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Identification, Distribution, and Toxigenicity of Obligate Anaerobes in Polluted Waters

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A seasonal occurrence of obligately anaerobic bacteria, predominantly of the genera *Bacteroides* and *Clostridium*, in a polluted water site has been observed. The number of anaerobes varied from 1.8×10^3 cells/ml in the warmer months to 10 cells/ml in winter. Several isolates were toxigenic, indicating a potential human health hazard.

Several species of facultatively anaerobic bacteria have been implicated as causative agents of infections resulting from exposure to polluted waters (1, 2, 5, 8, 10, 12, 16). There has been little mention, however, of potential health hazards presented by waterborne, obligately anaerobic bacteria for humans, although some, primarily *Clostridium*, have been incriminated in diseases of fish (3, 17). Obligate anaerobes, especially *Bacteroides* and *Clostridium* spp., are potential human pathogens and, if present in polluted waters, may constitute an added health risk, especially to swimmers and professional divers who must work in polluted aquatic environments.

A diver training site, where exercises are conducted througout the year, was sampled periodically to determine whether obligately anaerobic microorganisms comprise a portion of the microflora and, if present, represent health risks for humans.

Water samples collected at selected stations 1 m below the surface and 1 m above the bottom, as well as sediment samples collected with a grab sampler, were obtained from the Anacostia River, Washington, D.C., where the diver training school is located. Depth of the water at the sampling site was 20 ft (6.1 m). For comparison, seawater samples (salinity of 32‰) were collected at sites of ongoing diving operations in the New York Bight and Jamaica Bay, New York. Dissolved oxygen (dO_2), temperature, salinity, and turbidity were measured immediately at the time of collection (15).

All media and dilution blanks were prereduced by incubation at 25°C in an anaerobic glove box (Germ Free, Inc., Miami, Fla.), transported to the sampling site in anaerobic GasPak jars (BBL Microbiology Systems, Cockeysville, Md.), inoculated, placed in GasPak jars to maintain the reduced state, and returned to the laboratory for further processing in an anaerobic glove box. Anaerobiosis in the glove box was maintained with a gas mixture of 85% N_2, 10% CO_2, and 5% H_2 constantly circulated through HEPA filters over palladium catalysts (9) in the presence of resazurin solutions as indicators of anaerobiosis (18).

Total anaerobic counts (TAC), including obligate and facultative anaerobes, of water and sediment samples were determined by diluting in VPI anaerobic salt solution (9), plating on supplemented (9) anaerobic brain heart infusion (BHI) agar (BBL), and incubating under strict anaerobic conditions for 24 h at 25°C, followed by 24 h at 35°C to minimize temperature shock. Obligately and facultatively anaerobic bacteria were differentiated by twice replicating these plates and incubating one set of plates anaerobically in an atmosphere containing 10% CO_2 at 35°C and the other anaerobically, as described above, for 48 h. Obligate anaerobes were identified on the basis of Gram stain, morphology, gas-liquid chromatography of metabolic by-products, and the Minitek (BBL Microbiology Systems) anaerobic identification scheme (7, 9).

Assays for cytotoxic effects in the Y-1 adrenal cell system were performed with supplemented (9) BHI broth cultures as the inoculum after stationary incubation under anaerobic conditions at 35°C for 24 h (D. R. Maneval, S. W. Joseph, S. T. Donta, R. Grays, and R. R. Colwell, J. Tissue Culture Methods, in press). These cultures, containing 10^8 to 10^9 cells/ml, were also examined for enterotoxin-like activity in the rabbit ligated ileal loop (10, 15).

Figure 1 illustrates the seasonal fluctuation of dO_2, temperature, TAC, and obligate anaerobic counts (OAC) in the water column and sediment at the Anacostia River. As noted, the dO_2 and

temperature values fluctuated, ranging from 1 to 11 ppm and 0.5 to 28°C. Throughout the year, the river water was highly turbid, i.e., Secchi disk transparency of 1/6 to 1/3 m.

TAC and OAC of the water samples also varied, ranging from 2×10^2 to 9×10^3 cells/ml and from 10 to 1.8×10^3 cells/ml, respectively, which could be related to changes in dO_2 and temperature. The obligately anaerobic population, in general, ranged from 2 to 10% of the TAC, with only a few exceptions. The highest TAC (9×10^3 cells/ml) and OAC (5×10^2 to 1.8×10^3 cells/ml) occurred during the warmer months of the year, when the dO_2 values ranged from 1 to 5 ppm. Sediment TAC and OAC fluctuated similarly, but were substantially higher than counts in the water column (Fig. 1A and B). The lowest OAC occurred when dO_2 values approached 10 ppm, and T was below 10°C (Fig. 1 A–C).

In comparison, the TAC of water samples collected at the New York Bight site ranged from 10^3 to 10^4 cells/ml; however, obligate anaerobes were detected only at a station located near a sewage outfall in Jamaica Bay (dO_2 of 11 ppm), where they comprised 40% of the TAC. The sediment TAC from New York Bight samples ranged from 10^6 to 10^8 cells/g and, as noted above, obligate anaerobes were not detected, except in Jamaica Bay, where 20% of the sediment microflora was obligately anaerobic. The absence of obligate anaerobes in the New York Bight samples may be due, in part, to high salinity and comparatively low fecal pollution, as demonstrated by low coliform counts (data not presented).

The distribution and tentative identification of predominantly obligate anaerobes isolated from the Anacostia River are listed in Table 1. The majority of isolates were either bacteroides or clostridia. That bacteroides (6) were isolated primarily from the water column is significant since levels of superoxide dismutase are high in *Bacteroides*, thus supporting findings that this enzyme is important to the survival of organisms in oxygenated environments (6, 13).

Although several of the isolates were cytotoxic for Y-1 adrenal cells, activity usually was not detectable beyond a 1:2 broth dilution, indicating relatively weak toxigenicity as tested by this system (Table 2). Several of the *Bacteroides*, *Clostridium*, and *Fusobacterium* strains, and a single isolate of *Butyrivibrio*, elicited fluid accumulation in rabbit ligated ileal loops (Table 2), indicating that these organisms are capable of producing enterotoxic substances. The findings for *Clostridium* are consistent with those previously reported for strains isolated from diarrheal cases (4, 11). Conversely, isolates of *Peptococcus* and *Streptococcus* displayed neither cytotoxic nor enterotoxic activity.

TABLE 1. *Identification and distribution of anaerobic bacterial species isolated from the Anacostia River*

Organism	No. of isolates		
	Top water	Bottom water	Sediment
Bacteroides			
B. coagulans	—[a]	—	1
B. capillosus	—	2	—
B. distasonis	5	2	—
B. eggerthii	—	1	—
B. furcosus	1	—	—
B. multiacidus	—	3	2
B. oralis	2	4	—
B. putredinis	1	—	—
B. ruminicola subsp. brevis	8	1	—
B. ruminicola subsp. ruminicola	8	1	1
B. vulgatus	1	—	—
Bacteroides spp.	12	8	7
Clostridium			
C. aurantibutyricum	—	—	1
C. butyricum	—	—	6
C. clostridiiforme	—	—	2
C. hastiforme	1	—	1
C. histolyticum	2	—	—
C. indolis	—	—	7
C. nexile	—	—	1
C. perfringens	—	—	4
C. putrefaciens	1	—	—
C. sordellii	—	1	—
C. subterminale	—	1	1
C. tertium	—	1	3
Clostridium spp.	3	—	4
Bifidobacterium			
B. animalis	—	1	—
B. breve	—	1	—
B. longum	—	1	—
B. magnum	1	—	—
Butyrivibrio fibrisolvens	—	1	—
Eubacterium spp.	1	—	1
Fusobacterium			
F. mortiferum	—	—	3
F. prausnitzii	—	—	1
F. russi	1	—	2
Fusobacterium spp.	1	—	—
Lactobacillus plantarum	—	1	—
Peptococcus			
P. acnes	—	1	—
P. magnus	—	—	1
Peptococcus spp.	2	—	—
Sarcina spp.	1	—	—
Streptococcus			
S. intermedius	3	—	—
S. mutans	1	—	—
Streptococcus spp.	2	—	—

[a] —, None.

In conclusion, obligately anaerobic bacteria,

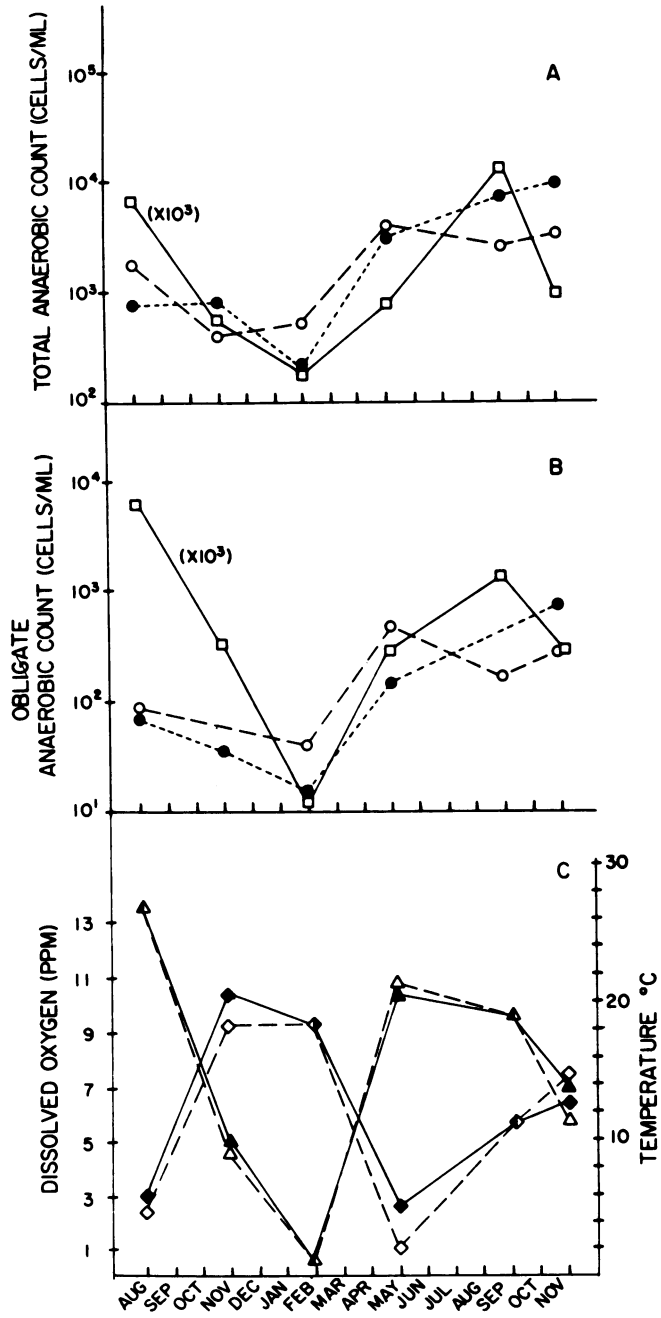

FIG. 1. *(A) TAC in top water (●), bottom water (○), and sediment samples (□) × 10^3 from the Anacostia River. (B) OAC in top water (●), bottom water (○), and sediment samples (□) × 10^3 from the Anacostia River. (C) dO_2 levels in top water (◆) and bottom water (◇) and temperature levels in top water (▲) and bottom water (△) from the Anacostia River.*

TABLE 2. *Toxigenicity of obligate anaerobes isolated from polluted waters*

Organism	Cytotoxicity for Y-1 adrenal cells[a] (no. positive/no. isolates tested)	Fluid accumulation in rabbit ligated ileal loops	
		No. positive/no. isolates tested	Avg fluid accumulation[b]
Bacteroides spp.	10/24	6/27	(0.61)
Butyrivibrio spp.	0/1	1/1	(1.0)
Clostridium spp.	2/10	3/9	(0.35)
Eubacterium spp.	1/2	ND[c]	ND
Fusobacterium spp.	2/7	3/5	(0.60)
Peptococcus spp.	0/4	0/1	—[d]
Sarcina spp.	1/1	ND	ND
Streptococcus spp.	0/3	0/3	—

[a] Cytotoxicity-cell lysis or cell shrinkage, crenation, vacuolization.

[b] Milliliters of fluid accumulated per centimeter of intestine, average of positive strains.

[c] ND, Not done.

[d] —, None.

some of which were shown to be cytotoxic or enterotoxic or both, were isolated from a polluted water site, suggesting that these organisms should be given further attention as potential hazards to swimmers and divers who work in polluted waters.

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