

SANITARY SIGNIFICANCE OF THE BACTERIAL FLORA
OF THE BRACKISH WATER CLAM, RANGIA CUNEATA,
IN ALBEMARLE SOUND, NORTH CAROLINA

A Thesis

Presented to

the Faculty of the Department of Biology
East Carolina University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Biology

by

Paul G. Comar

April, 1979

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ABSTRACT

Paul G. Comar. SANITARY SIGNIFICANCE OF THE BACTERIAL FLORA OF THE BRACKISH WATER CLAM, RANGIA CUNEATA, IN ALBEMARLE SOUND, NORTH CAROLINA. (Under the direction of Dr. Bernard E. Kane, Jr. - Department of Environmental Health and Dr. Donald B. Jeffreys - Department of Biology) Department of Biology, April 1979.

Rangia cuneata is a brackish water clam found commonly in the estuaries of the Atlantic and Gulf Coasts. There has been recent interest in North Carolina in commercial harvesting and processing of this clam for human consumption. This research was a microbiological investigation of Rangia to determine whether its bacterial flora, which is of greater density than other edible shellfish, posed a unique threat to public health.

Clams, water, and sediment were collected monthly for one year from four sites in Albemarle Sound. Microbiological examination of samples included enumeration of bacteria indicative of fecal contamination as well as screening for potentially pathogenic bacteria. The analysis of 48 Rangia samples showed a median fecal coliform most probable number (MPN) of only 80 FC/100g, considerably below the 230 FC/100g standard of federal and state regulation. Under these regulations a maximum of 10% of the samples may exceed 330 FC/100g, but 21% of the Rangia collections exceeded this value. In most clam samples exceeding 330 FC/100g, the total coliform values for the overlying water were also in excess of those allowable for shellfish growing waters. These findings indicate

that Rangia did not maintain high levels of these indicator organisms, but rather reflected the bacteriological quality of the ambient water.

Standard plate counts (SPC) for clams approached and sometimes exceeded 500,000 SPC/g. This value is used by some markets as an indicator of bacterial spoilage of a product. The SPC is of little sanitary significance in evaluating freshly harvested shellfish unless the number of fecal indicator organisms or pathogenic bacteria are considered. There was no statistical correlation between clam SPC's and either the numbers of indicator organisms or pathogens detected.

Potentially pathogenic bacteria, when recovered, were at very low, non-infective levels. Neither Salmonella nor Shigella were confirmed in any clam samples. Staphylococcus was detected in only 5 of 40 Rangia samples at values far below those which produce significant levels of enterotoxin. Vibrio counts as high as 4,500/g of clam were recorded. However, this bacterium is very common in marine and estuarine waters and shellfish. While particular strains of V. parahaemolyticus can cause gastrointestinal disorder, outbreaks of foodborne disease attributed to this organism are infrequent in this country.

These findings indicate that when Rangia cuneata is harvested from approved waters, there is no unique threat to public health caused by its bacterial flora. Large scale routine coliform testing should be continued to supplement and verify the coliform results reported in this research.

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INTRODUCTION

Rangia cuneata (Gray) is a brackish water clam found most commonly in the upper reaches of estuaries in the eastern United States and Central America where the salinity is 0-15⁰/oo. It has long been present in Gulf Coast estuaries and has recently become abundant in Atlantic Coast estuaries from Maryland to Florida (Pfitzenmeyer and Drobeck, 1964; Godwin, 1968; Hopkins and Andrews, 1970). A study by Cain (1975) showed that in one stretch of the James River in Virginia, Rangia comprised 95% of the benthic biomass. This mollusc is an important link in the food chain of oligohaline to mesohaline estuaries, converting detritus to biomass (Darnell, 1958; Tenore et al., 1968). Rangia serves as a food source for fish and crustaceans (Suttkus et al., 1954). Cain (1975) cites a report by Wass and Wright that Rangia are also a major component in the diet of various waterfowl.

Fossil Rangia have been harvested for many years along the Gulf Coast. The shell is used in industry for roadbed and for its chemical content. Rangia have been eaten by humans in a number of localities (Singley, 1893; Speck and Dexter, 1946; Hopkins et al., 1973). While conducting the field investigations for this study, I interviewed several North Carolinians who often used Rangia in chowders. Hoese (1972) indicated that a \$6 million per year industry could be sustained in a section of southwestern Louisiana if both shell and meat were sold. The estimate by Hoese was based on a shell price of \$3.50 per cubic yard and a meat value of \$0.30 per pound.

According to Chestnut and Porter (1976), a sizeable Rangia industry operated in North Carolina from 1964 to 1972. They report that Rangia

were harvested at an average of 40 to 50 bushels per fisherman per day in areas of Albemarle Sound and the Pamlico and Neuse Rivers. Such quantities were taken by hand rake and small dredge, but other methods of harvesting may have produced greater yields. Bailey (1970) quotes Haven as saying that 1 to 2 bushels per minute were lifted by hydraulic dredge in an experimental venture from a location in the James River, Virginia. During a six year period from 1966 to 1971, over 325,000 pounds of Rangia meats were landed from North Carolina waters. An average price of \$0.30 per pound of meat was paid to fishermen for Rangia (Lyles, 1968). This is the figure used by Hoese (1972) in estimating the value of Rangia stock in Louisiana waters.

Rangia was marketed in at least two different forms. A breaded product was sold to several motel and restaurant chains both within and outside North Carolina, and reports were made of its excellent flavor (Anonymous, 1964). Large quantities of Rangia were canned in ocean clam liquor and sent to a seafood market in New York. The clams were then seasoned, canned in cocktail sauce, and sold as a seafood cocktail. The North Carolina company involved, Willis Brothers Seafood, termed the business "profitable" (Willis Brothers Seafood, 1978).

There were two reasons for termination of the Rangia industry in 1972. First, on one occasion a shipment of about 10,000 pounds of clam meat was rejected by public health authorities in New York because of excessively high standard plate counts (Willis Brothers Seafood, 1978). The second and major reason was an extensive closure of Rangia growing-area waters in eastern Albemarle Sound by state shellfish sanitation authorities (Chestnut and Porter, 1976). These waters were reopened to

shellfishing in October of 1976, and renewed interest in marketing Rangia developed.

Shellfish are filter feeders and are often consumed raw or lightly heated. As a consequence they have the potential of transmitting water-borne diseases. Therefore, commercial harvest and marketing are carefully controlled to protect consumers. This control consists primarily of the cooperative effort of state regulatory agencies with the National Shellfish Sanitation Program of the Food and Drug Administration. An important element of this certification is the bacteriological monitoring of shellfish and shellfish growing waters to demonstrate the absence of fecal contamination. This is done by assaying for the presence of bacteria routinely found in sewage. Tests have been developed to quantify a group of bacteria associated with the intestines and feces of warm-blooded animals. This group, the coliforms, was shown to be indigenous to the intestinal tract (Escherich, 1885), and its use as an indicator of fecal contamination of water supplies was soon recognized.

The coliform group includes bacteria of various genera. It refers to aerobic and facultatively anaerobic, gram-negative, non-sporeforming rods which ferment lactose with gas formation within 48h at 35°C (APHA, 1975). Detection of these organisms in water is presumed to indicate fecal contamination. Thus, a potential health hazard exists because of the possibility of the presence of pathogenic organisms found in intestinal discharges.

Since the work of Escherich, many modifications of the coliform indicator system have taken place. There has been controversy over the fact that the coliform group was too inclusive. Some coliforms are not

of fecal origin (Eijkmann, 1904; Thomas and Hobson, 1955; Geldreich, 1964), and thus are not of sanitary significance. Another assay was designed to enumerate only those coliforms of fecal origin (Eijkmann, 1904). The current fecal coliform test (Hajna and Perry, 1943) is a modification of the Eijkmann method.

Total and fecal coliform testing now form the basis by which shellfish growing waters and shellfish themselves are evaluated for sanitary quality. It is possible to assay for pathogenic microorganisms, but a margin of safety is provided by the indicator groups. The indicators will be present whenever domestic sewage is present, while the pathogens may not be.

Another bacterial analysis performed on shellfish is the standard plate count (SPC). The SPC does not indicate the sanitary quality of a product in relation to fecal contamination. Rather, it is a measure of the total number of bacteria per gram of material tested that grow aerobically on a non-selective medium. Freshly harvested shellfish often have relatively low SPC's. Poor sanitary quality in handling and processing of the product may allow initially low numbers of bacteria to replicate at an exponential rate and result in high SPC's. The SPC is not an indicator of the likely presence of pathogens, but it is a measure of possible spoilage of a product by an increase in the total number of bacteria.

It is important to realize that the vast majority of food poisoning outbreaks in the United States, including those involving shellfish, are caused by improper handling, insufficient heat processing, or inadequate refrigeration (Bryan, 1975). Shellfish eaten raw may contain pathogens filtered from the water. It is for this reason that routine surveys are required to classify water safe for shellfishing. Shellfish from approved

waters may occasionally contain very low, non-infective numbers of pathogens. Shellfish free from pathogens when harvested may also become contaminated with such bacteria as Salmonella, Shigella, and Staphylococcus by workers carrying these microorganisms. If the product is then subjected to inadequate cooking and refrigeration, the number of bacteria may increase exponentially to numbers sufficient to cause illness in man. Thus, sufficient hygienic practices must be used to maintain the sanitary quality of freshly harvested shellfish.

Growing commercial interest in Rangia as a food product has led to scrutiny of its safety from a microbiological standpoint. In 1976, a preliminary investigation by the Shellfish Sanitation Program of the N.C. Department of Human Resources indicated the presence of high SPC's in North Carolina Rangia. My investigation of the clam began through an appeal from the Shellfish Sanitation Program for university research to ascertain the bacteriological quality of Rangia.

The objective of this research was to verify the high total bacterial counts and to determine whether these counts were of sanitary significance. Bacterial levels were monitored in clams, water, and sediment taken from Albermarle Sound, North Carolina, and the influence of seasonality and other factors on the numbers and kinds of bacteria was established. This work included enumeration of indicator bacteria as well as screening for potentially pathogenic bacteria.

Description of Study Area

Albermarle Sound is a bar built estuary located in the northeastern region of North Carolina near the Virginia border (Fig. 1). The sound averages 13 km in width and extends 89.5 km from its mouth near Roanoke

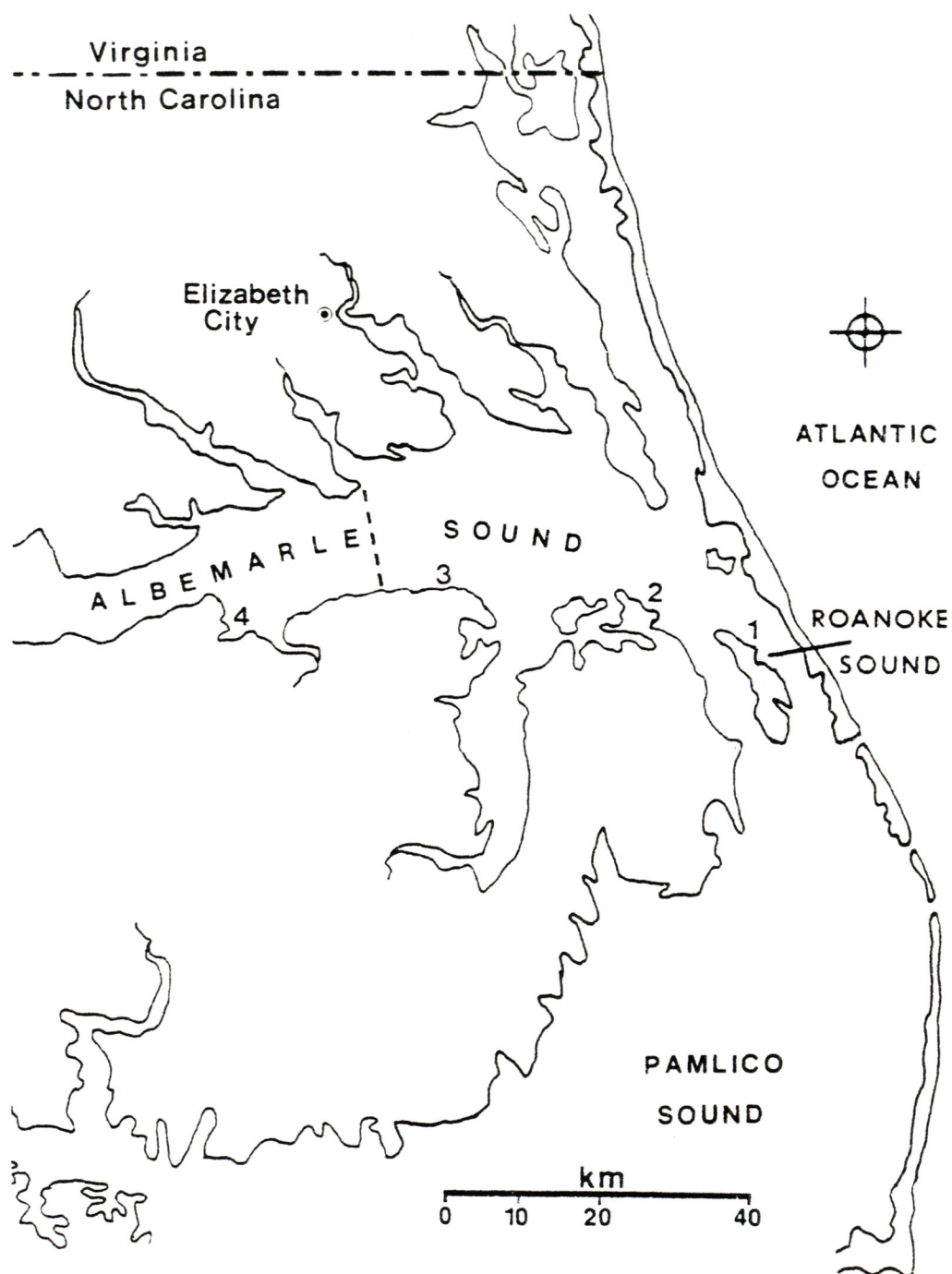


Figure 1. Location of Albemarle Sound in northeastern North Carolina. Water west of dotted line is closed to shellfishing.

Island to the N.C. route 17 bridge at Edenton. Its area is 1160 km², and the mean depth is 4.5 m (N.C. Wildlife Commission, 1969). Albemarle Sound divides at Roanoke Island into Roanoke and Croatan Sounds which are contiguous with the Atlantic Ocean at Oregon Inlet, some 37 km to the south. Eight rivers flow into the sound: the Perquimans, Little, Pasquotank, North, Alligator, Scuppernong, Roanoke, and Chowan (Fig. 2). The latter two, located at the western end of the estuary, are the largest tributaries. The watershed of the Roanoke is about 25,100 km², while the Chowan drains approximately 12,700 km² (Bowden and Hobbie, 1977). The annual precipitation for the region is 115-140 cm.

Albemarle Sound is oligohaline and characteristically less than 4‰ for most of the year. Higher salinities were recorded sometimes in Roanoke Sound during the study period. Surface temperatures ranged from 0-34°C. There is usually abundant dissolved oxygen in the bottom waters, even during summer months. The absence of significant stratification of salinity, temperature, and dissolved oxygen in Albemarle Sound is a distinctive trait when compared with the Pamlico and Neuse estuaries to the south (Bowden and Hobbie, 1977).

The majority of watershed in the region of Albemarle Sound is either wetland, upland, forest, or farmland. Corn, tobacco, peanuts, soybeans, and cotton are the chief cash crops. There is very little urbanization and industrialization of the area. However, this picture has been changing steadily in the past two decades with the addition of growing numbers of recreational homes. Elizabeth City, with a population of about 10,000 is the largest town proximal to the estuary. Wood and paper production is the major industry in the region (Clay et al., 1975).



Figure 2. Location of sampling sites in Albemarle Sound.

MATERIALS AND METHODS

Sampling Stations and Duration

Clam, water, and sediment samples were collected monthly from July 1977 through June 1978 at three stations in Albemarle Sound and one in Roanoke Sound (Fig. 2). These sites were chosen to ascertain any variation in bacterial counts caused by 1) the sanitary quality of overlying water as surveyed by State authorities, 2) differences in salinities and 3) proximity to areas of human habitation. Stations 1, 2, and 3 were in water open to shellfishing; station 4 was in a closed area. The dotted line across Albemarle Sound (Fig. 2) indicates the boundary of the area farthest up the sound which is closed to shellfishing based on sanitary surveys of water quality. The average salinity at Roanoke Island (station 1) during the study period was $5.8^{\circ}/\text{oo}$ (range $1\text{--}18^{\circ}/\text{oo}$), while that of the Bull Bay site (station 4) was $0.7^{\circ}/\text{oo}$ (range $0\text{--}2^{\circ}/\text{oo}$). Stations 1 and 4 were situated near residential areas of both permanent and recreational homes using septic tanks for waste disposal. There was no evidence of discharge from these systems to the sound. Stations 2 and 3 were more remote, located 1 km or more from any dwelling.

Field Collections

Samples of clams, water, and sediment were taken near the shore in water approximately 1 m deep. Clams were collected with a clam rake having tines 20 mm apart and openings in the attached wire basket about 25 mm wide. Therefore, clams smaller than 25–30 mm in length were not collected. The ranges in length of clams collected at the various sites were approximately 35–65 mm at station 1, 30–40 mm at station 2, 25–35 mm at station 3, and 30–50 mm at station 4. At least 20 clams were taken per station on

each sampling date. The clams were then sealed in plastic bags for transport. Water samples were collected in autoclaved polypropylene bottles at a depth of 0.5 m. Sediment was sampled using chlorine washed polycarbonate core tubes with an inner diameter of 45 mm. All samples were placed immediately on ice for transport back to the laboratory.

Air and water temperature and salinity were recorded at each station with a YSI Model 33 S-C-T meter. The pH of the water was taken with a Corning Model 3 pH meter. Dissolved oxygen was measured by Winkler titration, azide modification (APHA, 1975).

River discharge data were obtained from the U.S. Geological Survey in Raleigh, N.C. The major tributaries, the Roanoke and Chowan, were selected as the rivers to monitor. Gage stations were chosen that were closest to Albemarle Sound, with Roanoke Rapids serving as the station on the Roanoke River. There was no monitoring facility on the Chowan River in North Carolina. Therefore, two streams that empty into the Chowan near its mouth, Ahoskie Creek and Poteçasi Creek, were used to estimate flow in the Chowan River basin. A total river discharge figure for the sample date and three previous days was calculated from these three gage stations. This value was then used as the single discharge reading for the sample date. The individual discharges were weighted according to the size of the drainage basins. This calculation gives equal weight to each of the three gage stations in computing the total discharge value for the sample date. This system does not imply that bacteria in the sound on a given date were those washed down from these and other distant gage stations a few days earlier. However, a relatively high river discharge value is presumed to indicate greater freshwater drainage into the sound from its total watershed.

Laboratory Analyses

All clam, water, and sediment samples were analyzed for total coliforms (TC), fecal coliforms (FC), and standard plate counts (SPC). The water was also tested for fecal streptococci (FS). Clam samples were screened for potential enteric pathogens of the genera Salmonella, Shigella, Vibrio, and Staphylococcus. This pathogen screening was done for all clam samples except those collected in January.

Flow diagrams of the bacteriological analyses performed are depicted in Figs. 3, 4, and 5. All samples were kept iced and processed within 24h of collections. Media used in this study are listed in Appendix B.

At least 12 clams were shucked and blended per station in accordance with Recommended Procedures for the Examination of Sea Water and Shellfish (APHA, 1970). For collections of smaller clams, 15-18 were shucked to obtain the required 50g of liquor and meat. An equal amount, by weight, of sterile phosphate buffered dilution water was added prior to blending to make pipetting possible. Decimal dilutions to 10^{-4} g/ml were made in phosphate buffered water. Decimal dilutions to 10^{-3} ml/ml were done on water samples. In sediment processing 100g was aseptically weighed out and diluted with an equal amount of buffered dilution water in a sterile jar. The mixture was shaken for about 15 seconds, and dilutions then made to 10^{-4} g/ml.

Standard Plate Count.--One ml from the various dilutions of clam, water, and sediment samples was inoculated into standard methods agar (SMA) plates (Fig. 3). Bacterial colonies were counted after 48h and multiplied by the dilution factor to obtain the SPC per gram of clam and sediment or per ml of water.

CLAM FLOW CHART

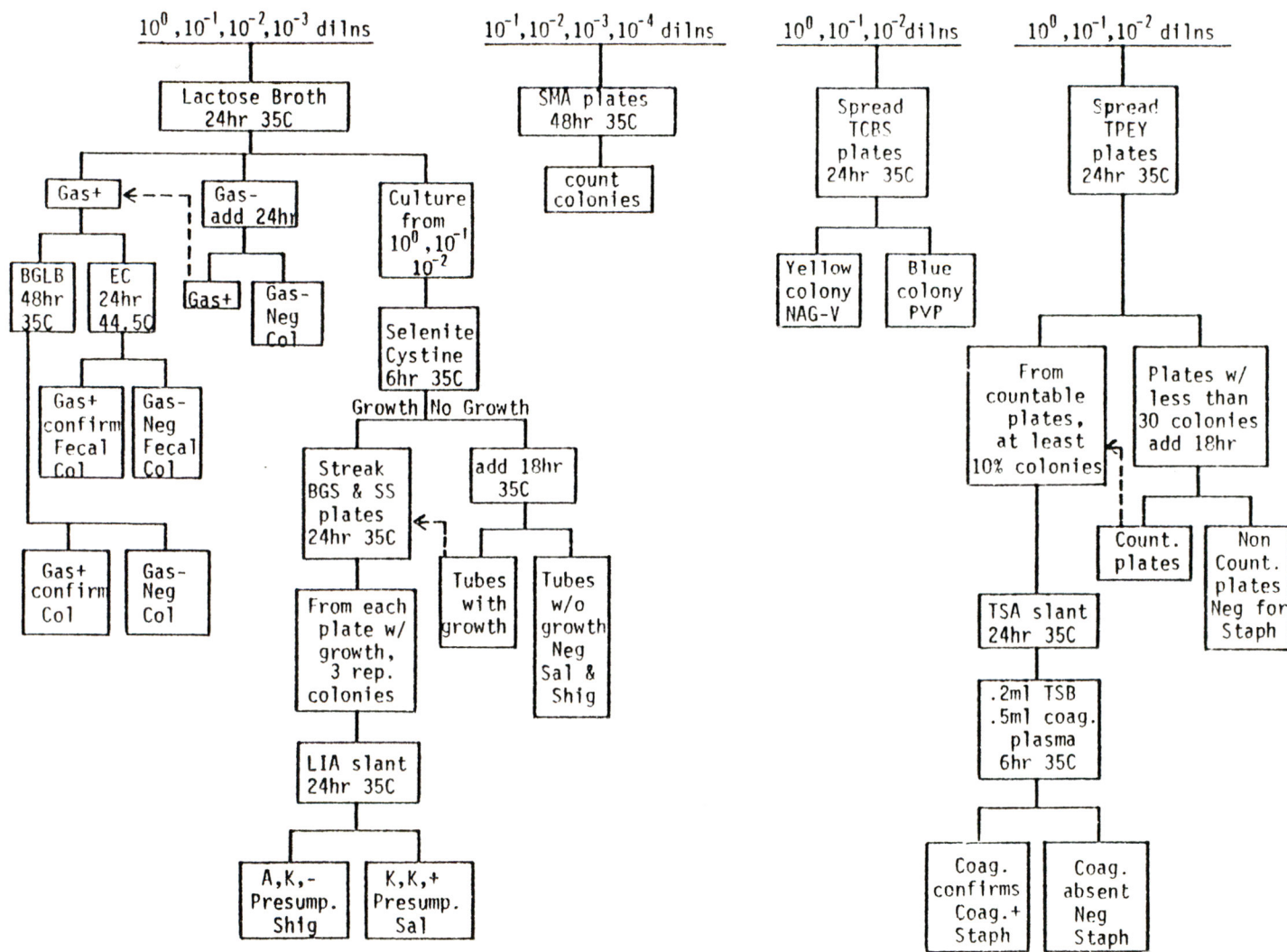


Figure 3. Bacteriological procedures used in the analysis of Rangia meats.

Indicator Organisms.--Standard 5-tube multiple dilution procedures were employed for the determination of a most probable number (MPN) of TC, FC, and FS (APHA, 1975). Lactose broth served as the presumptive coliform medium (Fig. 3). Transfers from gas positive tubes were made into brilliant green lactose bile (BGLB) broth for confirmation of TC's and into EC broth for confirmation of FC's. Azide dextrose broth was used as the presumptive and ethyl violet azide (EVA) the confirmatory media for FS enumeration (Fig. 4).

Vibrio.--Thiosulfate citrate bile salts sucrose (TCBS) agar was used as the selective medium for enumeration of vibrios (Fig. 3). Volumes from the clam homogenate and dilutions equal to 0.1g, 0.01g, and 0.001g were spread on TCBS plates. Consideration was given to using salted buffered dilution water recommended to enhance recovery of Vibrio parahaemolyticus. This strategy was rejected because the clams were collected from water that is, for osmotic purposes, essentially fresh (4⁰/oo). Results of Vibrio analyses were recorded as the average count/g of clam. In this system 1 colony detected on a single 0.1g plate was equivalent to 10 colonies per gram of clam meat. Since there were 4 stations per sample date, the lowest count that could be obtained was 2.5/g. When these bacteria were not detected in a sample, the value recorded was <2.5/g. Yellow colonies on TCBS were recorded as nonagglutinable vibrios (NAGV). These are vibrios which have the yellow colonial growth on TCBS typical of V. cholerae, but do not agglutinate in group 1-0 antigen antiserum. Five of these isolates were sent to the Center for Disease Control (CDC) in Atlanta, Georgia, soon after the first sample date in July 1977. They were confirmed as NAG vibrios. No additional samples were sent to CDC throughout the

WATER FLOW CHART

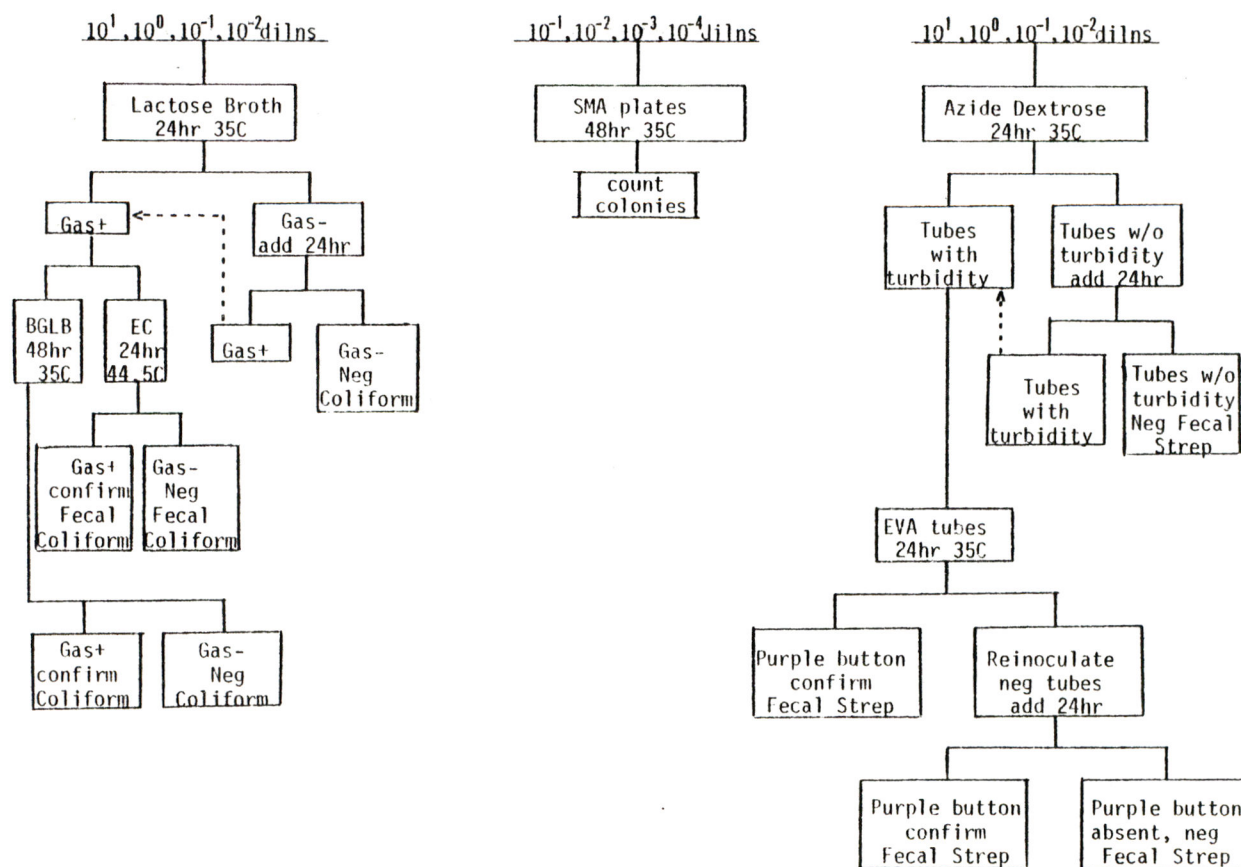


Figure 4. Bacteriological procedures used in the analysis of ambient water.

remainder of the study period due to the volume of samples that agency was receiving from other sources. Blue-green colonies were recorded as presumptive V. parahaemolyticus (PVP). This is a presumptive enumeration based on the selectivity of the medium.

Staphylococcus.--Plates of tellurite polymyxin egg yolk (TPEY) agar were employed for the enumeration of staphylococci (Fig. 3). These plates were inoculated in the same manner as the TCBS plates for Vibrio analysis, and enumeration procedures were identical to those used in quantifying vibrios. Representative gray to black colonies were analyzed by coagulase testing, and the number of coagulase positive staphylococci were recorded. These procedures are outlined in Examination of Foods for Enteropathogenic and Indicator Bacteria (Lewis and Angelotti, 1964). In the final six months of testing, brain heart infusion (BHI) broth was substituted for Trypticase soy broth (TSB) as growth medium during coagulase testing. This was done to adhere more closely to methods recommended in Bacteriological Analytical Manual (FDA, 1976).

Salmonella and Shigella.--Two procedures were employed at different times in the study for detection of Salmonella and Shigella. The original scheme, used through December 1977, is both a detection and enumeration method (Lewis and Angelotti, 1964). This scheme (Fig. 3) used lactose broth for pre-enrichment, inoculated quantitatively for enumeration by the multiple tube MPN technique. Transfers were made from lactose broth to selenite cystine, a selective enrichment for Salmonella and Shigella. Selenite cystine tubes showing growth were used to streak Salmonella-Shigella (SS) agar plates for detection of Shigella and brilliant green

sulfadiazine (BGS) plates for growth of Salmonella. One or two representative colonies were then streaked and stabbed into lysine iron agar (LIA) slants. Butt and slant reactions and H_2S production were recorded in that order as acid (A) or alkaline (K) and $^+\text{H}_2\text{S}$ production. Readings of K, K, + were recorded as presumptive Salmonella and A, K, - as presumptive Shigella. Each of the original lactose broth tubes were then recorded as positive or negative for the presence of Salmonella and Shigella, and an MPN was determined.

By December 1977, presumptive Salmonella had been found on only one sampling date, and no Shigella had been recorded. Therefore, beginning in February 1978, a procedure which is more sensitive for detection but does not permit enumeration was adopted (FDA, 1976). In this procedure additional clams were shucked and homogenized directly in selenite cystine enrichment broth. This reduces overgrowth of Salmonella and Shigella by other bacteria that can occur in lactose broth.

From the selenite cystine enrichment, a variety of agar plates were streaked-bismuth sulfite (BS), SS, and BGS for Salmonella culture and xylose lysine desoxycholate (XLD), desoxycholate-citrate (DC), and Levine eosin methylene blue (L-EMB) for growth of Shigella. LIA slants were inoculated and read as before. Presumptive Salmonella and Shigella growths on LIA were streaked to obtain pure cultures. An oxidase test (Fig. 5) utilizing dimethyl-p-phenylenediamine hydrochloride was performed on these isolates (MacFaddin, 1976). Oxidase negative (absence of purple color) cultures were inoculated into Enterotubes (Roche Diagnostics, Nutley, N.J.), a multi-media rapid identification system. Reactions were recorded, and the presence or absence of Salmonella and Shigella was ascertained.

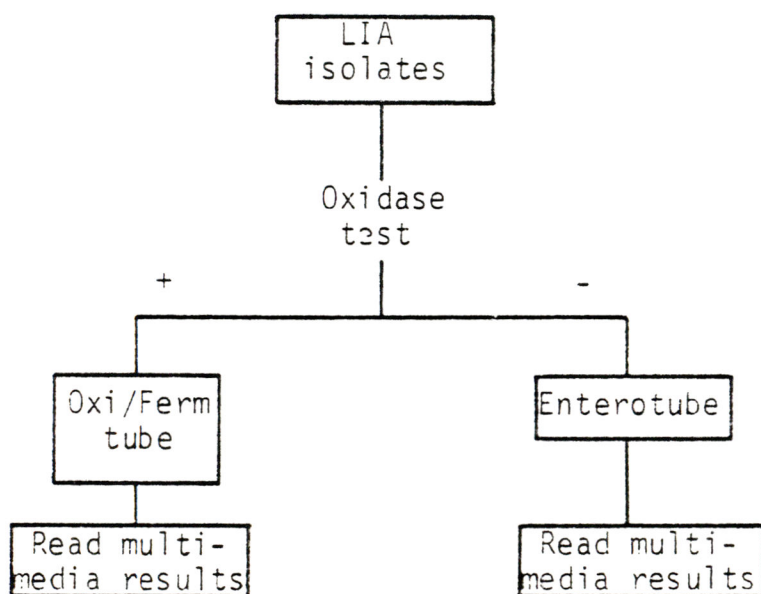


Figure 5. Diagnostic method used in confirmation of presumptive Salmonella and Shigella cultures.

RESULTS AND DISCUSSION

Standard Plate Count

Freshly harvested, properly stored shellfish other than Rangia destined to be marketed in North Carolina usually may be expected to have standard plate counts less than 10,000 SPC/g (G. Gilbert, 1978). Standard plate counts higher than this were recorded in Rangia throughout the study period (Table 1), confirming findings of the state shellfish sanitation authorities. Geometric means of the counts ranged from 31,000 SPC/g (October) to 4,900,000 SPC/g (December). There was no statistical correlation between these total counts and month or water temperature. There was, however, a relationship between the clam SPC and site (station number) over the entire 12 month study. The SPC's were higher from stations with lower salinities, reaching a geometric mean of 640,000 SPC/g at station 4 in water closed to shellfishing (Table 1).

The difference in SPC between Rangia and oysters could be related to the specific habitats of each mollusc. Oysters inhabit the mesohaline to polyhaline regimes of an estuary, whereas Rangia are found in fresher water. While the biocidal effect of saltwater on enteric organisms has been well documented (Ketchum et al., 1952; Carlucci and Pramer, 1960), there is little or no evidence in the literature that the SPC decreases with increased salinity. As levels of enteric and soil bacteria decline at higher salt concentrations, aquatic halophilic bacteria, such as some species of Vibrio, increase in number.

The habitats of Rangia and oysters differ not only in salinity, but also in bottom type. Oysters are epifauna, i.e., attached surface dwellers.

Table 1. Geometric means (10^x) of SPC's in Rangia meats by month and by sampling site from July 1977 through June 1978.

Month	Site	$\frac{\text{SPC}}{\text{g}}$	log	mean log (x)	10^x
JULY	1	100,000	5.000	4.746	56,000
	2	20,000	4.301		
	3	32,000	4.505		
	4	150,000	5.176		
AUG				4.798	63,000
SEPT				5.144	140,000
OCT				4.494	31,000
NOV				4.859	72,000
DEC				6.691	4,900,000
JAN				4.472	30,000
MAR				5.280	190,000
APR				5.470	300,000
MAY				5.688	470,000
JUNE				5.186	150,000

Site	mean log (x)	10^x
1	4.771	59,000
2	4.818	66,000
3	5.064	120,000
4	5.809	640,000

They fuse by means of an adhesive fluid to hard substrates slightly above the sediment surface where tidal currents prevent heavy sedimentation and burial of the animals. Rangia clams are infauna, soft bottom burrowers, living just below the sediment-water interface. Therefore, in feeding, oysters filter water that contains less sediment than the water filtered by Rangia.

The SPC of upper layer estuarine sediment in Chesapeake Bay is generally 10 to 1000 times greater than that of the ambient water (Sayler et al., 1975; Kaneko and Colwell, 1975; Carney et al., 1975). Similar results were found in this study of Albemarle Sound. In addition, there was a significant correlation ($P < 0.01$) between clam SPC and sediment SPC (Table 2). Thus, it is quite possible that Rangia have higher SPC's than oysters because these clams filter water containing a greater concentration of bacteria-laden suspended sediment.

Bacterial spoilage of shellfish is caused by exponential rates of increase by bacteria in the product. Therefore, the onset of spoilage may be detected by analyzing the meats for the number of bacteria that grow aerobically on standard methods agar (APHA, 1975). Shellfish that are improperly handled, processed, or refrigerated or products near the end of their shelf life will generally show an increase in SPC over freshly harvested shellfish. Sanitation authorities at some markets use 500,000 SPC/g as an indicator of product spoilage by bacteria.

Clams taken during this investigation, as previously noted, sometimes showed counts near or occasionally exceeding this number (Table 1). However, these were fresh clams harvested, iced immediately, and analyzed within 24h of collection. These relatively high total bacterial counts

Table 2. List of significant linear correlations.

Factors	Number of observations (n)	Correlation coefficient (r)	Regression line equation
Log clam SPC vs. Log sediment SPC	48	0.583 **	$y = 0.523x + 1.445$
Log clam FC vs. Log water TC	48	0.323 *	$y = 0.376x + 0.952$
Log clam FC vs. Log water FC	48	0.739 **	$y = 0.740x - 0.517$
Log clam FC vs. Log sediment FC	48	0.335*	$y = 0.123x + 1.203$
Log PVP vs. Water temp.	36	0.640 **	$y = 0.052x + 0.020$
Log NAGV vs. Water temp.	36	0.587 **	$y = 0.067x + 0.105$
Log PVP vs. Salinity	36	0.649 **	$y = 0.126x + 0.607$
Log NAGV vs. Salinity	36	0.653 **	$y = 0.178x + 0.792$
Log PVP vs. River discharge	36	-0.551 **	$y = -478x + 1198$
Log NAGV vs. River discharge	36	-0.547 **	$y = -345x + 1223$
Log PVP vs. Log clam FC	44	0.359 **	$y = 0.454x + 0.231$
Log NAGV vs. Log clam FC	44	0.460 **	$y = 0.806x + 0.006$
Log PVP vs. Log water FC	44	0.449 **	$y = 0.567x + 0.586$
Log NAGV vs. Log water FC	44	0.464 **	$y = 0.815x + 0.816$

*P<0.05

**P<0.01

then are not evidence of product spoilage, but rather indicate a naturally high bacterial flora in this clam. In addition, there was no positive correlation between clam SPC's and the number of indicator organisms or potential pathogens. The absence of a correlation here indicates that the high SPC's were not synonymous with similar increases in the number of fecal bacteria.

Indicator Organisms

In the FDA Shellfish Sanitation Program, the fecal coliform limit for marketable shellfish is a median MPN of 230 FC/100g, with not greater than 10% of the samples exceeding 330 FC/100g. This 12 month collection of 48 samples had a median MPN of only 80 FC/100g, but 10 of 48 or 21% of the samples exceeded 330 FC/100g (Fig. 6). A closer look at these counts provides interesting observations.

First, the highest clam FC values were recorded at station 1, with a median MPN of 220 FC/100g and 33% of the collections exceeding 330 FC/100g. This finding is somewhat in conflict with expected results of lower FC values at higher salinities (Ketchum et al., 1952; Carlucci and Pramer, 1960) and longer exposure in both time and distance from sources of river discharge farther up the sound (Kittrell and Furfari, 1963; Lin et al., 1974; Faust et al., 1975).

There are two factors that may have contributed to these higher FC densities. The water at this collection point is isolated from easy mixing with the rest of Roanoke Sound by a spit of sand about 50 m off-shore running parallel to the shore for at least 100 m. Thus, there is a lagoon formed which could provide containment of surface water runoff.

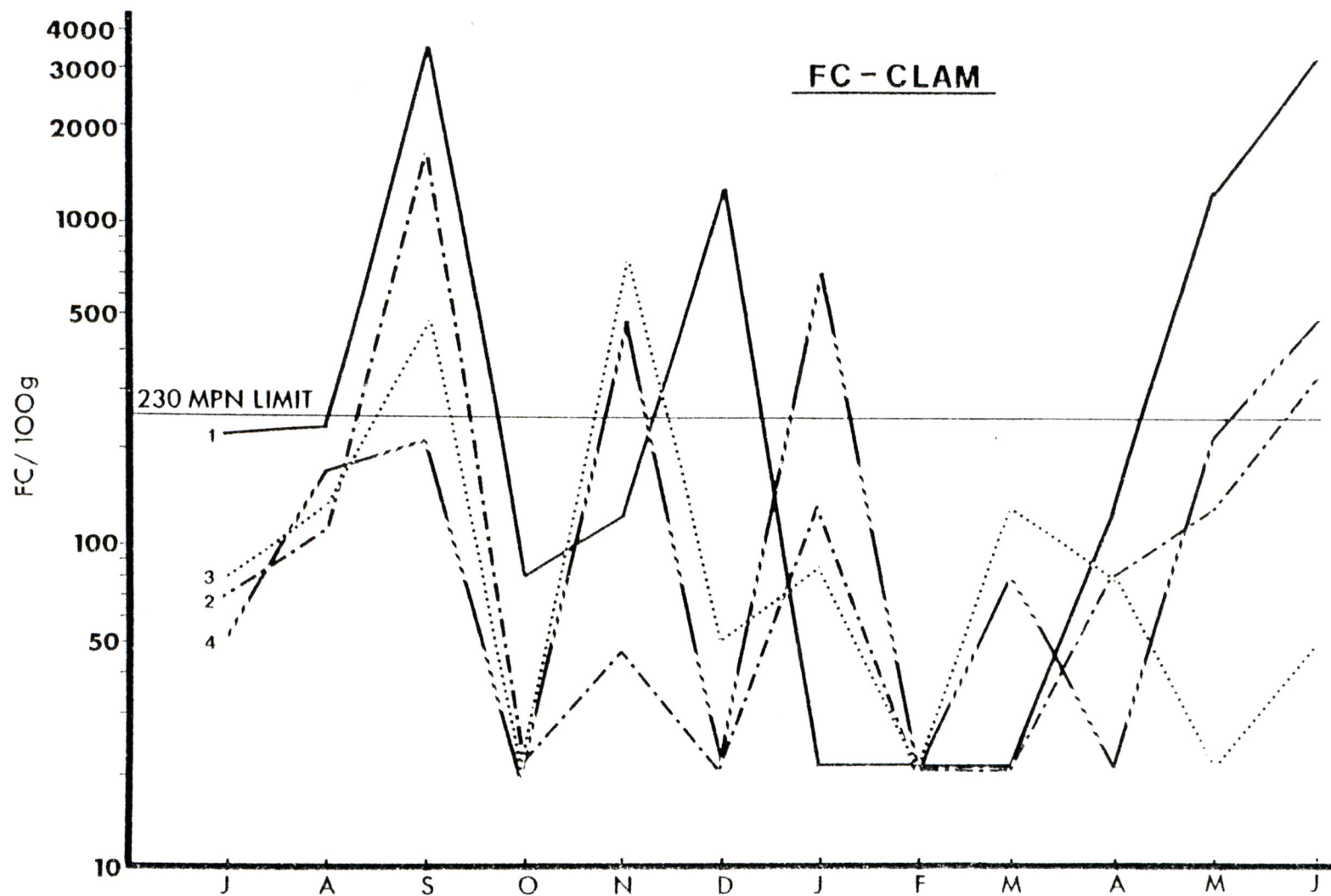


Figure 6. Fecal coliform levels in Rangia meats for the four sampling sites from July 1977 through June 1978.

There are also a number of homes along the shore utilizing septic tanks. While a chance exists that one of these systems could be malfunctioning and releasing coliforms to the lagoon, the shellfish sanitation officer responsible for shoreline surveys indicated that the location of the tanks and disposal fields make this possibility for contamination remote.

A second observation concerning fecal counts is that Rangia clams met the state FC standard for a five-month period of the study. From January through May, 1978, the median MPN for the 20 samples was 80 FC/100g, with only 2 of the 20 or 10% exceeding 330 FC/100g. It is impossible to state that this is a seasonal pattern of counts occurring annually which might allow for harvesting during these months. The possibility exists, but there are no continuous data from previous years. The counts may be highly variable, changing weekly or even daily. More frequent sampling over a longer period might provide an answer.

In North Carolina, in compliance with the FDA Shellfish Sanitation Program, water is classified for shellfishing based on a total coliform standard. The total coliform limit for water approved for shellfishing is a median MPN of 70 TC/100ml, with not greater than 10% of the samples exceeding 230 TC/100ml. It is worthwhile noting that in 8 of the 10 clam samples exceeding 330 FC/100g, the total coliform counts on the water were in excess of 70 TC/100ml.

As stated earlier, permanent and vacation homes lined the shore near stations 1 and 4. Stations 2 and 3 were more remote, situated at least 1 km from any dwellings. Only 3 of the combined 24 clam collections from stations 2 and 3 were in excess of 330 FC/100g. On each of these occasions, the overlying water total coliform counts were greater than 70 TC/100ml.

These results indicate two relationships. First, as might be expected, there were lower number of FC's at sites more distant from areas of human habitation. Second, the association between clam FC counts and water TC values is significant ($P < 0.05$) (Table 2). A highly significant correlation ($P < 0.01$) was established between clam fecal and water fecal counts, and another correlation ($P < 0.05$) existed between clam fecal and sediment fecal values (Table 2). Although water at stations 1, 2, and 3 is approved for shellfishing based on routine monitoring by the Shellfish Sanitation Program, periodic fluxes of higher TC and FC values in the water are accompanied by higher levels of these bacteria in the clams. These findings suggest that Rangia did not concentrate and maintain FC's in their digestive systems, but rather reflected the bacteriological quality of the overlying water and to a lesser extent the surrounding sediment.

At the Eighth National Shellfish Sanitation Program Workshop, a recommendation was made to change the standard used for evaluation of shellfish growing waters from the TC limit already mentioned to a median fecal coliform MPN of 14 FC/100ml, with not more than 10% of the samples exceeding 43 FC/100ml (Hunt and Springer, 1975). Andrews et al. (1976) found that both TC and FC standards for growing areas were acceptable as indicators for the presence of Salmonella in the hard clam, Mercenaria mercenaria. These same researchers conducted another study involving the Eastern oyster, Crassostrea virginica (Andrews et al., 1975). They found that although both standards provided adequate indication of Salmonella, increased detection of Salmonella more closely paralleled increases in the number of FC's rather than TC's.

The results of this study cannot substantiate a preference for using

either coliform test as an indicator of Salmonella because of the lack of confirmed Salmonella in any clam samples. However, the results do indicate a stronger correlation between clam FC and water FC counts ($r = 0.739$) than between clam FC and water TC values ($r = 0.323$). I have not determined a "safe" FC standard for shellfish growing waters. A detailed study of such an investigation (Hunt and Springer, 1975) was previously cited. Because fecal coliforms are more indicative of true fecal contamination and based on the strong correlation between clam and water FC counts recorded here, it seems logical that a FC standard for growing-area waters should be investigated.

A factor not studied directly in this research was the influence that water runoff from livestock areas might have on FC levels in Albemarle Sound. Faust (1976) found that runoff from rural watersheds contributed substantial numbers of FC's to a subestuary of Chesapeake Bay. This body of water was much smaller in volume than Albemarle Sound; and at its opening into Chesapeake Bay, the number of fecal coliforms recovered was less than 10 FC/100ml in 11 of 12 samples. During the course of my collections, I noticed that some small farms along the southern shore of the sound raise pigs and chickens. However, there were no statistical correlations between clam, water, or sediment FC counts and calculated river discharge values. Furthermore, it seems unlikely that FC's released directly into the sound as runoff would have much effect on the sanitary quality of Rangia due to the large receiving body of water and subsequent dilution of bacteria.

Vibrio

Reports of food poisoning by vibrios are a relatively recent develop-

ment. Epidemiological studies of these outbreaks show that contaminated seafood is almost always involved. Most cases are caused by Vibrio parahaemolyticus, although a number of cases of diarrhea, have been attributed to nonagglutinable vibrios (NAGV) (Hughes et al., 1978). NAGV's are those which biochemically resemble V. cholerae but do not agglutinate in V. cholerae antiserum. Vibrio parahaemolyticus was first identified as a causative agent of food poisoning by Fujino et al. (1953) and has since been recognized as the major cause of food poisoning in Japan (Sakazaki, 1969). Although confirmed outbreaks of vibrio-related gastroenteritis in the United States are relatively low, such reports have been increasing in recent years (Barker, 1974).

Vibrio parahaemolyticus is a normal inhabitant of coastal waters and estuaries and has also been isolated from a variety of shellfish (Vanderzant et al., 1970; Bartley and Slanetz, 1971; Colwell et al., 1973; Sizemore et al., 1975). Pathogenic V. parahaemolyticus demonstrates hemolysis on high-salt blood agar. This method of confirmation is known as the Kanagawa test, named for the Japanese laboratory where it was developed. Most marine strains have been shown to be harmless to man. Sakazaki et al. (1968) found 99% of 3,370 marine and seafood isolates to be Kanagawa negative. Barker (1974) reported that Sakazaki also found no gastroenteritis in volunteers injected with doses of up to 10^9 Kanagawa-negative V. parahae-
molyticus.

Organisms presumptively identified as vibrios, both NAGV and PVP, were enumerated in the analysis of Rangia meats (Fig. 7). The counts followed a characteristic pattern of greater numbers in warm water during the summer and lower levels during the winter months (Baross and Liston,

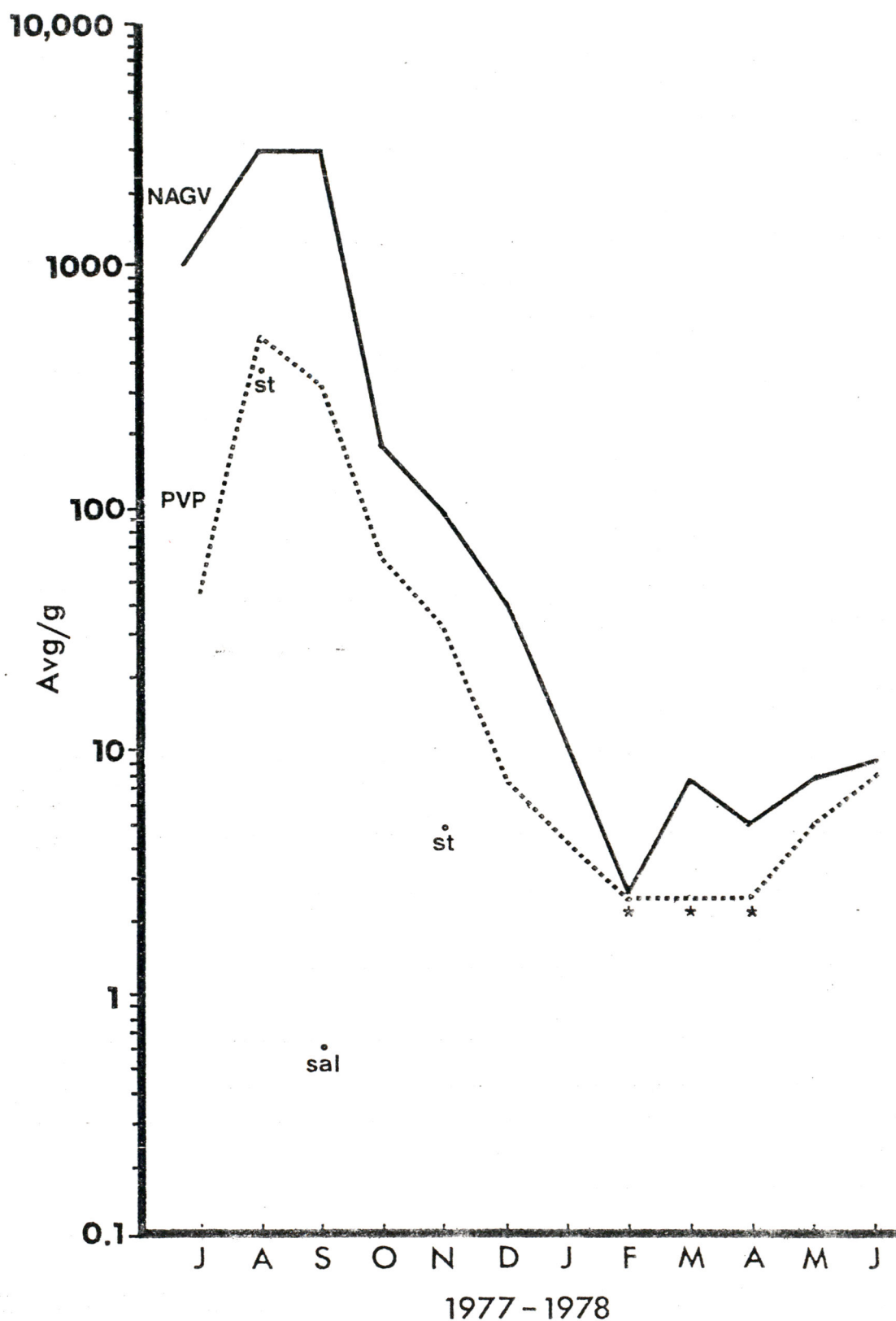


Figure 7. Potential pathogens levels in *Rangia* meats for combined monthly samples from July 1977 through June 1978. Starred points indicate *Vibrio* values < 2.5/g.

1970; Kaneko and Colwell, 1973). Table 2 shows significant correlations ($P < 0.01$) between vibrio density and water temperature. Because the vibrios enumerated were estuarine, their numbers decreased with a drop in salinity. Significant positive correlations ($P < 0.01$) were recorded between vibrio counts and salinity and negative correlations between vibrio values and river discharge (Table 2). There were also strong correlations ($P < 0.01$) between numbers of vibrios in clam meat and both clam and water FC levels (Table 2). Aquatic vibrios are not indigenous to the human intestinal tract; therefore, the relationship with fecal indicators may have been caused by similar responses of these bacteria to environmental stimuli. A factor that may have influenced the vibrio-FC correlation in this study was the spit of land at station 1 mentioned earlier. The spit could have been responsible for containment of fecal coliforms entering the water at this site. Also, station 1 had the highest salinity of the four sites and, therefore, would have been most conducive to survival and growth of vibrios. Based upon this observation, the relationship between vibrios and fecal coliforms may be purely coincidental and possibly not significant without these conditions. In a bacteriological study of oysters, seawater, and sediment from the coast of Australia, no relationship was found between FC counts and numbers of vibrios (Desmarchelier, 1978).

As stated earlier in this report, I did not confirm these isolates as positive vibrios due to laboratory limitations and the inability of the Center for Disease Control to cooperate in the identifications. Kaneko and Colwell (1975) reported the numbers of confirmed V. parahaemolyticus to be nine times lower than those shown to be presumptive on TCBS agar.

Such results indicate the actual numbers of V. parahaemolyticus in Rangia meats may be considerably lower than the values reported in my study.

The incidence of V. parahaemolyticus food poisoning in the United States is far below that of Japan. Because proper cooking temperatures destroy this bacterium, the difference in outbreaks between the two countries may lie in the fact that the Japanese eat much of their seafood raw (Barker, 1974). The first report of V. parahaemolyticus gastroenteritis in the United States occurred in 1971 (Molenda et al., 1972). It was not until February, 1978 that a Kanagawa-positive strain was actually isolated from food implicated in a case of food poisoning (Spite et al., 1978). Records compiled by the Center for Disease Control for the years 1971-1976 show 13 reported outbreaks of food poisoning caused by V. parahaemolyticus in the United States (CDC, 1972-1977). This number is much lower than the 198 outbreaks of food poisoning by Salmonella and 288 outbreaks by Staphylococcus during the 1971-1976 period. However, when considering outbreaks of food poisoning caused strictly by shellfish, V. parahaemolyticus is the major etiologic agent. Shellfish was the vehicle of transmission in 12 of the 13 vibrio outbreaks, but only 2 of the staphylococcal and 1 of the salmonella outbreaks involved shellfish. While V. parahaemolyticus is now recognized as a significant cause of gastroenteritis outbreaks in the U.S., none of the outbreaks through 1976 were attributed to consumptions of raw products, such as, oysters and clams. A single case of probable food poisoning by V. parahaemolyticus in raw oysters has since been reported (Spite et al., 1978). Sufficient cooking temperatures, elimination of cross contamination, and adequate refrigeration should prevent any health hazards posed by this bacterium.

Salmonella and Shigella

The isolation of Salmonella from natural waters and shellfish has been reported often in the last decade. Brezenski and Russomanno (1969) in a study of Raritan Bay, New Jersey, found salmonellae in greater than 50% of those water samples with FC densities over 2,000 FC/100ml. Salmonellae were also detected in the meats of the hard clam, Mercenaria mercenaria. With the exception of one sampling station, shellfish with a geometric mean FC count of 1,000 FC/100g or more contained salmonellae. In another estuarine study in New Hampshire, Salmonella was isolated with low level regularity from both oysters and overlying water (Slanetz et al., 1978). Such findings often occurred in oysters taken from water meeting the coliform standards for shellfish harvesting. Other investigations report survival (Fair and Morrison, 1967; Gallagher and Spino, 1967; Brezenski and Russomanno, 1969) and even multiplication (Hendricks and Morrison, 1967) of salmonellae in various aquatic systems. Recovery of Shigella from water or freshly harvested shellfish is rare (D.A. Hunt, 1978).

Because of this evidence in other studies, we screened clam meats for both Salmonella and Shigella in addition to enumeration of bacteria indicative of fecal contamination. Cultures presumptively identified as Salmonella were found on only one sampling date. The 11 colonies detected that month were not subjected to further tests to confirm them as salmonellae. The MPN procedure used at that time in the study yielded a salmonellae count of 0.6 sal/g of clam (Fig. 7). This quantity of Salmonella, even if confirmed, is very low in regard to minimum infective dose for these organisms. In most cases of salmonellosis, millions of bacteria must be ingested

(Bryan, 1975). In February, 1978, eight isolates produced reactions on LIA slants presumptive for Shigella. Because this bacterium had not been detected before this time, the cultures were subjected to further diagnostic procedures (Fig. 5). All eight presumptive shigellae were found to be false positive.

Staphylococcus

Coagulase positive staphylococci, those staphylococci most commonly causing illness in man, were found in 5 of 40 clam samples. Four of these samples were from the August, 1977 collection in which clams from each of the four stations contained this bacterium. The average number detected that month for the four sample sites was 380 st/g (Fig. 7). This value included those isolates found to be weakly coagulase positive. Most estimates indicate that it takes over one million staphylococci to produce enough enterotoxin to cause intestinal disorder in humans (Jay, 1978).

The very low incidence of Staphylococcus, unconfirmed scant recovery of Salmonella, and complete absence of Shigella in Rangia make statistical correlations between these microorganisms and indicator bacteria or environmental conditions impossible. However, it should be emphasized that there were practically no pathogens at the level of indicator organisms detected. These negative findings may be caused by two factors. First, as mentioned earlier, there is very little urbanization of the Albemarle region. Most of the area is either forest, cropland, or marsh. The two major tributaries, the Roanoke and Chowan Rivers, at the sound's western end are not heavily polluted by large municipal effluents. Since fecal waste is considered the principal source of Salmonella and Shigella, there conceivably would be fewer numbers entering this aquatic system than one which is more heavily

populated and industrialized. Second, Albemarle Sound is a large body of water. The dilution of such pathogens is presumably great enough to make them very difficult to detect, even in low numbers. Therefore, at these low values there appears to be little public health risk from these micro-organisms.

CONCLUSIONS

This study has confirmed the presence of high SPC's in Rangia meats as sometimes reported by state shellfish sanitation authorities. Counts ranging from tens of thousands to tens of millions per gram were recorded on clam meat. A limit of 500,000 SPC/g has been used by some markets to prevent the sale of seafood products spoiled by exponential increases in the total number of bacteria. High SPC's detected in Rangia were in freshly harvested clams. Therefore, these counts are not indicative of product spoilage, but rather suggest a naturally high bacterial flora.

The SPC is of little public health significance in freshly harvested shellfish when no correlation exists between the SPC and fecal coliform or potential pathogen counts. No correlation was observed between clam SPC's and the levels of fecal coliforms or potential pathogens in this study. A total of 48 Rangia samples showed a median fecal coliform MPN of just 80 FC/100g, but 21% of the samples did exceed the upper federal limit of 330 FC/100g. This fraction drops to 17% when data from station 4 in water closed to shellfishing is omitted. In most samples with FC counts exceeding 330 FC/100g, the TC quality of the overlying water was poor. This indicates that Rangia did not maintain unusually high coliform levels, but rather reflected the changing bacteriological quality of the water. More frequent sampling from approved growing-area waters would provide more information on any real public health problem indicated by levels of fecal coliforms.

One of the most important findings of this investigation was the low recovery of potential pathogens from Rangia. Vibrios were found in the

range of thousands per gram of meat in the summer months, but this value is by no means unusually high. It is well documented that vibrios are indigenous to coastal waters and regularly found in a variety of marine and estuarine organisms. Outbreaks of foodborne disease attributed to particular pathogenic strains of V. parahaemolyticus and NAGV's are infrequent in this country. There were no confirmed recoveries of either Salmonella or Shigella from clam meat. Coagulase positive Staphylococcus was isolated in only 5 of 40 clam samples at values far below the level necessary to cause gastrointestinal disorder.

Some objections to marketing Rangia come solely from those claiming the clam has a poor flavor. It was noted earlier in this report that Rangia has been consumed in a variety of areas for many years and that markets have been established in the past for various forms of the product. I feel that it is the responsibility of commercial interests to ascertain or resolve any market barriers concerning flavor. Other studies also recognize the very real market potential of Rangia (Hopkins et al., 1973; Chestnut and Porter, 1976).

It cannot be stated that the bacterial flora of Rangia cuneata presents no threat to public health. Potential health hazards exist in the consumption of any shellfish, raw or processed. My findings of approximate compliance with fecal coliform standards and low numbers of potential pathogens indicate that Rangia cuneata poses no unique public health risk. More frequent coliform testing of clams taken from a greater number of sites in approved growing-area waters should be continued to supplement and verify the baseline data generated by this research.

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APPENDICES

Appendix A: Glossary of Abbreviations

C	clam
°C	degree(s) Celsius
CDC	Center for Disease Control
cm	centimeter(s)
DO	dissolved oxygen (ppm)
FC	fecal coliform
FDA	Food and Drug Administration
FS ₃	fecal streptococcus
ft ³ /sec	cubic feet per second
g	gram(s)
h	hour(s)
km ₂	kilometer(s)
km ²	kilometer(s) squared
m	meter(s)
ml	milliliter(s)
mm	millimeter(s)
MPN	most probable number
NAGV	nonagglutinable vibrio
P	probability level
ppm	parts per million
°/oo	parts per thousand
PVP	presumptive <u>Vibrio parahaemolyticus</u>
r	correlation coefficient
RD	river discharge (ft ³ /sec)
S	sediment
SAL	<u>Salmonella</u>
SALIN	salinity (°/oo)
SHIG	<u>Shigella</u>
SPC	standard plate count
ST	<u>Staphylococcus</u>
STA	station
TC	total coliform
TEMP	temperature (°C)
W	water
**	no data

Blank spaces in appendix columns indicate tests not performed on given samples.

Appendix B: Laboratory Media and Reagents

Agar

Bismuth sulfite (BS)
Brilliant green sulfadiazine (BGS)
Desoxycholate citrate (DC)
Enterotube (Roche Diagnostics, Nutley, N.J.)
Levine eosin methylene blue (L-EMB)
Lysine iron agar (LIA)
Salmonella-Shigella (SS)
Standard methods agar (SMA)
Tellurite polymyxin egg yolk (TPEY)
Thiosulfate citrate bile salts sucrose (TCBS)
Trypticase soy agar (TSA)
Xylose lysine desoxycholate (XLD)

Broth

Azide dextrose
Brain heart infusion (BHI)
Brilliant green lactose bile (BGLB)
EC
Ethyl violet azide (EVA)
Lactose
Selenite cystine
Trypticase soy broth (TSB)

Reagents

Dimethyl-p-phenylenediamine hydrochloride (1%)
Rabbit coagulase plasma

Other

Phosphate buffered dilution water

Appendix C: Summary of Physical Data

STA	AIR TEMP	WATER TEMP	SALIN	DO	pH	RD
<u>Date: 071777</u>						
1	30	29	10.5	6.8	7.1	327
2	30	31	3.0	7.4	7.2	
3	32	31	2.5	6.9	7.2	
4	31	32	2.0	8.0	7.6	
<u>Date: 081577</u>						
1	33	29	18.0	7.4	7.4	85
2	35	30	9.0	6.6	7.2	
3	28	29	2.0	7.1	7.5	
4	24	29	0.0	7.8	6.8	
<u>Date: 091077</u>	**	**	**	**	**	102
<u>Date: 100977</u>	**	**	**	**	**	67
<u>Date: 110577</u>						
1	22	19	10.8	7.0	7.2	531
2	20	19	7.4	7.4	7.2	
3	21	19	4.0	7.3	7.6	
4	20	19	2.0	7.2	7.6	
<u>Date: 120377</u>						
1	13	11	6.0	9.5	7.4	405
2	12	11	3.5	10.0	7.6	
3	13	12	2.5	10.0	7.6	
4	17	13	1.5	9.9	7.8	
<u>Date: 010578</u>						
1	11	4	4.0	12.2	7.3	744
2	10	5	3.5	12.4	7.5	
3	11	5	3.0	12.4	7.6	
4	11	6	0.5	12.1	7.6	

Appendix C: (continued)

STA	AIR TEMP	WATER TEMP	SALIN	DO	pH	RD
<u>Date: 021178</u>						
1	3	1	2.0	12.8	7.5	941
2	3	0	2.5	13.2	7.6	
3	3	2	1.0	13.4	7.6	
4	8	4	0.5	13.2	8.2	
<u>Date: 031278</u>						
1	19	6	1.0	11.5	7.4	2575
2	22	8	1.5	12.2	7.4	
3	18	8	1.0	12.4	7.7	
4	20	9	0.0	12.2	7.8	
<u>Date: 041578</u>						
1	15	16	1.5	9.2	7.3	535
2	19	17	0.5	9.4	7.8	
3	24	19	0.2	10.0	7.7	
4	22	20	0.0	9.3	7.9	
<u>Date: 051178</u>						
1	21	19	2.0	8.5	7.5	**
2	21	19	0.5	8.8	7.8	
3	23	22	0.5	9.2	7.9	
4	22	24	0.0	8.6	8.4	
<u>Date: 061178</u>						
1	24	23	2.0	8.2	7.6	**
2	25	24	1.0	8.5	7.8	
3	26	24	0.5	8.3	8.1	
4	28	25	0.0	8.3	8.2	

Appendix D: Summary of Bacteriological Data

STA	TC 100g	FC 100g	SPC g	FS 100g	ST g	PVP g	NAGV g	SHIG g	SAL g
<u>Date: 071777</u>									
1-C	1700	220	100,000		**	40	1100	<0.2	<0.2
1-W	17	4	240	<2					
1-S	20	<20	1,400						
2-C	3300	70	20,000		**	10	2200	<0.2	<0.2
2-W	33	23	650	33					
2-S	130	20	1,900						
3-C	330	80	32,000		**	70	780	<0.2	<0.2
3-W	8	8	130	8					
3-S	50	50	5,400						
4-C	80	50	150,000		**	50	2.5	<0.2	<0.2
4-W	5	5	170	<2					
4-S	<20	<20	3,500						
<u>Date: 081577</u>									
1-C	6300	230	72,000		310	580	1800	<0.2	<0.2
1-W	33	33	830	49					
1-S	230	<20	6,200						
2-C	2200	110	28,000		620	1100	790	<0.2	<0.2
2-W	94	17	360	8					
2-S	460	<20	3,000						
3-C	4900	130	43,000		400	100	4500	<0.2	<0.2
3-W	130	7	**	46					
3-C	330	<20	2,200						
4-C	3300	170	180,000		130	200	**	<0.2	<0.2
4-W	170	13	360	33					
4-S	20	<20	2,100						
<u>Date: 091077</u>									
1-C	7000	3500	210,000		<2.5	300	3800	<0.2	0.2
1-W	280	350	9,000	220					
1-S	20	<20	12,000						
2-C	3500	1700	61,000		<2.5	940	3000	<0.2	0.9
2-W	1300	170	3,300	79					
2-S	80	20	280,000						
3-C	35000	490	49,000		<2.5	40	1900	<0.2	0.7
3-W	79	33	4,400	220					
3-S	60	<20	2,200						
4-C	7000	210	600,000		<2.5	40	780	<0.2	0.7
4-W	49	33	340	2					
4-S	130	20	5,800						

Appendix D: (continued)

STA	TC 100g	FC 100g	SPC g	FS 100g	ST g	PVP g	NAGV g	SHIG g	SAL g
Date: 100977									
1-C	2400	80	14,000		<2.5	90	130	<2.5	<2.5
1-W	330	17	770	230					
1-S	170	40	14,000						
2-C	330	<20	27,000		<2.5	130	150	<2.5	<2.5
2-W	23	<2	120	13					
2-S	50	50	1,300						
3-C	1100	<20	10,000		<2.5	30	240	<2.5	<2.5
3-W	49	2	360	49					
3-S	170	<20	4,000						
4-C	170	<20	250,000		<2.5	<2.5	190	<2.5	<2.5
4-W	8	<2	140	33					
4-C	50	<20	3,800						
Date: 110577									
1-C	1100	220	59,000		19	90	150	<2.5	<2.5
1-W	490	330	3,000	330					
1-S	790	50	25,000						
2-C	790	50	24,000		<2.5	30	210	<2.5	<2.5
2-W	49	11	670	240					
2-S	20	<20	7,500						
3-C	4900	790	60,000		<2.5	10	30	<2.5	<2.5
3-W	330	130	360	130					
3-S	210	40	2,100						
4-C	3500	490	320,000		<2.5	<2.5	<2.5	<2.5	<2.5
4-W	<2	<2	760	<2					
4-S	790	50	4,300						
Date: 120377									
1-C	2300	1300	930,000		<2.5	<2.5	100	<2.5	<2.5
1-W	13	13	570	13					
1-S	80	50	500,000						
2-C	230	<20	5,400,000		<2.5	10	20	<2.5	<2.5
2-W	13	2	120	<2					
2-S	80	<20	100,000						
3-C	790	50	15,000,000		<2.5	20	40	<2.5	<2.5
3-W	23	2	310	2					
3-S	490	20	640,000						
4-C	1100	20	7,700,000		<2.5	<2.5	<2.5	<2.5	<2.5
4-W	13	5	500	<2					
4-S	1100	50	380,000						

Appendix D: (continued)

STA	TC 100g	FC 100g	SPC g	FS 100g	ST g	PVP g	NAGV g	SHIG 25g	SAL 25g
Date: 010578									
1-C	460	20	4,500		**	**	**	**	**
1-W	5	<2	130	**					
1-S	90	<20	26,000						
2-C	170	110	31,000		**	**	**	**	**
2-W	2	2	59	**					
2-S	270	130	53,000						
3-C	1700	80	12,000		**	**	**	**	**
3-W	140	14	440	**					
3-S	20	<20	6,500						
4-C	7900	700	460,000		**	**	**	**	**
4-W	1400	49	3,200	**					
4-S	230	80	28,000						
Date: 021178									
1-C	120	20	12,000,000		<2.5	<2.5	<2.5	0	0
1-W	220	<2	1,000	<2					
1-S	490	<20	20,000,000						
2-C	160000	<20	4,000		<2.5	<2.5	<2.5	0	0
2-W	8	<2	1,000	2					
2-S	70	<20	8,200						
3-C	54000	<20	34,000		<2.5	<2.5	<2.5	0	0
3-W	49	<2	3,100	2					
3-S	170	20	15,000						
4-C	7000	<20	1,100,000		<2.5	<2.5	<2.5	0	0
4-W	110	8	2,100	2					
4-S	130	<20	6,600						
Date: 031278									
1-C	170	<20	26,000		<2.5	<2.5	30	0	0
1-W	310	7	13,000	2					
1-S	<20	<20	16,000						
2-C	220	20	85,000		<2.5	<2.5	<2.5	0	0
2-W	49	2	3,200	<2					
2-S	20	<20	20,000						
3-C	790	130	3,300,000		<2.5	<2.5	<2.5	0	0
3-W	130	33	17,000	220					
3-S	330	70	17,000						
4-C	92000	80	180,000		<2.5	<2.5	<2.5	0	0
4-W	350	22	57,000	40					
4-S	50	<20	9,900						

Appendix D: (continued)

STA	TC 100g	FC 100g	SPC g	FS 100g	ST g	PVP g	NAGV g	SHIG 25g	SAL 25g
Date: 041578									
1-C	330	130	41,000		<2.5	<2.5	20	0	0
1-W	33	5	500	2					
1-S	90	<20	17,000						
2-C	790	80	720,000		<2.5	<2.5	<2.5	0	0
2-W	2	2	630	<2					
2-S	40	<20	8,400						
3-C	1100	80	320,000		<2.5	<2.5	<2.5	0	0
3-W	130	7	580	<2					
3-S	330	50	30,000						
4-C	92000	<20	800,000		<2.5	<2.5	<2.5	0	0
4-W	79	2	360	<2					
4-S	<20	<20	2,900						
Date: 051178									
1-C	4900	1300	140,000		<2.5	10	20	0	0
1-W	490	79	1,300	4					
1-S	330	130	110,000						
2-C	160000	130	160,000		<2.5	10	<2.5	0	0
2-W	1400	4	1,100	2					
2-S	790	<20	40,000						
3-C	≥240000	20	190,000		<2.5	<2.5	<2.5	0	0
3-W	33	5	380	<2					
3-S	330	20	3,600						
4-C	≥240000	210	11,000,000		<2.5	<2.5	10	0	0
4-W	33	34	1,300	11					
4-S	80	20	7,600						
Date: 061178									
1-C	3300	3300	66,000		<2.5	20	30	0	0
1-W	79	2	**	<2					
1-S	80	<20	14,000						
2-C	160000	330	73,000		<2.5	10	<2.5	0	0
2-W	13	2	1,200	2					
2-S	220	40	17,000						
3-C	2300	50	180,000		<2.5	<2.5	<2.5	0	0
3-W	49	5	5,200	2					
3-S	20	<20	2,700						
4-C	1700	490	640,000		<2.5	<2.5	<2.5	0	0
4-W	330	26	7,500	2					
4-S	140	50	3,300						