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Comparison of the In Situ Survival and Activity of *Klebsiella pneumoniae* and *Escherichia coli* in Tropical Marine Environments

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Abstract. A near-shore coastal mangrove island receiving untreated sewage and a coastal cove receiving rum distillery effluent in Puerto Rico were examined for their ability to support survival and activity of *Klebsiella pneumoniae* and *Escherichia coli*. Pure cultures of both bacteria were monitored for 96 hours in situ at both locations using membrane diffusion chambers. *K. pneumoniae* survived at all sites as measured by AODC and Coulter Counter direct counts. However, at the mangrove island less than 20% of the *K. pneumoniae* population was active (AODC) after the first 3 hours and less than 10% of this population was respiring (INT). In contrast, the coastal area which was receiving rum distillery effluent was able to maintain 40% of the *K. pneumoniae* population in an active state with 90% respiring. The *E. coli* population declined by two orders of magnitude at the mangrove island, but remained unchanged at the rum distillery outfall. The *E. coli* population had a higher proportion of active cells and respiring cells than *K. pneumoniae* at all sites. At the rum distillery site, the *E. coli* population was remarkable in that 95% remained active and 99% were respiring. This study suggests that, when sufficient organic loading exists, *E. coli*, a "nonsurvivor," can overcome the bactericidal effects of tropical marine waters. *K. pneumoniae*, a "survivor," could survive under all conditions but could not maintain the activity or respiration that the *E. coli* population could, even when high organic loads were present. Morphological changes related to nutrient stress in the tropical marine environment were apparent in *E. coli*, but not in *K. pneumoniae*. Based on physiological activity *E. coli* is just as much a "survivor" as *K. pneumoniae* in tropical marine waters.

Introduction

Since 1914, the United States has used coliforms as the standard indicator of human pathogens in recreational waters. However, recently the U.S. Environ-

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mental Protection Agency (EPA) presented a radical change from this tradition and recommended that "fecal coliforms should be used as the indicator organism for evaluating the microbiological suitability of recreational waters" [8]. As discussed by Cabelli et al. [8], of 50 states, 5 U.S. territories, and the District of Columbia, only 12 used coliforms to regulate quality of primary contact marine recreational waters, while 27 used fecal coliforms. A major switch is now being made to fecal coliforms as the indicator of choice in temperate marine recreational waters. Unfortunately, more and more, the "maximum contaminant levels" established for temperate areas have been accepted without question by tropical nations. Despite this trend, there is a growing body of evidence that the underlying assumptions of even the fecal coliform assay are not valid in tropical climates [6, 24].

Three of the basic underlying assumptions for using fecal coliforms as indicators of human pathogens are: there are no organisms of extraenteral origin that can give a positive fecal coliform reaction, fecal coliforms die off similarly to human pathogens, and fecal coliforms cannot grow and survive for extended periods in the marine environment [6]. It is assumed that very few organisms, e.g., *Escherichia coli* and thermotolerant *Klebsiella pneumoniae*, can give a positive fecal coliform reaction [3, 8]. Previous studies have classified *E. coli* as a nonsurvivor in marine environments and *K. pneumoniae* as a survivor [33]. However, only density changes were monitored in these studies, and only viable plate count methods were used to determine densities. As shown recently by Xu et al. [34] in microcosms, *E. coli* and *Vibrio cholerae* can survive and remain viable but nonculturable under conditions simulating temperate marine environments. These findings raise serious doubts regarding the efficacy of using fecal coliforms for maximum contaminant level regulations for primary contact marine recreational waters. Also, none of the previous studies has considered how the unique water qualities of tropical marine waters may affect survival and viability of indicators and pathogens.

Puerto Rico, with its tropical climate, is a North American center for year-round recreational marine water activities, e.g., swimming, fishing, sailing, scuba diving, snorkeling. The industrialization of Puerto Rico has also created gross contamination by a variety of pollutants, and sewage treatment facilities have deteriorated to the point where the best service offered frequently is only primary sewage treatment [10, 17, 19, 32]. Adequate regulations for microbiological maximum contaminant levels for Puerto Rico and other tropical nations are of both public health and economic (tourism) importance. The present study examines the survival and physiological activity of *E. coli*, a "nonsurvivor," and *K. pneumoniae*, a "survivor" and virulent human pathogen [33]. Both bacteria were studied in situ in polluted and unpolluted marine environments of Puerto Rico.

Materials and Methods

Study Sites

La Gata Island is located on the inner-shelf of the southwest coast of Puerto Rico at 17°57'N and 67°02'W near the small fishing village of Parguera (Fig. 1). There is one high and one low tide

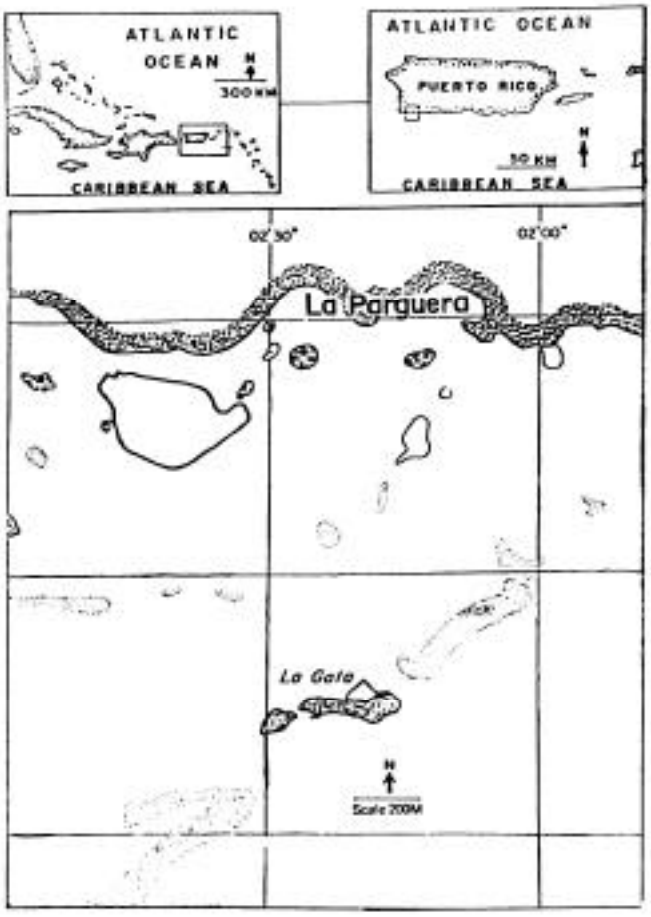

Fig. 1. Map of study sites at La Gata, Puerto Rico.

daily with a range of less than 0.33 m. Parguera receives an average rainfall of 100 cm annually which is offset by an annual average evaporation rate of 250 cm [1]. During 1980, La Gata was inaugurated as a recreational area by the Department of Natural Resources. During 1981 a total of 85,909 persons visited the island with an average of 7,160 persons monthly and 239 persons daily (Department Natural Resources, personal communication). Installation of two toilets created a raw sewage outfall on the island. Dye studies conducted by our laboratory showed that 20 min after the dye cone was flushed the dye could be detected in the surrounding waters. After 1 hour, the dye had spread to 50% of the island's north coast, and within 2 hours it covered the entire north coast including the area reserved for swimming.

Ensenada de Boca Vieja (18°27'48"N, 66°08'42"W) is a protected cove immediately adjacent to San Juan Bay, Puerto Rico (Fig. 2). This cove has a tidal range of 0.5 m, shoreline length of 855 m, mean depth of 4 m, surface area of 94,643 m^2, and received 1.4×10^6 l day^{-1} of untreated effluent from a single rum distillery [14]. An underground pipe connected the discharge point with the rum distillery 1 km to the southwest. Shore currents moved the effluent plume in a westerly direction, parallel with the shoreline. On rare occasions the wind was from the northwest and created a current that moved the waste plume in an easterly direction, so that it tended to accumulate in the apex of the cove.

Fig. 2. Map of study sites at Ensenada de Boca Vieja, Puerto Rico.

Water Analysis

Measurements were taken in situ for conductivity, salinity, pH, dissolved oxygen, light intensity, and temperature. The pH was measured with a digital pH meter, model 201 (Orion Research, MA) and dissolved oxygen with a model 57 DO meter (Yellow Springs Instrument Company, Yellow Springs, OH). A model 33 S-C-T meter (Yellow Springs Instrument Company, Yellow Springs, OH) was used to measure conductivity and salinity. Turbidity, alkalinity, hardness, and ammonia measurements were done in the field using a Mini Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, NY) [2]. Light intensity was measured in the field with an underwater photometer (Protomatic, Dexter, MI). For chlorophyll A determination, water samples were placed in amber-colored plastic bottles and analyzed at the laboratory using the trichromatic extraction method [2]. Other samples were fixed with mercuric chloride, sulfuric acid, and zinc acetate before being transported to the laboratory where they were analyzed for nitrate, sulfate, total phosphorus, and phosphate according to Standard Methods for Water and Wastewater Analysis [2].

Bacteriological Analysis

Samples of water were placed in sterile 180 ml Whirl-Pak bags (Nasco, Ft. Wilkinson, WI) and transported on ice to the laboratory. Samples were filtered using 0.45 μm pore size, 47 mm diameter, HA type membrane filters (Millipore Co., Bedford, MA). The filters were placed on double violet red bile (DVA) agar and incubated at 37°C for 24 hours. Following incubation, glistening lavender colonies 3–5 mm in diameter were counted as presumptive *K. pneumoniae* [9].

Random isolates were further characterized as *K. pneumoniae* using API-20E strips (Analytab Products Inc., Plainview, NY).

For fecal coliform counts, water samples were filtered through a sterile, 47 mm diameter, HC type membrane filter with a pore diameter of 0.7 μm (Millipore Corp, Bedford, MA). The filters were then placed on mFC medium (Difco, Detroit, MI) and incubated at 44.5°C for 24 hours. Blue colonies were counted as fecal coliforms. Direct cell counts for *K. pneumoniae* and *E. coli* in diffusion chambers were done using acridine orange staining and polycarbonate Nuclepore filters (0.20 μm pore size, 47 mm diameter) dyed with Sudan black (1:15,000), according to Hobbie et al. [20]. Half a milliliter of sample was filtered, and the filter was then stained with 0.01% acridine orange for 2 min. The filter was then placed on a microscope slide and examined with an epifluorescent microscope. Red fluorescing cells were assumed to be active in protein synthesis since the red fluorescence is caused by a dominance in RNA content. Cells with more DNA than RNA will fluoresce green [12]. Studies in our lab have shown that when *E. coli* and other bacteria are placed in distilled water, i.e., starved, the proportion of red fluorescing cells will decrease from 100 to 50% in 48 hours and to 10% in 72 hours. Simultaneous estimates of INT reduction show a decrease from 100 to 10% in 48 hours with no change from 48 to 72 hours. However, the coefficient of variation (CV) for the INT reduction is always greater than 100%, whereas the CV for the red fluorescing cells is less than 100%. Field studies conducted by our lab have shown that the number of red fluorescing *E. coli* remains high in tropical freshwaters as does the proportion of respiring cells [10]. The field studies also showed that activities measured by AODC were higher and less variable than activities measured by INT reduction [10]. This is expected for a facultative anaerobe like *E. coli*. Indeed, this same study showed that *Bifidobacterium adolescentis*, a microaerophile, had virtually no detectable INT reduction at any time, whereas the proportion of red fluorescing cells always exceeded 50% [10]. All of this indicates that the proportion of red fluorescing cells in AODC is a quick and accurate way to determine physiological activity in situ. Caution, however, must be used to maintain an exact acridine orange concentration and staining time in order to make accurate comparisons between samples.

Total number of *K. pneumoniae* and the number involved in respiration were determined using the technique of Zimmerman et al. [36]. The technique is based upon the assumption that the electron transport system of respiring organisms reduces 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan. Respiring bacteria accumulate INT-formazan intracellularly, which appears as dark granules under a light microscope. To 0.5 ml of water sample, 0.05 ml of INT (0.2% w/v) was added and incubated 20 min in the dark at in situ temperature. To stop the reaction, 0.3 ml of formaldehyde (37%) was added. The sample was later filtered and stained with acridine orange as described above. The filter was examined through the epifluorescent microscope for fluorescing cells that also contained INT-formazan granules; positive cells were presumed to be respiring.

Survival Studies

For survival studies of *K. pneumoniae*, plexiglass diffusion chambers, a modification of McFeters and Stuart [26] with 100 ml capacity, were used with a 0.45 μm pore size, nylon-reinforced Versapor membrane filters (Gelman Instrument Co., Ann Arbor, MI) as a diffusion surface [5, 13, 21]. O-rings were added to chambers to reduce leakage and contamination. Pure cultures of *K. pneumoniae* were grown in 5% tryptic soy broth at 37°C for 24 hours. The cells were then harvested by centrifugation and resuspended in filter-sterilized phosphate-buffered saline (pH 7). Cell density was determined with a model ZF Coulter Counter (Coulter Electronics, Hialeah, FL) and adjusted to a concentration of 10^7 cells ml^{-1}. The bacterial suspension was placed in sterile diffusion chambers just before placing them at the study sites. At the study site, a total of five chambers were placed strategically at a depth of 1 m. Periodically 1.0 ml samples were taken from each chamber with a sterile syringe. Half a milliliter of each sample was fixed with 1.5 ml of phosphate-buffered formalin for later counting at the laboratory with a Coulter Counter (CC), as described by Hazen and Esch [18]. The other 0.5 ml was incubated with INT and fixed. The preserved sample

was then stored on ice for membrane filtration at the laboratory and subsequent total direct counts and activity measurements, as described above.

Data Analysis

The data were analyzed using prepared programs for Apple II Plus and IBM 370-148 computers. Factorial analyses of variance were used to test for differences between sites and collection times. Multiple correlation and regression analyses were used to determine relationships between parameters measured. Data were subjected to the appropriate transformation prior to statistical analysis according to Zar [35]. Any probability less than or equal to 0.05 was considered significant [35].

Results

Water Quality

The two sites at La Gata Island were similar in water quality (Table 1). In comparison, site A at Ensenada de Boca Vieja had significantly higher water temperature, nitrate and nitrite, phosphates, total phosphorus, turbidity, densities of fecal coliforms, and densities of *Klebsiella* spp. (Table 1). Also site A had significantly lower levels of dissolved oxygen, pH, and salinity. Many of the differences observed between the two sites were greater than one order of magnitude. However, during the diffusion chamber study the effluent discharge stopped at 48 hours, and within 3 hours there were no detectable differences between the two sites for any of the water quality parameters tested.

Enumeration of *K. pneumoniae* by DVA at the two La Gata sites revealed less than 10 CFU/100 ml. Counts for fecal coliforms were 0–300 CFU/100 ml, with all viable counts significantly higher along the north coast of La Gata Island. At Ensenada de Boca Vieja, DVA counts for *K. pneumoniae* were from 100–2,000 CFU/100 ml at the effluent point source (site A) but were always less than 80 CFU/100 ml only 100 m upcurrent (site B). Densities of fecal coliforms were two orders of magnitude higher at the effluent outfall, e.g., 800 CFU/100 ml compared to 0/100 ml.

Survival of Bacteria In Situ

At La Gata Island the densities of *K. pneumoniae*, as determined by CC counts of samples from the diffusion chambers, decreased significantly over time ($F = 6.11$, df = 12 and 25, $P = 0.001$) (Fig. 3). Densities of *K. pneumoniae* as determined by AODC were also significantly different over time ($F = 6.01$, df = 12 and 26, $P < 0.001$) (Fig. 4). The proportion of the *K. pneumoniae* population that was active as measured by AODC declined significantly during the first 9 hours ($F = 8.11$, df = 12 and 26, $P < 0.0001$) and then remained low (Fig. 5). The proportion of the *K. pneumoniae* population that was respiring as determined by INT, also declined significantly during the first 9 hours ($F = 5.10$, df = 12 and 26, $P < 0.001$) (Fig. 6). The initial decline (first 3 hours) in activity and respiration was greater than 70%. After this, both measurements

Table 1. Water quality of La Gata and Ensenada de Boca Vieja, Puerto Rico

Sites	ATEMP	WTEMP	DO	pH	Sal	NO_{2+3}	PO_4	TP	Chl A
LG-N	27.5 ± 0.4	29.0 ± 0.3	6.1 ± 0.7	7.6 ± 0.2	35 ± 1	0.045 ± 0.01	<.1	<.1	2.13 ± 2
LG-S	27.1 ± 0.6	29.0 ± 0.3	5.6 ± 0.2	7.5 ± 0.2	35 ± 1	0.045 ± 0.01	<.1	<.1	29.5 ± 5
EBVA	27.0 ± 2.0	33.4 ± 1.5	4.2 ± 1.3	5.1 ± 0.3	34.3 ± 4.0	4.8 ± 0.3	19.6 ± 15.5	26.8 ± 9.8	1,342 ± 5,495
EBVB	27.0 ± 2.0	27.3 ± 0.6	5.3 ± 1.7	7.6 ± 0.3	32.2 ± 3.	0.15 ± 0.12	<.1	<.1	133 ± 116

Sites	NH_4	Lup	LDN	Turb	DVA	FC
LG-N	2.1 ± 1.0	7,966 ± 1,836	1,423 ± 242	95.6 ± 2	0.04 ± .01	3 ± 1
LG-S	3.0 ± 1.0	6,500 ± 1,900	1,733 ± 405	94.1 ± 1	1.0 ± 0.5	0 ± 0.1
EBVA	ND	9,000 ± 2,000	5,000 ± 1,000	72 ± 2	20 ± 5	8 ± 1
EBVB	ND	1,000 ± 450	2,000 ± 500	98.5 ± 11.5	0.8 ± 0.1	0 ± 1

Abbreviations: WTEMP = water temperature (°C); ATEMP = air temperature (°C); DO = dissolved oxygen (mg ml^{-1}); Chl A = chlorophyll A (mg ml^{-1}); NO_{2+3} = nitrites + nitrates (mg $liter^{-1}$); PO_4 = phosphates, P, (μg $liter^{-1}$); TP = total phosphorus (μg $liter^{-1}$); sal = salinity (ppt); NH_4 = ammonia (mg $liter^{-1}$); Lup = incident light (foot candles); LDN = reflected light (foot candles); Turb = turbidity (% transmittance); DVA = *Klebsiella* (CFU ml^{-1}); FC = fecal coliforms (CFU ml^{-1}); LGN = La Gata North site, LGS = La Gata South site; EBVA = Ensenada de Boca Vieja site A, EBVB = Ensenada de Boca Vieja site B. (All values are means ±1 standard error, n = 9.)

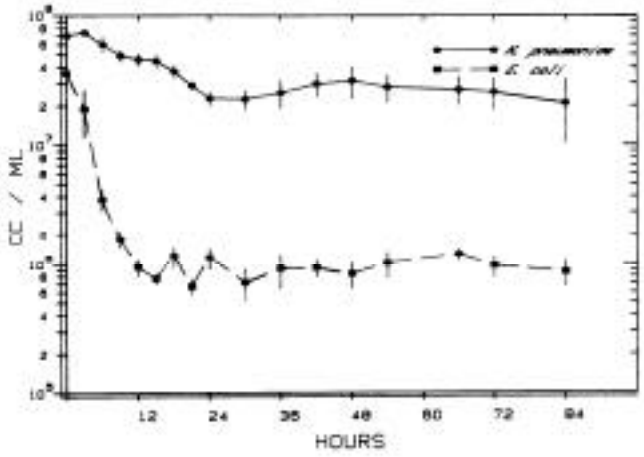

Fig. 3. Changes in total density as measured by Coulter County at La Gata Island. North and South sites were combined since there were no significant differences. (Mean ±1 standard error, n = 8.)

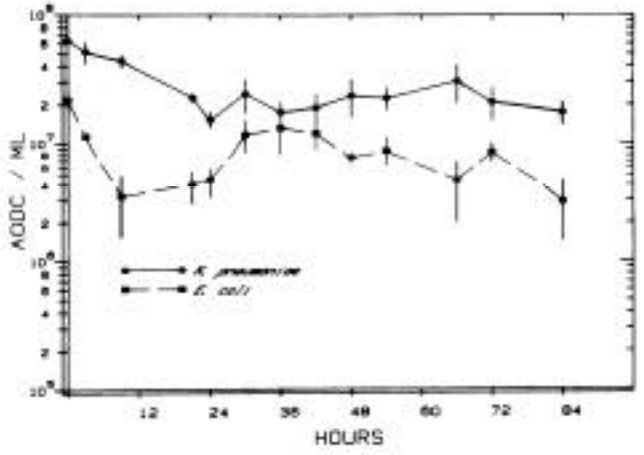

Fig. 4. Changes in total density as measured by AODC at La Gata Island. North and South sites were combined since there were no significant differences. (Mean ±1 standard error, n = 8.)

fluctuated between 1 and 30% without apparent pattern. None of the measurements were signficantly different by site.

Densities of *E. coli* in the diffusion chambers at La Gata Island, as determined by CC counts, declined significantly over time ($F = 40.7$, df = 16 and 34, $P < 0.0001$) (Fig. 3). The same was also true when densities of *E. coli* were determined by AODC, i.e., over time, differences were significant ($F = 3.47$, df = 7 and 16, $P < 0.05$) (Fig. 4). The activity of the *E. coli* population declined by 40% in the first 3 hours and then fluctuated between 1 and 35% with a large variability between samples (Fig. 5). The percentage of the *E. coli* population that was respiring changed significantly over time ($F = 9.6$, df = 6 and 14, $P < 0.005$) (Fig. 6). Like *K. pneumoniae*, the activity and respiration rate of the *E. coli* cells declined significantly only during the first 3 hours, i.e., more than 50%. During the subsequent samplings the variability in these measurements was even greater for *E. coli* than it was for *K. pneumoniae*. In comparison, *K. pneumoniae* had a significantly greater survival rate than *E. coli* at La Gata

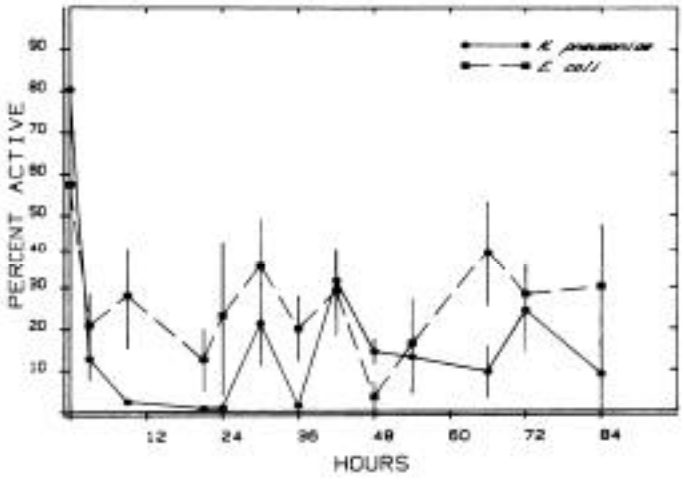

Fig. 5. Changes in % activity as measured by AODC at La Gata Island. North and South sites were combined since there were no significant differences. (Mean ±1 standard error, n = 8.)

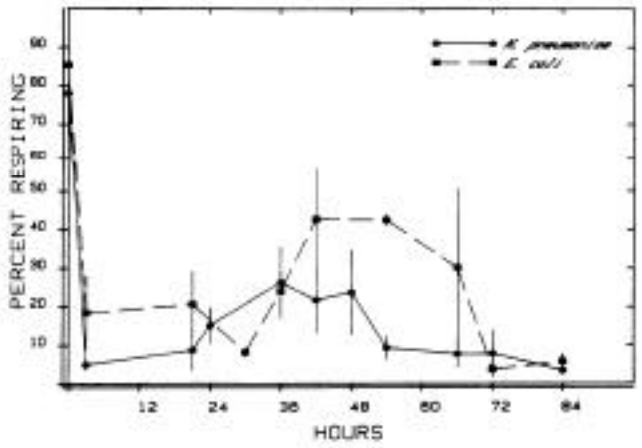

Fig. 6. Changes in % respiration as measured by INT at La Gata Island. North and South sites were combined since there were no significant differences. (Mean ±1 standard error, n = 8.)

Island; 21.4 and 0.025%, respectively, survived 96 hours. However, decline in activity in the remaining population as measured by AODC activity and INT respiration was greater for *K. pneumoniae* than it was for *E. coli* at these sites.

Densities of *K. pneumoniae* as determined by CC and AODC were not significantly different over time or between sites at Ensenada de Boca Vieja (Figs. 7 and 8). The percentage of the *K. pneumoniae* population that was active was significantly higher at site A ($F = 7.39$, df = 1 and 26, $P < 0.05$) for the first 48 hours during distillery effluent discharge (Fig. 9). After 48 hours there were no significant differences between sites, since the activity at site A declined by more than 30% 24 hours after the discharge was stopped. The percentage of *K. pneumoniae* that was respiring was not significantly different between sites because of large variability among samples (Fig. 10). After 48 hours there was a significant decline in the proportion of *K. pneumoniae* that was respiring ($F = 6.35$, df = 4 and 10, $P < 0.02$), largely resulting from the dramatic (70%) decline that occurred at site A only 6 hours after the discharge was stopped.

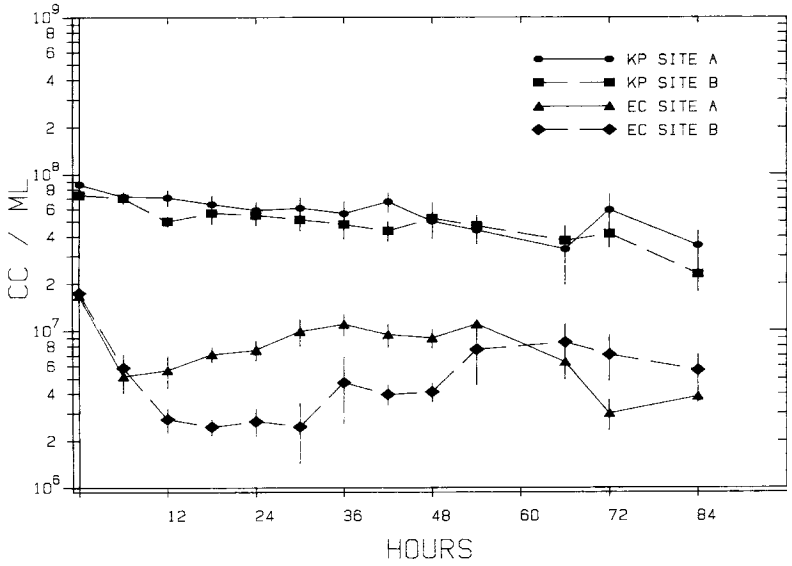

Fig. 7. Changes in total density as measured by Coulter Counter by site at Ensenada de Boca Vieja cove. Effluent at site A was shut off at 48 hours. (Mean ±1 standard error, n = 4.)

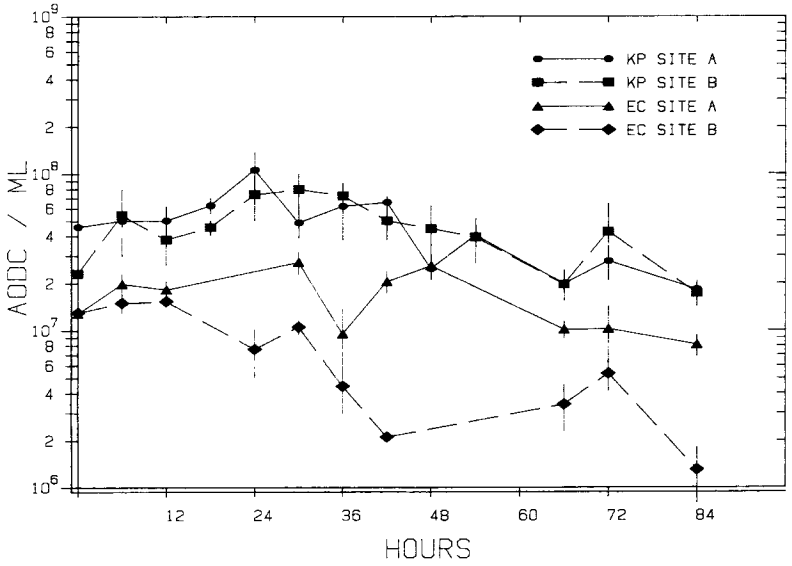

Fig. 8. Changes in total density as measured by AODC by site at Ensenada de Boca Vieja cove. Effluent at site A was shut off at 48 hours. (Mean ±1 standard error, n = 4.)

Densities of *E. coli* in the diffusion chambers at Ensenada de Boca Vieja were significantly higher at the effluent site as measured by CC ($F = 5.66$, df = 1 and 26, $P = 0.05$) but not at all times (Fig. 7). The percentage of the *E. coli* population that was active, as measured by AODC, was significantly higher at the effluent site until 48 hours ($F = 6.12$, df = 1 and 16, $P = 0.05$) and then declined significantly at the effluent site ($F = 7.3$, df = 9 and 22, $P < 0.0001$)

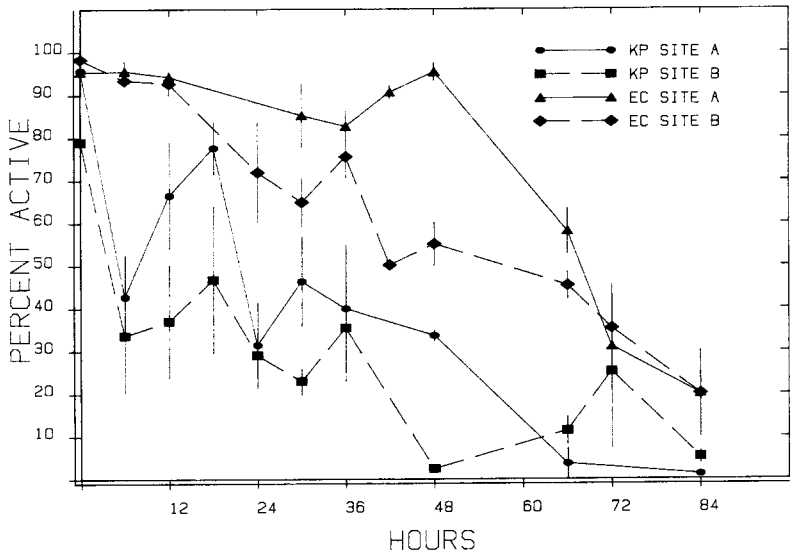

Fig. 9. Changes in % activity as measured by AODC by site at Ensenada de Boca Vieja cove. Effluent at site A was shut off at 48 hours. (Mean ±1 standard error, n = 4.)

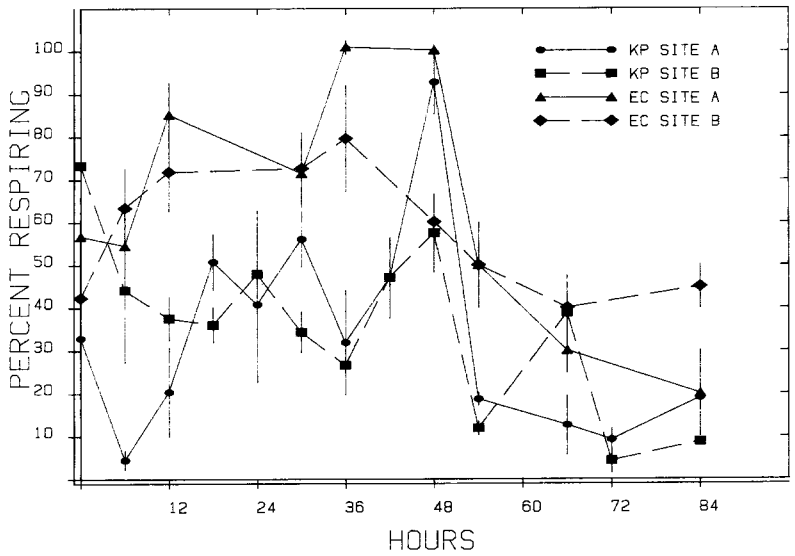

Fig. 10. Changes in % respiration as measured by INT by site for Ensenada de Boca Vieja cove. Effluent at site A was shut off at 48 hours. (Mean ±1 standard error, n = 4.)

(Fig. 9). The percentage of the *E. coli* population that was respiring was also significantly higher until the discharge was stopped ($F = 4.5$, df = 1 and 18, $P < 0.01$; Fig. 10). A 60% decline in the respiring population was observed only 6 hours after the discharge was stopped ($F = 4.5$, df = 8 and 18, $P < 0.01$).

In comparison, densities of *K. pneumoniae* in the chambers at Ensenada de

Boca Vieja did not show between-site differences as did the densities of *E. coli,* while the effluent was present. However, the activity of the *E. coli* population at both Ensenada de Boca Vieja sites was significantly higher than the activity of the *K. pneumoniae* population at those sites. In addition, the proportion of respiring cells of the *E. coli* population was also significantly greater than the proportion of *K. pneumoniae* cells that remained respiring. Activity and respiring counts for both bacteria declined more than 50% after 48 hours when the effluent discharge was stopped.

Densities of *K. pneumoniae,* as measured by AODC and CC, showed similar patterns of decline and variability in the diffusion chambers at La Gata and Ensenada de Boca Vieja. However, the proportion of the *K. pneumoniae* population that was active was 20–50% greater at Ensenada de Boca Vieja whereas the proportion that was respiring was 10–40% greater at this polluted site. The densities of *E. coli,* as measured by AODC and CC, at pristine La Gata declined much more rapidly than the densities of *E. coli* in chambers at effluent receiving Ensenada de Boca Vieja. Also, the proportion of the *E. coli* population that was active and respiring was 50% greater in diffusion chambers exposed to distillery effluent at Ensenada de Boca Vieja in comparison to chambers at La Gata. After the effluent discharge ceased at Ensenada de Boca Vieja, the differences in activity and respiration for both bacteria were not significantly different from La Gata Island.

Discussion

Water Quality

Dye studies showed that effluent from the two toilets on La Gata Island was well mixed along the north coast of the island 2 hours after being deposited. Thus, it is not surprising that the north coast site also had moderate levels of fecal coliforms even though the ambient water was toxic to coliforms, in terms of the synergistic effects of high dissolved oxygen, high light intensity, and high salinity. Nearly all known bacteria can survive these conditions for at least 2 hours [6]. Viable counts for *K. pneumoniae* and fecal coliforms were always higher along the north coast. However, recorded densities were below the recommended Maximum Contaminant Levels (MCL) for primary contact recreational waters [8].

Rum distillery effluent (mostos) in Ensenada de Boca Vieja has been shown to have serious effects upon the marine life not only at the point source of discharge but also adjacent areas [11, 16]. This and other studies [5] show that mostos significantly increases the concentration of nitrates, phosphates, total phosphorus, sulfates, and temperature. The amounts of nitrogen, phosphorus, and other inorganic compounds at the effluent point source are comparable to levels found in raw sewage. The BOD_5 for this rum distillery effluent is the highest ever reported for any effluent, $>32{,}000$ mg O_2 liter^{-1} [11]. Generally, site A at Ensenada de Boca Vieja is a strongly polluted site, and site B is a relatively pristine site. Site B at Ensenada de Boca Vieja was lower than any of the sites sampled for all inorganic nutrients, though chlorophyll A levels

were the highest at this site. Further proof that the effluent is the source of these between-site differences was seen when the effluent was shut off after 48 hours. Both sites at Ensenada de Boca Vieja were not different for all water quality parameters measured within 3 hours after the discharge was stopped.

Both sites at Ensenada de Boca Vieja had higher densities of viable *K. pneumoniae* and fecal coliforms than La Gata Island. However, the densities of *K. pneumoniae* were 10 times higher at the site receiving rum distillery effluent, and fecal coliform densities were 100 times greater at the effluent receiving site. The site that received rum distillery effluent always exceeded recommended MCL for fecal coliforms in primary contact recreational water, 200 CFU/100 ml [8]. Since *K. pneumoniae* can give a positive fecal coliform reaction [3, 8] it is suprising that fecal coliforms exhibited a greater difference between the two sites. The densities of *K. pneumoniae* reported for the rum distillery effluent-receiving waters are the highest ever reported for marine waters [22, 25, 27]. Many other investigators have shown that it is difficult to distinguish between clinical and environmental isolates of *K. pneumoniae* and that environmental isolates may be as virulent as clinical isolates [7, 13, 23, 25, 28, 29, 31]. The ability of *K. pneumoniae* to cause a variety of human diseases would suggest that tropical marine environments receiving heavy organic loads may be of public health concern [7, 28, 31].

Survival of Bacteria In Situ

Both direct count methods showed that the largest decline in *K. pneumoniae* densities occurred during the first 24 hours. Simultaneously, densities of *E. coli* in adjacent chambers showed a very precipitous decline to less than 0.1% of the initial density in only 12 hours. Studies by Vasconcelos and Swartz [33] also compared survival of *E. coli* and *K. pneumoniae* in marine environments in situ. They observed that *E. coli* did not survive as well as *K. pneumoniae*; however, they measured viable plate counts in low nutrient temperate marine waters (10.7°C) using a stirred diffusion chamber. The results for *E. coli* obtained by Vasconcelos and Swartz compared more favorably to our AODC density estimates than our Coulter Counter density estimates.

The differences between the AODC and Coulter counts for *K. pneumoniae* were negligible, although the differences between these counts for *E. coli* were greater than one order of magnitude. This observation is undoubtedly because of the morphological changes that occur in *E. coli* in the absence of changes in their ability to be cultured. As reported by others [4, 21, 30, 34] we observed shortening and condensation of the *E. coli* cells at all sites except the site receiving distillery effluent. After 24 hours of in situ exposure, 50% of the cells had transformed to micrococci, i.e., <1 μm. Since the CC counts were done with a 10 μm aperture, the micrococci could not be detected with the CC. The *K. pneumoniae* cells did not show any morphological changes at any time during the study and therefore did not demonstrate differences between CC and AODC at any of the sites.

Physiological activity of the bacteria in the diffusion chambers at La Gata Island as measured by AODC and INT declined by more then 60% after only

3 hours exposure for both bacteria. Previous studies observed that *K. pneumoniae* could survive nutrient poor, saline, high DO, intense sunlight, and high temperature environments [6, 8, 33]. The present study showed that *K. pneumoniae* was more rapidly inactivated than *E. coli* and could not maintain a level of activity that was any higher than *E. coli,* a low-survivor under the same conditions. Indeed, *E. coli* is generally classified as a nonsurvivor in marine environments [6, 8]. The apparent difference in actual cell survival of *K. pneumoniae* may be due to the ability of the capsule to resist extremes in water activity and pH. Recent studies in our lab have shown that some strains of *K. pneumoniae* can grow even in concentrated orange juice at pH 3.2, 58° Brix, and at temperatures as low as 4°C [15].

The survival in terms of density of *K. pneumoniae* at Ensenada de Boca Vieja was the same as La Gata, even though the rum distillery outfall site was significantly different in all the physical and chemical parameters measured. However, respiration and activity measurements showed that physiologically *K. pneumoniae* was much more active in the distillery discharge than in the waters of La Gata Island. Indeed, the percentage of the *K. pneumoniae* population that was active in the chambers at Ensenada de Boca Vieja initially declined by only 50%, whereas for the same period at La Gata Island the activity declined by nearly 70%. Also, the proportion of the *K. pneumoniae* population that was active in the chambers at Ensenada de Boca Vieja was greater at the outfall site only while the effluent was being discharged, i.e., during the first 48 hours of the study. At 48 hours, both the percentage of the *K. pneumoniae* that were active and the percentage that were respiring declined by more than 30% at the outfall. The rum distillery effluent did not increase survival of *K. pneumoniae* but did stimulate physiological activity allowing a larger proportion of the population in the chambers to remain active for a longer time.

The *E. coli* cells were much more responsive to the rum distillery effluent at Ensenada de Boca Vieja. Instead of rapidly declining as they did at La Gata Island, they did not significantly decrease in density for the first 48 hours at the effluent site. Immediately after the effluent discharge stopped, the density for *E. coli* at the outfall site began to decline. Chambers at the uncontaminated site had significantly lower densities of *E. coli* than chambers at the effluent site according to both direct count methods after only 18 hours. The decline in *E. coli* density at site B, however, was much less than either site at La Gata Island.

This last observation was reinforced by the much higher percentage of activity and respiration for *E. coli* at both sites of Ensenada de Boca Vieja. Again, bacteria at site B had significantly lower activity and percentage of respiring cells after only 18 hours; however, the activity and respiration were greater than 40% at both sites for the first 48 hours. This shows that although densities, activity, and respiration for *E. coli* were declining at site B, the adverse nature of the environment was not as great as La Gata Island. This was further emphasized by the observation that there was also a significant decline in respiration and activity for *E. coli* at site B after the effluent stopped at 48 hours. It would also seem that since water quality differences were negligible, the *E. coli* could respond to environmental changes that may not normally be measured.

These studies suggest that under high nutrient loading, *E. coli* cannot only survive extended periods in marine environments but also remain physiologically active. On the other hand, potential pathogens like *K. pneumoniae,* while appearing to survive under all conditions are nearly completely inactive physiologically. This became apparent after looking at changes in physiological activity in the diffusion chambers at these same sites. Other survival studies using *Aeromonas hydrophila* at these same study sites have shown that in the rum distillery effluent, *A. hydrophila* not only survives but proliferates, whereas in the other areas it declines dramatically [5]. The different survival characteristics of pathogens like *K. pneumoniae* compared to *E. coli* would seem to make fecal coliform bacteria poor indicators of the presence of pathogens in tropical marine environments, using the criteria of Bonde [6]. This study has further demonstrated that bacteria of enteric origin may respond to marine environments in a species-specific manner. Indeed, their density and physiological activity may be quite different for marine environments receiving effluents.

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