Table of Contents

List of Tables

List of Figures

Abstract

Elizabeth D. Hilborn. Risk of Gastrointestinal Illness from Consumption of Raw Clams: Results of a Clinical Trial (Under the Direction of Dr. Mark D. Sobsey)

A randomized clinical trial was performed to investigate the association between consumption of raw clams and development of gastrointestinal disease and infectious hepatitis in healthy volunteers. 938 volunteers were randomized into 3 groups. The first (control) group consumed no shellfish. The second group consumed shellfish harvested from National Shellfish Sanitation Program 'approved' growing waters in Narragansett Bay. The third group consumed shellfish from the same growing waters that were then depurated for 30 days to further reduce potential pathogens. Subjects were fed shellfish on 1 of 7 feeding dates then followed by telephone and written questionnaire for 60 days to assess rates of gastrointestinal illness and jaundice following exposure. Illness rates in volunteer subjects were assessed at 1, 2, 4, and 8 weeks after exposure.

The shellfish from the 2 sources were analyzed for presence of standard and proposed microbial indicators of fecal contamination. When measured at the harvest site, depurated clams had significantly ($p < 0.05$) fewer total coliforms, Enterococci and *C perfringens* than clams directly harvested from Narragansett Bay (Wilcoxon rank sum test, 2-tailed).

Rates of diarrhea reported for week 1 were significantly ($p < 0.05$) higher in the group exposed to clams derived directly from the Bay compared to diarrhea rates in subjects who ate depurated clams (Mantel-Haenszel X^2 test) or to diarrhea rates in controls (Fisher's exact test 2-tailed). Relative risk of diarrhea in the first week after ingestion of directly harvested raw clams, was 2.86 (95% C.I.: 1.00-8.14) compared to controls and 2.21 (1.09-4.46) compared to those who ate depurated clams. No subjects reported jaundice during the follow-up period.

When illness rates of subjects were analyzed with levels of microbial indicators in clam meat measured in R.I., Enterococci enumerated by the BEAA method were significantly associated with subsequent development of diarrheal illness during the first week of follow-up (Mantel-Haenszel X^2 test, $p < 0.05$). When *E. coli,* enumerated by the MF method at the feeding site in N.C, was categorized into 3 levels, X^2 analysis for trend indicated that there was a significant association ($p<0.05$) between E.coli and diarrheal illness.

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CHAPTER I

INTRODUCTION

Bivalve molluscan shellfish such as Eastern oysters (*Crassostrea virginica*), mussels (*Mytilus edulis*) and hard clams (*Mercenaria mercenaria*) are filter feeders, deriving nutrients from particulates in the water of their habitat. In the course of feeding, they may concentrate pathogens and other contaminants in their digestive systems and in other tissues at levels 10 to 100 times greater than in the surrounding water (4). Extensive cooking will destroy pathogens, but many persons enjoy eating the whole animal raw or partially cooked, thereby exposing themselves to potential infection.

Early in the twentieth century, consumption of contaminated molluscan shellfish resulted in large typhoid fever outbreaks along the eastern seaboard of the United States. In 1925, in response to this threat to the public health, regulations were proposed to limit harvesting of molluscan shellfish to areas free of contamination by sewage outflow (8). General adherence to shellfish growing water (coastal waters where shellfish are produced) and shellfish microbiological standards adopted by states through the National Shellfish Sanitation Program (NSSP) has stopped the large typhoid epidemics, but other pathogens have emerged as public health concerns.

A variety of food-borne pathogens are known to be associated with consumption of raw molluscan shellfish and many of these come from fecal contamination of shellfish growing waters. The human enteric viruses, including Norwalk and Norwalk-like viruses and hepatitis A virus are the most important viral pathogens implicated in shellfish-borne outbreaks (8,45,33). Enteric viruses may be infective in very low numbers (32,30), and agents such as Norwalk virus are difficult to detect in shellfish and environmental samples. In addition, some species of bacteria, such as *Vibrio* sp. are a public health threat, especially to those with liver dysfunction or immunosuppression.

Norwalk virus

Norwalk Virus (NV) and related Small Round Structured Viruses (SRSV) may be the most common viral agents of shellfish-associated illness. NV and related small round viruses are members of the Caliciviridae family (13). All virions are about 30-35 nm in diameter, contain single stranded, +-sense RNA of about 7.6 K nucleotides in length, and have an icosahedral capsid containing a single type of polypeptide of about 60 kilodaltons (44). Transmission is typically by the fecal-oral route, although there is a report of suspected aerosolization of viral particles occurring, which led to widespread infection.(42) Outbreaks have been associated with ingestion of shellfish (19, 45, 29, 5), swimming in contaminated water, consumption of contaminated well water (25), person to person contact and ingestion of commercially produced ice (3). Norwalk and Norwalk-like viral infections are characterized by acute gastroenteritis within 24-48 hours of exposure. Illness is generally self-limiting in healthy individuals, ranging in severity from mild abdominal discomfort to severe diarrhea and/or vomiting of 12-60 hours duration (22).

Human seroprevalence to Norwalk virus is widespread, even seemingly, in infants. Gray surveyed 3,250 serum samples collected in England from 1991-1992 and found seroprevalence ranging from 24.6% in 6-11 month old infants, to 89.7% in persons over 60 years of age (12). Lefkowitz reported no significant difference in seroprevalence between 3 groups of subjects chosen for high, moderate and no shellfish consumption. All groups were approximately 70% seropositive to Norwalk virus, strengthening the argument that contaminated water, the environment and person to person contact may be the primary routes of transmission (26).

Presence of antibody to NV is not uniformly associated with a decreased risk of illness upon subsequent exposure to virus (21). Some studies have suggested that persons with preexisting antibody titers to NV may actually be more likely to develop clinical illness after exposure to the virus (2, 11).

Hepatitis A virus

Hepatitis A virus (HAV) is a 27-nm nonenveloped RNA virus in the family Picornaviridae. The primary route of transmission is the fecal-oral route. Virus is shed

primarily through the feces and may remain infective for up to 2 weeks in fecal material. The virus is very persistent in shellfish and in environmental media, including seawater, wastewater and sediments. HAV infects only human and nonhuman primates, hence zoonotic transmission is negligible in the US (15).

Molluscan shellfish may serve as vehicles when they become contaminated after harvest by infected food handlers (30), or when they bioconcentrate HAV from fecally contaminated water during active filter feeding. When infective virus particles in the shellfish are ingested by a human host, HAV infection may occur. HAV infection results in an infectious hepatitis characterized by periportal necrosis accompanied by an infiltration of mononuclear cells (15). The disease has a mean incubation period of 4 weeks a range of 3-6 weeks. Clinical illness in adults is characterized by fever, nausea, vomiting and jaundice. Duration of illness is generally 1-2 weeks and severity of illness increases with age (15). Duration of clinical hepatitis may also be prolonged in immunocompromised individuals.

HAV has a single serotype and seroprevalence increases with age. Age adjusted seroprevalence of anti-HAV in the US population in 1980 was 40.4% for males and 42.4% for females (43). Presence of anti-HAV confers active immunity for long periods of time, sometimes for life (15). Approximately 25,000 cases of Hepatitis A are reported annually in the US (30). Risk factors associated with illness listed in descending order of importance are: day-care center contact, personal contact with a HAV infected individual, consumption of contaminated food or water, international travel and parenteral drug abuse. Approximately 42% of cases had no identifiable source of illness (43). It's estimated that 19-25% of all Hepatitis A illnesses may be contracted after consumption of contaminated raw shellfish (38).

Vibrio sp.

Vibrio species of bacteria in the family *Vibronaceae*, are important sources of shellfish-borne disease. *Vibrios* are halophilic, gram negative, curved or rod-shaped bacteria that thrive in warm, coastal and estuarine waters. *Vibrio* species that have been associated with illness from shellfish consumption include: *Vibrio* type O1 *cholerae, V.* non-O1 *cholerae, V.parahaemolyticus* and *V. vulnificus*.

Consumption of raw oysters has been linked with outbreaks of gastroenteritis due to Non-O group 1 *Vibrio cholerae* (30,50). In 1986 (24) and 1991, there were cases of cholera (due to type O1 *Vibrio cholerae)* associated with consumption of raw oysters harvested from the Gulf of Mexico and Ecuador. *V. parahaemolyticus* is a common cause of seafood-borne gastroenteritis in healthy persons (20). *V. parahaemolyticus* infection may be characterized by diarrhea, nausea, vomiting and abdominal pain. The incubation period is about 4 - 96 hours with duration of illness of approximately 72 hours (36). Generally *V. parahaemolyticus* gastroenteritis is self-limiting in healthy individuals. *Vibrio vulnificus,* is an important pathogen primarily in those who are immunosuppressed or have chronic liver disease. *V. vulnificus* may cause fatal systemic infections (*Vibrio* septicemia) with a mortality rate of greater than 50% (23,30,46). *Vibrio* non-O1 *cholerae* and *V. parahaemolyticus* infection may also occasionally produce a fatal outcome in immunocompromised individuals (23).

All of the *Vibrio* species associated with shellfish-borne illness are naturally occurring in the marine environment. *Vibrios* generally favor warm coastal and estuarine waters. In addition, of these *Vibrio* species, *V. parahemolyticus* is most commonly isolated from feces and may be transmitted by fecal contamination of shellfish both before and after harvest (30).

Public health impact

The Centers for Disease Control and Prevention (CDC) reported 128 outbreaks and 3747 cases of shellfish-borne illness in the 10 year period from 1978-1987. This constitutes 31% of all seafood associated outbreaks and 66% of all seafood-borne cases reported to the CDC during this time period (30). CDC's data was derived from food-borne disease outbreak reports from state health departments. Rippey and Verber report 361 outbreaks and more than 5797 cases of shellfish associated gastroenteritis and hepatitis in the US from 1979-1988 (35). Rippey and Verber's data are derived from a variety of sources: state and local health department files, books, news accounts, CDC reports, Public Health Service files, case histories and archival reports (30). (Table 1)

Table 1: **Shellfish-borne Illness, (1978-1987)**

	CDC*		NETSU**
	Outbreaks	Cases	Cases
Naturally Occurring Aquatic Agents			
Vibrio parahemolyticus	15	176	52
Vibrio cholerae O1	2	14	13
Vibrio cholerae non-O1	2	11	120
Vibrio vulnificus	-	-	100
Vibrio mimicus	-	-	5
Vibrio hollisae	-	-	5
Vibrio fluvialis	-	-	5
Plesiomonas	-	-	18
Aeromonas	-	-	7
Infectious Agents Associated with Fecal Pollution of Water			
Hepatitis, unspecified	-	-	1,645
Hepatitis A	7	33	45
Salmonella (nontyphoidal)	3	80	-
Shigella	4	77	84
Campylobacter	-	-	16
Norwalk and Norwalk-like viruses	2	42	82
Non-A, non-B hepatitis	-	-	1
Infectious Agents Associated with Processing and Preparation			
Clostridium perfringens	2	28	-
Staphylococcus aureus	1	9	-
Bacillus cereus	2	6	-
Total outbreaks/cases[1]	40	476	2,198
Unknown agents[2]:	88	3,271	5,098

* Centers for Disease Control and Prevention, Atlanta, Georgia

** Rippey and Verber, Northeast Technical Services Unit, FDA, Shellfish Sanitation Branch, Davisville, Rhode Island.

Table 1, cont.

1 outbreaks/cases of known etiology

2 Toxins, allergies, unknown infectious agents

From: 'Seafood Safety', from the Committee on Evaluation of the Safety of Fishery Products, National Academy of Sciences, 1991.

The actual number of persons who become ill every year due to consumption of contaminated molluscan shellfish is estimated to be much greater than those cases recorded (30). This is due to mild, undiagnosed or misdiagnosed illness and under reporting by the currently passive surveillance system. Shellfish-borne illness causes appreciable economic costs to society via losses due to morbidity, mortality, medical expenses and decreased productivity.

Chronically ill or immunocompromised individuals may be at higher risk of illness from infectious shellfish-borne agents. The agents that pose the greatest public health risk from raw shellfish consumption are probably the *Vibrio spp.* Non-O1 *V. cholerae* has been isolated from up to 37% of all US oysters sampled during the warmer months of the year. Diagnosed infection rates are in the range of 0.5-1.0 cases/100,000 population per year (30). *Vibrios* may cause severe diarrhea in healthy persons who become infected, but immunocompromised individuals or those with liver disease are at risk for systemic infections. In these individuals the humoral immune system is ineffective in the fight to overcome the bacteria's rapid multiplication in the circulatory system. The result is a massive *Vibrio* bacteremia/septicemia.

There is a need to assess the incidence of gastrointestinal disease and hepatitis following consumption of raw shellfish of known microbial quality which are harvested from approved waters. Shellfish-borne epidemic disease is well documented (19, 50, 45, 22), but studies designed to characterize the endemic disease risk from ingestion of shellfish from approved waters have not been performed. Many outbreak investigations have been incomplete because of unknown or poorly characterized microbial quality of the shellfish involved. Many outbreaks are related to recreational shellfishing or are associated with shellfish from sources where the water and/or shellfish quality is unknown (30,19,39).

Indicator bacteria

In response to large, shellfish-borne outbreaks of Typhoid Fever in the 1920's, the National Shellfish Sanitation Program (NSSP) was created as a cooperative effort between shellfish producing states, the Public Health Service and the shellfish industry. The program proposed shellfish growing water quality regulations, which restricted harvest of shellfish from potentially hazardous growing waters. Harvest restrictions included: growing areas near major sources of pollution, areas where sewage may contaminate the waters, and in areas where microbiolgical tests demonstrate growth of coliform bacteria from analysis of 1cc of growing water (8). Coliform bacteria as a group, were considered indicators of fecal contamination of the growing water. These standards, including the use of coliform indicator organisms, were adopted by NSSP participating states in 1925. These restrictions helped to curtail the large typhoid fever outbreaks seen in the early 20th century.

Today, modifications of those first techniques to identify fecal contamination in growing waters are utilized. Growing water standards are based on analysis of total coliforms or fecal coliforms (thermotolerant coliform bacteria which are most likely to inhabit human and animal gastrointestinal tracts) present in 100 ml of water. Presently though, there is strong evidence that bacterial indicators of fecal contamination of growing waters are not correlated with enteric virus and *Vibrionaceae* concentrations in shellfish (9, 47) or growing waters (10, 7). In general, the enteric viruses persist in seawater for longer periods of time than indicator bacteria (8). This means that a growing area recently reopened to shellfishing may still have elevated levels of enteric viruses which may pose a risk to the public health. Outbreaks of HAV (31), non-O group 1 *Vibrio cholerae* (50) and presumptive NV (5) have all been associated with consumption of shellfish harvested from NSSP approved areas. Although most human illness associated with consumption of fecally contaminated shellfish is due to viral pathogens. The bacterial indicators are still used, as there are currently no practical methods to evaluate viral contamination of shellfish growing waters and reliable virus indicator organisms have not been established (8,30).

Presently we do not have an ideal indicator organism of fecal contamination in marine environments, but ideal characteristics (40) would be:

-Present and easily detectable in the water when fecal contamination is present.
-Numbers of indicator organism correlate with amount of fecal contamination.
-Indicator numbers stable in the marine environment. Persistence must equal or exceed fecal pathogens, but will not increase numbers or grow once introduced into seawater.
-Absent when there is a lack of fecal contamination.
-Dose-response relationship, between numbers of indicator organism and risk of human enteric illness.
-Not pathogenic for humans.

Potential (candidate) indicator organisms include:

-Enterococci: a human-feces-specific group of fecal streptococci classified within Lancefield's serological Group D. Organisms include: *S. faecalis*, and *S. faecium* (14).
-*Clostridium perfringens*: an anaerobic spore-forming bacterium of fecal origin which may be more persistent in seawater than enteric pathogens.
-Coliphages: viruses which infect coliform bacteria. Coliphages may be more resistant to chlorine than coliform bacteria and thus may be better indicators for enteric virus contamination (14).
-F-specific coliphages and somatic coliphages have both been proposed as potential indicators of fecal contamination.

Shellfish Microbial Standards and Analytical Methods

The standards for shellfish growing water quality are based on the amounts of total or fecal coliforms present. To maintain approved growing water status the total coliform median Most Probable Number (MPN) must not exceed 70 per 100 milliliters (ml)., with $\leq$10% exceeding an MPN of 230 per 100 ml. Alternatively, the fecal coliform median MPN must not exceed 14 per 100 ml., with $\leq$10% exceeding a MPN of 43 per 100 ml.(8). These bacteria have historically functioned as indicators of fecal contamination of bathing, drinking and shellfish growing waters. Although coliforms and *E. coli* may reflect levels of bacteria from direct fecal

contamination or after sewage treatment processes, counts may also be elevated in organically enriched waters. Some total and fecal coliforms may have non-fecal environmental sources such as vegetation and soil.

The standards to assess microbial quality of shellfish are based on the Most Probable Number (MPN) of fecal coliforms or *Escherichia coli* (*E. coli*) organisms per 100 grams of shellfish meat. In addition, the Standard Plate Count (SPC) is used to assess the number of aerobic bacterial organisms per gram of shellfish meat. The current recommended limits are 230 fecal coliforms or *E. coli* per 100 grams shellfish meat, and a SPC of less than 500,000 per gram meat. Shellfish are rejected if any sample contains greater than 330 fecal coliform or *E. coli* organisms per 100 grams of meat or if 2 of 5 samples contain greater than 230 organisms per 100 grams. Rejection also occurs if any one shellfish meat sample exceeds 1,500,000 SPC or 2 of 5 samples are greater than 500,000 SPC (8).

Depuration

Because of the health risks associated with fecal contamination of shellfish, depuration or controlled purification (cleansing of shellfish by allowing them to feed and purge contaminants in clean water), has become a common practice. Controlled purification was developed in Europe in the early 20th century in response to shellfish-borne outbreaks of illness (32). In the US today, a number of commercial depuration facilities are producing shellfish for wholesale and retail markets.

Generally, the process of depuration consists of holding shellfish in a tank of clean seawater for 48-72 hours in conditions that encourage pumping and feeding behavior in the animals. Depuration facilities may utilize recirculating water systems or continuous flow through systems. In either case, seawater is pumped from a clean source or is filtered and purified before it contacts the shellfish.

Important physical parameters for effective depuration by shellfish are: temperature, pH, salinity, turbidity, as well as phytoplankton and dissolved oxygen content of the water (37). Optimal temperature requirements for elimination of pathogens vary by pathogen and shellfish species. At approximately 20°C, Hard clams have been shown to eliminate

E. coli, fecal coliforms, poliovirus and Coxsackievirus B4 to nondetectable levels within 48 hours (32). In one study, 6 days of depuration of Hard clams at 16°C, achieved a 1 log reduction in S-13 coliphage concentration. When the temperature was increased to 24°C, phage levels were negligible after 72 hours (32). *V. parahaemolyticus* is most rapidly eliminated at 15°C (32, 37). The pH of seawater should be maintained in the range of pH 7-8.4 (32). Optimal salinity's for depuration of *E. coli* and polio virus from Hard clams vary from 22-31 ppt.(32). Turbidity is due to suspended particulates in the water column. The water must contain sufficient micronutrients for the shellfish to feed, but excessive turbidity attenuates ultraviolet (UV) light penetration (37), compromising the water disinfection process. Dissolved oxygen (DO) varies with temperature and salinity, but must be sufficient to allow normal physiologic functioning of the shellfish.

Microbiological quality of the water must be monitored to prevent introduction of bacterial contamination. Fecal coliforms are monitored in the inflow water, but are not useful as indicators of enteric viral contamination (32, 4). In the US, UV light disinfection is the method of choice to reduce microbial pathogens in the inflow water (4). This method has the advantage of sanitization without the use of chemical agents which may impair physiologic function of the shellfish or which may produce harmful residues in their meat.

Typically, in the US, shellfish destined for depuration are harvested from areas defined as no worse than restricted by the NSSP. Total coliforms must be < 700/100ml seawater, fecal coliforms must be < 88/100ml seawater (32). Shellfish depuration for 48-72 hours effectively reduces levels of enteric bacterial organisms, but is less effective at reducing enteric virus pathogen loads (4). Depuration is not a substitute for careful application of the principles of shellfish sanitation.

Current indicators of fecal contamination of shellfish growing waters are unreliable for predicting risks of shellfish-borne illness due to non-bacterial pathogens, notably enteric viruses. Viruses are more persistent in shellfish habitats and shellfish than are enteric bacteria, including the coliform indicators. The current classification system for establishing approved waters may not assure a low risk of viral presence in shellfish that are harvested from areas impacted by substantial sources of human fecal wastes, despite low levels of coliform bacteria in the water and the shellfish. This study was intended to quantify the risks of gastrointestinal illness associated with ingestion of raw shellfish

(Hard clams) harvested from approved growing waters, as well as consumption of shellfish derived from the same source, then subjected to substantial controlled purification in clean seawater.

Study objectives

- To investigate the association between ingestion of raw Hard clams of different microbiological quality and risks of subsequent enteric illness in volunteer consumers.

- To determine if clams harvested directly from NSSP approved growing waters impacted by substantial fecal pollution sources, pose a measurable risk of gastrointestinal illness or hepatitis if consumed raw, and if the risk is lower from clams subjected to further microbial purification (depuration).

- To determine if the current microbial standards for sanitation of shellfish growing waters, which assess indicator bacteria (total and fecal coliforms), are sufficient to protect against gastrointestinal disease or hepatitis in persons who ingest the shellfish derived from them.

- To determine if the current microbial standards for sanitation of molluscan shellfish, which utilize indicator bacteria (fecal coliforms, *E. coli* and aerobic plate count bacteria), are sufficient to protect against gastrointestinal disease or hepatitis in persons who ingest the shellfish.

- To investigate if levels of *E. coli, Clostridium perfringens*, Enterococci, *Vibrio* species, Standard plate count bacteria, F-specific coliphage or somatic coliphages are better indicators of fecal contamination of shellfish than total or fecal coliforms. To determine which indicators, if any, are predictive of development of gastrointestinal disease in human volunteer consumers of raw Hard clams.

CHAPTER II
RESEARCH DESIGN AND METHODS

Overview

The study was a randomized clinical trial designed to investigate the association between consumption of raw Hard clams of two different microbiological qualities, and subsequent development of gastrointestinal disease and hepatitis in healthy volunteers recruited from the University of North Carolina at Chapel Hill community.

Nine hundred eighty five volunteers were block randomized into 3 groups. Approximately 150 subjects were assigned to each of 7 feeding dates. The control group did not ingest clams. The second group ingested raw clams harvested from approved growing waters, then depurated for 30 days directly after harvest to further reduce pathogenic microorganism levels. Group three ingested shellfish harvested directly from the same approved growing waters.

Microbiological parameters for shellfish growing waters and shellfish were within NSSP guidelines, and therefore met all State and Federal guidelines for wholesomeness and purity. Based on their sanitary quality, the shellfish were similar to those which may be purchased from wholesale sources, at retail outlets or restaurants. The study protocol was reviewed and approved by the Committee for Research Involving Human Subjects.

Growing waters and the shellfish harvested from them were analyzed for bacterial and bacteriophage indicators of fecal contamination. The shellfish were analyzed both at the harvest site, and then again after transport to the feeding site to ascertain if there was any difference in bacteria or bacteriophage levels after 12-18 hours in transport. The levels of standard and proposed microbial indicators of pathogenic organisms were measured in subsamples of each batch of shellfish ingested by each group.

Rates of illness were calculated from data obtained in 4 written follow up questionnaires. In addition, telephone interviews were administered to each subject to cross check data provided on the 1st follow-up questionnaire, as well as to encourage continued participation in the study.

Subject selection

Recruitment was a continuous process over the 6 month-long study period to accommodate monthly feeding dates. Advertisements in the University of North Carolina at Chapel Hill student newspaper ran during the entire 6 month study period. Flyers were posted on campus bulletin boards in an attempt to recruit maximal numbers of potential study participants. Because of the methods of notifying potential subjects of the study, the majority of participants were obtained from the University community. Subjects were continuously recruited and assigned to the next scheduled feeding that could accommodate their schedule. Approximately 150 persons per feeding date were recruited for a total of 7 feeding dates. A total of 985 persons were initially recruited.

Selection criteria included subjects in good health, with no severe chronic disease or disorders of the gastrointestinal tract, hepatobiliary, or renal / urinary systems. Subjects could not currently be taking antibiotics, immunosuppressive drugs or chemotherapeutic agents. Participants must have been at least 18 years of age.

All subjects signed a consent form and agreed to complete and return the 4 follow-up questionnaires, subjects were given a $25.00 participation payment for completion of the study. Other stipulations of participation in the study included abstention from eating raw shellfish from any source in the 30 days before and after the feeding date to avoid misclassification of the subjects' exposure status.

Study design

A randomized clinical trial was conducted with feeding dates encompassing the 6 months between July, 1988 to January 1989. Because there can be seasonal variation in the levels of microbial contamination of the shellfish and their growing waters, the study consisted of 7 separate feeding dates, between July 15, 1988 and January 30, 1989, approximately 1 per every calendar month, dependent on clam availability.

For the entire study, a total of 985 subjects were randomized by blocks of 12 subjects into 3 exposure groups; controls, high and low exposure. The control consisted of 197 unfed subjects. The subjects in the low exposure group ingested 6 uncooked clams

derived from approved Narragansett Bay, Rhode Island waters, and then depurated for 30 days in Rhode Island before shipment to NC. The high exposure group subjects ingested 6 uncooked clams harvested directly from approved waters in the West Middle Bay region of Narragansett Bay, Rhode Island.

The subjects were followed for development of gastrointestinal illness or jaundice for a total of 60 days after the feeding date by means of 4 written follow-up questionnaires prepared and returned by the subjects at 1 week, 2 weeks, 4 weeks and 8 weeks post exposure.

All subjects were contacted between 6 and 10 days after the feeding date by telephone. This initial follow-up effort served 2 purposes. All subjects were reminded to return their questionnaires, and ill subjects were reminded to contact our staff physician for free medical services and specimen collection. A brief oral questionnaire was administered during which we established; current stool frequency and if subjects experienced any abdominal discomfort, vomiting, diarrhea, fever or other symptoms. If so, dates, times and frequency of symptoms. Analysis and results of the oral questionnaire will not be addressed here.

Subject exposure methods

Approximately 150 subjects were assembled monthly at the University of North Carolina Hospitals' cafeteria in Chapel Hill, North Carolina. Each subject was randomly assigned a group (Control, High or Low Exposure) within blocks of 12 subjects, by using a random number table. Identification numbers were assigned which included a exposure category code. A color-coded card and 4 questionnaires in stamped, self-addressed envelopes were distributed to each participant. Colored cards designated group assignation to aid subjects in identification of their own group's feeding table.

Control subjects left the cafeteria unfed, but some chose to consume one of the soft drinks provided for all participants. The control group ingested no clams because to effectively sterilize raw clams by radiation or heat, the organoleptic quality is markedly altered, possibly rendering the clams unpalatable. Therefore it was elected to maintain

unfed controls given the impossibility of blinding participants as to control status and have them ingest shellfish.

Chilled clams which were maintained at <7.2^0 C (<45^0 F) were washed in tap water, scrubbed, then opened at the site. The Low and High Exposure group subjects (all blinded as to exposure status) were each served 6 raw, shucked clams along with their choice of cocktail sauce, hot pepper sauce or lemon wedges from their respective, separate color coded tables. A subject had to present his/her colored card to receive their 6 clams to help prevent returns for second helpings. All clams were consumed within 60 minutes of removal from refrigeration. Subjects ingested no other food or drink at the feeding site. All subjects were instructed to eat their clams in the hospital cafeteria and were observed by study coordinators.

Narragansett Bay

Narragansett Bay is a large estuary in eastern Rhode Island, fed by the Providence River to the north, the Woonasquatucket and the Pawtuxet Rivers to the west, and the Taunton River to the east where it flows past Fall River, Massachusetts. The bay is bounded on the north by the large metropolitan area of Providence/Pawtucket, R.I.. Warwick, R. I. is situated on the western shore of the mid bay. Historically, the upper Narragansett Bay has been heavily impacted by the textile and jewelry industries in Providence, some operating since the early 1900's. Today, the major sources of pollution in Narragansett Bay are sewage and wastewater flowing into the bay, jewelry and metallurgy industrial pollution, and some chemical and pharmaceutical manufacturing.

The upper Bay has been closed to shellfishing for years due to pollution (Figure 1). The upper bay which encompasses the prohibited area is the portion of the bay north of a line drawn between Conimicut Point on the western shore and Nayatt Point on the eastern shore. This line demarcates the prohibited area to the north from the conditionally approved area to the south. A line drawn across the mid bay from the southernmost tip of Warwick Neck across to the northern point of Prudence Island

demarcates conditionally approved waters to the north and approved waters to the south. The conditionally approved portion of the bay is closed whenever rainfall equals or exceeds 0.5 inches within a 7 day period. The approved and conditionally approved areas remain the most important area for commercial shellfishing as the environmental conditions are most favorable for growth of Hard clams. The lower reaches of the bay, south of Quonset Point on the western shore and Sandy Point on Prudence Island, do not have shellfish populations large enough to sustain a commercial fishery (48). All growing areas are monitored by the state of Rhode Island to ensure bacterial indicator organisms are within NSSP sanitation guidelines. The clams for this study were all harvested from the approved area approximately 1/2 mile south of Warwick Neck and about 1/4 mile west of Prudence Island.(Figure 1 (28))

Narragansett Bay is similar to other northern East Coast estuaries which have historically produced large numbers of shellfish. Portions of Massachusetts Bay and Buzzard's Bay, Massachusetts, parts of Long Island Sound and New York Bay, New York have all had shellfish growing areas adversely impacted by urban development, antiquated waste disposal methods and industrial water pollution. Although these bodies of water share similar histories of adverse impacts from coastal development, each is unique due to surface water flow patterns, variations in industrial discharges, and patterns of development.

Growing water sampling: Narragansett Bay shellfish collection site

Harvest sites were surveyed and divided into quarters. A 1 liter water sample was collected aseptically in a sterile bottle by local technicians from each quarter at a depth of 1 meter. Water sample containers were tightly capped and placed immediately on ice (maintained at 4^0 C), and transported to the US Food and Drug Administration, Northeast Technical Services Unit laboratory in Kingstown, R.I. for analyses. Levels of total and fecal coliforms, *E. coli*, *Clostridium perfringens* and Enterococci species were measured approximately every 2 weeks throughout the study period including at the time of harvest to aid in assessment of dynamic water quality conditions. Water quality data was not analyzed and it will not be addressed in this document.

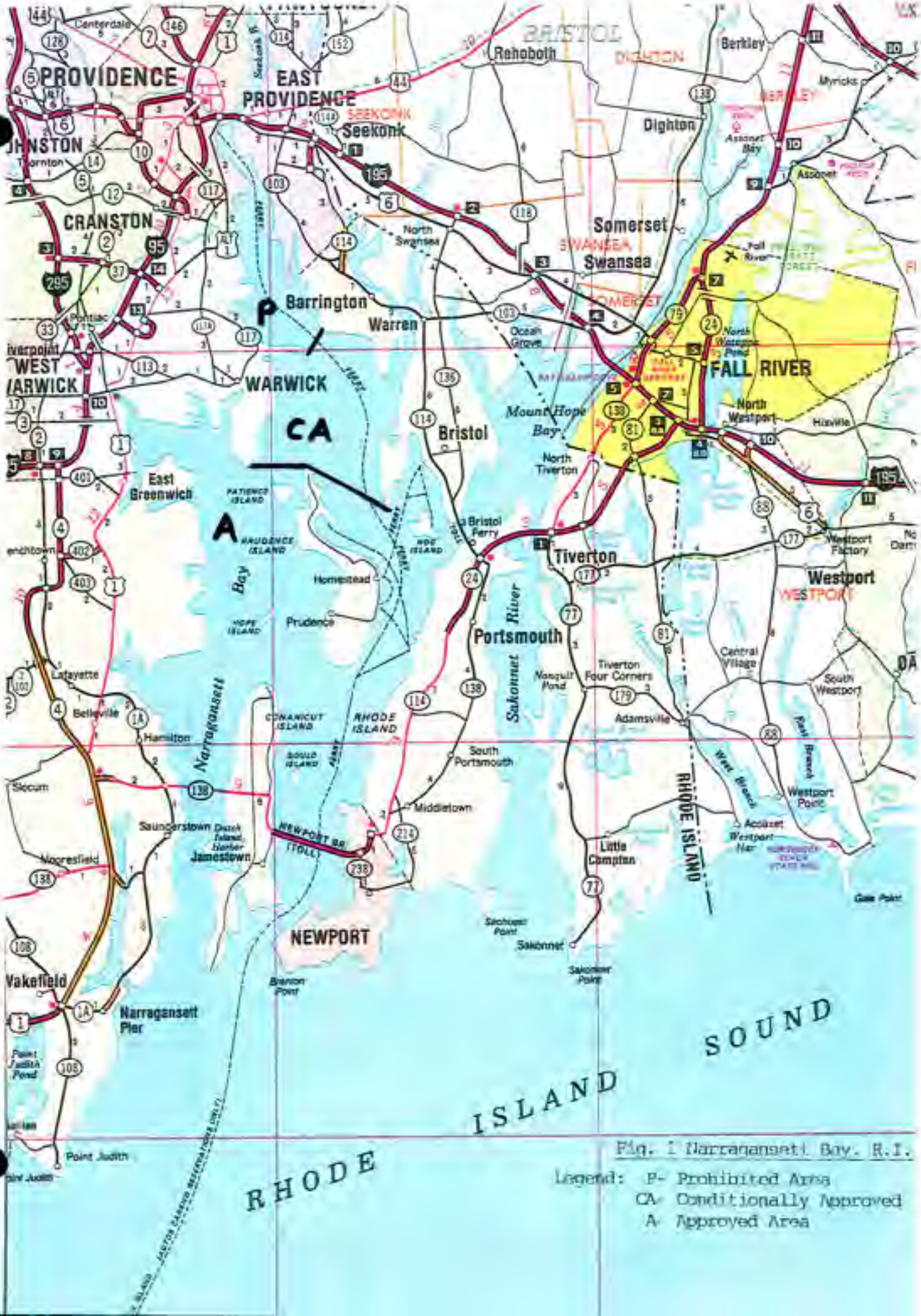


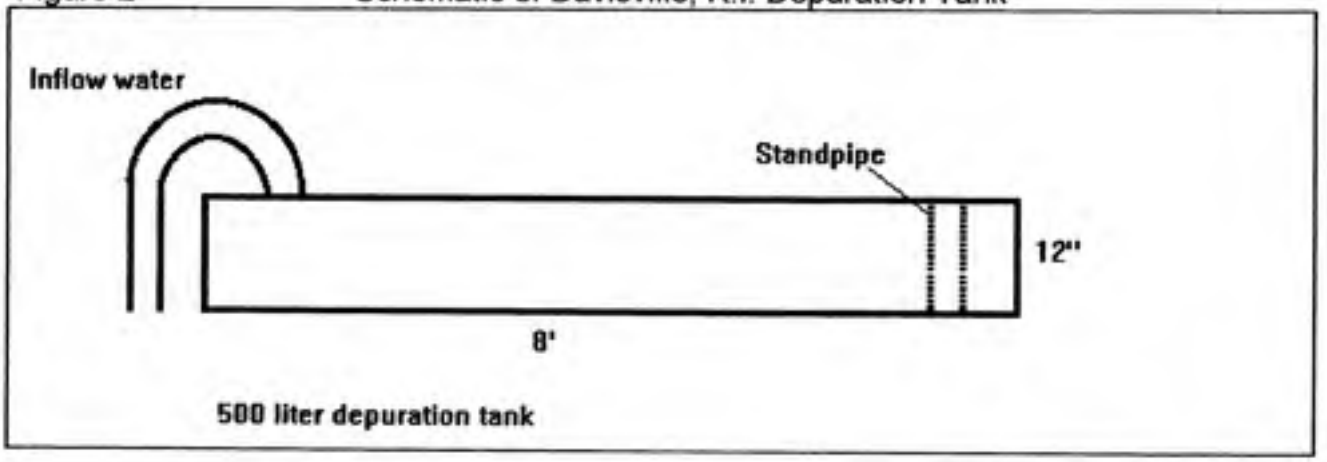
Fig. 1 Narragansett Bay, R.I.

Legend: P- Prohibited Area
CA- Conditionally Approved
A- Approved Area

Depuration of study clams

The Davisville, Rhode Island depuration facility was utilized solely to purify the clams used in this study. This is a pilot scale depuration plant owned and operated by the US Food and Drug Administration, Northeast Technical Services Unit as a research and training facility. The facility had a capacity of approximately 500 liters with a flow rate of 20 liters / minute in a flow through system with inflow water derived from Narragansett Bay (Figure 2). Bay water was disinfected with UV radiation before entering the depuration tank. Fecal coliforms were measured in the water after disinfection. Inflow water sanitization was designed to maintain zero fecal coliforms per 100ml water. The temperature was maintained at a minimum of 10^{0} C. Salinity ranged from 28-30 parts per thousand. Dissolved O_2 (DO) was not monitored, but the surface area to water volume ratio was high and apparently precluded problems with low DO. Approximately 350 clams per 30 day period were maintained in a monolayer held off the bottom of the depuration tank on stainless steel screens. Clams were maintained in the depuration facility for a minimum of 30 days before harvest for each feeding dates (34).

Figure 2 Schematic of Davisville, R.I. Depuration Tank



Water microbiologic procedures

Total and fecal coliforms and *E.coli* were enumerated by the Most Probable Numbers (MPN) method (1) using five tubes and four dilutions of lauryl sulfate lactose broth (LSLB) which was incubated at 35° C. After 48 hours, tubes were examined and scored as presumptively positive if growth appeared and gas had formed. Positive cultures were transferred into brilliant green lactose bile broth (BGLBB), then tubes were incubated at 35° C for 48 hours. Coliform bacteria were confirmed after tubes were examined and found positive for growth and gas formation. Fecal coliforms were analyzed by transferring bacteria from growth and gas positive tubes of LSLB into EC broth supplemented with 100ug/ml MUG. Tubes were incubated at 44.5° C for 24 hours. Tubes were then examined for growth plus gas to score for fecal coliforms, and examined by means of a 366 nm wavelength ultraviolet light source for the fluorescent metabolites of MUG produced by *E. coli* (49).

Clostridium perfringens were quantified by a MPN method, using five tubes and three dilutions. Each tube contained 10ml of whole milk and 0.2g iron powder (41, 49).

Enterococci were quantified by a MPN method using five tubes and three dilutions of azide dextrose broth which were incubated at 35° C for 48 hours. Tubes which showed turbidity were transferred onto modified mE agar, which was incubated at 41° C for 24 hours. Blue colonies were counted as confirmed Enterococci (49).

Clam harvest, storage and transport procedures

Clams were harvested from the Bay by means of a boat dredge or removed directly from the depuration facility at monthly intervals throughout the study period. Harvested clams were scrubbed, washed in potable water and sorted at the site. The shellfish were rapidly cooled to <7.2 ° C and packed on 'blue ice' type frozen chill packs to maintain a chilled state and minimize microbial growth en route to the feeding site (8). Shellfish were transported overnight from the harvest sites to the North Carolina Hospitals' cafeteria in Chapel Hill, North Carolina via overnight air express service. Upon arrival a portion of the

shellfish were removed for microbiological analysis. Remaining clams were maintained at <7.2° C in the hospital cafeteria refrigeration units until they were opened at the feeding site. Clams were consumed within 60 hours of harvest. (Table 2)

Table 2 **CLAM SAMPLING AND MONTHLY FEEDING DATES OF STUDY**

Rhode Island Sample Dates	North Carolina Sample Dates	Feeding Dates
July 11, 1988	July 12, 13, 1988	July 14,1988
August 29, 1988	August 30, 31,1988	September 1,1988
September 16, 19, 1988	September 20, 21, 1988	September 22, 1988
October 17, 1988	October 18, 19, 1988	October 20, 1988
November 14, 1988	November 15, 16, 1988	November17,1988
December 12, 13, 1988	December 14, 15, 1988	December14,1988
January 30, 1989	January 31, February 1, 1989	February 2, 1989

Shellfish microbiologic methods

Shellfish were analyzed for total and fecal coliforms, *Escherichia coli (E. coli)*, *Clostridium perfringens*, Enterococci, *Vibrio* species and Standard (aerobic) plate count bacteria. F-specific bacteriophages (FPH) grown on *Salmonella typhimurium* strain WG49 and *E. coli* F Amp, as well as Somatic Coliphages (SOMCPH) cultured on *E. coli* C, were enumerated and assessed as indicator organisms of the human enteric viruses (16).

Clams were washed and scrubbed a second time before processing for analysis. Clams were shucked manually and clam meat was collected aseptically and homogenized. Serial dilutions of each sample homogenate were prepared and used in MPN procedures. Diluents varied for each assay and are detailed in the following description of methods for each assay. The aerobic plate count assay was performed utilizing phosphate-buffered peptone water (1). Clam homogenate was diluted with peptone tween buffer for Membrane Filter (MF) methods developed by Dufour and Hopkins for oyster analysis (6, 49).

Total and fecal coliforms and *E. coli* were quantified by the MPN method as described for the water analysis.

In addition, *E. coli* were enumerated by the MF method. Clam homogenates were digested with 5% trypsin at 37^0 C for 20 minutes (49). One gram of digested clam tissue was filtered through 5 um poresize prefilters then 0.45 um poresize filters. Triplicate 0.45 um filters were incubated on mTEC agar at 35^0 C for 90 minutes. The filters were then incubated at 44.5^0 C for 20 hours. At that time, filters were transferred to pads soaked with urea substrate. All yellow urease-negative colonies were counted as *E. coli* (6, 49).

Enterococci were quantified by the MF procedure. Triplicates of filters used to process clam homogenate digested with trypsin were placed on mE agar and incubated at 41^0 C for 48 hours. The filters were then transferred to Esculin Iron Agar (EIA) and reincubated at 41^0 C for 24 hours. Red colonies possessing underlying black zones in the agar were counted as confirmed Enterococci (6, 49).

Enterococci were also enumerated by the MPN method. Trypsin-digested clam homogenate was incubated in Azide Dextrose Broth (ADB) tubes at 37^0 C for 48 hours. Material was transferred from presumptive positive tubes, then filtered through 0.45 um poresize filters, filters were placed on mE agar then incubated at 41^0 C for 24 hours. Blue streaks were scored as confirmed enterococci by the MPN method. In addition, material from presumptive positive ADB tubes was transferred to Bile Esculin Azide Agar (BEAA) and incubated at 35^0 C for 24 hours. Growth was scored as confirmed enterococci by the BEAA method.

Clostridium perfringens were also quantified by a MF method. Triplicate filters of clam homogenates which had been trypsin-digested were incubated on mCP agar at 41^0 C for 18 hours. Yellow presumptive colonies that turned pink or red after exposure to ammonia fumes were scored as confirmed *C. perfringens*.(6, 49)

Aerobic plate counts were performed by pouring molten plate count agar over dilutions of clam homogenate and incubated at 35^0 C for 48 hours (49, 1).

Bacteriophages were enumerated in the clams by mixing clam homogenate with molten agar medium inoculated with one of three phage hosts: *E. coli* C, a SOMCPH phage host, *Salmonella typhimurium* WG-49, a FPH phage host, or *E.coli* F Amp, an ampicillin-resistant FPH phage host (17). The mixture was then pipetted onto triplicate bottom agar plates for each host. After incubation at 37^0 C for 18 hours, plaques were counted (49).

Vibrio spp. were quantified by a MPN procedure using five tubes, and four dilutions of alkaline peptone water which were incubated at 35^0 C for 16 hours. Tubes were then transferred to TCBS agar plates and incubated for 24 hours at 37^0 C. Green or yellow colonies were presumed positive for *Vibrios* (49).

Enteroviruses were recovered from a 50 gram clam homogenate sample utilizing an adsorption-elution-precipitation method modified by a terminal clarification step (49). Samples of concentrated virus extract were inoculated into a series of 75 cc flasks containing confluent layers of MA-104 (rhesus monkey kidney derived) and Buffalo Green Monkey kidney (African Green Monkey kidney derived) cell cultures. After a 1 hour adsorption period, cultures were supplemented with maintenance medium and incubated at 37 C for 14 days. The cells were checked frequently for virus-induced cytopathogenic changes (CPE). Cell cultures were frozen after 14 days, then pooled lysates derived from each line of cells were inoculated into the other cell culture line for 14 days. Cell cultures which exhibit CPE were confirmed for the presence of enteroviruses by repassage of 0.1ml of cell lysate into a new culture of the same cell line that demonstrated cytopathology (49).

CHAPTER III
RESULTS

Clam microbiology

Clam microbiology data from RI and NC were log transformed to approximate normality. Geometric means and arithmetic ranges were reported. Differences between geometric means of organisms per 100 grams clam meat in the Narragansett Bay vs. Depurated samples were analyzed utilizing the Wilcoxon Rank Sum test (2-tailed). The p-value was calculated at the 95% confidence level, (Table 3).

TABLE 3 Microbiologic Analysis of Shellfish [1]

	N	Site 1, Mean (Range)	Site 2, Depurated Mean (Range)	Site 1 vs. 2 Mean [2]	p-value [3]
Total coliforms, MPN (RI)	12	71.54 (2-700)	8.59 (2-33)	0.92	0.05
Total coliforms, MPN (NC)	14	4.86E4 (5.0E3-2.3E5)	5.200E4 (5.0E3-2.3E5)	-0.03	0.90
Fecal coliforms, MPN (RI)	14	1.97 (<1.8 - 9)	1.54 (<1.8 - 4.5))	0.11	0.55
Fecal coliforms, MPN (NC)	14	5.04 (<9-9)	5.65 (<9-20)	-0.05	0.94
E. coli, MF(RI)	14	<50 (<50)	<50 (<50)	0.0	0.99
E. coli, MF(NC)	14	30.16 (<17-1050)	22.96 (<17-167)	0.12	0.90
Enterococci, MF (RI)	14	<50 (<50)	<50 (<50)	0.0	0.99
Enterococci, MF (NC)	14	36.95 (<17-1017)	16.93 (<17-717)	0.34	0.17
Enterococci, MPN (RI)	14	8.74 (<1.8-445)	1.72 (1-11)	0.71	0.06
Enterococci, MPN (NC)	14	81.32 (9-700)	83.45 (<9-1500)	-0.01	0.99

TABLE 3, cont. Microbiologic Analysis of Shellfish [1]

	N	Site 1, Mean (Range)	Site 2, Depurated Mean (Range)	Site 1 vs. 2 Mean [2]	p-value [3]
Enterococci, BEAA (RI)	12	10.10 (<1.8-88)	1.74 (1-11)	0.76	0.08
Enterococci, BEAA (NC)	12	119.57 (9-1500)	185.00 (<9-2900)	-0.19	0.81
C. perfringens, MF (RI)	14	29.25 (<50-75)	<50 (<50)	0.07	0.40
C. perfringens, MF (NC)	14	<17 (<17)	<17 (<17)	0.0	0.99
C. perfringens, MPN (RI)	14	106.08 (10-703)	11.79 (2-500)	0.95	0.03
C. perfringens, MPN (NC)	14	17.49 (<9-150)	10.02 (<9-110)	0.24	0.39
Coliphage Assays:					
-E. coli F Amp, (RI)	14	39.60 (<5-627)	3.30 (<2.5 - 44)	1.08	0.04
-E. coli F Amp, (NC)	14	48.97 (<12-1250)	14.03 (<8-62)	0.54	0.22
-E. coli C, (RI)	14	21.89 (<5 -275)	2.18 (<2.5 - <5)	1.00	0.004
-E. coli C, (NC)	14	49.15 (10-700)	22.03 (<8-1400)	0.35	0.48
-S. typhimurium (RI)	14	33.07 (<5 - 1090)	3.07 (3.3- 8)	1.03	0.04
-S. typhimurium (NC)	14	49.17 (<12-1225)	11.69 (<8-120)	0.62	0.29

Table 3, cont.

Analyses performed Only in NC[1]	N	Site 1, Mean (Range)	Site 2 Depurated Mean (Range)	Site 1 vs. 2 Mean[2]	p-value[3]
Aerobic Plate Count	14	4.69E7 (1.32E7-2.84E8)	4.06E7 (2.02E6-6.57E8)	0.06	0.99
Vibrio sp.	14	3.62E4 (400-1.6E6)	6.51E4 (900-1.6E6)	-0.25	0.70
Salmonella	13	<2.0 (<4 - <6.7)	<2.0 (<4 - <6.7)	-0.004	0.99
Shigella	13	<2.0 (<4 - <6.7)	<2.0 (<4 - <6.7)	-0.004	0.99
Enterovirus	14	<2.0 (<2)	<2.0 (<2)	0.0	0.99

1 = Organisms/100 grams, geometric means, arithmetic ranges

2 = Difference between means of logged values; positive = Site 1 > Site 2

3 = p-value based on Wilcoxon rank sum test (two-tailed) applied to log data, Site 1 vs. Site 2

Significant differences in mean levels of indicator organisms between Narragansett Bay and Depurated clams were noted for samples analyzed at the harvest site in Rhode Island (RI). Of the bacterial indicators, total coliforms and *C. perfringens* (by the MPN method) were significantly different at the 95% confidence level. All three coliphage assays demonstrated significant differences in coliphage levels between Narragansett Bay and Depurated clams when measured in RI, suggesting that our extended depuration times (30 days in this case) were effective in reducing coliphage indicators as well as total coliforms and *C. perfringens*.

Differences between mean levels in these indicator organisms were not found after transport to North Carolina (NC) despite strict transport protocol adherence to minimize time-temperature abuse, which is known to degrade shellfish quality (8). In addition, there were apparent increases in levels of total coliforms, Enterococci (MPN, BEAA methods) and coliphages based on NC assay results compared to those in RI assays.

The apparent increase in number of coliphages before and after transport may be explained by differences in technique between the two sites. Those detected in S typhimurium WG49 and *E. coli* F-Amp require F-pili to reproduce, and it its known that F-pili are only produced by actively growing bacteria at > 30^{O} C. Hence, these F-specific coliphages cannot replicate in an environment that is not heavily contaminated with feces. SOMCPH phages are believed to be more able to reproduce in the environment (16). *C. perfringens* (MPN method) data support the argument of variations in technical procedures since mean levels of this organism are lower in NC than those measured in RI.

Volunteer characteristics

Subjects who entered the study, but were found later to possess a characteristic that would normally have excluded them, were excluded from the analysis. Exclusion criteria included subjects with severe chronic disease or disorders of the gastrointestinal tract, hepatobiliary, or renal / urinary systems. Subjects could not currently be taking antibiotics, immunosuppressive drugs or chemotherapeutic agents. Subjects were asked to refrain from ingestion of other raw, molluscan shellfish for 30 days before and after the feeding date. Participants must have been at least 18 years of age. All subjects agreed to return 4 follow-up questionnaires at 1 week, 2 weeks, 4 weeks and 8 weeks after the feeding date.

A total of 30 persons were excluded for systemic antimicrobial therapy. Also excluded were 11 persons who failed to return any follow up questionnaires. A total of 5 subjects ingested raw shellfish in addition to the controlled exposure of the study. Two persons with hepatitis at the time of recruitment were excluded. One subject was excluded for active Giardiasis, 1 for systemic corticosteroid therapy and 1 subject for lack of a colon. One subject did not meet the minimum age requirements of the study and 1 provided no information on age. Therefore of the 985 persons recruited, 53 persons were excluded for 56 instances of not meeting selection criteria or for nonresponse to follow-up questionnaires. A total of 932 subjects (94.6% of initial subjects recruited), were included in final analysis after exclusions and deletions (Table 4).

EXCLUSIONS, Table 4

1	Age<18
1	No age recorded
1	No colon
1	Giardia infection
1	Systemic corticosteroid therapy
2	Hepatitis at the time of initial questionnaire
5	Ingested raw shellfish in addition to clam study feeding
11	No questionnaire returns
30	Systemic antimicrobial therapy
53	Total exclusions

A total of 932 subjects met all selection criteria and returned at least one follow-up questionnaire. Of these 932, 5 subjects (0.54%) returned only 1 questionnaire, 7 subjects (0.75%) returned only 2 questionnaires, and 18 subjects (1.93%) returned only 3 questionnaires. Of the 932 subjects included in the analysis, 902 subjects (96.78%) returned at least 4 questionnaires.

Subjects were similar across exposure groups with respect to age, sex, initial number of stools per day and number and ages of children as recorded in the initial questionnaire (Table 5). Mean ages, initial number of stools and number of children were analyzed using Student's T-test or Satterthwaite's T-test. Gender of subjects was analyzed by the X^2 test. There were no significant differences at the 95% confidence level between groups in these variables.

TABLE 5
INITIAL CHARACTERISTICS OF SUBJECTS IN CLAM STUDY

	NARRAGANSETT	DEPURATED	CONTROLS
N	365	384	183
Age, years (mean±SD)	31.45 ±10.74	31.45 ±11.16	31.07 ±9.21
Sex = Female (%)	57.0	51.0	59.6
Stools/day (mean±SD)	1.27 ±0.56	1.21 ±0.53	1.21 ±0.56
Age of 1st child (N, mean±SD)	119, 13.18±9.83	139, 12.55±10.99	51, 12.29±8.17
Age of 2nd child (N, mean±SD)	71, 16.86±10.02	83, 17.80±10.09	28, 18.36±7.94
Age of 3rd child (N, mean±SD)	20, 23.70±10.05	24, 23.71±9.62	7, 28.57±7.25

Initial N=985
Final N=932 after exclusions and deletions

94.62% of initial subjects included in final analysis

Rates of illness

Rates of gastrointestinal illness (diarrhea, vomiting, abdominal pain and credible illness) were calculated for the 3 study groups. No subjects reported jaundice. Diarrhea, vomiting and abdominal pain were all self defined and self reported by the subjects. Credible illness was defined by a positive report of any two of three types of illness in the same questionnaire by one individual subject. For example, diarrhea *and* abdominal pain or vomiting *and* diarrhea would be scored as credible illness in that subject.

Questionnaires from the first week of follow-up were analyzed separately from weeks 2-8 to identify probable viral gastroenteritis or *Vibrio sp.* gastroenteritis that would occur within approximately 4 days of exposure. We excluded those with pre-existing gastrointestinal disease from illness analyses for the first week of follow-up. A total of 6 individuals were excluded from the first week's analysis due to lack of information about initial stool characteristics. No subjects reported 3 or more loose stools per day (our case definition of diarrhea) on the initial subject questionnaire, so none were excluded for pre-existing diarrhea.

Statistical analysis revealed significant differences at the 95% confidence level in rates of diarrheal illness during the first week of follow-up between those subjects in the High Exposure group compared to either controls or those in the Low Exposure category. Probability values were calculated utilizing the Fisher Exact test and the Mantel-Haenszel X^2 test respectively. (Table 6).

Relative risk of gastrointestinal illness was compared among the 3 study groups.(Table 6) Subjects in the High Exposure group were almost 3 times more likely to develop diarrhea during the first week of follow-up than controls. In addition, High Exposure group subjects were approximately 2 times more likely to experience diarrhea during the first week of follow-up than Low Exposure group subjects

TABLE 6
ILLNESS RATES FOLLOWING SHELLFISH INGESTION

1-7 days (6 Exclusions)

				Relative Risk (95% C. I.)		
Illness	Control	DEP	NAR	N vs. C	D vs. C	D vs. N
Diarrhea	2.22%	2.88%	6.35%	2.86 * (1.00-8.14)	1.30 (0.42-4.01)	2.21 ** (1.09 4.46)
Vomiting	0.55%	0.26%	0.28%	0.50 (0.03-7.90)	0.47 (0.03-7.49)	1.06 (0.07-16.81)
Abdominal Pain	3.89%	4.71%	5.25%	1.35 (0.58-3.15)	1.21 (0.52-2.85)	1.11 (0.59-2.09)
Credible Illness	1.67%	1.05%	1.93%	1.16 (0.30-4.43)	0.63 (0.14-2.78)	1.85 (0.55-6.26)

No significant difference (at the 95% confidence level) in illness rates between groups was found during analysis of the 2-8 week follow-up data. (Table 6, cont.)

Table 6 cont.

8-60 days

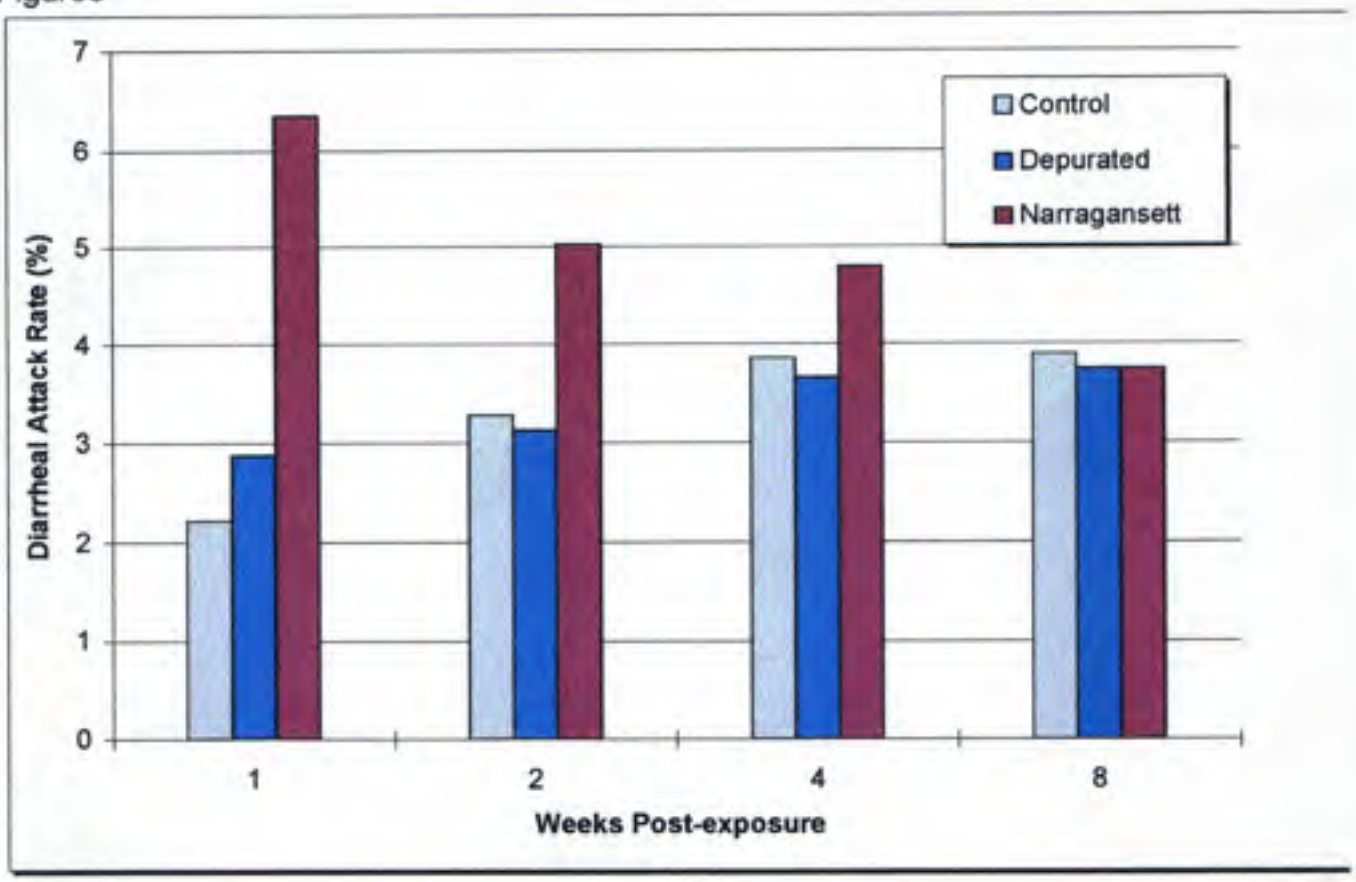
Illness	Control	DEP	NAR	Relative Risk N vs. C	D vs. C	D vs. N
Diarrhea	3.67%	3.51%	4.52%	1.23 (0.74-2.06)	0.96 (0.56-1.62)	1.29 (0.85-1.95)
Vomiting	0.92%	1.05%	0.66%	0.72 (0.23-2.25)	1.15 (0.41-3.24)	0.63 (0.25-1.58)
Abdominal Pain	3.85%	3.07%	3.01%	0.78 (0.46-1.34)	0.80 (0.47-1.36)	0.98 (0.61-1.57)
Credible Illness	1.47%	1.84%	1.70%	1.15 (0.51-2.64)	1.25 (0.56-2.82)	0.92 (0.49-1.72

* = $0.01 < p < 0.05$, Fisher Exact Test

** = $0.01 < p < 0.05$, Mantel-Haenszel X^2 Test

Diarrheal illness attack rates for each group are graphically illustrated in (Figure 3).

Figure3



Relationship between volunteer illness rates and clam microbiology

Rates of diarrheal illness in exposure groups were analyzed with clam microbiology data from the RI harvest site (Table 7) and the NC feeding site (Table 8) to determine if specific microbial indicators of fecal contamination were associated with subsequent development of diarrheal illness in the first week of follow-up in exposed subjects.

Microbial indicators levels in clams were generally categorized into nondetectable (< values) / detectable levels. In the case of total coliforms and *Vibrio* sp. of bacteria, three categories were created in an attempt to be more representative of the large range of measured values. Once levels of all the microbial indicators measured in the shellfish analysis were established, then rates of diarrheal illness in the subjects (exposed to those same batches of shellfish) were calculated for each level. The Fisher Exact test was utilized to calculate probability values to determine if the rates of illness associated with each level of microbial indicator were significantly different at the 95% confidence level.

TABLE 7

Clam Quality As A Predictor of Diarrhea (0-7 days) in Volunteers

Clam Quality **(RI harvest site)**

Indicator	Organisms/100 grams	Ill subjects/ total subjects (%)		p-value
Total coliforms, MPN	<4	9 / 302	(3.00%)	p=0.22
	≥4	25 / 530	(4.72%)	
Fecal coliforms, MPN	<230	38 / 924	(4.11%)	p=0.99
E. coli, MF	all<50	38 / 924	(4.11%)	p=0.99
Enterococci, BEAA	<1.8	12 / 451	(2.66%)	p=0.03
	≥1.8	20 / 357	(5.60%)	
C. perfringens, MPN	<4	9 / 297	(3.03%)	p=0.25
	≥4	29 / 627	(4.63%)	
C. perfringens, MF	<50	35 / 887	(3.95%)	p=0.19*
	≥50	3 / 37	(8.11%)	
Coliphage E. coli C	<5	20 / 619	(3.23%)	p=0.55
	≥5	18 / 305	(5.90%)	
Coliphage S. typhimurium	<5	21 / 602	(3.49%)	p=0.19
	≥5	17 / 322	(5.28%)	
Coliphage E. coli F Amp	<5	21 / 602	(3.49%)	p=0.19
	≥5	17 / 322	(5.28%)	

p values based on Mantel-Haenszel X^2 test

* p value based on Fisher Exact test, 2-tailed

Data collected at the RI harvest site, demonstrate that the presence of enterococci (BEAA method), were significantly associated with subsequent development of diarrheal illness in subjects during the first week of follow-up.

Clam Quality As A Predictor of Diarrhea (0-7 days) TABLE 8

Clam Quality (NC FEEDING SITE)		**Ill subjects/**		
Indicator	**Organisms/100 grams**	**total subjects (%)**		**p-value***
Total coliforms, MPN	<10,000	9 / 284	(3.17%)	X^2 test for
	≥10,000 &			trend
	<100,000	23 / 524	(4.39%)	p=0.31
	≥100,000	6 / 116	(5.17%)	
Fecal coliforms, MPN	<230	38 / 924	(4.11%)	p=0.99
E. coli, MF	<17	10 / 378	(2.65%)	p=0.06
	≥17	28 / 546	(5.13%)	
E. coli, MF	<330	36/ 877	(4.10%)	p=0.99**
	≥330	2 / 47	(4.26%)	
E. coli, MF	<17	10./ 378	(2.65%)	X^2 test for
	≥17 &			trend
	<100	17 / 389	(4.37%)	p=0.02
	≥100	11 / 157	(7.01%)	
Enterococci, BEAA	<9	8 / 233	(3.43%)	p=0.61
	≥9	25 / 575	(4.35%)	
Enterococci, MF	<17	19 / 558	(3.41%)	p=0.18
	≥17	19 / 366	(5.19%)	
C. perfringens, MPN	<9	20 / 505	(3.96%)	p=0.80
	≥9	18 / 419	(4.30%)	

Clam Quality As A Predictor of Diarrhea (0-7 days) TABLE 8 page-2-

Clam Quality (NC FEEDING SITE)		**Ill subjects/**		
Indicator	**Organisms/100 grams**	**total subjects (%)**		**p-value***
C. perfringens, MF	<17	38 / 924	(4.11%)	p=0.99
Coliphage E. coli C	<12	19 / 472	(4.03%)	p=0.89
	≥12	19 / 452	(4.20%)	
Coliphage S. typhimurium	<12	22 / 512	(4.30%)	p=0.75
	≥12	16 / 412	(3.88%)	
Coliphage E. coli F Amp	<12	17 / 460	(3.70%)	p=0.53
	≥12	21 / 464	(4.53%)	
Aerobic Plate Count	<5E7	26 / 652	(3.99%)	p=0.77
	≥5E7	12 / 272	(4.41%)	
Vibrio sp.	<1E3	10 / 296	(3.38%)	X^2 test for trend
	≥1E3 & <1E5	18 / 355	(5.07%)	p=0.84
	≥1E5	10 / 273	(3.66%)	

*p values based on Mantel-Haenszel X^2 test

**p values based on Fisher's Exact test, two-tailed

E. coli (enumerated by the MF method at the feeding site in N.C.) were analyzed by three different categorical methods: detect / nondetect levels, <330 / ≥330, (data not shown) and then by 1 log differences. When *E. coli,* , was categorized into 3 levels, X^2 analysis for trend indicated that there was a significant association ($p<0.05$) between *E.coli* and diarrheal illness. The NSSP guideline for E. coli contamination is <330 organisms per 100 grams clam meat. Only 1 clam sample derived from Narragansett Bay was found to exceed this value, with only 2 of 47 persons who ate these clams developing illness in the first week of follow-up.

All statistical analyses were performed with SAS software, Cary, North Carolina and Epi Info software, Stone Mt., Georgia.

CHAPTER IV
DISCUSSION

The clinical trial design optimized measurement of exposure of subjects to shellfish-associated pathogens. Previous reports of shellfish associated illness have either described outbreak investigations (19, 50, 45, 22) or individual case reports (23, 24, 46). To our knowledge, this was the first study to control human exposure to these pathogens via ingestion of raw clams in a clinical trial. Our follow-up period was designed to encompass the incubation period of gastrointestinal illness caused by study exposure to Norwalk and Norwalk-like viruses as well as *V. parahaemolyticus* and HAV. The pattern of diarrheal illness reported during the first week of follow-up, which was significantly associated with exposure to clams harvested from Narragansett Bay, was consistent with the clinical picture of viral or *Vibrio* gastroenteritis.

There was no significant association between levels of *Vibrio* sp. contamination and subsequent diarrhea in the first week of follow-up. If vibrio species were indeed important etiologic agents of clam associated gastroenteritis, the lack of association with Vibrio numbers may be due to the phenomenon of the viable but non-culturable state. *Vibrio sp.* may enter into a quiescent, but still potentially infective form in response to hostile environments, such as the marine environment (48). This phenomenon might cause underestimation of the actual levels of *Vibrios* present in the clams.

By investigating microbial quality of the shellfish, it was possible to examine the relationship between traditional and proposed indicator bacterial levels and risk of illness. The data suggest that measurement of Enterococci in clams at the time of harvest, may be a useful indicator of enteric virus or *Vibrio* contamination of the shellfish. In addition, *E. coli,* although nondetectable when measured in the clams at the harvest site, proved to be significantly associated with risk of diarrheal illness when clams were analyzed after transport to the feeding site.

Although F-specific bacteriophages have been correlated with enteric virus concentrations in environmental samples (18), there was no significant association between detectable levels of F-specific coliphage in the clams and risk of diarrheal illness in the subjects who ate them.

Limitations

In any study involving volunteers, self selection bias may present a problem as far as generalizability is concerned. Our study may have attracted volunteers who were already habitual consumers of raw shellfish and viewed our study as an opportunity to obtain a 'free' shellfish meal. If frequent shellfish consumers develop protective levels of antibody to hepatitis A virus, *Vibrio sp.*(26) or other shellfish associated pathogens, illness rates in these volunteers may be lower than rates would be in a population composed of persons with no or low exposure to raw shellfish. These variations in immunity which may be found in frequent shellfish consumers may differ from the target population, thus rates of illness associated with shellfish consumption in the general population may be higher.

The working case definition of diarrhea was $\geq$3 loose stools/day. This definition was used to categorize subjects in the initial questionnaire to determine if they would be included in the first week of analyses or would be excluded because of pre-existing diarrhea. No persons met our definition of pre-existing diarrhea. During the follow-up questionnaires, no data were gathered for stools/day. However, data were gathered for diarrhea as a dichotomous variable (yes, no). If persons reported yes for diarrhea in the initial questionnaire, but were not excluded for pre-existing diarrhea due to not meeting our case definition, there was a potential for misclassification of illness dependent on the subject's responses during the follow-up period. There might be potential for misclassification bias if these persons continued to report diarrhea for the first week of follow-up. Their continuing illness would be recorded as illness following exposure. If persons with pre-existing illness in the Control group reported diarrheal illness in the first week of follow-up, this would tend to bias our results towards the null. . If persons with pre-existing illness in the Exposure groups reported diarrheal illness in the first week of follow-up, this would tend to bias our results away from the null.

In order to assess the effect misclassification of pre-existing illness may have had on follow-up illness rates, all persons who reported 'y' to the variable diarrhea were identified and their follow-up responses examined. Seven persons self-reported diarrhea when given a yes / no query, but were included due to lack of meeting the formal case definition of diarrhea. Upon examination of the follow-up illness rate data, of the 7 persons who reported 'yes' for diarrhea in the initial questionnaire, only 2 reported 'yes' for diarrhea in any follow-up questionnaire. One of these subjects was randomized to the Control group

and reported diarrhea in the third week of follow-up. The second subject was included in the Low Exposure group and reported diarrhea in the first week of follow-up. A total of 11 persons in the Low exposure group reported diarrhea in follow-up questionnaire 1. Nine percent of this category, then, may be affected by misclassification of illness. There was no way to assess by these data if that subject's diarrhea was continuous or was separated by some days between responses in the initial and follow-up questionnaires.

In addition, we wished to validate reports of diarrheal illness to ascertain how closely subject self-reports of diarrheal illness met our case definition. The diarrheal frequencies and comments from the first 250 subjects (27% of total subjects) enrolled in our study were examined. This subset of subjects was determined to be similar to the entire study group on the basis of age and baseline stools/day (Student's T test or Satterthwaite's T-test, 95% confidence level). A total of 38 of these subjects reported diarrhea at some time during the follow-up period. Ten of these (26%), did not report frequency of diarrhea. Of the 28 individuals who did report frequency of diarrhea, 17 (or 61%), reported 3 or more episodes of diarrhea per day. Eleven (39%) recorded 'y' for diarrhea, but reported <3 episodes of diarrhea per day, thus not meeting our initial case definition. In future studies this potential for misclassification of illness might be avoided by printing the case definition by the affirmative choice on the questionnaire.

A source of confounding may be other exposures to agents of gastroenteritis or hepatitis through water, person to person transmission or other foods. Norwalk virus has been associated with consumption of raw shellfish, but in 1992, Lefkowitz (26) reported no significant difference in seroprevalence between 3 groups of subjects chosen for high, moderate and no shellfish consumption. All groups were approximately 70% seropositive to Norwalk virus, strengthening the argument that contaminated water, the environment and person to person contact may be the primary routes of exposure. Our subjects were randomized to exposure groups. Analysis of subject characteristics reveal no significant difference at the 95% confidence level between mean exposure group age, sex, baseline stool frequency and number of children, suggesting thorough randomization of subjects. Presumably, randomization helped eliminate confounding effects from variables that may affect rates of illness such as age, presence of preschool children in the household or source of drinking water.

Control subjects were not blinded as to exposure status due to the inherent organoleptic changes that occur when pathogens are eliminated in shellfish meat by heat, irradiation or other means of sterilization. Because of this limitation, we chose not to feed our controls rather than chance an imperfect sterilization procedure which may introduce misclassification bias. Since the controls were not blinded, differential reporting of subsequent illness may have occurred between the control and exposed subjects, with exposed subjects being more likely to anticipate, monitor and report disease. This differential reporting of illness would tend to bias our results away from the null.

It is important to note that the depurated clams are not representative of depurated clams which may be available commercially. The clams in this study were depurated for a minimum of 30 days. Commercial depuration facilities depurate shellfish for a total of 48-72 hours. Studies of shellfish depurated for this shorter period of time have suggested that depuration does not significantly decrease enteric virus levels. However, subjects who ate the depurated clams of this study, had significantly lower rates of diarrheal disease compared to subjects who ate the nondepurated clams although the clams were derived from a common source. These results suggest that long-term (30 day) depuration of shellfish, significantly reduces the consumer's risk of exposure to gastrointestinal pathogens. Furthermore, commercial depuration facilities are allowed to utilize clams from growing waters classified as restricted. In contrast, the depurated clams of this study were derived from the same approved waters in Narragansett Bay as those in the High Exposure category. Therefore, we started with cleaner clams than do most commercial facilities. The combination of a cleaner source of shellfish before depuration in addition to depuration for 30 days, probably resulted in our Low Exposure category clams containing fewer enteric pathogens than commercially depurated clams. Thus the risk of illness associated with depurated clams of this study may be an underestimate of the risk of gastrointestinal illness associated with consumption of commercially depurated clams.

CHAPTER V
CONCLUSIONS

The results of this study suggest that there is an almost 3 times greater risk of gastrointestinal illness from the consumption of raw clams from NSSP approved sources, compared to no shellfish consumption. In addition, the risk of illness in those who ingested clams depurated for 30 days was not significantly different than the baseline risk in the non-consuming control group. The timing of diarrheal illness was suggestive of Norwalk or Norwalk-like virus or *Vibrio* gastroenteritis, onset of which would be expected within the first 4 days after exposure. The risk of HAV infection was less clearly elucidated since none of the volunteers developed jaundice or statistically significant nausea or vomiting after the initial first week of follow-up. The follow-up period was sufficient (8 weeks) to detect new cases of HAV (incubation period of 3-6 weeks) associated with our raw clam exposure.

For clam microbiology data collected at the RI harvest site, the presence of Enterococci (BEAA method), were significantly associated with the subsequent development of diarrheal illness in subjects during the first week of follow-up, suggesting that this proposed indicator may be useful in the regulation of shellfish sanitation. Since these analysis examined nondetect / detect levels of Enterococci, more work needs to be performed to elucidate unsatisfactory levels of Enterococci in shellfish after harvest.

There was a statistically significant X^2 analysis for trend in the enumeration of *E. coli* (enumerated by the MF method at the feeding site in N.C.), when analyzed with rates of diarrhea in the first week of follow-up. Categorization of the organism into nondetect / detect levels was not significantly associated with development of diarrheal illness in volunteer subjects. Since our clams had low levels of E. coli, only 1 sample was greater than the NSSP limit for contamination of <330 organisms per 100 grams clam meat. Only 47 subjects were exposed to these clams with 2 subsequent diarrheal illnesses in the first week of follow-up. So, although this last type of categorization seemed obvious from a regulatory standpoint, we did not have the power in our study to detect a significant association. Therefore the role of *E. coli* as an indicator of enteric virus or *Vibrio* contamination of shellfish, is unclear in our study.

Levels of total coliforms, *C. perfringens* (by the MPN method), and all three coliphage assays were significantly different in depurated vs. nondepurated clams derived from approved growing waters, when measured in RI. The data suggest that these indicators may be useful in assessing depuration efficacy of enteric viruses and *Vibrio sp.* in clams. The subjects who consumed depurated clams experienced rates of gastrointestinal illness similar to controls, suggesting that the extended depuration time (30 days) was effective in reducing pathogen levels as well as indicator species.

Our study results suggest that extended depuration times may be efficacious in reducing the risk of shellfish-associated illness for those persons who choose to eat raw clams. Other measures of shellfish sanitization such as irradiation or thorough cooking, change the texture of the shellfish and are objectionable to some consumers. Extended depuration times, though costly, may provide consumers what they desire; safer raw shellfish.

It is important to note that the shellfish derived directly from Narragansett Bay were representative of shellfish that might be purchased at a restaurant or retail outlet. This factor combined with a minimally restricted study population should enhance the generalizability of the study's findings to all healthy consumers of raw clams.

Improved shellfish sanitation regulations accompanied by strict enforcement, are essential to reduce shellfish associated morbidity and mortality. Special precautions are needed to protect the growing numbers of chronically ill and immunosuppressed persons in the population. In the immediate future as our population ages, and persons who are now Human Immunodeficiency Virus seropositive develop Acquired Immunodeficiency Syndrome, the number of persons at highest risk from shellfish acquired illness will only increase. To meet this growing public health challenge it is necessary to more accurately characterize the risk associated with consumption of raw shellfish so that appropriate policy measures may be implemented.

Ideas for future investigation

Obtain paired serologies to Vibrio sp., HAV, Norwalk virus before and after exposing subjects to shellfish. Seroprevalence rates in the study population may then be compared

to rates described in other populations. This would aid in assessment of the external validity of the study. Such information would facilitate calculation of a more precise estimate of the risk of gastrointestinal illness associated with consumption of raw shellfish.

Levels of enterococci enumerated by the MPN (BEAA) method in this study's depurated clams were significantly associated with subsequent diarrheal illness in the first week of follow-up in those subjects exposed to them. This association was based on detect / nondetect levels of enterococci. Further studies are needed to characterize enterococci as an indicator of shellfish microbiological quality.

It would be informative to have future studies investigate the health risks associated with ingestion of raw shellfish that has been extensively depurated. This may be a promising technique that could minimize the risk of gastrointestinal illness for raw shellfish lovers.

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