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1 Survival and Activity of Streptococcus
2 faecalis and Escherichia coli in
3 Petroleum-Contaminated Tropical Marine
4 Waters

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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 unoiled 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 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 (2, 6, 7). 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 (2, 7). These viral pathogens are increasingly associated with cases of gastroenteritis where the etiological agent is unknown (16). 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 (5, 25). 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 and Kenner (13) 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 (21). Thus diet alone can affect the proportion of

1 enterococci found in human fecal material. Evison and James (10) also
2 reported differences in survival patterns of fecal coliforms and fecal
3 streptococci depending on the temperature. They observed that fecal
4 streptococci survive better than fecal coliforms at low temperatures
5 ($< 4^{\circ}\text{C}$); however, at temperatures above 20°C E. coli survive better
6 because of the rapid die-off rates of the fecal streptococci. Gordon and
7 Fliermans (14) demonstrated that E. coli could survive for much longer
8 periods of time in a lake receiving thermal effluent. Thus,
9 environmental factors might also significantly affect the recovery and
10 proportion of fecal coliforms and fecal streptococci in environmental
11 samples.

12 Environmental factors like temperature, solar radiation, seasonal
13 variability, and concentration of nutrients are quite different in tropical
14 aquatic systems as compared to temperate aquatic systems. Bigger (4)
15 first reported the growth of coliforms in tropical waters in 1937, while
16 in 1939 Ragavachari and Iyer (32) showed that coliforms can survive
17 for several months in natural tropical river waters. Recent studies in
18 Puerto Rico (19) showed that the survival of fecal coliforms increased in
19 marine and freshwater systems possibly because of the high nutrient
20 concentrations present. Other tropical studies have also shown that the
21 fecal coliforms (E. coli) may survive better in tropical freshwater than in
22 marine waters (11, 12, 20). Studies in Puerto Rico have demonstrated
23 high numbers of fecal coliforms in pristine rain forest streams where
24 there is no apparent fecal contamination source (8, 33).

25

1 Several studies have even suggested that E. coli may be part of
2 the normal aquatic flora in the tropics (11, 12, 20). Thus, high counts of
3 total coliforms and fecal coliforms in water supplies do not necessarily
4 indicate recent fecal contamination. The reliability of coliforms and
5 fecal coliforms as indicators of recent fecal contamination in tropical
6 waters is questionable when no identifiable source of fecal
7 contamination has been detected (8). This has suggested to us that the
8 fecal streptococci might be a more suitable alternative indicator of fecal
9 contamination in tropical waters. The present study examines the
10 ability of E. coli and S. faecalis to survive in a tropical marine
11 environment contaminated with petroleum.

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MATERIALS AND METHODS

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3 **Study Site.** Yabucoa Bay is on the southeastern corner of the
4 island of Puerto Rico. This man-made harbor was heavily oiled in 1978
5 with bunker C oil from a 105 m barge (30). Continuous traffic of oil
6 tankers enriches the hydrocarbon load in this area in addition to the
7 primary treated effluents from an oil refinery. Two sites were selected
8 for chamber studies (Fig. 1). Site A was adjacent to the harbor bridge,
9 where the oil tankers load or unload crude oil and its derivatives. Site B
10 was located 100 m away on the other side of the bay and was
11 presumably much less contaminated.

12 **Water Analysis.** Air and water temperature, pH, and
13 conductivity were measured in situ with a model 4041 Hydrolab
14 Surveyor (Hydrolab Corp., Austin, TX). Dissolved oxygen (DO) was
15 measured using the Winkler method (1). Water samples were collected
16 in dark plastic bottles and small amounts of a preservative - sulfuric
17 acid, zinc acetate, or mercuric chloride - were added before
18 transporting them to the laboratory where they were analyzed for
19 nitrates plus nitrites, sulfates, and total phosphorus using Standard
20 Methods for Water and Wastewater Analysis (1). One liter of water was
21 collected in an amber-colored glass bottle and analyzed for chlorophyll
22 A. Also, water samples were collected in dark plastic bottles and
23 transported to the laboratory to test for alkalinity, ammonia, and
24 turbidity (1). All samples were immediately placed on ice for transport
25 to the laboratory.

1 **Survival Studies.** Pure cultures of E. coli ATCC 11775 and
2 S. faecalis CBSC 15-5600A were grown in nutrient broth at 37°C for 24
3 h. The cells were harvested by centrifuging and were washed in 0.85%
4 sterile phosphate-buffered saline (pH 7). The final concentration was
5 adjusted to 10^8 cells ml⁻¹ using a model ZM Coulter Counter (Coulter
6 Electronics, Hialeah, FL). The final bacterial suspensions were placed in
7 sterile membrane diffusion chambers just before immersion in the site.
8 (For more details see Biamón and Hazen (3), Hazen and Esch (19), López
9 et al. (28)).

10 **Total Direct Counts and Cell Activity.** Bacterial cell densities
11 were determined by acridine orange direct counts (AODC) and Coulter
12 Counter counts (CC) as before (27). Respiring cells were determined
13 according to Zimmermann et al. (40). To determine the total INT
14 reduced by the bacterial population, an INT-formazan alcohol extraction
15 was performed after Jeffrey and Paul (21). Aliquots of the same water
16 samples, used to determine the number of respiring cells above, were
17 filtered using 0.2- μ m-pore size, 25-mm-diameter polycarbonate
18 membranes. The membranes were placed in clean glass containers and
19 1 ml of ethanol (95%) was added to extract the intracellular INT-
20 formazan. The ethanol extracts were clarified by filtration through a
21 Millex-GV 0.22- μ m filter, and formazan content was determined by
22 measuring the absorbance at 485 nm. INT-reduced-per-bacterial-cell
23 was calculated as suggested by Kurath and Morita (23).

24 Cells active in DNA synthesis were determined by
25 microautoradiography following Tabor and Neihof (35), using

1 [3H]thymidine (10 mCi/mmol) as the substrate. Water samples (1 ml)
2 from the chambers were taken and exposed to [3H]thymidine for 2.5 h
3 in the dark. After incubation, samples were fixed with 0.1 ml of 10 %
4 phosphate-buffered formalin. The water samples were stored on ice
5 and transported to the laboratory for analysis. Aliquots (0.1 ml) of the
6 water samples were filtered using 0.2- μ m pore size, 25-mm-diameter
7 polycarbonate membranes. The membranes were placed face-down on
8 a gel-matrix on microscope slides. After 24 h, membranes were
9 removed and slides exposed for 3 days to nuclear track emulsion, NTB2
10 (Eastman Kodak Co., NY). Slides were then examined by
11 epifluorescence/transmitted brightfield microscopy. Scintillation counts
12 were performed with aliquots of the same water samples to measure
13 the uptake of [3H]thymidine by the bacterial population over time. ATP
14 concentrations were measured with the luciferin-luciferase test (34)
15 using a liquid scintillation counter model LS 7500 (Beckman Instrument
16 Inc., Palo Alto, CA). ATP per bacterial cell was calculated as suggested
17 by Kurath and Morita (23).

18 **Data Analysis.** Programs developed for the Apple IIe and
19 Macintosh computers were used for statistical tests. Two-way analysis
20 of variance was used to test for differences between sites and over
21 time. Multiple correlation analyses were performed to establish the
22 relationship between bacteria and water quality. Heteroscedastic data
23 were made more homoscedastic using the appropriate transformation
24 prior to analysis. Any statistical probability less than or equal to 0.05
25 was considered significant (39).

RESULTS AND DISCUSSION

1 **Water Quality.** Representative water quality data are given in
2
3 Table 1. Concentrations of total organic carbon were greater than 4.03
4 mg/l at both sites (data not shown). These high concentrations of
5 organics are undoubtedly caused by petroleum contamination. The
6 other water quality parameters indicate that both sites were highly
7 productive compared to uncontaminated marine coastal areas in Puerto
8 Rico (28, 38). The low concentrations of nutrients (PO_4 , NO_{2+3}) observed
9 suggest that this bay is limited by concentrations of these nutrients.
10 This observation was further substantiated by microcosm studies in
11 which phosphorus and/or nitrogen was added to water from the bay
12 (Fuentes, F. A., 1987, Doctoral Dissertation, Department of Biology,
13 University of Puerto Rico, Río Piedras; Fuentes, manuscript in
14 preparation). Supplemented microcosm bacterial densities increased by
15 3 log values in 3 days, while unsupplemented microcosm densities
16 remained the same.

17
18 **Survival.** No significant differences were found in the densities
19 of S. faecalis or E. coli by site as determined by AODC and CC (Fig. 2 and
20 3). However, S. faecalis and E. coli cell densities declined significantly
21 over time for AODC ($F = 12.1$, $df = 14$ and 90 , $P < 0.001$; $F = 12.5$, $df = 14$
22 and 60 , $P < 0.001$; S. faecalis and E. coli, respectively) and CC ($F = 14.6$, df
23 $= 14$ and 90 , $P < 0.001$; $F = 18.6$, $df = 14$ and 60 , $P < 0.001$; S. faecalis and
24 E. coli, respectively). Density estimates by AODC were one order of
25 magnitude greater than Coulter Counter estimates due to the greater

1 sensitivity of the AODC method. Both techniques showed the same
2 trends and indicated only a 1 log decrease over the 72 h period
3 observed. Thus S. faecalis and E. coli were surviving in situ in this
4 environment. Several previous investigations have reported that high
5 temperature and high salinity environments are stressful to enteric
6 bacteria and some pathogens (9, 17, 27, 30, 33). However, several
7 studies (3, 14, 25, 27, 37) have shown that enteric bacteria can
8 overcome the bactericidal effects of seawater and temperature if the
9 environment is high in nutrients. Yabucoa Bay has high concentrations
10 of total organic carbon caused by petroleum contamination. This
11 apparently increases the survivability of E. coli and S. faecalis. In
12 addition, the gross morphological changes observed to occur in cells of
13 several bacteria exposed to marine environments were not observed in
14 this study (9, 27, 30, 33, 38).

15 Activity. S. faecalis and E. coli cell activities, as determined by
16 the percentage of cells active in protein synthesis (AODC), were
17 significantly different over time ($F = 4.9$, $df = 14$ and 90 , $P < 0.001$ and F
18 $= 2.5$, $df = 14$ and 60 , $P < 0.02$, respectively; Fig. 4). No evident pattern
19 in S. faecalis protein synthesis was observed at site A even though the
20 number of active cells was lower by the end of the study. High
21 percentages of E. coli and S. faecalis cells were active in protein
22 synthesis at both sites. Although some fluctuations were observed, the
23 percentage of E. coli cells active in protein synthesis was as high at the
24 end of the study as at the beginning. In general, S. faecalis and E. coli
25 protein synthesis was higher at site A, where total organic carbon and

1 total phosphorus were in higher concentrations, than at site B. This
2 suggests that high concentrations of nutrients in marine waters can
3 increase survival and activity.

4 The percentage of fermenting S. faecalis cells was not significantly
5 different by site; however, it decreased significantly over time ($F = 15.0$,
6 $df = 14$ and 90 , $P < 0.001$) at both sites (Fig. 5). The percentage of
7 respiring E. coli cells declined significantly over time ($F = 4.6$, $df = 14$
8 and 60 , $P < 0.001$; Fig. 5). Differences in the interaction between site
9 and time were also observed ($F = 4.1$, $df = 14$ and 60 , $P < 0.001$). The
10 percentage of E. coli and S. faecalis active in respiration (E. coli) or
11 fermentation (S. faecalis) declined slowly during the study. S. faecalis
12 fermenting cells decreased more rapidly at site A, and after 3 days (69
13 h) only 2% of S. faecalis cells were fermenting. However, at site B,
14 S. faecalis cells were observed to increase in activity at 24 h, although
15 only 5% of the cells were active in fermentation at the end of the
16 survival study. These measurements correspond to respiration rates
17 found in more oligotrophic, temperate, marine environments (40) and
18 would seem to indicate that both S. faecalis and E. coli decreased in
19 activity over time.

20 Two basic problems reportedly occur with the microscopic
21 observation of INT-formazan granules: the concentration of INT reduced
22 by some metabolically active bacterial cells may not be sufficiently high
23 to form visible granules (40), and intracellular INT-formazan granules
24 may be dissolved by immersion oil within a short period of time (28,
25 36). In both cases there might be a significant underestimation of the

1 bacterial population that is metabolically active. Because
2 dehydrogenase enzymes should not be active in dormant cells, INT-
3 reduced-by-individual-cells was calculated as a function of respiring or
4 fermenting cells, as suggested by Kurath and Morita (23). When the
5 activity is examined in this way, we see that both S. faecalis and E. coli
6 activity significantly increase over time ($F = 2.8$, $df = 14$ and 90 , $P <$
7 0.005 ; $F = 6.3$, $df = 14$ and 60 , $P < 0.001$; S. faecalis and E. coli,
8 respectively; Fig. 6). Indeed E. coli reduced more INT per cell at site A
9 than at site B ($F = 11.0$, $df = 1$ and 60 , $P < 0.005$; Fig. 6). This further
10 suggests a higher population-carrying capacity at site A. The slight
11 decline in cell density observed in both bacteria left the remaining
12 population more active, suggesting some environmental adaption had
13 occurred. Jones and Simon (22) found that planktonic and benthic
14 bacteria in an English Lake contained from 1×10^{-11} g to 9×10^{-11} g of
15 INT-formazan per cell, which is somewhat greater than most of the
16 amounts of INT reduced by S. faecalis and E. coli per cell in our study.
17 However, the habitats studied by Jones and Simon (22) were freshwater
18 and probably higher in nutrient concentrations than Yabucoa Bay.

19 Significant increases over time were also observed in ATP per cell
20 for both S. faecalis ($F = 4.8$, $df = 3$ and 24 , $P < 0.02$; Fig. 7) and E. coli ($F =$
21 4.6 , $df = 3$ and 16 , $P < 0.05$; Fig. 7). Thus ATP also suggests that, after
22 the initial decline, the remaining portion of both populations was much
23 more active than the initial population. ATP per cell ranged from an
24 average of 2.16×10^{-12} μ g to 1.59×10^{-10} μ g for E. coli and an average
25 of 2.12×10^{-12} μ g to 1.39×10^{-9} μ g for S. faecalis. This is somewhat

1 lower than the range found by Hamilton and Holm-Hansen (16) of $0.5 \times$
2 $10^{-9} \mu\text{g}$ to $6.5 \times 10^{-9} \mu\text{g}$ for marine bacteria in the log phase of growth
3 and by Kurath and Morita (23) for a marine Pseudomonas sp. in a
4 survival study. However, the bacteria used in this study were enteric in
5 origin and should have lower physiological activities than bacteria of
6 marine origin.

7 The percentage of S. faecalis cells active in DNA synthesis as
8 determined by microautoradiographs was significantly different over
9 time ($F = 9.2$, $df = 6$ and 42 , $P < 0.001$), but only slightly higher at the
10 end of the study at both sites. Site B had a lower level of synthesis
11 activity; however, the percent of active cells was lower than 15% at all
12 times (Fig. 8). Significant differences in the interaction between site
13 and time were also observed ($F = 3.9$, $df = 6$ and 42 , $P < 0.02$).
14 [^3H]thymidine uptake by S. faecalis increased significantly over time at
15 both sites ($F = 3.1$, $df = 6$ and 42 , $P < 0.05$) but no significant differences
16 were observed by site (Fig. 9). The percentage of E. coli cells active in
17 DNA synthesis decreased significantly over time ($F = 6.9$, $df = 6$ and 28 ,
18 $P < 0.001$, and $F = 5.1$, $df = 6$ and 28 , $P < 0.05$, microautoradiography
19 and [^3H]-thymidine uptake, respectively; Figs. 8 and 9). The percentage
20 of S. faecalis cells active in DNA synthesis was higher than E. coli after
21 24 h at site A. However, other metabolic activities were frequently
22 higher for E. coli during the same period at site A. This suggests that
23 although protein synthesis and respiration were higher for E. coli, the
24 S. faecalis cells were just as active. This was further supported by the
25 slightly higher concentrations of ATP per cell seen for S. faecalis.

1 Studies by Xu et al. (38), and Roszak et al. (33) showed that some
2 bacteria could be "viable but nonculturable", a concept which suggests
3 that most of the bacterial population, if not all, that can be seen when a
4 water sample is being examined under the microscope must be alive
5 even though part of it can not be cultured on artificial media. Roszak et
6 al. (33) observed that after 48 h starvation Salmonella enteritidis did
7 not grow on non-selective media, although cell densities greater than
8 10^4 ml^{-1} were obtained by AODC, fluorescent antibody (FA) and direct
9 viable counts (DVC). Colwell et al. (9) showed that a pathogenic Vibrio
10 cholerae that lost the ability to grow on conventional culture media not
11 only remained as virulent to rabbits as a non starved strain, but
12 regained their ability to grow in selective and nonselective media.
13 These findings reinforce the necessity of other methods to accurately
14 determine bacterial activity in aquatic systems. Our results point out
15 the importance of measuring several physiological parameters
16 simultaneously for different bacteria in order to correctly determine the
17 effect of ambient water quality on the physiological status of the cells
18 in situ.

19 This study showed that S. faecalis and E. coli can survive and
20 remain active in petroleum contaminated tropical marine waters. This
21 does not support previous investigations that have suggested that fecal
22 streptococci survive better than fecal coliforms in marine waters. Thus,
23 the fecal streptococci do not represent a better alternative to fecal
24 coliforms in tropical marine waters. Because coliforms have also been
25 demonstrated not to fulfill the requisites of a good indicator of fecal

¹ pollution in tropical areas (8, 11, 19, 26, 27, 37), maximum biological
² contaminant levels for tropical waters should be based upon direct
³ monitoring of one or more resistant pathogens rather than the indicator
⁴ organisms currently used, i. e., coliforms or fecal coliforms.

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FIGURE LEGENDS

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- 3 Figure 1. Map of sampling sites in Puerto Rico.
- 4 Figure 2. Survival of Streptococcus faecalis and Escherichia coli at sites
- 5 A and B of Yabucoa Bay by Acridine Orange Direct Count
- 6 (Mean $\pm$ one standard error, n=4).
- 7 Figure 3. Survival of Streptococcus faecalis and Escherichia coli at sites
- 8 A and B of Yabucoa Bay by Coulter Count (Mean $\pm$ one
- 9 standard error, n=4).
- 10 Figure 4. Activity of Streptococcus faecalis and Escherichia coli at sites
- 11 A and B of Yabucoa Bay by Acridine Orange staining (Mean $\pm$
- 12 one standard error, n=4).
- 13 Figure 5. Activity of Streptococcus faecalis and Escherichia coli at sites
- 14 A and B of Yabucoa Bay by INT respiration (Mean $\pm$ one
- 15 standard error, n=4).
- 16 Figure 6. Activity of Streptococcus faecalis and Escherichia coli at sites
- 17 A and B of Yabucoa Bay by INT-formazan per cell (Mean $\pm$
- 18 one standard error, n=4).
- 19 Figure 7. Activity of Streptococcus faecalis and Escherichia coli at sites
- 20 A and B of Yabucoa Bay by ATP per cell (Mean $\pm$ one standard
- 21 error, n=4).
- 22 Figure 8. Activity of Streptococcus faecalis and Escherichia coli at sites
- 23 A and B of Yabucoa Bay by microautoradiography (Mean $\pm$
- 24 one standard error, n=4).
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1 Figure 9. Activity of Streptococcus faecalis and Escherichia coli at sites
2 A and B of Yabucoa Bay by incorporation of [³H]-thymidine
3 (Mean $\pm$ one standard error, n=4).
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Table 1. Water physicochemical parameters by sampling site.

Parameter	ATEMP	WTEMP	DO	pH	ALKAL	NO ₂₊₃
Site A	25.8 ± 1.7	29.1 ± 0.2	6.0 ± 0.1	7.3 ± 0.1	100 ± 0	0.07 ± 0.00
Site B	25.0 ± 1.5	28.1 ± 0.3	6.1 ± 0.1	7.5 ± 0.1	97 ± 3	0.06 ± 0.01

Parameter	TP	SO ₄	Chl A	COND	SAL	NH ₄
Site A	0.01 ± 0.00	1.84 ± 0.11	2.61 ± 0.38	504 ± 2	34.5 ± 0.4	2.34 ± 0.09
Site B	0.00 ± 0.00	1.96 ± 0.03	3.07 ± 0.53	510 ± 2	34.2 ± 0.1	2.25 ± 0.10

All values are mean ± one standard error (n = 7), ATEMP = air temperature (°C), WTEMP = water temperature (°C), DO = dissolved oxygen (mg/L), COND = Conductivity (x1000 umohs/cm), SO₄ = sulfates (x1000 mg/L), Alkal = Alkalinity (mg/L CaCO₃), Sal = salinity (ppt), NH₄ = ammonium (mg/L), NO₂₊₃ = nitrites plus nitrates (mg/L), TP = total phosphorus (mg/L), ChlA = chlorophyll A (mg/L).