DISTRIBUTION OF ACANTHAMOEBA (PROTOZOA: ACANTHAMEOEBIDAE) IN MARINE AND FRESHWATER SEDIMENTS

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Introduction

Small free-living amoebae of the genus Acanthamoeba are well-known inhabitants of soil and water. The first described species, A. castellanii, was more or less discovered accidentally when it was observed on an agar plate streaked with a soil-sample that was being studied for bacteria and fungi. Very large numbers of the amoebae may be grown in the laboratory on agar plates and in sterile liquid culture medium. Although various species of Acanthamoeba have been known to science for over 50 years, intensive research on them began in the 1950's. Jahnes et al (1957) first observed Acanthamoeba in cultures of monkey kidney cells, and Culbertson et al (1959) found that amoebae from such cultures could kill laboratory animals when inoculated intranasally. In the mid 1960's it was discovered that several species of Acanthamoeba were responsible for disease or death in humans and domestic or wild animals. Griffin (1978) has published an extensive review on the role of free-living amoebae in human and animal disease. The genus Acanthamoeba has attracted worldwide attention and cases of disease have been reported from many countries including the United States, Russia, Korea, England, India, etc.

Sawyer (1970) discovered that A. castellanii and A. polyphaga would grow experimentally on full-strength oceanic seawater and produce cysts which had lost their diagnostic characters. Later, Sawyer (1971) described the first marine species, A. griffini in honor of Dr. Joe Griffin, Armed Forces Institute of Pathology, who made the original isolation from a sediment sample collected in Long Island Sound. The observation that marine waters were a new environmental source for Acanthamoeba then led to discovery that a pathogenic species, A. culbertsoni, was present in sewage sediments of the Atlantic Ocean near New York State (Sawyer et al, 1977). Singh & Das (1972) previously had shown that several species of Acanthamoeba were present in municipal sewage wastes in India. Davis et al (1978) cultured seawater samples from the open ocean and reported that Acanthamoeba was one of three genera which were observed most commonly in their study.

The discovery of both pathogenic and non-pathogenic species of Acanthamoeba in the marine environment suggested that a major study should be made to determine whether or not there is an association between the presence of sewage wastes in the sea and the presence of potentially pathogenic Acanthamoeba. Our report summarizes the results of a comprehensive study on the distribution of Acanthamoeba in sediments from inshore, coastal, and offshore environments. Our choice of study areas with differing characteristics included the St. Martins River-Isle of Wight Bay complex which represents a shallow fresh- to seawater gradient; the New York Bight apex which represents a nearshore marine habitat which receives both municipal and barged sewage, and the Philadelphia-Camden sewage disposal site which represents an offshore impacted environment with sewage-free adjoining waters. The present report provides the first extensive account of the distribution of

Acanthamoeba species in oceanic bottom-sediments and demonstrates that such amoebae are potential residents of the ocean when bacteria, including the coliform group, are available as a source of food.

Methods

Sediment samples in or distant from the Philadelphia-Camden acid or sewage dump-sites were collected during 5 separate Environmental Protection Agency cruises (Table 1). A total of 177 sediment subsamples were taken aseptically from bottom grabs made with a Smith-McIntyre sampler. Subsamples were transferred to sterile 60-mm plastic dishes and stored on ice until processed in the laboratory. A total of 36 subsamples were made in the New York Bight apex during 3 cruises with personnel from the Food and Drug Administration (Table 2). The subsamples were taken aseptically from bottom grabs made with a Ponar grab sampler and stored on ice until processed. A total of 77 subsamples were collected from the St. Martins River-Isle of Wight complex during 4 sampling trips (Table 3). The samples were collected with a Ponar grab sampler, or with an improvised device made up of a short length of pipe attached to a wooden handle. The device was designed for use in shallow-water stations which did not require the use of a boat.

Dishes containing sediment samples were weighed before and after transferring small amounts of sediment to culture dishes in order to determine the weight of sediment used in each culture. Culture procedures and media were the same as described previously by Sawyer *et al* (1977). In some cases each sediment was placed on agar media made with distilled water and on media made with low salinity river water (4 - 8 o/oo) to estimate whether or not the composition of culture media influenced the number of cultures that were positive for amoebae. Occasionally 2 or 3 grab samples were taken from the same station to estimate how frequently amoebae could be isolated from a single location. All cultures were examined several times a week to check for the growth of amoebae and cultures which were negative after 2 weeks were discarded. Identifications based on cyst characteristics were made on original cultures when only one species was present. Subcultures were made when more than one species was present to obtain pure cultures and facilitate identifications.

Species of *Acanthamoeba* that were identified as known pathogens were grown at 40° C to confirm their ability to grow at temperatures approximating or exceeding mammalian body temperatures. Selected strains were donated to the American Type Culture Collection, Rockville, Maryland, or to the Communicable Disease Center, USPHS, Atlanta, Georgia, for animal pathogenicity tests. Amoebae which did not have the identifying characters for known species were studied further to determine if they were new to science.

Results

Twenty-two of 177 stations sampled (12 percent) in the offshore Philadelphia-Camden dump-site yielded cultures of Acanthamoeba (Table 1), including two pathogenic species A. culbertsoni and A. hatchetti. It was of interest to note that only 2 of 119 control stations yielded amoebae while 17 of 42 stations in or near the sewage spoil area were positive. Most of the stations in the sewage area which yielded Acanthamoeba also were positive for either fecal or non-fecal coliform bacteria (Table 4). Positive cultures from stations identified as in or near the acid-waste site (3 of 16 stations) were of interest since coliform bacteria, if present, were below detectable limits with the methods employed. The location of all stations sampled in the vicinity of the sewage dump-site is shown in Fig. 1. Analyses of the data showed that 40 percent of the sewage stations yielded Acanthamoeba in contrast to less than 2 percent for the non-sewage stations and 19 percent for the acid-waste site.

Twenty-four of 36 stations sampled (67 percent) in the New York Bight apex (Tables 2 and 5) yielded cultures of Acanthamoeba, including the two pathogenic species. Bacteriological studies showed that 33 of the stations were positive for non-fecal coliforms and 23 of them were positive for fecal-coliforms; station locations are shown in Fig. 2. In this sampling area 29 of the 36 stations were located in the sewage disposal site or in the closed area and 7 stations were not in close proximity to either area; the bacteriological studies showed 6 of the 7 stations harbored coliforms. All stations sampled were located in or near the area of the Bight apex that has been closed to shellfishing and none of them could be considered as control stations.

Twelve of 19 stations sampled (63 percent) in the St. Martins-Isle of Wight Bay complex yielded cultures of Acanthamoeba, including the two pathogenic species and one new species (Lewis and Sawyer, in press). The river-bay system (Fig. 3) had very few characteristics in common with the ocean-dumping stations other than being recognized as a polluted environment which has been closed to the harvesting of shellfish. All of the stations ranged from only 1 to 9 feet in depth, from 0 to 30 o/oo salinity; sediments ranged from foul, black muds to clean sand. The 6 stations located in the Isle of Wight Bay were sampled in from 1 to 3 replicates to yield a total of 25 subsamples and only 2 of them were positive for Acanthamoeba. In contrast to the bay stations, the 13 river stations were sampled to yield 52 subsamples of which 30 (60 percent) were positive for the amoebae.

Comparative analyses of data from the 3 different collecting sites supported one singularly important hypothesis, i.e., the presence of pathogenic or non-pathogenic species of Acanthamoeba depends, in part, on the availability of suitable bacteria as food organisms. The requirement for such food sources is met, in part, by the introduction of sewage-associated bacterial species. The recovery of amoebas from sediments that are not impacted by sewage additions appeared to depend on the presence of bacteria which occur naturally as a consequence of natural biogenic decay of organic materials, a phenomenon that is enhanced artificially by the introduction and decay of sewage-related materials.

Discussion

The design of our studies had a twofold purpose, (1) to demonstrate that the ocean disposal of barged sewage wastes alters the natural microbial flora of the seabottom in such a way as to enhance the recovery of pathogenic and non-pathogenic amoebae from impacted collection sites, and (2) to show that the distribution of such amoebae in the marine environment depends on the presence of an adequate supply of bacterial food organisms whether or not such bacteria are associated with sewage input.

The recovery of pathogenic and non-pathogenic species of Acanthamoeba from the marine environment, and the relationship of their distribution to the ocean disposal of sewage sludge, was observed more precisely in the Philadelphia-Camden study area than in the New York sewage dump-site or the St. Martins River-Isle of Wight Bay complex. The distribution of fecal and non-fecal coliform bacteria in sediments from the offshore study area showed precisely that such bacteria were introduced in the sewage sludge and not by potential run-off from municipal discharges. In contrast, the New York Bight apex study area showed distribution patterns indicative of bacterial input from both sewage sludge and municipal discharges. Thus, many stations in the Philadelphia-Camden area could be considered suitable as control sites while none of the stations in the Bight apex were suitable as controls. Both dump-sites were similar with respect to salinity in the sense that they were not subject to dilution by freshwater run-off. The St. Martins River-Isle of Wight complex provided parameters for analyses that were not present in the ocean stations: (1) it was not impacted by sewage sludge deposited by barges, (2) water depth ranged from only 1 to 9 feet in depth, (3) certain stations were impacted by residential or commercial pollution, (4) stations ranged from salt marshes to polluted muds, and (5) historical records showed that fecal coliforms and very low levels of dissolved oxygen were found in the low salinity to freshwater uppermost stations.

Cumulative data derived from the Philadelphia-Camden and New York Bight study areas showed that less than 2 percent of the non-sewage impacted samples (2/119) yielded cultures of Acanthamoeba. In marked contrast, 54 percent of the sewage impacted stations (40/74) were positive for the amoebae. The results are remarkable since the amount of sediment inoculated onto agar plates seldom exceeded 0.5 g in weight. When the vastness of the seabottom and the wide distribution in station location is taken into account, it is apparent that our approach is extremely conservative. Regardless of salinity, water depth, coliform bacteria, etc., 58 of 232 stations (25 percent) were positive for one or more species of Acanthamoeba.

Characteristics of the Philadelphia-Camden Dumping Grounds. The sewage disposal site situated 40 miles offshore from Maryland, Delaware, and New Jersey has been utilized only since 1972. In contrast to the New York Bight apex station which has been used for over 40 years, this site rarely yields bottom sediments which uniformly are made up of black silty sludge. Thus, there does not yet appear to be an extensive sludge "blanket" overlaying the bottom sediments. Furthermore, fecal and non-fecal coliform sediment MPN's vary from less than 2 to over 300 bacteria, suggesting that such sewage contamination is erratic as a result of the "youth" of the dump-site. In this study area high bacterial counts during one cruise may have reflected recent dumping activity, and low counts obtained during other cruises may have reflected differences in the dispersion patterns of settled sewage. In spite of differences in sediment characteristics and the numbers of countable coliform bacteria, a very high rate of recovery for Acanthamoeba species was recorded for the area. In the immediate vicinity of the dump-site bottom, bacteriogical studies and studies on the presence of amoebae indicate that sludge may have spread to the northeast and southwest of the dump-site. In contrast, other stations situated some distance from the sewage site consistently were negative for coliforms and rarely yielded cultures of the amoebae.

Characteristics of the New York Bight Apex Dumping Grounds. The sewage disposal site situated approximately 12 miles from the shores of Long Island Sound, New York has been used for sludge dumping for about

40 years. Bottom sediments taken from the site have no resemblance to naturally occurring seabottom and have been described as having the appearance of "black mayonnaise." Fecal coliform sediment MPN's ranging to several thousand are not unusual. Measurable amounts of the human fecal sterol "coprostanol" also are present throughout the area that is influenced by barged sewage sludge. Shoreward in all directions from the dump-site varying numbers of coliform bacteria are present in sediments as a result of land-based sewage discharges. Acanthamoeba routinely was isolated from the dump-site and from stations situated between the site and the adjacent shoreline. In marked contrast, similar amoebae rarely were obtained from sediments shoreward from the Philadelphia-Camden disposal site.

Characteristics of the St. Martins River-Isle of Wight Bay Complex. The river-bay complex which runs inland from Ocean City, Maryland was selected for study because it represented a system that is used extensively for recreational, residential, industrial, and agricultural purposes. The most inland stations that were sampled were in or near the town of Bishopville where the river is considered to be highly polluted and subject to concern by state health personnel. Stations downriver from Bishopville as the river empties into the Isle of Wight Bay were bordered by farms, residential communities, salt marshes, a dead-end canal at Ocean Pines, etc. Additionally, stations along the selected transect represented a full range of salinity from oceanic to brackish or freshwater and sediments ranged from foul, black mud to clean mud or sand. The study area, although not impacted by sewage-sludge dumping practices, was chosen to provide an inland study area with known pollutants, including fecal coliform bacteria which, theoretically, could be associated with the presence of Acanthamoeba. Thus, the river-bay system was studied to determine whether point sources of pollution could be identified as sources for amoebae which might be flushed downriver and seed the coastal marine seabottom.

General Conclusions. The first report of the presence of potentially pathogenic *A. culbertsoni* from the marine environment (Sawyer *et al*, 1977) suggested that there might be a cause-and-effect relationship between the ocean disposal of sewage wastes and the introduction of sewage-associated pathogenic protozoans into otherwise clean bottom sediments. Such a relationship must be studied further because barged sewage sludge has not been examined for the presence of the amoebae or their resistant cyst stages. Our extensive survey data clearly demonstrate, however, that there is a high correlation between the presence of fecal and non-fecal coliforms in bottom sediments and the successful isolation of Acanthamoeba from the marine and freshwater environments. The Philadelphia-Camden sewage disposal site may be considered as a geographical source for Acanthamoeba since it is approximately 40 miles offshore and 40 percent (17/42) sewage-associated sediment samples yielded the amoebae. In contrast, less than 2 percent (2/119) of the stations sampled from the periphery of the dump-site to less than 1 mile from shore yielded cultures of amoebae. It is evident from studies in the Philadelphia-Camden area that sewage-induced bacterial loading of the seabottom has measurable effects on the distribution of Acanthamoeba in the sea.

Conclusions based on the findings in the Philadelphia-Camden dump-site have been confirmed by further studies made in the New York Bight apex near New Jersey and New York. Thirty-six stations that were sampled either in the dump-site or in adjacent waters that have been closed to shellfishing yielded a 67 percent recovery of Acanthamoeba (24/36 stations). The association of Acanthamoeba with coliform bacteria was confirmed by the fact that in the New York stations 92 percent (33/36) of the stations harbored non-fecal coliforms, and 64 percent (23/36) harbored fecal coliforms. Bacteriological studies showed that coliforms were widely distributed in bottom sediments that were taken shoreward from the dump-site.

Results of the study in the St. Martins River-Isle of Wight complex, although obtained in an entirely different ecosystem from that of the sewage dump-sites, supported our hypothesis that bacterial loading influenced the frequency with which Acanthamoeba could be recovered from bottom sediments. Culture studies showed that the presence of the amoebas was independent of water depth, salinity, and sediment characteristics. The studies showed, however, that sediments which yielded the amoeba most frequently were those that were in close proximity to sources of human and industrial activity.

The final, and most important, conclusion to be reached from our studies concerns the fact that the presence of Acanthamoeba in the coastal or oceanic environment does not depend exclusively on the introduction of bacteria-laden sewage sludge. Davis et al (1978) isolated amoebae from waters of the open ocean and found that species of Acanthamoeba were among the most frequently isolated organisms of the identifiable protozoa encountered in their study. Superficially, the data suggest that species of Acanthamoeba appear to be among the most common inhabitants of the marine environment. Rather curiously, however, among the 76 species of marine amoebae included in a recent key to the marine amoebae (Bovee and Sawyer, 1979), and among the 13 species isolated by Davis et al (1978), only members of the genus Acanthamoeba are known to form resistant cysts. Thus, it is possible that a culture which yields Acanthamoeba may have originated from a single cyst and prevalence or frequency data would not serve as an indication of the abundance or population density of Acanthamoeba. There remains a very important requirement that known amounts of bottom sediment or seawater be analyzed to determine how many of the amoebae are present per unit of weight of sediment or volume of seawater. There appears to be very little doubt that population densities will be determined, in part, by the extent of bacterial loading in the test sample.

Summary

1. Pathogenic or non-pathogenic species of Acanthamoeba may be isolated from bottom sediments, the open ocean, and the nearshore environment.

2. The opportunity or chance of obtaining positive cultures depends on the presence of resistant cyst stages in the sample, or on the presence of suitable bacteria that provide a source of food.

3. The disposal of barge-delivered sewage wastes in the marine environment introduces fecal coliform bacteria into marine ecosystems which, otherwise, would harbor only naturally-occurring marine species.

4. It is not yet known whether Acanthamoeba species are present in barge-delivered sewage wastes, or whether bacteria present in the waste material encourage the growth of protozoan species to levels which exceed naturally-occurring ambient levels.

5. Most of the well-known species of Acanthamoeba are not limited in their distribution by factors such as salinity, water depth, and sediment characteristics.

6. Marine habitats which harbor fecal coliform bacteria have a 50 percent or better chance of harboring Acanthamoeba than those which are not impacted by sewage-disposal practices.

7. Several species of Acanthamoeba which thrive at mammalian body temperatures and are known to cause disease or death in laboratory animals and man were isolated from each of the 3 areas employed in the present study.

8. The Philadelphia-Camden sewage dump-site situated 40 miles from shore harbors geographically isolated species of Acanthamoeba which rarely are found at shoreward stations which do not yield countable numbers of coliform bacteria.

9. Species of Acanthamoeba may prove to be of equal or greater value than coliforms for measuring the persistent effects of unnatural loading of the marine environment by bacteria.

10. Less than 1 g of sediment was used for isolating Acanthamoeba. Further studies with larger amounts of sediment are likely to show that the amoebae are considerably more prevalent in the ocean than is documented in our report.

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Table 1. Summary of Environmental Protection Agency Cruises to Philadelphia-Camden Sewage Disposal, Acid-Waste and Control Stations[1]

Name of Cruise	Date	Control Stations	Acid-Waste Stations	Sewage Stations	Total No. Positive Stations[2]
RIDGERUNNER	Apr 1978	1/60	2/7	4/14	7/81
DEKKO	Aug 1978	1/5	-	1/4	2/9
HELMALEE	Sep 1978	0/18	0/4	4/9	4/31
FDA[3]	Oct 1978	0/7	-	1/5	1/12
ENFIN	Apr 1979	0/29	1/5	7/10	8/44
Totals		2/119	3/16	17/42	22/177

1 No. Stations Positive for Acanthamoeba/No. Stations Sampled

2 No. Stations Positive for Acanthamoeba/No. Stations with Coliform Bacteria = 10/20 excluding DEKKO for which bacteriology was not done.

3 FDA Cruise - a cruise made outside of the established sewage disposal site for purpose of determining horizontal spread of sewage as indicated by fecal and non-fecal coliform counts.

Table 2. Summary of Cruises in New York Bight Apex Within or Near Area Closed to Shellfishing.[1]

Trip No.	Date	No. Samples in Closed Area	No. Stations Outside of Closed Area	Total No. Positive Stations[2]
1	Jul 1978	5/8	-	5/8
2	Dec 1978	3/5	2/5	5/10
3	Jun 1979	13/16	1/2	14/18
Totals		21/29	3/7	24/36

1 No. Stations Positive for *Acanthamoeba*/No. Stations Sampled.

2 No. Stations Positive for *Acanthamoeba*/No. Stations with *Acanthamoeba* and Coliform Bacteria = 22/24.

Table 3. Summary Data from 4 Collecting Trips to the St. Martins River-Isle of Wight Bay Complex.[1]

Station Number	Type of Station[2]	Positive or Negative for *Acanthamoeba*	No. Positive/ No. Replicates
1	3	Pos.	1/3
2	1	Pos.	2/4
3	2	Pos.	5/5
4	2	Pos.	4/4
5	2	Pos.	2/4
6	3	Pos.	1/5
7	3	Neg.	0/5
8	3	Neg.	0/4
9	2	Pos.	3/5
10	1	Pos.	2/5
11	1	Neg.	0/1
12	1	Pos.	1/4
13	1	Pos.	4/4
14	1	Neg.	0/4
15	2	Pos.	4/4
16	2	Neg.	0/4
17	2	Pos.	3/4
18	3	Neg.	0/4
19	3	Neg.	0/4

1 Summary of 4 trips - 19 stations with 77 subsamples (See below).

2 Type 1 - more than 3 miles from Bishopville, MD - 4/6 positive; 9/22 replicates
Type 2 - within 3 miles of Bishopville, MD - 6/7 positive; 21/30 replicates
Type 3 - Isle of Wight Bay, MD - 2/6 positive; 2/25 replicates

Table 4. Summary of Environmental Protection Agency Stations Positive for Acanthamoeba With or Without Countable Coliform Bacteria.

Cruise Name	Date	Station No. and Type[1]	Total Coliform Count	Fecal Coliform Count
RIDGERUNNER	Apr 1978	NB-1 (1)	< 10	< 10
"	"	11 (3)	>230	>230
"	"	206 (3)	160	>230
"	"	407 (2)	< 10	< 10
"	"	414 (2)	< 10	< 10
"	"	420 (3)	22	51
"	"	423 (3)	22	91
DEKKO	Aug 78	206 (3)	N.D.	N.D.
"	"	33 (1)	N.D.	N.D.
HELMALEE	Sep 78	1K3 (3)	120	91
"	"	423 (3)	22	10
"	"	E (3)	< 10	< 10
"	"	2 (3)	< 21	< 21
FDA	Oct 78	97 (3)	2400	33
ENFIN	Apr 1979	2 (3)	77	< 37
"	"	20 (2)	< 37	< 37
"	"	203 (3)	37	< 37
"	"	210 (3)	37	< 37
"	"	211 (3)	< 37	< 37
"	"	E-18 (3)	< 37	< 37
"	"	E-12 (3)	< 37	< 37
"	"	206 (3)	< 37	< 37

[1] (1) = Control Stations, 2/119 positive for Acanthamoeba; no coliform bacteria
(2) = Acid-Waste Site, 3/16 positive for Acanthamoeba; no coliform bacteria
(3) = Sewage Dump-site, 17/42 positive for Acanthamoeba; 10/17 with coliform bacteria

Table 5. Summary of New York Bight Apex Stations Positive for <u>Acanthamoeba</u> With or Without Countable Coliform Bacteria

Date	Station No. and Type[1]	Total Coliform Count	Fecal Coliform Count
Jul 1978[2]	2 (DS)	170	< 20
	3 (DS)	78	45
	4 (DS)	160,000	3,300
	5 (DS)	4,600	700
	6 (DS)	460	40
Dec 1978[3]	2 (DS)	270	37
	4 (DS)	> 7,000	270
	5 (DS)	630	37
	6 (CA)	630	37
	10 (CA)	630	37
Jun 1979[4]	53 (CA)	49	4.5
	62 (CA)	4.5	< 2
	103 (OCA)	< 2	< 2
	104 (CA)	< 2	< 2
	754 (CA)	13	< 2
	756 (CA)	79	6.8
	757 (CA)	13	< 2
	761 (CA)	14	< 2
	764 (CA)	7.9	2
	765 (CA)	33	2
	790 (CA)	2	< 2
	791 (CA)	4	< 2
	792 (CA)	330	6.8
	1385 (CA)	2	< 2

1 (DS) - Sewage disposal site (8/24)
(CA) - Not a disposal site but closed to shellfishing (15/24)
(OCA) - Outside of closed area (1/24)

2 Five of eight stations yielded amoebae; 8/8 stations positive for coliform bacteria.

3 Five of ten stations yielded amoebae; 10/10 stations positive for coliform bacteria.

4 Fourteen of eighteen stations yielded amoebae; 15/18 stations positive for coliform bacteria; 12/14 stations positive for amoebae also positive for coliform bacteria and 3 stations positive for coliforms but negative for amoebae.

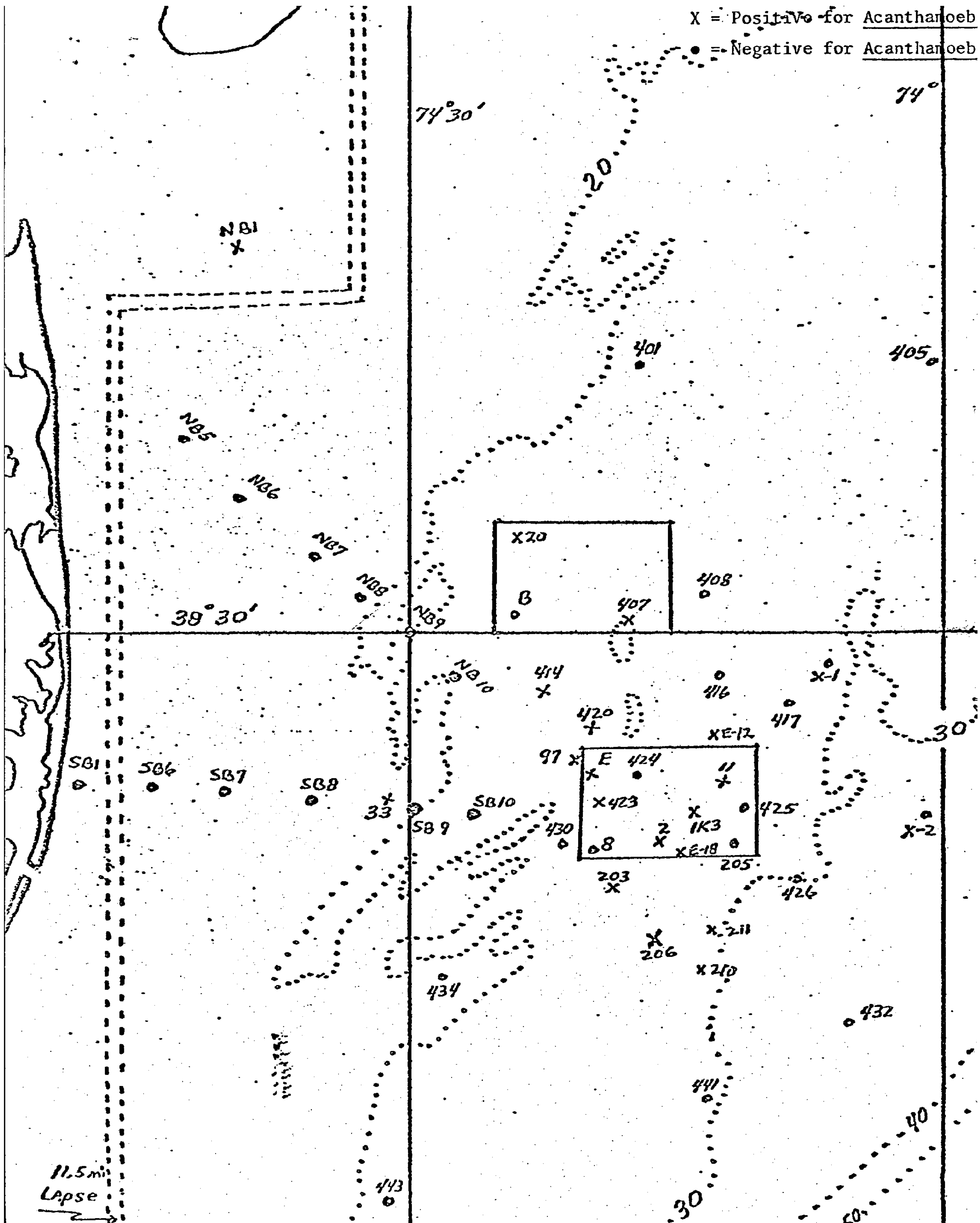

Fig. 1. Representative stations sampled for *Acanthamoeba* during 5 EPA-FDA cruises to the Philadelphia-Camden dump-site.

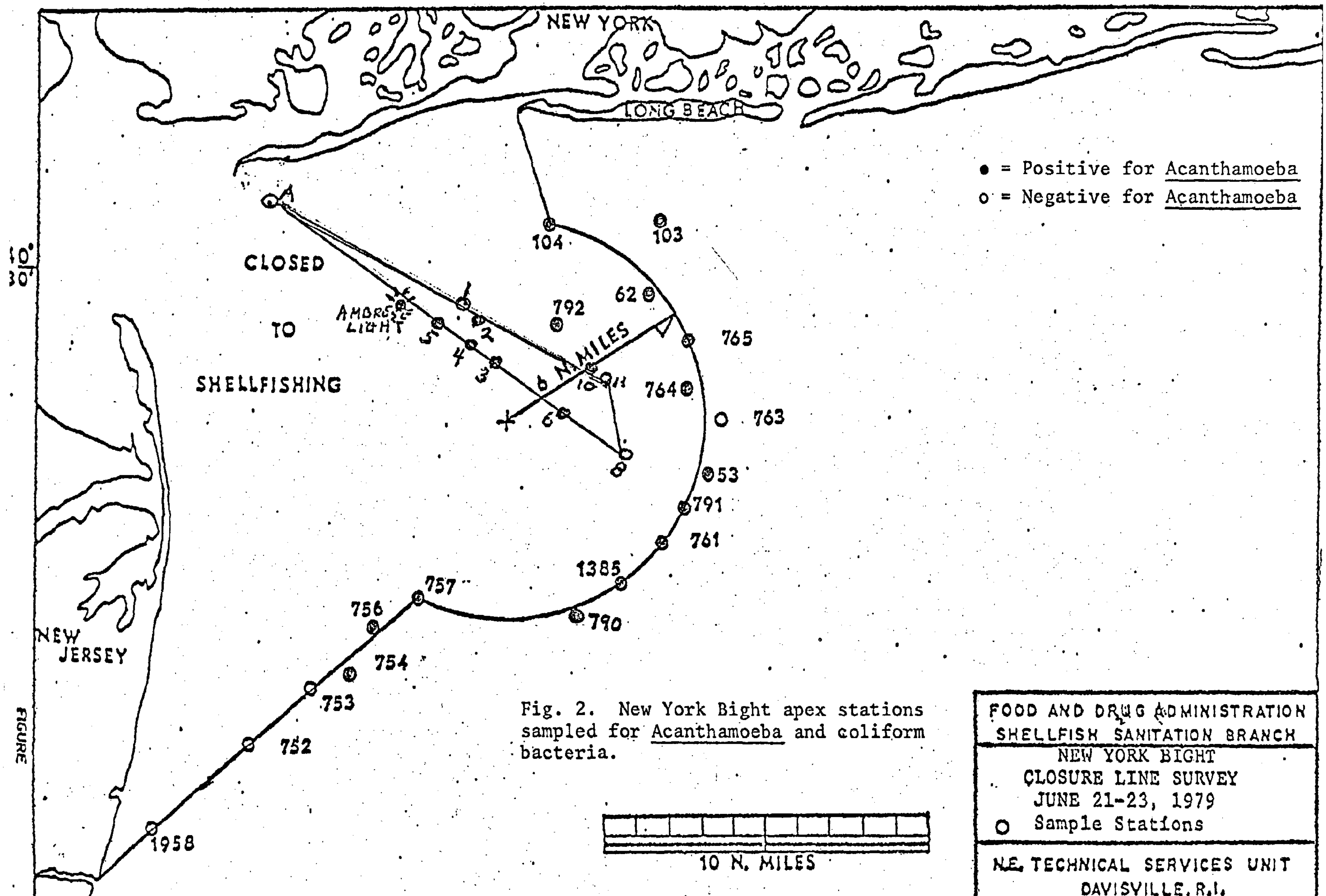

Fig. 2. New York Bight apex stations sampled for *Acanthamoeba* and coliform bacteria.

FIGURE

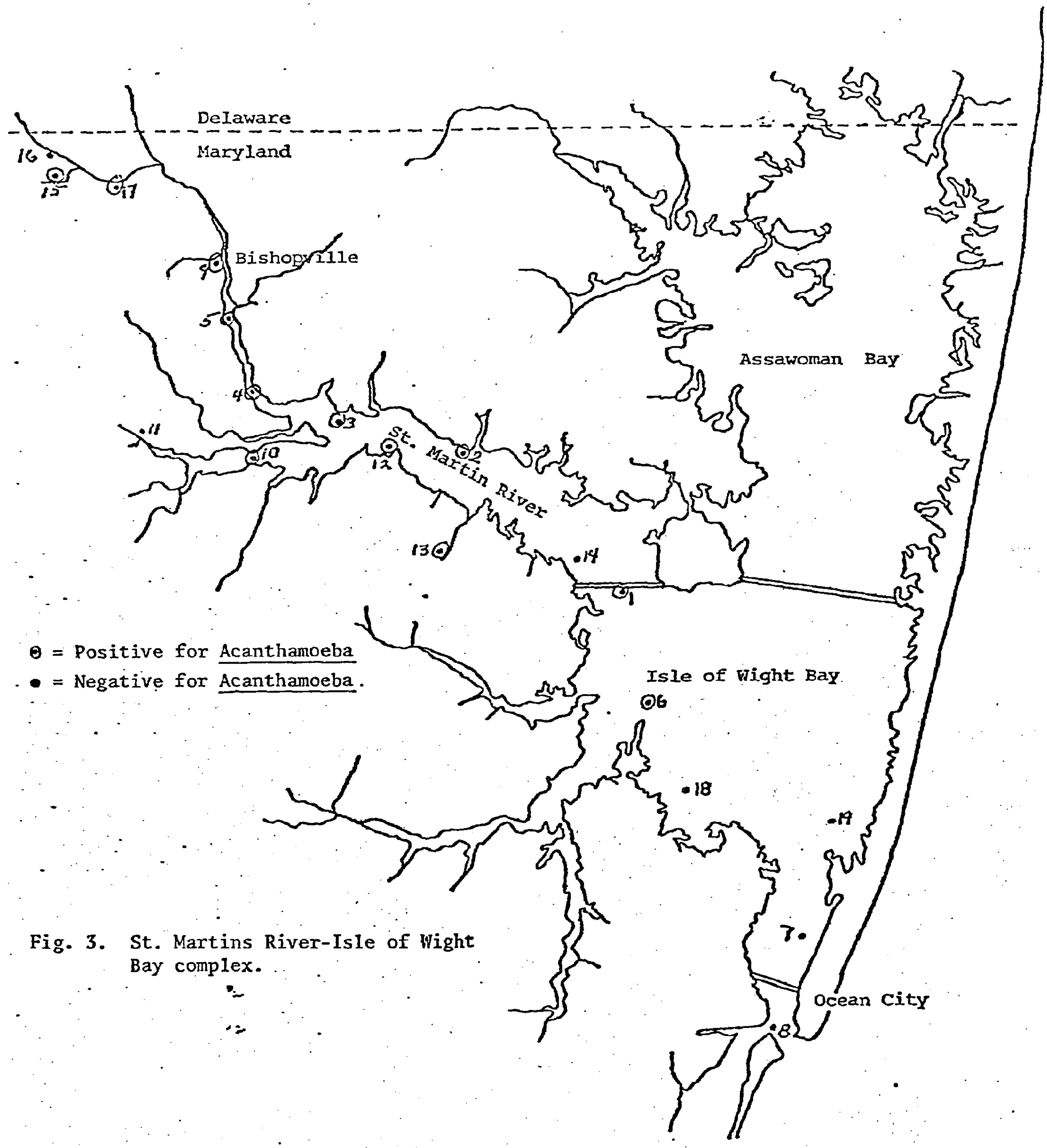

Fig. 3. St. Martins River-Isle of Wight Bay complex.