**Clostridium perfringens** in the Environment

JACK R. MATCHES, JOHN LISTON, AND DONALD CURRAN

Institute for Food Science and Technology, College of Fisheries, University of Washington, Seattle, Washington 98195

Received for publication 7 February 1974

*Clostridium perfringens* was isolated from samples collected in Puget Sound in the state of Washington and areas considered as possible sources of these organisms to Puget Sound. The distribution of *C. perfringens* in the total *Clostridium* population was determined for fish gut contents and sediments collected in highly polluted and less polluted areas, sewage samples, freshwater sediments, and soils. The greatest numbers of *C. perfringens* were obtained from marine sediments collected near the sewage outfall at West Point. Fewer isolates were made from fish collected from less polluted stations, although the number of *C. perfringens* remained high in sediments from other Puget Sound stations. The proportion of *C. perfringens* in the total *Clostridium* populations varied between 56 and 71% for sewage samples and only 0.4 to 4.1% for freshwater sediments and soil samples. Only 25 *C. perfringens* isolates out of 137 from fish guts, or 18%, were identifiable serologically and these fell into 12 groups. *C. perfringens* were fed to fish and the fish were sacrificed after varying lengths of time. The number of *C. perfringens* increased slightly in the gut during the first 24 h and then the numbers decreased rapidly for the next 120 h.

*Clostridium perfringens* is more widely spread than any other pathogenic bacterium. Its principal habitats are the soil and the intestinal contents of man and animals (3, 18). This organism has been recognized since the late 1800's, when reports linked it with food poisoning; however, it was not until 1945 that *C. perfringens* foodborne illness was reported (9). The illness received considerable attention in Great Britain in 1953 (7) and has been recognized as a very important food poisoning organism in the United States since that time (3, 8).

The presence of *C. perfringens* has been reported by a number of workers in soils (14, 17, 19, 20) and in marine sediments (1, 2, 5, 10, 11, 16). This work was done to determine its presence in Puget Sound, an area used both for sports and for commercial fishery and recreation, and also areas which may contribute clostridia to Puget Sound.

**MATERIALS AND METHODS**

**Sampling: fish stomach and gut contents.** Fish were caught by the College of Fisheries research vessel MV Commando with an otter trawl which collects fish lying on the bottom and swimming within 3 to 4 feet (ca.0.914 to 1.21 m) of the bottom. Immediately after the trawl net was retrieved, the fish were removed and the desired species were selected. Fish stomach and gut contents were removed from fish with sterile instruments. The surfaces of the fish were washed with water and swabbed with 70% ethanol. The belly wall of the fish was cut with a pair of sterile scissors. The stomach and gut were removed with scissors and scalpels sterilized by soaking in alcohol and flaming. When large fish like the Pacific dogfish were taken, one gut was large enough for a sample. Smaller fish like English and Dover sole have a small gut, and a composite of contents from five fish was collected in sterile bottles. Samples were held in ice until used.

**Sediments.** Sediments were collected aboard the MV Commando with a gravity geological corer containing a 3.8-cm plastic liner. The corer was sterilized by rinsing with 70% ethanol before use. Approximately equal quantities of water and sediment at the interface were removed from the plastic sleeve and placed in sterile jars. Samples collected aboard the research vessel were either processed in the laboratory aboard the vessel immediately after collection on cruises of several days, or returned to the laboratory in the College of Fisheries for processing on 1-day trips. Samples were refrigerated until processed, usually 4 to 6 h.

**Sewage.** Samples of sewage were collected from Seattle's West Point sewage disposal plant by plant employees during their normal daily sample collection. Samples collected from this primary treatment plant included sludge, unchlorinated and chlorinated effluent and sludge, plus chlorinated effluent. Samples were collected in sterile large-mouth bottles and held refrigerated until processed in the laboratory, a period of approximately 4 h.
Freshwater sediment and soils. Samples of sediment from a freshwater stream and soil from the surrounding forests and farm land were collected in conjunction with other studies. These samples were collected from the Snohomish River system, which empties into Puget Sound at the city of Everett. Samples were collected along approximately 70 miles (ca. 112.6 km) of stream from within 2 miles (ca. 3.2 km) of the crest of the Cascades down to Puget Sound. Most of the samples were collected along the Sound of stream not affected by tidal action. Samples of sediment collected from the river in water greater than 12 inches deep (ca. 0.3 m) were collected with a mud snapper (Kahl Scientific Instrument Corp., San Diego, Calif.). In the upper reaches of the stream with less than 12 inches of water, samples were collected from the stream banks, forest floor, and cultivated land with tills and spoons sterilized by soaking in alcohol and by flaming. Samples were refrigerated as soon as collection as possible and held until returned to the laboratory, usually no longer than 6 to 8 h.

Sampling stations. Sampling stations in Puget Sound and off the coast of Washington were selected for the College of Fisheries training cruises along the research vessel and other sampling programs. The West Point sampling station is located off Seattle's Metro sewage disposal plant, which dumps up to 2.5 x 10^9 gallons (ca. 8.5 x 10^9 liters) per day, and for this reason considered a polluted area. The remaining stations located in northern Puget Sound were considered less polluted. These stations were selected to represent different hydrographic conditions and are listed elsewhere (10, 11). Freshwater sediments and soil were collected along the Snohomish River system at stations selected for other studies. Sewage samples were collected from the settling tanks at the sewage disposal plant and at sampling ports were collected through the treatment plant.

Enumeration and identification. Blood agar plates were prepared with blood agar base (BBL), made up with 5% outdated whole citrated human blood. This medium was found to support excellent growth of both aerobic and anaerobic organisms collected from both freshwater and marine areas and also permitted the testing of the hemolysis reactions. Blood agar plates were either prepared and used immediately or poured in advance, the surface was dried, and the plates were prereduced by storage in anaerobic jars. Serial decimal dilutions of samples were prepared, using freshly prepared 0.1% peptone water made up with 50% seawater and 50% distilled water for the other samples. Earlier studies (12) showed that higher anaerobic counts were obtained from marine sediments using 50% seawater than distilled water in both diluent and media. One-tenth-milliliter quantities of the appropriate dilutions were spread on the surface of the blood agar with sterile glass rods, and plates were incubated anaerobically or incubated aerobically exposed to the atmosphere, both at 30 °C until colonies were large enough to be picked easily, usually 5 to 7 days. Clostridium species were isolated by either picking all colonies on a plate incubated anaerobically, or randomly selecting a percentage of the colonies. All isolated colonies were purified by streaking and were tested for both aerobic and anaerobic growth on McIlvaine and Toabe egg yolk medium (10). Isolates were Gram-stained to test for gram-positive rods and the presence of spores. Lecithinase-positive organisms growing only anaerobically were subjected to further testing in these studies for identification of C. perfringens. From the anaerobic populations isolated, the total Clostridium populations were identified and will be described in a later publication.

No distinction was made between active vegetative cells and resting spores in these studies because we were interested in total numbers present and the potential hazard, rather than the physiological state of the organisms.

The media and methods used for the identification of the clostridia were the reactions suggested in the literature (4, 18) and methods used in our laboratory. Reactions used in identifying C. perfringens were: acid from glucose, lactose, maltose, sucrose, and trehalose; no acid from mannnitol and salinic; gelatin and meat digested, with meat turning pink; stormy fermentation in milk (clot and gas); indole negative; nitrate reductions in approximately 80% of cultures; lecithinase positive, lipase negative; beta hemolysis of human blood and nonmotile. Although some variations were noted, cells under microscope examination were short with squared ends. When present, spores were subterminal; however, demonstration of sporulation was not possible with most isolates. All samples were incubated in Brewer Gas Pak (H₂ + CO₂) Anaerobic Jars (BBL) to ensure anaerobic conditions.

Feeding studies. Survival of C. perfringens in fish gut was determined by feeding cultures of the test organism to a test animal, the staghorn sculpin (Leptocotlus armatus), an animal found in the same environment as the fish caught and sampled for gut contents. Although the animals could be forced or injected with cultures of C. perfringens, using a syringe, a natural feeding method was desired to reduce stress on the animal. Small Coho salmon (Oncorhynchus kisutch), 5 to 7 cm in length, were obtained from a local state fish hatchery. Small volumes of C. perfringens cultures containing vegetative cells were injected into the gut area of the dead salmon. The same number of cells was inoculated into each salmon, the inoculated salmon were dropped into the aquarium, and the staghorn sculpins, being aggressive and active feeders, quickly consumed the entire inoculated fish and were then transferred to other aquaria. The fish were sacrificed after varying time periods and the stomach and gut contents were weighed and blended in a Waring blender with sterile diluent (0.1% peptone in seawater and distilled water). The total number of C. perfringens surviving was calculated. The number of cells in the inoculum was determined by spreading 0.1-ml quantities of serial decimal dilutions on blood agar.

RESULTS

The stomach and gut contents of otter trawl-caught fish were examined for total bacterial
numbers by incubating both aerobically and anaerobically. Colonies picked from the anaerobically incubated count plates were purified and studied for morphological and biochemical features. Total plate counts of organisms growing both aerobically and anaerobically on blood agar at 30°C, the types of fish samples, and the number of fish per sample are shown in Table 1. Total numbers of bacteria growing both aerobically and anaerobically varied per gram of gut contents by several orders of magnitude. Aerobic counts had a range of 2,000 to 32 million, and the anaerobic counts had a range from 1,000 to over 6 million. The averages obtained for both aerobic and anaerobic counts from fish collected from the different sampling areas are of the same magnitude. The average aerobic counts were 2,600,000 and 1,600,000 organisms/g of gut contents for West Point and other sampling stations, respectively. Average anaerobic counts for the same two areas were 630,000 and 580,000 bacteria/g of gut contents, respectively.

Samples of sediment and fish gut contents, collected from the sampling stations in Puget Sound and off the coast of Washington, were tested for the presence of C. perfringens (Table 2). The largest percentage was found in gut contents from fish caught from the West Point sewage disposal outfall diffuser pipe area. Fewer C. perfringens were isolated from fish collected at other sampling stations some distance from the source of contamination. Fish samples collected off the coast of Washington out to depths of 850 m, surprisingly, did not yield C. perfringens. Sediment samples collected from Puget Sound stations contained Clostridium populations of which C. perfringens made up 38.3 and 26.2% for West Point and other sampling stations, respectively. Sediments collected off the coast of Washington, like fish gut contents, did not yield C. perfringens. Fish caught at the West Point station contained a higher percentage of C. perfringens in their gut contents than did the sediments collected from the same area. This may be due to the organisms or material used as feed by fish harboring C. perfringens in larger numbers as a result of the food chain.

C. perfringens is not a member of the normal flora of fish; however, these organisms and other pathogens can contaminate fish caught in polluted water (6). Since fish can pick up organisms on the surface of skin and gills and the gut during feeding, the survival of C. perfringens was studied. The results of three experiments in which duplicate fish were sacrificed at each sampling time are shown in Fig. 1. When sampled 1 h after feeding, the feed had not been digested. The numbers of C. perfringens increased 1 log during the first 24 h and then declined rapidly during the next 120 h. Further change was not detected after incubation for 288 h. The aquarium temperature was 18.7°C, which is above the minimal growth temperature for C. perfringens, making the 1-log increase possible.

C. perfringens isolates were serotyped at the Center for Communicable Diseases, Atlanta, Ga. Of a total of 137 cultures serotyped only 25, or 18%, were identified as known serotypes of C. perfringens (Table 3). One hundred cultures were collected from the West Point sampling

| Table 1. Total counts obtained from fish gut contents during 1988 |
|--------------------|-----------------|-----------------|-----------------|
| Date               | Fish type       | No. fish per sample | Count per single sample |
|                    |                 | Aerobic count     | Anaerobic count   |
|--------------------|-----------------|-----------------|-----------------|
| Fish collected at West Point sampling station | | | |
| January            | English Sole*   | 4               | 1,000,000       | 25,000          |
| January            | Ratfish*        | 4               | 32,000,000      | 6,300,000       |
| February           | Dover Sole      | 3               | 250,000         | 130,000         |
| February           | Ratfish         | 4               | 160,000         | 20,000          |
| April              | Ratfish         | 4               | 100,000         | 5,000           |
| May                | Pacific Dogfish*| 1               | 8,000           | 2,500           |
| May                | Ratfish         | 5               | 2,000           | 1,000           |
| May                | Sand Sole       | 4               | 25,000          | 16,000          |
| July               | English Sole    | 2               | 16,000          | 3,200           |
| July               | Pacific Hake    | 2               | 16,000          | 10,000          |
| July               | Ratfish         | 3               | 160,000         | 40,000          |
| July               | Ratfish         | 3               | 2,000,000       | 2,000,000       |
| July               | Rockfish*       | 4               | 3,200,000       | 800,000         |
| August             | Rockfish        | 3               | 100,000         | 50,000          |
| August             | Sand Sole       | 6               | 160,000         | 100,000         |
| Average            |                 |                 | 2,600,000       | 630,000         |
| Fish collected at other sampling stations | | | |
| January            | English Sole    | 4               | 320,000         | 2,500           |
| January            | Pacific Cod     | 4               | 40,000          | 3,200           |
| January            | Pacific Dogfish | 2               | 400,000         | 2,000           |
| January            | English Sole    | 4               | 20,000          | 16,000          |
| February           | Pacific Cod     | 3               | 400,000         | 200,000         |
| February           | Dover Sole      | 3               | 250,000         | 130,000         |
| February           | Ratfish         | 4               | 160,000         | 20,000          |
| February           | Sablefish*      | 4               | 16,000,000      | 4,000,000       |
| April              | Pacific Hake    | 5               | 10,000          | 2,500           |
| April              | Sand Sole       | 5               | 8,000           | 8,000           |
| July               | English Sole    | 5               | 2,500,000       | 2,500,000       |
| July               | English Sole    | 5               | 200,000         | 200,000         |
| July               | Rockfish        | 4               | 2,500,000       | 1,300,000       |
| August             | English Sole    | 5               | 25,000          | 10,000          |
| August             | Rockfish        | 5               | 630,000         | 320,000         |
| Average            |                 |                 | 1,600,000       | 580,000         |

* Parophrys vetulus.  
* Hydrologus coli.  
* Microstomus pacificus.  
* Squalus acanthias.  
* Poetitichys melanostictus.  
* Merluccius productus.  
* Sebastes species.  
* Gadus macrocephalus.  
* Anaoplopoma fimbria.
TABLE 2. Distribution of C. perfringens isolated from the marine environment

| Origin of samples          | No. of samples collected | No. of samples containing C. perfringens | No. of Clostridium isolates* | No. of C. perfringens isolated | C. perfringens in isolates (%) | Avg no. of C. perfringens per gram |
|---------------------------|--------------------------|-----------------------------------------|------------------------------|-------------------------------|--------------------------------|-----------------------------------|
| Fish gut contents*        |                          |                                         |                              |                               |                                |                                   |
| West Point                | 15                       | 15                                      | 160                          | 105                           | 66                             | $3.4 \times 10^4$                  |
| Other Puget Sound stations| 15                       | 5                                       | 168                          | 13                            | 7.7                            | $5.0 \times 10^4$                  |
| Off Washington coast      | 6                        | 0                                       | 15                           | 0                             | 0                               |                                   |
| Sediments                 |                          |                                         |                              |                               |                                |                                   |
| West Point                | 38                       | 38                                      | 115                          | 44                            | 38.3                           | $2.9 \times 10^3$                  |
| Other Puget Sound stations| 66                       | 10                                      | 61                           | 16                            | 26.2                           | $1.7 \times 10^3$                  |
| Off Washington coast      | 6                        | 0                                       | 142                          | 0                             | 0                               |                                   |

* A randomly selected portion of the Clostridium flora.
* Pooled fish samples (See Table 1).

![Graph](image)

Fig. 1. Numbers of C. perfringens inoculated into and recovered from staghorn sculpins.

station and only 21 were identified. Of the remaining 37 cultures from the other sampling stations, only 4 fell into identifiable groups.

Areas serving as possible sources of C. perfringens, including sewage from the West Point disposal plant and freshwater sediments and soil, were investigated (Table 4). Nine hundred and eighty-six Clostridium strains were isolated and from this population 254 C. perfringens were identified. The freshwater sediments and soil contained very low numbers of C. perfringens. This was even true of soil samples collected from farm land used during parts of the year as pasture for cattle. Sewage samples contained high levels of C. perfringens which was expected because the sewage was composed largely of domestic waste.

**DISCUSSION**

The size of the aerobic bacterial population in the gut of fish reported in these studies is within the range reported by other investigators (13). Little work has been done, however, with the populations growing anaerobically, which include both obligate and facultative anaerobes. The wide range in aerobic and anaerobic counts is not surprising, since feeding fish have been reported to contain high levels of organisms in their gut. The fast that normally occurs during spawning with many species, or other periods of nonfeeding, would reflect the low bacterial loads in the intestine of some fish.

Studies of the numbers of bacteria in Puget...
Sound sediments at the sediment-water interface have been published elsewhere (12) and range from $0.73 \times 10^4$ to $23.5 \times 10^4$ and $1.21 \times 10^4$ to $16.9 \times 10^4$ cells/ml of sediment-water slurry for anaerobic and aerobic counts, respectively. These data from 110 core samples collected over a 3-year period show the lack of seasonal variation. The 2-log variation in anaerobic counts can probably be accounted for by the composition and especially the organic content of the sediments collected. The highest counts were obtained from reduced mud samples and the lowest counts were from sand.

*Clostridium perfringens*, an important food poisoning organism, is ubiquitous. This organism has been isolated from marine sediments by several authors from both polluted and nonpolluted areas. In the studies reported here, greater numbers of *C. perfringens* were isolated from polluted areas than from less polluted areas. This is in agreement with the report of Bonde (1) that a close relationship between the amount of pollution and numbers of *C. perfringens* was always established and, although a few colonies generally will be found in unpolluted samples, the counts are always proportional to the pollution. In the Puget Sound studies, sediments collected near the sewer outfall contained a *Clostridium* population composed of 38.3% *C. perfringens*. At the other Puget Sound sampling stations *C. perfringens* made up 26.2% of the population. The number of *C. perfringens* isolated from sediments in these studies agrees very well with some of our other studies in which *C. perfringens* made up 37.3% of the *Clostridium* population (12). Samples of sediment collected off the coast of Washington just south of the Straits of Juan de Fuca at depths of 880 m were negative for the presence of *C. perfringens*. Bonde (1) reported an inverse relationship between numbers of *C. perfringens* in sediments and depth, with the highest counts ($>10,000/g$) in shallow water (0 to 5 m). Counts of as much as 1,000 to 10,000 at depths of 15 to 20 m, but below 20 m only very low counts were found.

In these studies *C. perfringens* made up a higher percentage of the total *Clostridium* population in both fish gut and sediments collected at the West Point sampling station than at the other Puget Sound sampling stations. Also, clostridia made up a higher percentage of the population growing anaerobically in samples collected at West Point than at the other sampling stations. It can be reasoned that Puget Sound waters, varying from estuarine to suboceanic, receive more pollution than waters off the coast of Washington. Puget Sound is a deep-water port with many seagoing ships and large numbers of pleasure boats, and is rapidly becoming surrounded by cities and housing developments. Although sewage disposal plants are operating, bacteria can still be isolated in large numbers from chlorinated sewage (Table 4). Fish feeding near areas receiving pollution such as sewage can pick up enteric bacteria on their skin and gills and in their intestines (6). Although there are little data available on the *Clostridium* flora of fish gut contents, *C. perfringens* has been isolated from the gut of fish. Bonde (1) found high numbers of *C. perfringens* in the gut of plaice, flounder, and mackerel, but small to nil numbers in other species.

The number of bacteria and *C. perfringens* in the gut contents was related to the amount of feed in the fish. The numbers of organisms per gram of gut contents were found to vary between species of fish and even between individuals within a species. The numbers of *C. perfringens* in the total *Clostridium* population in fish gut were higher than the numbers in

### Table 4. Total number of clostridia studied and number of *C. perfringens* isolated from freshwater sediments, soils, and sewage

| Origin of samples            | No. of Samples | Total no. of Clostridium isolates | No. of Samples containing C. perfringens | No. of C. perfringens | C. perfringens (%) |
|------------------------------|----------------|-----------------------------------|------------------------------------------|-----------------------|--------------------|
| Stream beds                  | 22             | 247                               | 1                                       | 1                     | 0.4                |
| Adjacent banks               | 15             | 145                               | 1                                       | 6                     | 4.1                |
| Forest floor                 | 10             | 100                               | 1                                       | 4                     | 4.0                |
| Farm land                    | 12             | 93                                | 2                                       | 2                     | 2.1                |
| Unchlorinated effluent       | 15             | 68                                | 15                                      | 48                    | 70.6               |
| Chlorinated effluent         | 15             | 75                                | 10                                      | 46                    | 61.3               |
| Sludge                       | 15             | 181                               | 19                                      | 102                   | 56.4               |
| Sludge plus chlorinated effluent | 3          | 77                                | 3                                       | 45                    | 58.4               |
| Total                        | 106            | 986                               | 52                                      | 254                   |                    |
sediment off the sewer outfall diffuser pipe. At the other Puget Sound stations the reverse was true. One conclusion that can be drawn from these data is that since *C. perfringens* is abundant in human feces (15) and much of the effluent is domestic waste large numbers of *C. perfringens* are being dumped into the marine environment (Table 4). Fish feeding in this environment, and especially near the bottom, may pick up higher levels of *C. perfringens* from this area than from the other cleaner areas.

Since *C. perfringens* was found to occur in fish gut contents, its survival in this environment was studied. Feeding fish will no doubt continue to pick up and discharge organisms. These studies show that one species of fish consuming *C. perfringens* during feeding will eliminate these organisms if they do not continue to feed. These data are interesting and further studies should be carried out.

Primary treatment sewage disposal plants add high levels of organic matter and bacteria to the environment. Another area contributing bacteria to the marine environment is runoff from land. The Snohomish River system, chosen in these studies, runs through different environments. Approximately the first 8 miles (ca. 12.9 km) of the stream from the source cascades down a timbered canyon through an area reached only on foot by trail. Below this area a few miles the canyon widens and the stream is flanked by farm land. The stream empties into Puget Sound at Everett, Wash. through an industrial area. The numbers of *C. perfringens* isolated along this stream system during both dry and wet weather were lower than was expected, and the samples positive for *C. perfringens* had an average count of 5.9 × 10⁷/g. It was felt that farm land used as pasture for cattle would harbor large numbers of *C. perfringens*, although Hobbs et al. (7) reported only 2 of 113 samples (1.7%) of cattle feces contained *C. perfringens*. Sediments collected from the stream bed contained only 0.4% *C. perfringens* in the total *Clostridium* population. This level increased to 4.1 and 4.0% in samples collected along the adjacent stream banks and forest floor. The level dropped to 2.1% in samples of soil collected from farm land.

The serotyping of *C. perfringens* from fish gut was carried out to determine if there was any correlation between these types and the serotypes occurring in food poisoning outbreaks in Seattle. However, only a small proportion (18%) of the strains could be typed and no correlation was established. Nevertheless the data do indicate the possibility of transport of *C. perfringens* directly into the human food system through fish caught in polluted areas.

ACKNOWLEDGMENTS
This research was supported by Public Health Service grants 5 R01 EF 00882 and 5 R01 FD 00292.

LITERATURE CITED
1. Bonde, G. J. 1967. Pollution of a marine environment. J. Water Pollut. Cont. Fed. 39:45-63.
2. Bonde, G. J. 1968. Studies on the dispersion and disappearance phenomena of enteric bacteria in the marine environment. Rev. Int. Oceanogr. Med. Tome IX:17-44.
3. Bryan, F. L. 1969. What the sanitarian should know about *Clostridium perfringens* foodborne illness. J. Milk Food Technol. 32:381-389.
4. Cato, E. P., C. S. Cummings, L. V. Holdeman, J. L. Johnson, W. E. C. Moore, R. M. Smibert, and L. D. Smith. 1970. Outline of clinical method in anaerobic bacteriology. The Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va.
5. Davies, J. A. 1967. Isolation and identification of *Clostridia* from North Sea sediments. J. Appl. Bacteriol. 32:164-169.
6. Guelin, A. 1952. Bacteriophage and enterobacteria chez les poissons de mer et le probleme des eaux polluees. Ann. Inst. Pasteur (Paris) 83:35-46.
7. Hobbs, B. C., M. E. Smith, C. L. Oakley, and G. H. Warrack. 1953. *Clostridium welchii* food poisoning. J. Hyg. 51:75-101.
8. Hobbs, B. C. 1969. *Clostridium perfringens* and *Bacillus cereus* infections. In H. Rieman (ed.), Food borne infections and intoxications. Academic Press Inc., New York.
9. McClung, L. S. 1945. Human food poisoning due to growth of *Clostridium perfringens* (C. welchii) in freshly cooked chickens: preliminary note. J. Bacteriol. 59:229-231.
10. McClung, L. S., and R. Toabe. 1947. The egg yolk plate reaction for the presumptive diagnosis of *Clostridium sporogenes* and certain species of the gangrene and *Botulinum* groups. J. Bacteriol. 53:139-147.
11. Matches, J. R., and J. Liston. 1975. Methods and techniques for the isolation and testing of *Clostridia* from the estuarine environment, p. 345-362. In L. H. Stevenson and R. R. Colwell (ed.), Belle W. Baruch Library in Marine Science, vol. 1. Estuarine Microbiol. Ecology. University of South Carolina Press, Columbia.
12. Matches, J. R., and J. Liston. 1974. Mesophilic clostridia in Puget Sound. Can. J. Microbiol. 20(1):1-7.
13. Shewan, J. M. 1961. The microbiology of sea water fish. In G. Borgstrom (ed.), Fish as food, vol. I. Academic Press Inc., New York.
14. Shinjo, T., and M. Ogata. 1965. Studies on the genus *Clostridium*. II. Clostridial flora in soil. Jap. J. Vet. Sci. 27:305-308.
15. Smith, H. W., and W. E. Crabb. 1961. The faecal bacterial flora of animals and man: its development in the young. J. Pathol. Bacteriol. 82:53-66.
16. Smith, L. D. 1966. The clostridial flora of marine sediments from a productive and a non-productive area. Can. J. Microbiol. 14:1301-1304.
17. Smith, L. D., and M. V. Gardner. 1949. The occurrence of vegetative cells of *Clostridium perfringens* in soil. J. Bacteriol. 58:407-408.
18. Smith, L. D., and L. V. Holdeman. 1968. The pathogenic anaerobic bacteria. C. C. Thomas Co., Fort Lauderdale, Fla.
19. Taylor, A. W., and W. S. Gordon. 1940. A survey of the type of *Cl. welchii* present in soil and in the intestinal contents of animal and man. J. Pathol. Bacteriol. 50:271-277.
20. Yamagishi, T., S. Ishida, and S. Nishida. 1964. Isolation of toxigenic strains of *Clostridium perfringens* from the soil. J. Bacteriol. 88:646-652.