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Effects of Pharmaceutical Wastes on Microbial Populations in Surface Waters at the Puerto Rico Dump Site in the Atlantic Ocean

E. R. PEELE, F. L. SINGLETON, J. W. DEMING, B. CAVARI, AND R. R. COLWELL*

Department of Microbiology, University of Maryland, College Park, Maryland 20742

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A series of cruises during 1979 and 1980 to the pharmaceutical dump site located 64 km north of Arecibo, Puerto Rico, in the Atlantic Ocean, was carried out to evaluate effects of wastes on the ecology of the microflora of surface waters of the dump site. In addition to bacteriological monitoring of the waste plume created by the release of wastes from the disposal barge, stations along a series of transects, extending north from coastal waters through and beyond the dump site, were sampled. Largest numbers of culturable bacteria on marine agar were found at stations closest to shore and in the vicinity of the dump site. Bacteria recovered on marine agar were predominantly *Vibrio* and *Aeromonas* spp., with the relative abundance of these organisms decreasing as gram-positive organisms (staphylococci, micrococci, and bacilli) became dominant in areas immediately affected by waste dumping. Total numbers of bacteria (determined by acridine orange direct counts [AODC]), which were relatively stable throughout the region, and a direct estimate of viable cells (DVC), i.e., those cells responsive to additions of yeast extract and nalidixic acid, were determined by acridine orange staining and epifluorescence microscopy. Heterotrophic bacterial activity, measured by the uptake (V_{max}) of ^{14}C-labeled amino acids, declined relative to distance from land. Increases in specific activity indices (DVC/AODC and V_{max}/AODC) were observed near the dump site. The composite results of this study, i.e., increased specific activities (determined by two methods), increased numbers of culturable marine bacteria, and marked alteration of the taxonomic composition of the culturable bacterial community in waters within and surrounding the Puerto Rico dump site, indicate demonstrable changes in the marine microbial community in the region used for waste disposal.

The importance of bacteria in the sea, as biogeochemical agents and as a significant component of the marine ecosystem, has long been recognized and, more recently, accorded increased attention. Bacteria as indicators of environmental alterations have been used to monitor the presence of domestic wastes (18), and they have been used recently to evaluate effects arising from massive oil spills and other catastrophic events (3). The usefulness of bacteria as indicators of environmental perturbations in the aquatic environment that result from chronic or low-level input of allochthonous materials has not been investigated extensively.

Problems of waste disposal have increased in severity in recent years, becoming in some cases of crisis proportion. The need for an immediate solution has made ocean dumping an attractive alternative to land disposal. However, there is little information on potential acute and chronic biological effects resulting from waste disposal activities, especially at open-ocean dump sites (11).

Surface waters of the Puerto Rico Trench area of the Atlantic Ocean presently serve as a dump site for pharmaceutical wastes and will do so until Puerto Rico completes a regional wastewater treatment system at Barceloneta, scheduled to begin operation in 1981. The amount of material dumped annually is estimated to be 3.2 $\times 10^8$ liters and includes wastes from seven pharmaceutical industries as well as a petrochemical plant. The wastes are collected in a common holding tank and, at intervals of 2 or 3 days, are transferred to barges which are towed to the dump site. The composite waste is released over an area of several square kilometers.

Assessment of the biological impact of ocean disposal of pharmaceutical wastes in this region is confounded by the distribution and persistence of the wastes in an area of several hundred square kilometers surrounding the dump site

proper and also by the variability in the composition of the waste. Industries contributing to the waste use different batch-type processes throughout the year, so that wastes are not of uniform composition (20). Results of analyses of composite waste samples indicate low metal concentrations, i.e., less than 1 ppm (1 mg/liter), but a relatively high organic content, about 1 to 4% by weight (T. O'Connor, personal communication). In general, the wastes are derived from fermentation and other processes involved in the production of antibiotics, sterols, and organic solvents.

To date, studies of the effects of pharmaceutical waste disposal on macroorganisms at the Puerto Rico site have been inconclusive (11). In view of their ubiquitous distribution, short generation times, and sensitivity to changes in the physical and chemical environment, bacteria may be the first component of the biota to reflect environmental perturbations. The objective of this study was to apply microbiological methods to evaluate effects on the bacterial community in surface waters of the Puerto Rico dump site.

MATERIALS AND METHODS

Sampling location. The pharmaceutical waste dump site is an area of approximately 500 km^2 about 64 km north of Arecibo, Puerto Rico. The dump site proper is bounded by coordinates shown in Fig. 1. Each waste dump is centered within a specific quadrant of the dump site, and wastes are discharged from moving barges at a rate of 70,000 liters/km.

Three cruises designed to study effects of dumping activity were made in October and December 1979 and February 1980, after a preliminary cruise in May 1979. Sampling stations are shown in Fig. 1. The October cruise focused on microbiological studies of the waste plume behind the disposal barge and on chemical analyses of surface waters from a sampling grid of 36 stations encompassing the dump site and a surrounding region of approximately 2.4×10^4 km^2. Detectable concentrations of volatile organic waste-specific compounds were present in all water samples collected, including those collected 144 km northeast of the dump site (J. M. Brooks et al., Abstr. Annu. Meet. Am. Soc. Limnol. Oceanogr., 1980). Because the boundaries of the waste-affected area could not be clearly defined, microbiological sampling sites for the December 1979 and February 1980 cruises were established in relation to land along transects extending from coastal waters, through the dump site, and into areas of the open ocean 64 km beyond the dump site. For all cruises, water temperatures were between 25.5 and 28.8°C. Salinities ranged from 34.6 to 36.1‰, increasing characteristically with distance from land.

Sample collection. Water samples were collected aseptically with sterile Niskin bags (General Oceanics, Miami, Fla.) and processed on board ship within 15 min of collection. Surface water samples (1-m depth) were collected at all stations.

Bacterial enumeration. Aerobic, heterotrophic marine bacteria in surface water samples were enumerated on marine agar 2216 (MA) (Difco Laboratories, Detroit, Mich.). Culturable heterotrophic bacteria which did not require marine salts for growth were enumerated by using plate count agar (PCA) (Difco). Sample volumes (1, 10, and 100 ml) were filtered in triplicate, using 0.2-μm nitrocellulose membrane filters (Scheicher & Schuell Co., Keene, N.H.), and the filters were placed on agar media. After inoculation, MA culture plates were incubated at 25°C for 7 days, and PCA plates were incubated at 25°C for 14 days before total colony-forming units were enumerated.

Samples of water, collected as described above with the Niskin sampler, were immediately preserved with Formalin (final concentration, 2%, vol/vol) for direct microscopic enumeration of bacteria. Direct counts were obtained by acridine orange staining and epifluorescence microscopy (5, 6, 9), using a Zeiss Standard 18 microscope equipped with an IV FL epifluorescence condenser, 100-W halogen lamp, BP450-490 band-pass filter, FT 510 beam splitter, and LP520 barrier filter (Carl Zeiss, Inc., New York, N.Y.). For each sample, a minimum of 10 randomly selected fields with ≥50 cells per field were counted to determine total cell numbers.

Total numbers of viable, substrate-responsive bacteria were determined by the method of Kogure et al. (12). Twenty-milliliter samples of seawater were enriched with 0.025% (wt/vol) (final concentration) yeast extract (Difco) and 0.002% (wt/vol) (final concentration) nalidixic acid (Sigma Chemical Co., St. Louis, Mo.). All samples were incubated in the dark at in situ temperatures for 6 h before fixation with Formalin (final concentration, 2%, vol/vol) for subsequent examination in the laboratory by acridine orange staining and epifluorescence microscopy.

Identification and classification of isolates. Ten colonies from one MA culture plate per sample per station were randomly selected for taxonomic analysis. The cultures were purified and subjected to a battery of tests (4) and identified to the genus level, using selected biochemical and morphological char-

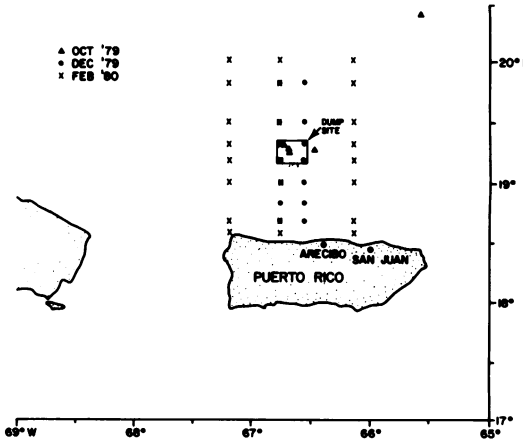

FIG. 1. *Pharmaceutical waste dump site, located 64 km north of Puerto Rico, and stations sampled in October 1979* (▲), *December 1979* (●), *and February 1980* (×).

acteristics described in *Bergey's Manual of Determinative Bacteriology* (2) and identification schema of Bain and Shewan (1) and Gibson et al. (7).

Heterotrophic activity. Heterotrophic activity was measured by the method of Hobbie and Crawford (8). Fifty-milliliter water samples, aseptically collected, were placed into sterile 125-ml Erlenmeyer flasks and supplemented with a ^{14}C-labeled amino acid mixture (57 mCi/matom of carbon; Amersham Corp., Arlington Heights, Ill.). Final concentrations of amino acids were 25, 50, 75, and 100 μg/liter. All concentrations, including controls, which were samples fixed with 0.5 ml of 30% trichloroacetic acid and 0.05% mercuric chloride in 40% Formalin, were duplicated for each sample site. After addition of the amino acid mixture, the flasks were sealed with serum stoppers fitted with a plastic cup containing a precombusted (450°C) glass fiber filter. The flasks were incubated at 25 ± 1°C for 3 h, and the reaction was terminated by injection of the trichloroacetic acid-mercuric chloride-Formalin mixture. Filter wicks were saturated with 0.2 ml of phenethylamine to absorb $^{14}CO_2$, and the flasks were incubated for 1 h with shaking.

After incubation, the filter wicks were placed in scintillation vials, and the water samples were filtered through 0.2-μm membrane filters (Millipore Corp., New Bedford, Mass.). After rinsing with 10 ml of filtered (0.2 μm) seawater, filters were placed in vials for transport to the laboratory at College Park, Md. In the laboratory, a 10-ml volume of scintillation cocktail (Amersham Corp.) was added to each vial, and radioactive counts were made by using a Beckman liquid scintillation spectrometer model LS-3155T. Calculations of V_{max}, the maximum uptake velocity for the substrate used and the microbial population of the water sample, were made according to the procedure of Hobbie and Crawford (8).

Specific activity indices. Based on concepts discussed by Wright (21) and a method developed by S. A. Orndorff and R. R. Colwell (17), indices were calculated to depict the relationship between microbial heterotrophic activity and number of bacteria present in a given sample (determined by acridine orange direct counts [AODC]). The index, V_{max}/AODC, utilized V_{max} as a measure of potential heterotrophic activity in relation to the number of bacteria determined by AODC. A second index, DVC/AODC, compared the number of viable bacteria (DVC) capable of utilizing yeast extract (12) and the total number of bacterial cells.

Other parameters. For the December 1979 and February 1980 cruises, the following parameters were also measured: total particulate adenosine triphosphate, chlorophyll *a*, total particulate organic carbon, total dissolved organic nitrogen and phosphorus, and dissolved nitrate, nitrite, ammonia, and phosphate. These parameters decreased characteristically with distance from land, and concentrations in surface waters in the vicinity of the dump site proper did not correlate with microbiological measurements or assist in explaining marked increases in total viable counts or specific activity indices.

RESULTS AND DISCUSSION

Enumeration of culturable bacteria. During the October 1979 cruise, two geographic control stations were sampled in addition to a timed-sequence sampling of the waste plume (Table 1). Total numbers of culturable, aerobic heterotrophic bacteria, enumerated on MA, were consistently low in the waste plume compared with stations east and northeast of the dump site proper. In contrast, numbers of bacteria cultured on PCA increased during the timed-sequence sampling.

In general, total numbers of culturable, aerobic heterotrophic bacteria on MA were relatively stable for samples collected along transects through the dump site region (for example, see Fig. 2). However, during December 1979, results revealed significant increases ($P \leq 0.05$) in numbers of culturable heterotrophs at stations within and due north (16 km) of the dump site proper. Counts for samples collected at stations 32 km north of the dump site were similar to those obtained in surface waters south of the designated dumping area.

During February 1980, large numbers of heterotrophic colony-forming bacteria obtained on MA were again recorded for stations north and northwest of the dump site, although largest numbers were obtained at a coastal station 6.4 km north of San Juan.

A significant portion of the culturable bacterial community did not require seawater, or media prepared with the major salts of seawater, for growth, as evidenced by the numbers of microorganisms growing on PCA (Fig. 2). The numbers of bacteria which grew on PCA were found to vary within and among transects as well as among sampling times. However, the largest numbers of bacteria growing on PCA were recovered from water samples collected at stations north of the dump site during December 1979. In February 1980, fewer bacteria were recovered on PCA than during the December cruise.

TABLE 1. *Bacteriological analysis of surface waters east and northeast of the dump site and in the waste plume (October 1979)*

Location	Bacterial concn[a]	
	MA	PCA
144 km NE of dump site	47	10
16 km E of dump site	>100	4
Waste plume (t = 2 h)[b]	7	18
Waste plume (t = 5 h)	6	24
Waste plume (t = 15 h)	6	102

[a] Colony-forming units/milliliter. Average standard deviation of plate counts was ±22% of the reported value, except where numbers were higher than anticipated by the filtration schedule (>100).

[b] The first sample in the waste plume was collected 2 h after the dump was initiated in order to allow for deployment of drogues used to track the plume.

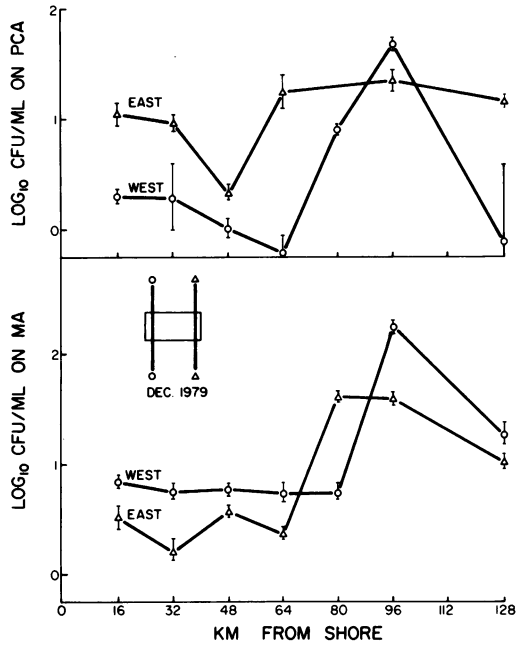

FIG. 2. *Total numbers of colony-forming units (CFU) per milliliter on MA and on PCA for surface water samples collected in December 1979 at stations along transects passing through the eastern (△) and western (○) ends of the dump site. Error bars represent standard deviations of the mean.*

Identification of bacterial isolates. Results of taxonomic analyses of data for pure cultures of randomly selected bacteria (10 strains/sample) that grew on MA revealed a predominance of *Vibrio* and *Aeromonas* spp. (Table 2). This group of organisms accounted for 74 to 86% of strains isolated during all cruises. *Pseudomonas* spp. occurred at low frequencies.

During December 1979, a surprisingly large number of the bacterial strains isolated on MA were gram-positive cocci (Table 2). Gram-negative bacteria (*Vibrio* and *Aeromonas* spp.) were dominant in surface water samples collected south of and within the dump site. However, at stations 16 km north of the dump site, the relative abundance of gram-negative bacteria decreased concomitant with an increase in the number of gram-positive organisms (Table 3).

Heterotrophic activity. A decline in heterotrophic bacterial activity, measured by uptake of ^{14}C-labeled amino acids, correlated with distance from land (Table 4). Highest uptake velocities were detected in samples collected at a coastal station 6.4 km north of San Juan.

Direct counts of bacteria and direct estimates of bacterial activity. No dramatic variations in total numbers of bacteria, determined by AODC, were noted among any of the water samples collected during this study. For example, during the December 1979 cruise, counts ranged from 1.93×10^5 to 3.24×10^5/ml (Fig. 3). However, the proportion of the total bacterial population which was active, i.e., responsive to substrate addition (12), ranged from 0.2 to 12.9%, depending on location. When DVC determined by the method of Kogure et al. (12) was related to total numbers of bacteria determined by AODC, the resulting activity indices suggested marked differences in specific activity among various water samples. Highest specific activities were recorded for samples collected within the dump site proper (Fig. 3).

TABLE 2. *Taxonomic distribution of bacterial strains isolated on MA from Puerto Rico Trench surface waters*

Genus	% of total isolates examined[a]		
	May 1979	October 1979	December 1979
Pseudomonas	1.3	8.1	2.2
Vibrio/Aeromonas	86.3	81.8	74.3
Photobacterium	3.1	0	1.4
Lucibacterium	0	4.0	0
Flavobacterium	0.9	5.1	3.5
Acinetobacter/Moraxella	3.5	1.0	2.1
Unknown gram-negative rods	0	0	1.4
Unidentified gram-positive cocci	0	0	15.0

[a] The total numbers of strains isolated and examined from samples collected in May 1979, October 1979, and December 1979 were 227, 99, and 140, respectively.

TABLE 3. *Distribution of gram-negative and gram-positive bacteria isolated on MA in surface waters of the study area (December 1979)*

Distance from shore (km)	% Gram positive	% Gram negative	% *Vibrio*
Eastern transect			
16	0	100	90
32	0	100	80
48	0	100	90
64	0	100	100
80	0	100	100
96	60	40	40
128	70	30	30
Western transect			
16	0	100	90
32	0	100	100
48	0	100	70
64	0	100	90
80	0	100	90
96	60	40	30
128	0	100	40

TABLE 4. *Heterotrophic bacterial uptake of amino acids in surface water samples collected in December 1979 and February 1980*

Distance from shore (km)	V_{max} (μg of amino acid/liter per h)		
	Transect 1[a]	Transect 2	Transect 3
6		0.0798	0.4537
16		0.0344	
32			
48	0.0250	0.0251	0.0192
64	0.0184	0.0106(0.0974)[b]	0.0158
80	0.0105	0.0063	
96	0.0116	0.0139	0.0182
112			
128	0.0104	(0.363)	0.0137
144		0.0104	

[a] Transect number refers to location respective to dump site: 1 is 16 km west of dump site, 2 is through the western side of the dump site, and 3 is 16 km east of dump site.

[b] Numbers in parentheses represent values obtained in December 1979. All other values were obtained in February 1980.

A comparison of total numbers of bacteria (AODC) and specific activities (DVC/AODC) for water samples collected in December 1979 with data from the same transect sampled in February 1980 indicated little seasonal variation between December and February (Fig. 4). In the absence of marked seasonal variations, specific activities based on V_{max}/AODC, obtained for two stations on the eastern transect sampled in December 1979, were compared with similar indices obtained for stations along the western transect sampled in February 1980. Specific activity indices based on V_{max}/AODC for two samples collected on the eastern transect were highest, in agreement with indices based on DVC/AODC (Fig. 5).

The oceanographic cruises described here were designed to detect effects of pharmaceutical waste dumping on the size, taxonomic composition, and metabolic activities of the bacterial community in the surface waters within and surrounding the Puerto Rico dump site. Until recently, the dump site was believed to be influenced by the Antilles Current, which flows west-northwest (10). The distribution of the wastes, therefore, was expected to be limited to the dump site and regions west of it. However, the results of recent current measurements and chemical analyses of surface water samples from an area encompassing 2.4×10^4 km^2 and including the dump site clearly indicate a pattern of stagnant water masses and the persistence of low levels of wastes in this region (see Materials and Methods; T. Ichiye et al., Abstr. Annu. Meet. Am. Soc. Limnol. Oceanogr., 1980).

Culturable bacterial counts obtained in this area were compared with available data from other cruises conducted in neighboring regions of the Atlantic Ocean. Orndorff and Colwell (16) found that, along a transect through the Sargasso Sea, the culturable bacterial counts in surface waters ranged from 1 to 7/ml. Earlier cruises in the general area of the Puerto Rico Trench yielded similar results (R. R. Colwell, *R/V Eastward* cruise reports, 1972–77). Within the dump site region, however, at a latitude similar to those of stations sampled by Orndorff and Colwell (16), culturable heterotrophic bacteria were significantly more abundant than in the Sargasso Sea (Fig. 2). Largest numbers of colony-forming bacteria were found at stations in and north of the dump site proper.

The direct counts of bacteria appeared to be relatively stable throughout the study area, but changes in the specific activity indices, DVC/AODC and V_{max}/AODC, indicated variations in bacterial heterotrophic activity that appeared to be related to dumping activities, again by geographic implication.

The culturable bacterial community of the surface waters in and around the dump site was found to be dominated by *Vibrio* and *Aero-*

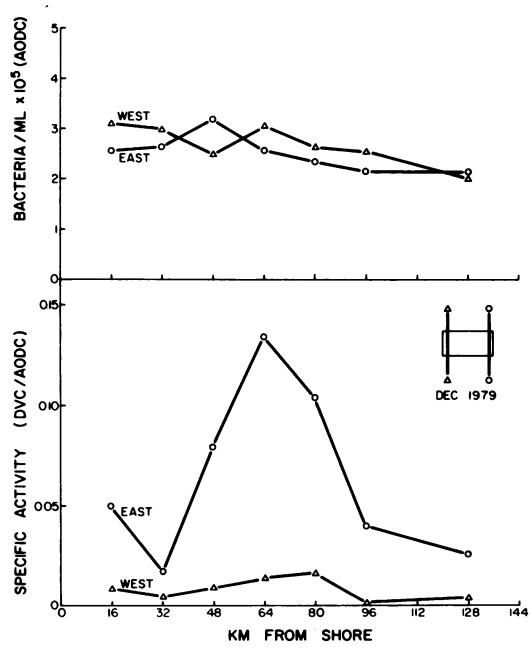

FIG. 3. *Total numbers of bacteria per milliliter, determined by AODC, and specific activities, calculated as the ratio of DVC determined by the Kogure et al. method (12) and total counts (AODC), for surface water samples collected in December 1979 at stations along transects passing through the eastern* (○) *and western* (△) *ends of the dump site.*

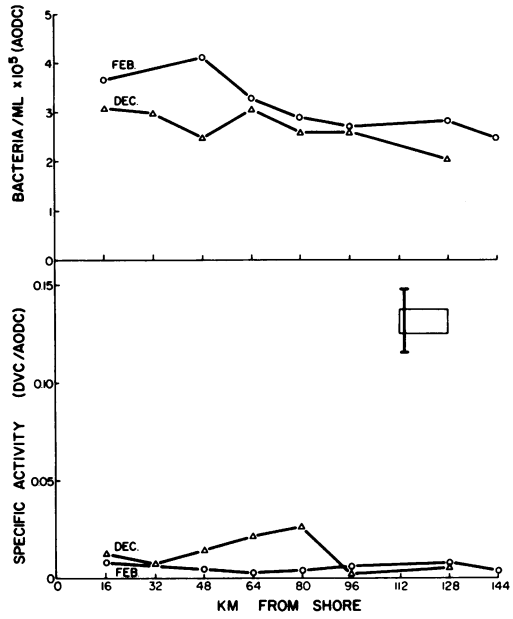

FIG. 4. *Total numbers of bacteria per milliliter, determined by AODC, and specific activities, calculated as the ratio of DVC determined by the Kogure et al. method (12) and total counts (AODC), for surface water samples collected in December 1979 (△) and February 1980 (○) at the same stations along a transect passing through the western end of the dump site.*

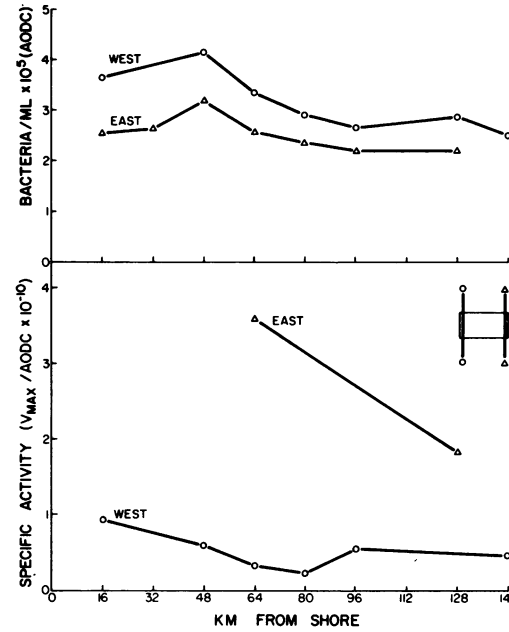

FIG. 5. *Total numbers of bacteria per milliliter, determined by AODC, and specific activities, calculated as the ratio of maximum uptake velocity of ^{14}C-labeled amino acids (V_{max}) and total counts (AODC), for surface water samples collected in December 1979 at stations along a transect passing through the western end of the dump site (○) and collected in February 1980 at two stations along a transect passing through the eastern end of the dump site (△).*

monas spp. (Table 2), an unexpected finding, since it is generally accepted that the bacterial community of open-ocean waters contains a predominance of pseudomonads. Sieburth (19) conducted a survey of 381 samples collected to depths of 1,200 m at 47 offshore stations in the Atlantic, Caribbean, and Pacific. His results showed that *Pseudomonas* spp. were dominant in the Atlantic and Caribbean samples. Similarly, Murchelano and Brown (15) found that, in Long Island Sound, *Pseudomonas* spp. were dominant, accounting for 40.6% of the culturable bacteria. The most common genera of bacteria in ocean waters have been reported to be *Pseudomonas*, *Vibrio*, *Flavobacterium*, *Arthrobacter*, *Caulobacter*, *Hyphomicrobium*, *Cytophaga*, *Acinetobacter*, and *Photobacterium* (G. Jones and R. R. Colwell, manuscript in preparation). Within the dump site area, however, *Pseudomonas* and *Flavobacterium* spp. were found to occur at very low frequencies (<9%) (Table 2). This observation, coupled with the dominance of *Vibrio* and *Aeromonas* spp. (80 to 90%) in the bacterial community, suggests that the bacterial community of the surface water at the dump site has undergone either alteration or a succession not reported previously.

Bacteria in seawater samples capable of growth on media usually used for culture of freshwater bacteria were also isolated, enumerated, and characterized. For stations located directly north of the dump site, a large percentage of the bacteria cultured on PCA were determined to be gram-positive cocci (Table 3, Fig. 2). It is generally accepted that the majority of bacteria found in open-ocean waters are gram-negative organisms (22). Kriss (13, 14) reported isolation of gram-positive organisms, i.e., *Micrococcus* spp., from water and sediment samples collected in the Black Sea, but did not conclude that they comprised a significant portion of the natural flora. In this study, gram-positive isolates were identified as *Micrococcus* spp. and *Staphylococcus* spp., including *Staphylococcus epidermidis* (data not shown). Staphylococci accounted for the majority (90%) of the strains isolated on PCA during December 1979. It is possible that the gram-positive cocci were introduced with the pharmaceutical wastes disposed at the dump site. The presence of unusually large numbers of gram-positive cocci, isolated on both MA and PCA, in the vicinity of the dump

site proper, the increase in viable counts on PCA observed in a waste plume studied in October 1979 (Table 1), and the presence of viable, gram-positive cocci in all waste samples examined to date (data not shown), provide evidence in support of such a hypothesis.

The composite results of this study, i.e., increased specific activities (determined by two methods), increased numbers of culturable marine bacteria, and marked alteration of the taxonomic composition of the culturable bacterial community in waters within and surrounding the Puerto Rico dump site, indicate demonstrable changes in the marine microbial community in the region used for waste disposal.

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