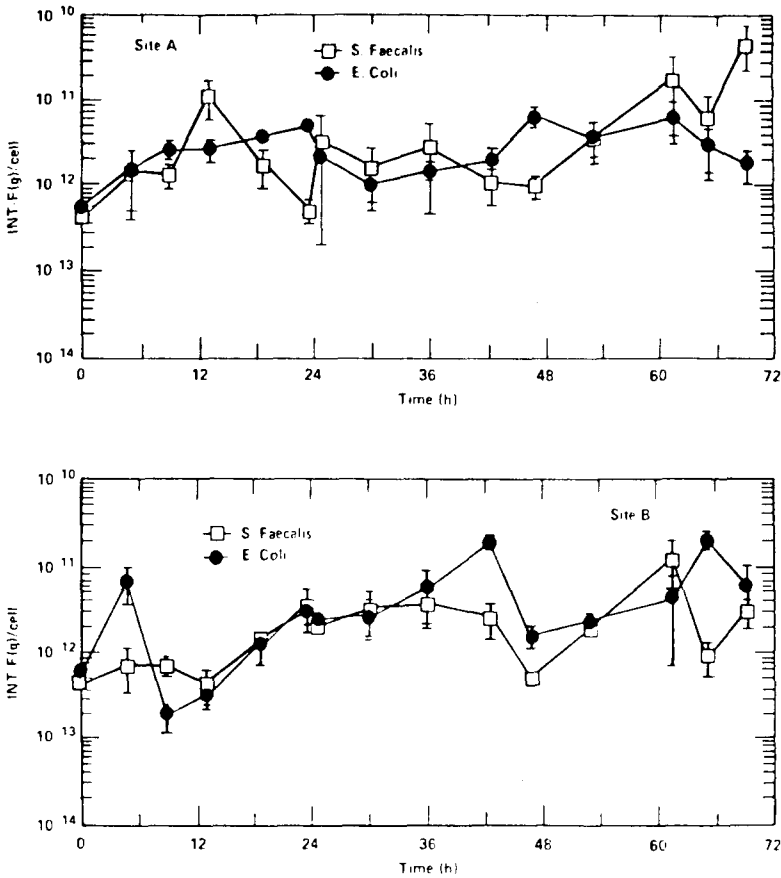


Fig. 6. Activity of *Streptococcus faecalis* and *Escherichia coli* at sites A and B of Yabucoa Bay by INT-formazan per cell, using AODC for total cell numbers (mean \pm one standard error, $n = 4$).

(Newell, 1984; Tabor & Neihof, 1982b). In both cases there might be a significant underestimation of the bacterial population that is metabolically active. Because dehydrogenase enzymes should not be active in dormant cells, INT-reduced-by-individual-cells was calculated as a function of respiring or fermenting cells, as suggested by Kurath & Morita (1983). When the activity is examined in this way, we see that both *S. faecalis* and *E. coli* activity significantly increase over time ($F = 2.8$, $df = 14$ and 90 , $P < 0.005$; $F = 6.3$, $df = 14$ and 60 , $P < 0.001$; *S. faecalis* and *E. coli*, respectively; Fig. 6). Indeed, *S. faecalis* reduced more INT per cell at site A than at site B ($F = 11.0$, $df = 1$ and 60 , $P < 0.005$; Fig. 6). This further suggests a higher population-carrying capacity at site A. The slight decline in cell density observed in both bacteria left the remaining population more active,

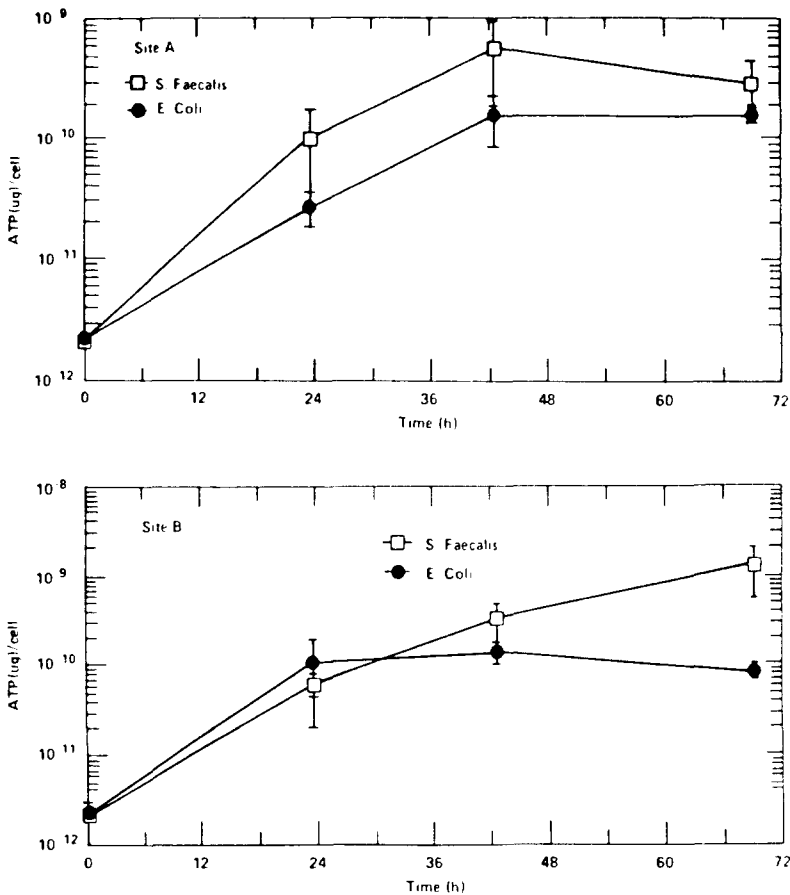


Fig. 7. Activity of *Streptococcus faecalis* and *Escherichia coli* at sites A and B of Yabucoa Bay by ATP per cell, using AODC for total cell numbers (mean \pm one standard error, $n = 4$).

suggesting some environmental adaptation had occurred. Jones & Simon (1979) found that planktonic and benthic bacteria in an English lake contained from 1×10^{-11} g to 9×10^{-11} g of INT-formazan per cell, which is somewhat greater than most of the amounts of INT reduced by *S. faecalis* and *E. coli* per cell in our study. However, the habitats studied by Jones & Simon (1979) were freshwater and probably higher in nutrient concentrations than Yabucoa Bay.

Significant increases over time were also observed in ATP per cell for both *S. faecalis* ($F = 4.8$, $df = 3$ and 24 , $P < 0.02$; Fig. 7) and *E. coli* ($F = 4.6$, $df = 3$ and 16 , $P = 0.05$; Fig. 7). Thus ATP also suggests that, after the initial decline, the remaining portion of both populations was much more active than the initial population. ATP per cell ranged from an average of 2.16×10^{-12} μ g to 1.59×10^{-10} μ g for *E. coli* and an average of 2.12×10^{-12} μ g to

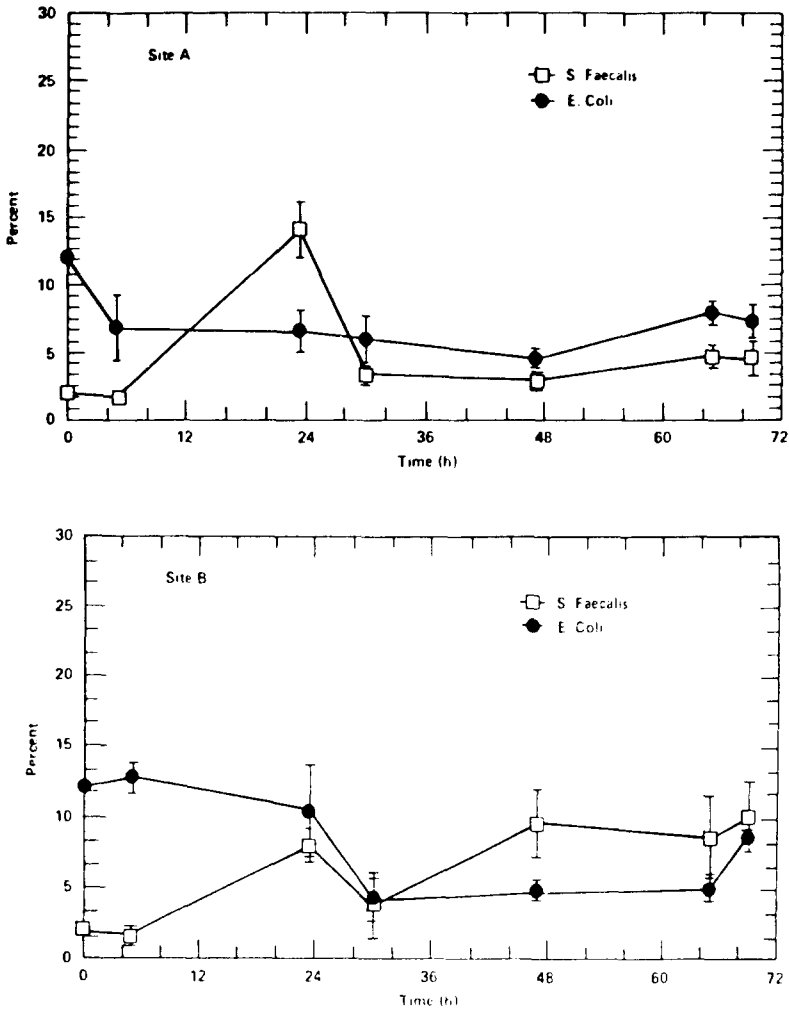


Fig. 8. Activity of *Streptococcus faecalis* and *Escherichia coli* at sites A and B of Yabucoa Bay by microautoradiography, percentage of acridine orange fluorescing cells with [³H]thymidine positive granules (mean \pm one standard error, $n = 4$).

$1.39 \times 10^{-9} \mu\text{g}$ for *S. faecalis*. This is somewhat lower than the range found by Hamilton & Holm-Hansen (1967) of $0.5 \times 10^{-9} \mu\text{g}$ to $6.5 \times 10^{-9} \mu\text{g}$ for marine bacteria in the log phase of growth and by Kurath & Morita (1983) for a marine *Pseudomonas* sp. in a survival study. However, the bacteria used in this study were enteric in origin and should have lower physiological activities than bacteria of marine origin.

The percentage of *S. faecalis* cells active in DNA synthesis as determined by microautoradiographs was significantly different over time ($F = 9.2$, $df = 6$ and 42 , $P < 0.001$), but only slightly higher at the end of the study at

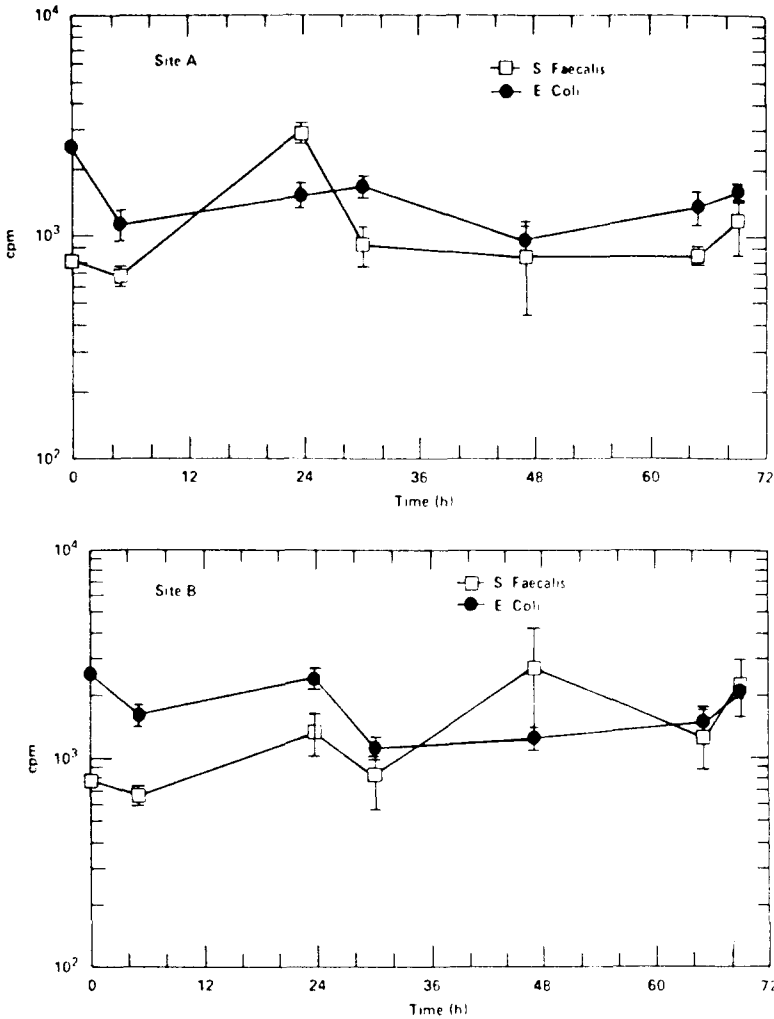


Fig. 9. Activity of *Streptococcus faecalis* and *Escherichia coli* at sites A and B of Yabucoa Bay by incorporation of [³H]thymidine, using AODC for total cell numbers (mean ± one standard error, n = 4).

both sites. Site B had a lower level of synthesis activity; however, the percent of active cells was lower than 15% at all times (Fig. 8). Significant differences in the interaction between site and time were also observed ($F = 3.9$, $df = 6$ and 42 , $P < 0.02$). [³H]thymidine uptake by *S. faecalis* increased significantly over time at both sites ($F = 3.1$, $df = 6$ and 42 , $P < 0.05$) but no significant differences were observed by site (Fig. 9). The percentage of *E. coli* cells active in DNA synthesis decreased significantly over time ($F = 6.9$, $df = 6$ and 28 , $P < 0.001$, and $F = 5.1$, $df = 6$ and 28 , $P < 0.05$, microautoradiography and [³H]-thymidine uptake, respectively; Figs 8 and 9).

The percentage of *S. faecalis* cells active in DNA synthesis was higher than *E. coli* after 24 h at site A. However, other metabolic activities were frequently higher for *E. coli* during the same period at site A. This suggests that although protein synthesis and respiration were higher for *E. coli*, the *S. faecalis* cells were just as active. This was further supported by the slightly higher concentrations of ATP per cell seen for *S. faecalis*.

Studies by Xu *et al.* (1982), and Roszak *et al.* (1984) showed that some bacteria could be 'viable but nonculturable', a concept which suggests that most of the bacterial population, if not all, that can be seen when a water sample is being examined under the microscope must be alive even though part of it cannot be cultured on artificial media. Roszak *et al.* (1984) observed that after 48 h starvation *Salmonella enteritidis* did not grow on non-selective media, although cell densities greater than 10^4 ml^{-1} were obtained by AODC, fluorescent antibody (FA) and direct viable counts (DVC). Colwell *et al.* (1985) showed that a pathogenic *Vibrio cholerae* that lost the ability to grow on conventional culture media not only remained as virulent to rabbits as a non-starved strain, but regained their ability to grow in selective and nonselective media. These findings reinforce the necessity of other methods to accurately determine bacterial activity in aquatic systems. Our results point out the importance of measuring several physiological parameters simultaneously for different bacteria in order to correctly determine the effect of ambient water quality on the physiological status of the cells *in situ*.

This study showed that *S. faecalis* and *E. coli* can survive and remain active in petroleum contaminated tropical marine waters. This does not support previous investigations that have suggested that fecal streptococci survive better than fecal coliforms in marine waters. Thus, the fecal streptococci do not represent a better alternative to fecal coliforms in tropical marine waters. Because coliforms have also been demonstrated not to fulfil the requisites of a good indicator of fecal pollution in tropical areas (Carrillo *et al.*, 1985; Fujioka & Shizumura, 1985; Hazen *et al.*, 1987; López-Torres *et al.*, 1987, 1988; Valdés-Collazo *et al.*, 1987), maximum biological contaminant levels for tropical waters should be based upon direct monitoring of one or more resistant pathogens rather than the indicator organisms currently used, i.e. coliforms or fecal coliforms.

ACKNOWLEDGEMENTS

We thank Andrés Calvo and Jesús Santiago for their technical assistance. We are especially grateful to Gary A. Toranzos and Carl B. Fliermans who made many helpful suggestions to the manuscript. Sun Oil Company,

Yabucoa, graciously provided access to their facilities during the study. This work was supported by the Water Resources Research Institute of the University of Puerto Rico at Mayagüez and in part by Sea Grant R/LR-08-87-THA1 and by Public Health Service grants RR-2657 and RR-8102 from the National Institutes of Health. In addition, portions of the information contained in this paper were developed during the course of work under Contract No. DE-AC09-76SR00001 with the US Department of Energy.

REFERENCES

- American Public Health Association (APHA) (1985). *Standard Methods for the Examination of Water and Wastewater* (16th). American Public Health Association, Washington, DC.
- Berg, G. & Metcalf, T. G. (1978). Indicators of viruses in waters. In *Indicators of Viruses in Water and Food*. ed. G. Berg, Ann Arbor Science Publications, Ann Arbor, Mich., pp. 1-30.
- Biamón, E. J. & Hazen, T. C. (1983). The distribution and survival of *Aeromonas hydrophila* in tropical near-shore coastal waters receiving rum distillery effluents. *Water Res.*, **17**, 319-26.
- Bigger, J. W. (1937). The growth of coliform bacilli in water. *J. Pathol. Bacteriol.*, **44**, 167-211.
- Burlingame, G. A., McElhaney, J. & Pipes, W. O. (1984). Bacterial interference with coliform colony sheen production on membrane filters. *Appl. Environ. Microbiol.*, **47**, 56-60.
- Cabelli, V. J. (1983). Public health and water quality significance of viral diseases transmitted by drinking water and recreational water. *Water Sci. Technol.*, **15**, 1-15.
- Cabelli, V. J., Dufour, A. P., McCabe, L. J. & Levin, M. A. (1983). A marine recreational water quality criterion consistent with indicator concepts and risk analysis. *J. Water Pollut. Control Fed.*, **55**, 1306-14.
- Carrillo, M., Estrada, E. & Hazen, T. C. (1985). Survival and enumeration of the fecal indicator *Bifidobacterium adolescentis* and *Escherichia coli* in a tropical rain forest watershed. *Appl. Environ. Microbiol.*, **50**, 468-76.
- Colwell, R. R., Brayton, P. R., Grimes, D. J., Roszak, D. B., Huq, S. A. & Palmer, L. M. (1985). Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment, implications for release of genetically engineered microorganisms. *Biotechnology*, **3**, 817-20.
- Evison, L. M. & James, A. (1975). *Bifidobacterium* as an indicator of human fecal pollution in water. *Prog. Water Technol.*, **7**, 57-66.
- Fujioka, R. S. & Shizumura, L. K. (1985). *Clostridium perfringens*, a reliable indicator of stream water quality. *J. Water Pollut. Control Fed.*, **57**, 986-92.
- Fujioka, R. S., Hashimoto, H. H., Siwak, E. B. & Reginald, H. F. (1981). Effect of sunlight on survival of indicator bacteria in seawater. *Appl. Environ. Microbiol.*, **41**, 690-6.
- Geldreich, E. E. & Kenner, B. A. (1969). Concepts of faecal streptococci in stream pollution. *J. Water Pollut. Control Fed.*, **41**, R336-52.

- Gerba, C. P. & McLeod, J. S. (1976). Effect of sediments on the survival of *Escherichia coli* in marine waters. *Appl. Environ. Microbiol.*, **32**, 114–20.
- Gordon, R. W. & Fliermans, C. B. (1978). Survival and viability of *Escherichia coli* in a thermally altered reservoir. *Water Res.*, **12**, 343–52.
- Goyal, S. M. (1984). Viral pollution of the marine environment. *CRC Crit. Rev. Environ. Contr.*, **14**, 1–32.
- Hamilton, R. D. & Holm-Hansen, O. (1967). Adenosine triphosphate content of marine bacteria. *Limnol. Oceanogr.*, **12**, 319–24.
- Hanes, N. B. & Fragala, R. (1976). Effects of seawater concentrations on survival of indicator bacteria. *J. Water Pollut. Control Fed.*, **39**, 97–104.
- Hazen, T. C. & Esch, G. W. (1983). The effect of nitrogen fertilizer factory and pulp mill effluent on the distribution and abundance of *Aeromonas hydrophila* in Albemarle Sound, North Carolina. *Appl. Environ. Microbiol.*, **54**, 31–42.
- Hazen, T. C., Santiago-Mercado, J., Toranzos, G. A. & Bermúdez, M. (1987). What do water fecal coliforms indicate in Puerto Rico? A review. *Bul. PR Med. Assoc.*, **79**, 189–93.
- Hill, M. J., Drasar, B. S., Aries, V., Crowther, J. S., Hawksworth, G. & Williams, R. E. O. (1971). Bacteria and aetiology of cancer of large bowel. *Lancet*, **1**, 95–100.
- Jeffrey, W. H. & Paul, J. H. (1986). Activity of an attached and free-living *Vibrio* sp. as measured by thymidine incorporation, *p*-iodonitrotetrazolium reduction & ATP/DNA ratios. *Appl. Environ. Microbiol.*, **51**, 150–6.
- Jones, J. G. & Simon, B. M. (1979). The measurement of electron transport system activity in freshwater benthic and planktonic samples. *J. Appl. Bacteriol.*, **46**, 305–15.
- Kurath, G. & Morita, R. Y. (1983). Starvation-survival physiological studies of a marine *Pseudomonas* sp. *Appl. Environ. Microbiol.*, **45**, 1206–11.
- Lavoie, M. C. (1983). Identification of strains isolated as total and fecal coliforms and comparison of both groups as indicators of fecal pollution in tropical climates. *Can. J. Microbiol.*, **29**, 689–93.
- Lessard, E. J. & Sieburth, J. McN. (1983). Survival of natural sewage population of enteric bacteria in diffusion and batch chambers in the marine environment. *Appl. Environ. Microbiol.*, **45**, 950–9.
- López-Torres, A. J., Hazen, T. C. & Toranzos, G. A. (1987). Distribution and *in situ* survival and activity of *Klebsiella pneumoniae* in a tropical rain forest watershed. *Curr. Microbiol.*, **15**, 213–8.
- López-Torres, A. J., Prieto, L. & Hazen, T. C. (1988). Comparison of the *in situ* survival and activity of *Klebsiella pneumoniae* and *Escherichia coli* in tropical marine environments. *Microb. Ecol.*, **15**, 41–57.
- Newell, S. Y. (1984). Modification of the gelatin-matrix method for enumeration of respiring bacterial cells for use with salt-marsh water samples. *Appl. Environ. Microbiol.*, **47**, 873–5.
- NOAA (1979). *The Peck Slip Oil Spill*. ed. J. H. Robinson, NOAA special report, Office of Research and Development, Washington, DC.
- Novitsky, J. A. & Morita, R. Y. (1976). Morphological characterization of small cells resulting from nutrient starvation of a psychrophilic marine *Vibrio*. *Appl. Environ. Microbiol.*, **32**, 617–22.
- Ragavachari, T. N. S. & Iver, P. V. W. (1939). Longevity of coliform organisms in water stored under natural conditions. *Indian J. Med. Res.*, **26**, 877–83.

- Rivera, S., Hazen, T. C. & Toranzos, G. A. (1988). Isolation of fecal coliforms from pristine sites in a tropical rain forest. *Appl. Environ. Microbiol.*, **54**, 513-7.
- Roszak, D. B., Grimes, D. J. & Colwell, R. R. (1984). Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. *Can. J. Microbiol.*, **30**, 334-8.
- Stanley, P. E. & Williams, S. G. (1969). Use of liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Anal. Biochem.*, **29**, 381-92.
- Tabor, P. S. & Neihof, A. R. (1982a). Improved microautoradiography method to determine individual microorganisms active in substrate uptake in natural waters. *Appl. Environ. Microbiol.*, **44**, 945-53.
- Tabor, P. S. & Neihof, A. R. (1982b). Improved method for determination of respiring individual microorganisms in natural waters. *Appl. Environ. Microbiol.*, **43**, 1249-55.
- Valdés-Collazo, L., Schultz, A. J. & Hazen, T. C. (1987). Survival of *Candida albicans* in tropical marine and freshwaters. *Appl. Environ. Microbiol.*, **53**, 1762-7.
- Xu, H.-S., Roberts, N., Singleton, F. L., Attwell, R. W., Grimes, D. J. & Colwell, R. R. (1982). Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microb. Ecol.*, **8**, 313-23.
- Zar, J. H. (1984). *Biostatistical Analysis*. Prentice-Hall, Inc., Englewood Cliffs, NJ.
- Zimmermann, R., Iturriaga, R. & Becker-Birck, J. (1978). Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. *Appl. Environ. Microbiol.*, **36**, 926-34.