Human Pathogens in the Environment

In Situ Survival and Activity of Pathogens and their Indicators

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INTRODUCTION

The presence of pathogens in water was suggested by the earliest microscopists. Leeuwenhoek (1678) drew organisms found in canal water that resemble vibrios, though he did not make any connection between these observations and disease. Snow (1854) is generally acknowledged as having made the first description of an epidemic that provided proof that water was the contagion. However, it was not until the work of Koch and his institute that procedures were established for examining sources and pathways of infections. For more than 100 years we have looked for reliable methods to determine whether water is fit for human use, for a historical review see Bonde (4).

Since 1914, the United States has used coliforms as the standard indicator of human pathogens in recreational waters. However, recently the U. S. Environmental 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" (5). As discussed by Cabelli et al. (5), in 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 has been occurring to fecal coliforms as the indicator of choice in marine recreational waters. Despite this trend, there is a growing body of evidence that the underlying assumptions of even the fecal coliform assay are not valid in marine waters (4). The present study examines the in situ survival of several pathogens and indicators simultaneously at trophically diverse marine sites using two direct count methods and several different measures of activity.

MATERIALS AND METHODS

Study sites. La Gata island is a mangrove island in southeastern Puerto Rico (10). Ensenada de Boca Vieja is a protected cove immediately adjacent to San Juan Bay, Puerto Rico that recieves effluent from the world's largest rum distillery (3). Boqueron lagoon is a mangrove lagoon in southwestern Puerto Rico which receives effluent from two primary sewage treatment plants (see I. Lopez de Cardona, 1984, M. S. Thesis, University of Puerto Rico, Rio Piedras for details). Palomonitos island is a coral island on northeastern Puerto Rico (see N. Perez Rosas, 1983, M. S. Thesis, University of Puerto Rico, Rio Piedras for a detailed description). The refinery sites were located at Yubicoa Bay, Puerto Rico at a oil tanker loading station.

Water Analysis. Measurements were taken in situ for conductivity, salinity, pH, dissolved oxygen, light intensity, and temperature. Turbidity, alkalinity, hardness and ammonia measurements were done in the field using a Mini Spectronic 20 (Bausch and Lomb, Rochester, NY). Light intensity was measured in the field with an underwater photometer (Protomatic, Dexter, MI). Chlorophyll A, nitrate, sulfate, total phosphorus and phosphate were determined according to standard methods for water and wastewater analysis (2) see Biamon and Hazen (3) for details.

Bacteriological analysis. Direct cell counts for bacteria in diffusion chambers were done by AODC (16). Red and green fluorescing cells were recorded as active and inactive bacteria, respectively, as described in Carrillo et al (6). Frequency of dividing cells were measured by recording the number of bacteria cells that appeared elongated or the number of yeast with buds. Total number of bacteria and the number involved in respiration were determined by measuring reduction of 2-(p-iodophenyl)-3-(p-nitro phenyl)-5-phenyl tetrazolium chloride (INT) to INT- formazan using the technique of Zimmerman et al. (16).

Survival studies. For survival studies of bacteria, viruses, and yeast plexiglass diffusion chambers were used with a 0.45 μm pore size, nylon reinforced Versapor membrane filters (Gelman,

Instrument Co., Ann Arbor, MI) as a diffusion surface (3, 9). For virus survival studies an additional 0.0001 μm pore size polycarbonate filter was placed inside the other filter. Cell densities of pure cultures of the tested bacteria were determined with a model ZF Coulter Counter (Coulter Electronics, Hialeah, FL) and adjusted to a concentration of 10^7 cells ml^{-1}. Lastly, the bacterial suspension was placed into the sterile diffusion chamber just before placing them at the study sites. At the study site, a total of 5 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 as described by Hazen and Esch (7). 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. 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. Heteroscedastic data were made more homoscedastic using the appropriate transformation prior to analysis. Any probability less than or equal to 0.05 was considered significant (15).

RESULTS AND DISCUSSION

The survival of pathogens and their indicators can be quite different. This is especially evident when examining marine environments that receive different effluents, or are in different trophic states. Oligotrophic marine waters appear to support high densities of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Candida albicans* (Table 1). Yet *Vibrio cholerae*, *Aeromonas hydrophila*, *Streptococcus faecalis*, *Escherlichia coli* and Polio virus show reductions in density of 90% or more in only 24 h, in these same environments. These direct count densities; however, can give quite different results depending on the method employed and the bacteria being studied. When using AODC or other epifluorescent staining methods none of the bacteria densities above changes dramatically. However *V. cholerae*, and *E. coli*. show great changes in density when they are measured by particle counters (Table 1).

These changes are due to morphological changes in the cells, that decrease their detectability by the counter. Other investigators have demonstrated in laboratory marine microcosms that these morphological changes, shortening and condensation of the cell, occur commonly in these bacteria in response to nutrient stress (2, 9, 11, 14). In our studies after 24 h of in situ exposure, 50% of the cells had transformed to micrococci ie. <1 μm.

Many previous studies (4) have demonstrated that viable counts of many pathogens decrease rapidly when exposed to marine waters. Though most of these studies were done in the laboratory a few were done in situ (13). It is interesting to note that the in situ rates of survival of *E. coli* and *K. pneumoniae* in marine environments observed by Vasconcelos and Swartz (13) were significantly lower than the laboratory studies done by others (4, 9, 11). As shown recently for microcosms by Xu et al. (14). *E. coli* and *Vibrio cholerae* can survive and remain viable but non-culturable under conditions simulating marine environments. Colwell and her colleagues will report in this symposium that plate counts may have little connection with overall viability and or ability to infect animals and man. They have demonstrated that non-culturable *V. cholerae* are still viable and can still infect animals. This suggests that culturability can not be relied upon for determining survivability of some pathogens or indicators like *E. coli* in marine waters. Thus much of our previous reliance upon plate counts for determining indicator and pathogen density and hence water usability by animals and man is in doubt. At a time when our water resources continue to deteriorate in quality it seems that our ability to determine the presence of pathogens may be inadequate.

Measurements of in situ activity can provide information on pathogen and indicator viability and ecological potential; however, great caution must be used in method selection. Each method is highly dependent on the underlying assumptions of the test. *Klebsiella pneumoniae*, *P. aeruginosa*, and *C. albicans* appear to survive well by a number of direct cell count techniques (Table 1). However, when these same bacteria examined for percent activity as measured by the ratio of red to total fluorescing cells, they are no more active than *A. hydrophila*, *S. faecalis*, *V. cholerae*, or *E. coli* in these same environments (Table 2). Indeed, even those bacteria which undergo morphological changes have a similar proportion of active cells. The underlying assumption of this measurement is that red fluorescing cells have higher proportions of RNA:DNA then green flourescing cells. We further assume that cells with high proportions of RNA:DNA are active in protein synthesis.

Despite the large number of assumptions made

Table 1. Survival of bacteria in Diffusion Chambers in Marine Environments

	Time									
	12		24		48		72		96	
	AODC	CC	AODC	CC	AODC	CC	AODC	CC	AODC	CC
S. faecalis										
refinery – 1	93.9	83.5	57	56.2	30.6	23	16	14.3	ND	ND
refinery – 2	87.1	108.2	100.1	70.9	40.5	23.7	23.8	14.7	ND	ND
E. coli										
refinery – 1	167.4	64.4	85.7	67.1	60.9	23.7	39.2	5	ND	ND
refinery – 2	89.5	64.9	66.7	60.6	45.2	22.6	34.5	12.7	ND	ND
lagoon	50	6	46	5.2	60	.6	50	.7	2.5	.7
lagoon + sewage	106	6.4	81	6.1	87	.8	47	.9	2.2	.7
mangrove – 1	ND	2.1	4.6	6.6	6.4	2.2	ND	2.7	ND	3
mangrove – 2	ND	2.1	5.1	2.5	5	1.5	ND	3.6	ND	2.7
coral reef	248	37.9	221	28.4	12.8	34.7	ND	ND	ND	ND
sediments	ND	ND	31.5	57.8	14.3	247.5	ND	ND	ND	ND
destillery	ND	34.2	ND	43.3	ND	63	ND	59.2	ND	41
V. cholerae										
coral reef	84.6	81	203.1	74	178.5	74	ND	ND	ND	ND
sediments	ND	ND	97	530	16.2	610	ND	ND	ND	ND
mangrove – 1	90.4	49.7	55.7	8.6	32.9	2.6	7.9	4.6	7.5	4.1
mangrove – 2	ND	ND	41.3	4	76.7	2.2	7.8	2.9	7.8	4.5
A. hydrophila										
distillery	ND	88.7	ND	117	ND	187	ND	ND	ND	ND
near shore	ND	32	ND	23	ND	18	ND	11	ND	ND
P. aeruginosa										
refinery – 1	38.3	96.4	50	85.7	38.3	14.4	35	14.9	ND	ND
refinery – 2	65.7	117	86.7	120.5	12.9	9.8	15	9	ND	ND
C. albicans										
refinery – 1	55.4	91.1	46.4	58	34.9	18.8	27.5	7.1	ND	ND
refinery – 2	73.4	189.8	91	159.1	25.8	25.2	19	29.7	ND	ND
distillery	ND	96.9	ND	126.2	ND	169.2	ND	141.5	ND	120
near shore	ND	57.5	ND	48.8	ND	43.8	ND	53.8	ND	68.8
K. pneumoniae										
mangrove – 1	69	69	15	36	39	57	23	36	ND	ND
destillery	80	81	142	69	66.7	63	33.3	56	25	50
	PFU/ml		PFU/ml		PFU/ml		PFU/ml		PFU/ml	
Polio Virus										
lagoon	ND		34		11		0		0	
lagoon + sewage	17		2.7		0		0		0	

AODC = Acridine Orange Direct Counts, CC = Coulter Counter Counts, ND = Not Done, all numbers represents an average from 4 chambers expressed as the percentage of total cells remaining.

with this method and the necessity for rigid controls it does seem to agree with other methods of determining cell activity (Table 2). Indeed, in oligotrophic environments the percent activity drops rapidly and stays at or below 10% of the population. Marine waters receiving effluents and or classified as eutrophic do not cause the activity of *K. pneumoniae*, *V. cholerae*, *S. faecalis*, *A. hydrophila* or *E. coli* to decrease significantly (Table 2). We can also observe that those bacteria which undergo morphological changes in oligotrophic marine environments do not undergo these same changes in eutrophic environments. In fact when an effluent point source is removed *E. soli* and *K. pneumoniae* immediately decrease their activity and *E. coli* begins to undergo morphological changes (Figures 1–4, Table 1).

Activity in situ can also be measured by INT (16). This technique assumes that INT is reduced to INT-formazan by the cytochrome system and deposited as intracellular granules. This method further assumes that this reduction should be directly proportional to respiration and therefore

Table 2. Activity of Bacteria in Diffusion Chambers in Various Marine Environments

	Time									
	12		24		48		72		96	
	ACT	RESP	ACT	RESP	ACT	RESP	ACT	RESP	ACT	RESP
S. Faecalis										
refinery-1	78.4	21.8	32.7	10.8	64.5	14.9	37.2	5.4	ND	ND
refinery-2	89.9	7.2	86.0	12.0	66.8	6.6	51.3	2.1	ND	ND
E. coli										
refinery-1	79.9	11.3	69.1	18.6	91.5	11.1	66.8	16	ND	ND
refinery-2	83.2	27.8	83.4	21.1	88.3	11.1	81.6	9.8	ND	ND
sediments	ND	ND	86	41	36	9	ND	ND	ND	ND
coral	26	9	5	8	3	11	ND	ND	ND	ND
mangrove-1	7	ND	21	ND	4	48	11	4		2
mangrove-2	8	ND	19	ND	10	44	7	4		1.9
C. albicans										
refinery-1	(10.9)*	20.7	(9.7)	4.8	(3)	4.4	(6.5)	3.9	ND	ND
refinery-2	(12.1)	8.5	(18.0)	8.7	(6.1)	5.2	(3.9)	2.9	ND	ND
P. aeruginosa										
refinery-1	(2.6)	5.9	(7)	3.1	(4.7)	4.9	(8)	3.5	ND	ND
refinery-2	(8.4)	5	(5.9)	9.6	(3.0)	4.7	(1.0)	7.6	ND	ND
K. pneumoniae										
mangrove-1	2.5	5	10	3.5	16	18	22	3.5	ND	ND
distillery	45	20	18	40	17	58	8	25	ND	12.0
V. cholerae										
sediments	ND	ND	92	27	59	12	ND	ND	ND	ND
coral	79	30.6	89	8	39	1	ND	ND	ND	ND
mangrove-1	21	1.6	14	0.8	17	2.9	40	20.3	17	1.6
mangrove-2	ND	ND	16	0.5	25	2.7	42	22.0	10	5.1

* FDC = frequency of dividing cells, ACT = AODC activity, RESP = respiring, ND = Not Done, all numbers represents an average from 4 chambers expressed as percentage.

cell activity. Again oligotrophic marine environments caused rapid decreases in the percentage of respiring bacteria especially those undergoing morphological changes. However, it should be noted that many pathogens and indicators could rely completely upon fermentation. Indeed this was observed in *S. faecalis* and *E. coli* in an environment receiving oil refinery effluents (Table 2). The percent activity was significantly higher than the percentage respiration. In addition the difference between the direct count methods did not indicate morphological changes,

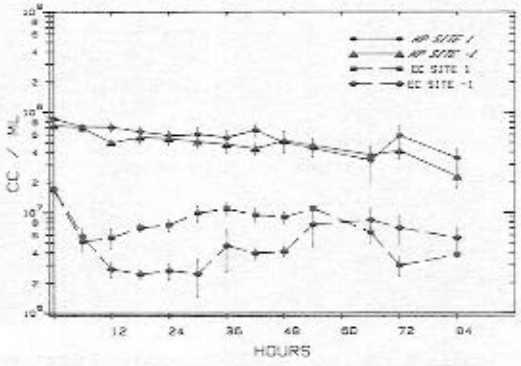

Fig. 1. Changes in total density as measured by Coulter Counter by site at Ensenada de Boca Vieja cove (Effluent at site 1 was shut off at 48 h, mean±one standard eror, n = 4).

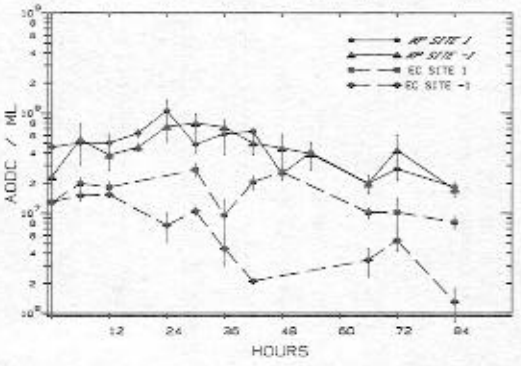

Fig. 2. Changes in total density as measured by AODC by site at Ensenada de Boca Vieja cove (Effluent at site 1 was shut off at 48 h, mean±one standard error, n = 4).

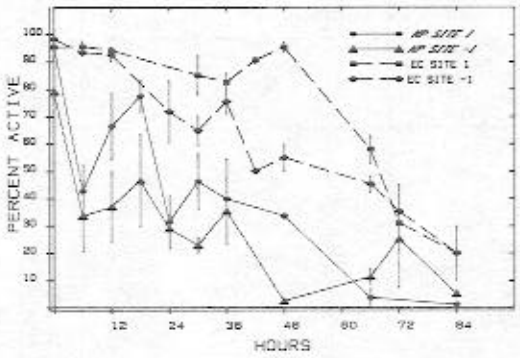

Fig. 3. Changes in percent activity as measured by AODC by site at Ensenada de Boca Vieja cove (Effluent at site 1 was shut off at 48 h, mean±one standard eror, n = 4).

further suggesting a more hospitable environment.

Transient changes in the environment can also result in changes in pathogen and indicator activity and survival, this was evident for *S. faecalis, C. albicans, P. aeruginosa,* and *E. coli* during tanker movement near the oil refinery. The percentage of respiration, percentage active and the frequency of dividing cells would increase by 5–10% when a tanker passed stirring up the anaerobic sediments momentarily increasing phosphorus content of the overlying water. *Vibrio cholerae* and *E. coli* survival and activity was also observed to be much higer when chambers were buried in coral sediments compared to the overlying water (Table 2).

Polio virus survival was significantly lower at a sewage out fall in a mangrove lagoon. Yet *E. coli* showed no difference at the same sites during the same samplings for these sites in terms of

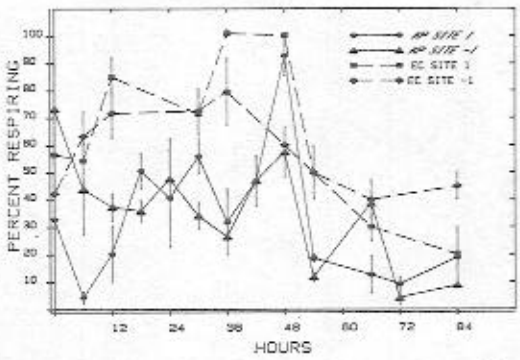

Fig. 4. Changes in percent respiration as measured by INT by site for Ensenada de Boca Vieja cove (Effluent at site 1 was shut off at 48 h, mean±one standard error, n = 4).

activity or density. Thus again, we observe that indicators do not accurately reflect survival of pathogens in some marine environments.

Other measurements of activity suh as frequency of dividing cells rely upon visual accuity and morphological changes that suggest division. This further assumes that only active populations will have high percentages of cells that appear to be dividing. This technique also indicates that under oligotrophic conditions that activity of *P. aeruginosa* and *C. albicans* decreases (Table 2), even though densities by either direct count method do not change (Table 1). The advantage of techniques used to measure activity in this study are that they do not involve any long preincubation or enrichment incubation and other techniques involving substrate uptake eg. microautoradiography (Table 3). At the refinery sites thymidine incorporation fluctuated overtime suggesting high activities; however, it must be remembered this involves a 2.5 h preincubation (12). In addition, all of the activity techniques used were used in conjunction with AODC so that direct cell counts were obtained simultanously. Since these methods are used with AODC we can also be sure that the activity is being measured in something that has cell morphology and fluoresces. Techniques that require enrichment and/or preincubation may show only the proportion of cells that can respond to those conditions and may not necessarily reflect the activity of the pathogens and indicators in situ.

Pathogens and their indicators survive differently in different marine environments. In fact *E. coli* which is the primary indicator of human

Table 3. Thymidine Incorporation

	Time			
	0	24	48	72
S. faecalis				
refinery-1	700	2223	2053	2270
refinery-2	700	4320	883	2011
E. coli				
refinery-1	3140	3103	1993	2235
refinery-2	3140	2671	2579	2711
C. albicans				
refinery-1	798	984	1837	1607
refinery-2	798	1752	463	1305
P. aeruginosa				
refinery-1	2200	8098	4110	2105
refinery-2	2200	4838	1617	2423

All numbers represents the average of CPM* from 4 chambers.
* CPM = Counts Per Minute.

fecal contamination in most countries seems to be much more effected by local marine environmental conditions than most pathogens. In fact it would see to survive much worse than pathogens in oligotrophic marine waters. This may in fact account for the findings of Cabelli et al (5) which showed fecal coliforms were the least reliable index of swimmer related disease. By looking at in situ marine survival of *E. coli* and different pathogens we can see that *E. coli* does not fit 4 of the 8 ideal requirements for an indicator organisms as described by Bonde (4): (1) it is not always present when the pathogens concerned are present, (2) it may be present when the presence of pathogenic organisms is not an imminent danger, (3) it may occur in much lower numbers than the pathogens, (4) it is not as resistant to the aqueous environment as the pathogens. Thus perhaps it is time we seriously reexamined our indicators and even considered looking for selected resistant pathogens directly rather than use indicators at all. We certainly need to use multiple methodologies for any measurements of density or activity. These changes may even improve public health in marine areas.

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