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INDICATIVE, PATHOGENIC, AND MARINE BACTERIA IN THE SEA

Studies of the occurrence and survival of sewage-origin bacteria in coastal waters and sediments continued this year at the marine microbiology laboratory at California State University at Long Beach. The goal of these studies has been to determine what relationships, if any, exist between the routinely sampled indicator bacteria (e.g., coliforms, fecal streptococci) and pathogenic bacteria (e.g., salmonella, staphylococci) in coastal waters. The objectives were to (1) improve and refine procedures for sampling and isolating pathogenic bacteria in seawater and sediment samples and (2) identify differences between laboratory survival tests and actual distribution of bacteria in the field. Mr. Daryl Osato, graduate student, and Mr. Chee Ho, student technician, assisted in field sampling and isolation procedures.

METHODS

The studies focused on six types of microorganisms: Total coliforms, fecal coliforms, fecal streptococci, *Staphytococcus auveus*, salmonella, and *Candida albicans*, a pathogenic fungi. Coliform and fecal streptococcus bacteria are normally present in fecal material and sewage and are generally not pathogenic; *Staphylococous aureus*, several species of salmonella and Shi-get to. and *Candida, albicans* cause a variety of diseases and lesions in man and other warm-blooded animals.

Water and sediment samples were taken during 1974 from near the outfalls of the Hyperion Treatment Plant—the 1-mile pipe (five sites) and the 5- and 7-mile pipes in Santa Monica Bay (three sites). The 1-mile outfall site was sampled because chlorinated secondary effluent is discharged there for a 4-hour period once a week (for cleaning purposes), thus providing an opportunity to observe bacteria before and after a discharge. Samples were also taken from deeper waters in the Santa Monica Basin (eleven sites) and from Dana Point, Orange County (three sites).

We determined the most probable numbers (MPN) of coliform bacteria (by lactose broth, EC broth, and EMB agar) and fecal streptococci (by fecal streptococci azide dextrose broth and ethyl violet broth) from 10 ml water samples and 1 gm sediment samples. Salmonella cells were obtained by an enrichment technique (using Dulcitol selenite broth, selenite cystine broth, Hektoen enteric agar, brilliant green agar. Salmonella/Shigella agar and XLD agar) using a series of 100- to 1,000-ml water samples. Salmonellae were identified by serotyping and biochemical tests. Isolation of pathogenic staphylococci was attempted with a Mannitol salt sorbic acid enrichment and Millipore filter technique, followed by placement on Staphylococcus Agar No. 110 and Vogel Johnson agar. Hemolysis of blood agar and coagulase tests were performed to confirm the identity of the isolates.

To test for pathogenic fungi, the samples were filtered through Millipore membranes to concentrate the microbial cells, and the membranes were placed on three different agar media for their primary isolation (of the three media tested, the brain/heart infusion agar showed the

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best results) . The isolates were tested for *Candida alblcans* by production of chlamydospores on corn meal agar and fermentation tests.

The counts for total aerobic and anaerobic terrestrial bacteria were made by the standard procedures; marine agar was used for the marine species that would grow in the presence of 3 percent salt. The anaerobes were incubated in Gas Pak anaerobic jars.

INDICATOR AND PATHOGENIC BACTERIA

Previous and recent laboratory studies indicate that the survival of Streptococcus fecatis and *Staphylococcus aureus* in filtered seawater is less than that of *Escherichia coli* and *Salmonella typhimurium; Candida albicans* is relatively long-lived (Table 1).

Comparison of mortality rates under these conditions would seem to indicate that coliform bacteria are not only a reasonable and conservative indicator for the more pathogenic forms in seawater, but that they could be an accurate indicator of salmonella bacteria. These observations also suggest that, although Streptococcus may not be a good indicator for salmonella, it may be quite indicative of *Staphylococcus aureus* mortality. Thus, one indicator organism may be insufficient for assessing the survival and distribution of all probable types of pathogenic bacteria in seawater.

Samples taken from various sites in Santa Monica Bay and from unchlorinated and chlorinated treated wastewaters suggest that the survival of fecal streptococci is higher— and that of coliforms lower—in the field than in the laboratory. Each 100 ml of unchlorinated primary effluent from the Hyperion Treatment Plant contains 4 to 150 $X10^6$ total coliform bacteria and more than 170,000 fecal streptococcus bacteria and always yields positive tests for salmonella. After secondary treatment and chlorination, discharged effluent contains about 500 coliforms and 170 fecal streptococci per 100 ml, few salmonella, and no *Staphytococcus aureus* or *Candida alb-icons*. When chlorinated secondary effluent is discharged through the nearshore, 1-mile outfall, most water samples within 0.5 km of the discharge contain 20 to 200 coliforms per 100 ml (well below the State standard of 1,000 per 100 ml) and no detectable salmonella or staphylococci; but fecal streptococcus counts may range from 20 to nearly 6,00 bacteria/100 ml (Table 2). These data suggest that diffusion of wastewater is low at this outfall. With no discharge for periods up to 5 days, fecal coliform counts remain low, but fecal streptococcus counts remain high. It is evident that, although chlorination is effective at initially reducing the numbers of coliforms, fecal streptococci, staphylococci, and salmonella, some attribute of the receiving waters seems to temporarily enhance survival of fecal streptococci.

Previous studies at the 1-mile outfall indicated that even while the outfall was not in use, fecal streptococci were present at the discharge site. However, it is possible that other sources may contribute to maintaining streptococcus populations at this site (such as runoff from nearby Ballona Creek or effluent from the 5-mile outfall).

Sediment samples from all stations around the 1-mile out-fall not only verified this trend but seemed to emphasize a relatively higher mortality of coliform bacteria compared to fecal streptococci (Table 2). Perhaps the sediments themselves serve as a latent source of streptococci to the overlying water column when the outfall is not in use.

To obtain some estimate of the relative concentration of pathogens in seawater and to estimate their relation to indicator organisms, it was necessary to sample in the vicinity of the 5-mile and 7-mile outfalls (which discharge unchlorinated effluents through a more efficient diffusion system) and to use the larger sample sizes indicated above. We used the widest possible range of environmental levels of indicators (i.e., in both water and sediment samples) to obtain some estimate of the relation between salmonella isolates and indicator bacteria.

Of the two pathogens studied, only salmonella was isolated from water and sediment samples along with coliform and fecal streptococcus bacteria. To summarize data for examination, we classified the samples according to four concentration ranges of indicator organisms and then entered the number and percent of samples positive for salmonella (Table 3). Salmonella were isolated in only up to 8 percent of the samples containing less than 1,000 indicator bacteria (coliform or fecal streptococcus) per 100 ml. However, salmonella could be detected in 25 to 75 percent of the samples containing in excess of 1,000 fecal coliforms/100 ml, and in 38 to 100 percent of the samples containing in excess of 1,000 fecal streptococci/ 100 ml. Our best estimate is that the maximum concentration of salmonella found was about one organism in 400 ml of water (0.25/100 ml) and about one salmonella per 20,000 indicator bacteria.

THE MARINE BACTERIA

Natural marine microorganisms appear to be affected by wastewater discharge. The number of bacteria from the chlorinated secondary effluent that could grow in a marine medium was only 420/ml, whereas their numbers in the surface seawater samples collected from the five stations on 24 July off the 1-mile pipe averaged 68,000/ml. Effluent flow had been off for 7 days when the samples were taken. The samples taken on the following day (25 July), while chlorinated secondary effluent was being discharged through the pipe, showed a count of 24,000/ml, which was about 35 percent of the population before the discharge. Similar observations were also made on the samples taken a week later. Before the discharge, the counts were 8,420/ml; when the pipe was turned on 2 days later, the counts were only 2,150/ml (39 percent of the predischarge population) . The general population of marine bacteria during this particular sampling period was lower than that of the previous week, probably due to a heavier than usual chlorination of the discharge. The population of marine anaerobic bacteria was also affected by the discharge of the chlorinated effluent. Their counts were higher just before the discharge and went down during the discharge.

The aerobic and anaerobic freshwater bacteria that grow in media prepared without seawater or salt were also checked during and after the discharge. The aerobic freshwater bacteria numbered about 60 cells/ml during the discharge (25 July), but the counts 24 hours prior to the discharge were less than 10/ml, except at the nearshore station to the south of the 1-mile pipe, which yielded 200 cells/ml. The numbers of anaerobic freshwater bacteria were less than 10 cells/ml in most of the samples tested.

Natural marine waters may contain hundreds of bacteria per milliliter, and marine sediments thousands per milliliter, as shown for Santa Monica Basin and Dana Point samples (Table 4). Surface waters (i.e., above the thermocline) contain in excess of 300 bacteria/ml (30,000/100 ml); midwater samples may contain less (10 to 100/ml), except near the sewage outfalls (where counts are often over 1,000/ml). There appear to be no striking differences in bacteria concentrations in sediments from Dana Point, Santa Monica Basin, or near the discharge sites in Santa Monica Bay (bottom of Table 4), but the concentration range is one to two orders of magnitude higher than in water samples of equivalent volume. Comparison of these data suggests that unchlorinated sewage enhances growth of native marine bacteria while chlorination depresses growth.

THE PATHOGENIC FUNGI, *CANDIDA ALBICANS*

Candida albicans, a pathogenic yeast-like fungus from the feces of man (recently found to be present in seawater), was investigated as a possible contaminant of local coastalwaters. The procedures we employed for its isolation failed to reveal presence of large numbers of pathogenic fungal species in the samples taken off the outfalls. Many species of *Candida* that resemble *C. albicans* were isolated repeatedly from the samples; however, none of the isolates was able to produce chlamydospores on corn meal agar, which is one of the decisive characteristics of this particular species. (In a 1974 report, one researcher was able to isolate thousands of *C. albicans* cells from estuarine waters in New York by using the fluorescent antibody technique. However, it is generally felt that the fluorescent antibody technique is probably not a very specific test for *C. albicans*.)

Considering its natural inhabitance in the feces of man and its occurrence and survival in the marine environment, *Candida albicans* is a promising indicative organism for fecal pollution, and the possibility of i'ts occurrence in the coastal waters of southern California deserves further study.

DISCUSSION

The concentrations of pathogenic bacteria such as *Salmonella*, *Shigella*, and *Staphylococcus aureus* in municipal wastewaters greatly depends on the occurrence of these organisms in human populations. Infections and enteric diseases rarely reach epidemic proportions in southern California, but there may be periods of relatively high or low concentrations. In contrast, bacteria used as indicators of bacterial contamination (coliforms, etc.) always occur in great quantities in sewage and are much less variable in abundance.

There are several possible reasons for the lack of pathogenic staphylococci in the Santa Monica Bay samples. First, the culture media designed for pathogenic staphylococci were overgrown by other types of marine bacteria. Obviously, the procedures for isolating staphylococci of human origin in marine water need to be refined. How-ever, this observation may indicate that there is considerable competition between large numbers of native marine bacteria and staphylococcus. In addition, many reports have demonstrated the occurrence of antistaphylococcal agents in seawater and other water sources. These agents were found to be produced by algae, diatoms, fungi, and other organisms.

It is also possible that *Staphylococcus aureus*, the primary pathogen sought in these studies, is present in exceedingly low numbers in final effluent as a result of competition with the large numbers of Gram-negative rods present in sewage. As in natural seawater, antistaphylococcal agents may also be generated in sewage waste-waters. The sources and fate of staphylococci, especially *S. aureus*, have yet to be fully evaluated.

Our data from 1974 and early 1975 suggested that, of the three pathogens studied, only salmonella were present in sufficient quantities to be detected in sewage and in ocean waters and sediments near the outfalls. Our best estimate is that there was about one salmonella bacteria in 400 ml of seawater from the most contaminated samples (e.g., coliform and fecal streptococcus in excess of 5,000 MPN per 100 ml). This corresponds to a minimum estimated ratio of about one salmonella per 20,000 indicator organisms (either fecal coliform or fecal streptococcus) in the most contaminated marine samples from Santa Monica Bay. This relationship is similar to that found in the sewage samples, but our precision must be considerably improved before we can determine actual fate and survival rates of salmonella in coastal waters.

Comparison of laboratory and field data indicate that fecal streptococci have a lower rate of survival than coliform bacteria in filtered seawater but that the reverse may be true in coastal waters. Selective predation and competition with marine organisms may be responsible for this.

Actual quantitative measurements of organisms such as salmonella in seawater will require samples even larger than we used, and the samples will have to be graded (e.g., 1, 10, 100 liters) or serially diluted in large volumes to make useful counts of these organisms.

Table 1. Estimates of mortality rates for five sewage-origin microorganisms Studied at 25°C and 1 atm pressure in filtered Seawater in laboratory.

Organism	% Mortality Day 4	% Mortality Day 7	Time to 90% Mortality (days
Candida albicans	20	24	29 – 33
Escherichia coli	40	51	23 – 26
Salmonella typhimurium	47	53	23 – 25
Streptococcus fecalis	70	86	9 – 10
Staphylococcus aureus	75	83	9 – 10

Table 2. Percent occurrence of indicator bacteria in samples taken near the 1-mile outfall, Santa Monica Bay, 1974*.

	Indicator Bacteria Count (MPN/100 ml)			
	<20 – 200	**201 - 1,000**	**1,001 – 5,000**	**>5,000**
Surface and Water Column				
Fecal streptococci				
Pipe on	58	25	13	5
Pipe off	83	13	3	0
Total coliforms				
Pipe on	78	20	3	0
Pipe off	100	0	0	0
Fecal coliforms				
Pipe on	100	0	0	0
Pipe off	100	0	0	0
Sediment **				
Fecal streptococci	0	0	60	40
Total coliform	87	13	0	0

*Sampling periods were 9-21 May and 24 July to 1 August.
**Values based on means of counts made both when the pipe was on and when it was off.

Table 3. Salmonella isolates from seawater and sediment sampoles collected at 5-mile and 7-mile outfalls at various total coliform ranges, fecal coliform range, and fecal streptococci ranges.

Analysis	Count (MPN/100 ml) <20 – 200	201 - 1,000	1,001 – 5,000	>5,000
Total Coliform Bacteria				
No. of samples analyzed	13	3	4	4
No. of samples positive for salmonella	1	0	2	2
Percentage	8	0	50	50
Fecal Coliform Bacteria				
No. of samples analyzed	13	1	8	2
No. of samples positive for salmonella	0	0	3	2
Percentage	0	0	38	100

Table 4. Concentrations (cells/ml) of total marine and freshwater aerobic and anaerobic bacteria in water and sediment samples from Dana Point, Santa Monica Basin and Santa Monica Bay.

	Dana Point	Santa Monica Basin	5-and 7Mile Outfall Area, Santa Monica Bay
Surface Water			
Marine aerobes	770	773	360
Freshwater aerobes	390	390	328
Midwater			
Marine aerobes	130	--	423
Freshwater aerobes	10	--	760
Sediment			
Marine aerobes	50,000	7,200	13,266
Freshwater aerobes	12,600	72,000	49,000
Marine anaerobes	20,800	8,800	2,475
Freshwater anaerobes	2,100	2,800	6,850