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Microbial Hazards Of Diving In Polluted Waters

A Maryland Sea Grant Publication
University of Maryland

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A Proceedings

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A Maryland Sea Grant Publication
University of Maryland
College Park

Publication Number
UM-SG-TS-82-01

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Welcoming Remarks

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Historically, NOAA's interest in polluted waters has focused on the effects of human activities on the world's oceans. But we have increasingly become concerned with the impact of polluted waters on our divers. In the early days, we attempted to treat the GI tract of our divers by rinsing out the tract with cognac or rum to remove the nasty taste of protective polluted water—a crude practice that didn't always work. More comprehensive protective efforts on our part seemed in order. With the help of the Undersea Medical Society and the NOAA medical review board, we instituted a program to better safeguard our divers. In general, the program has been effective; our divers have not had serious health problems. In the last several years, however, our activity in polluted waters has increased, especially in studies related to sewage dumping in the New York Bight. One project I was recently asked to review proposed to put divers in the Bight to about 60 feet and dump sludge on them from the sewage barge in order for the diver to sample the water during the dump and to film the dumping action underwater. In light of such proposals, we at NOAA chose to support research at the University of Maryland and the Naval Medical Research Institute to determine what kinds of microbial hazards exist and what the potential impact would be on the divers. Within NOAA, support came from the MUST Office, now the NOAA Undersea Material Program Office, directed by Jerry Umback of the NOAA diving office. In addition to NOAA's support, this workshop is also supported by the National Institute of Occupational Safety and Health (NIOSH), which has a charter interest in the health of workers during their occupational activities.

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Foreword

R.R. Colwell and **S.W. Joseph**

In response to an increased interest in problems engendered by our changing environment, a workshop was organized to examine microbial hazards encountered by divers in polluted waters, sponsored jointly by the National Oceanic and Atmospheric Administration (NOAA) and the National Institute for Occupational Safety and Health (NIOSH). The workshop, held in January 1981 at the headquarters of the Undersea Medical Society, Bethesda, Maryland, was attended by 35 scientists and engineers interested in the microbiology of polluted waters related to the safety of diving operations.

The first of four sessions was devoted to the operational aspects of diving. William Phoel, John J. Pennella, and R.L. Sphar discussed the equipment used in diving, procedures for decontamination, and illness episodes encountered among divers. A recurrent problem is that diving equipment that provides the maximum degree of safety for the diver also curtails his freedom of operation.

The later sessions on medical microbiology and the microbiology of polluted waters dealt with the major classes of microorganisms in waters, their importance as pathogens and as indicators of pollution, and their survival in the environment. Sheldon F. Gottlieb, James C. Coolbaugh, Emilio Weiss, Pierre-Marc Daggett, and Mark D. Sobsey reviewed, respectively, the problems of pollution associated with microorganisms, and current research on *Aeromonas, Legionella,* protozoa, and viruses. Sam Joseph and Rita Colwell discussed ongoing programs of research on the microbiology of polluted waters, as well as methodology used to assess the potential virulence of the isolated microorganisms. The hazards these microorganisms pose for divers was emphasized. Benzion Cavari, Richard W. Attwell, James Kaper, and Jorge Crosa reported on the various types of microorganisms, including eubacteria, Actinomycetes, and related species, found in estuarine and coastal waters. Techniques, both biochemical and genetic, which are employed for rapid characterization of microbial activity and those factors most commonly associated with virulence of water-borne pathogens were described.

Microbial pathogens—bacteria, viruses and parasites—present in polluted waters clearly pose potential hazards for divers. However, the magnitude of infectious disease problems for the diver needs to be established and new methods developed to assess the extent of microbial hazard(s) at a given diving site if cost-effective guidance is to be offered operational personnel in their choice of appropriate protective equipment and safety procedures. Microbiological surveys of areas where extensive diving operations are carried out should be undertaken to provide information concerning incidence of potentially pathogenic microorganisms and relevant information on pathogenicity and virulence. Only after these data are gathered and analyzed will it be possible to recommend procedures and equipment for minimal risk to divers who must carry out operations in polluted waters.

The workshop was notably successful in bringing together laboratory and field scientists, working divers and medical personnel to assess the risks and dangers of diving in polluted waters and to address the important problem of protective equipment. As to be expected, many questions were raised. For example: What changes do human pathogens undergo in the aquatic environment? Do they remain virulent under varying conditions of salinity, temperature, dissolved oxygen and variable nutrient concentrations? How does the pathogen gain entry to cause disease in humans? What disinfection procedures are needed for gear and for support personnel who handle diving equipment but do not themselves enter the polluted water? What precautions should a diver take when he prepares to enter a heavily polluted area?

The conclusions reached at the workshop were that microbial hazards do exist and that rational approaches to the problem are being developed, both in health safety precautions on the part of the diver and in new gear designed to minimize risk to the diver when actually in the water. Clearly, a new area of environmental microbiology has been defined which can be of major significance for the working diver.

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Introduction: An Ecological Perspective

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This workshop marks the initiation of a new area of research, perhaps a new sub-discipline of microbiology. A few studies have been done, which align somewhat the topics covered here. For example, pathogenic microorganisms, *per se,* have been studied for decades and the fundamental mechanisms of pathogenicity have been pursued by many workers in laboratories around the world. Many microbial ecologists have looked extensively at the distribution of several of the species of interest to us, since the survival of pathogens in drinking water, streams, rivers, and estuaries is currently of great interest. However, we bring together in this workshop available information interfacing and interlocking pathogenic microbiology and microbial ecology in order to determine gaps in knowledge about potential health effects for humans entering a contaminated aquatic environment. The risks, in general, are not known and perhaps not even appreciated. Some information concerning microbial hazards for divers in polluted waters in available in the scientific literature, but it has not yet been brought together for synthesis and analysis. Part of our task is to gather available information, including anecdotal, so that an assessment of hazards to divers can be made and the lacunae in our knowledge clearly identified.

Studies have been carried out which attempt to assess health risks for recreation swimmers and pioneering work has been reported by Cabelli and his colleagues.[1,2] However, professional divers, i.e., individual working divers, are today, more or less, in the category of "experimental animal", when they enter polluted waters to work. They have a job to do, viz. retrieval of gear lost overboard or underwater repairs to make. Entering polluted areas, especially sewer outfalls, involves some health risk but how much is simply not yet known. Thus, risks and hazards for divers and mechanisms for protecting them are the subjects of our discourse.

Developmental work on masks, breathing apparatus, helmets, and safety equipment for diving in hazardous areas is in progress. Rudimentary disinfection of gear is practiced, but the efficacy of the equipment and the disinfection procedures is undocumented, to date.

The kinds of questions to be asked should be from an ecological perspective, that is, from the environmental viewpoint, querying the importance of interactions of microorganisms with their environment and the effects of those microorganisms on humans. Do microorganisms pathogenic for humans undergo significant morphological and metabolic alteration during short- or long-term exposure to the aquatic environment? What are the effects of salinity, temperature and/or organic nutrient concentrations on microorganisms in a given aquatic environment? Are there synergistic effects of toxic chemicals on pathogenic properties of microorganisms? In a mixed industrial waste dumped in streams, harbors, estuaries or the ocean, is there a selection of specific organisms that possess potentially pathogenic properties? These are, indeed, difficult, but important, questions that need to be phrased in the context of the hazards they pose to divers. They represent the larger issues and problems becoming increasingly more important in the context of undersea habitats and offshore industry.

A key issue highlighted by this workshop is that of joining basic and applied research in order to speed development of gear and procedures to aid divers and the diving industry. Fundamental questions of microbial ecology, pathogenicity, immunology, efficacy of drugs for waterborne diseases, and treatment of diver infections acquired during diving operations have to be answered and the answers will come from basic research and application of available technology. Because the number of divers working in polluted waters is increasing, and the bodies of water that are polluted are also increasing, the risks to which divers are exposed, therefore, must increase. It can be fairly asked of an employer what precautions his working divers should take and what pay is appropriate to the occupational hazard. Thus, basic research and available technology are needed to solve these real problems.

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Every environment, terrestrial or aquatic, supports a complex, dynamic microbial community structure. What is not always readily appreciated is that many microorganisms that are potentially pathogenic for man are natural inhabitants of the environment. The presence of pathogens in our environment usually is considered a negative, or "unnatural" event. In fact, the concept of what is a pathogenic organism ought to be considered according to the function(s) and role(s) of an organism in its natural habitat. There are, of course, many kinds of pathogens, including the obligate and the opportunistic.

A good example of the naturally occurring potential pathogen is found within the major group of bacteria, the *Vibrionaceae,* which are inhabitants of estuarine, coastal, and marine environments. In such environments, one may not detect *Escherichia coli* by the usual culturing methods employed, but many species of *Vibrio* will readily be isolated, depending on the salinity of the water. Some *Vibrio* spp. are pathogenic for man, if ingested, and others if a laceration or wound is infected. Both *Vibrio* and *Aeromonas* spp., members of the *Vibrionaceae,* will, under conditions of high nutrient concentrations and elevated water temperature, grow to population sizes that are very large, since they are naturally occurring in the aquatic environment. Naturally occurring aeromonads, as vibrios, can be pathogenic, under certain circumstances.

Pathogens not naturally occurring, but instead, entering bodies of water from an external source, such as a sewage, include a variety of enteric pathogens, viz. *Salmonella* and *Shigella* spp., members of the *Enterobacteriaceae.* Some species of *Enterobacteriaceae,* as for example, *Klebsiella* spp., may comprise part of the natural microbial flora in certain geographical areas. From these brief examples, it is clear that an understanding of the environment is important in order to recognize those organisms which are part of the ecosystem, but, yet, pose a health hazard, as well as those entering via domestic or industrial wastes.

Factors to be considered in determining which bacterial species will be predominant in a given environment include salinity, temperature, heavy metal concentrations, etc. For example, in Baltimore Harbor of the Chesapeake Bay, mercury-resistant bacteria are present in large numbers. The major bacterial group found in the water and sediment is *Pseudomonas.*[3,4] Species diversity among bacteria is much reduced in a polluted environment such as Baltimore Harbor, resulting in a selection of species such as *Pseudomonas* which may be pathogenic. In addition, the majority of metal-resistant bacteria may also be antibiotic-resistant,[5] an important characteristic if these bacteria prove to be opportunistic pathogens.

Aquatic microorganisms often demonstrate a seasonality in distribution, another factor important in assessing a health hazard. Divers may be in greater risk at certain times of the year.[6] For example, a recognized human pathogen, *Vibrio parahaemolyticus,* is present in large numbers in the water column of estuaries during the warmer months of the year, i.e., June through November.

The organism survives during the winter in sediment.[7] Thus, its distinct seasonality in distribution suggests that, during the late summer months, divers in estuarine or harbor areas should be aware of the possibility of infection with *V. parahaemolyticus* and related vibrios.

A characteristic of many aquatic microorganisms is their ability to attach to surfaces, including the respiratory mucosa of a diver. Many bacteria demonstrate very strong attachment to submerged solid surfaces, notably the shells of crabs, oysters, and other macrofauna of estuaries and coastal waters. Attachment may not necessarily be specific, with almost any surface serving as an attractive settling site for the bacteria. A diver would offer good surfaces for attachment, either the diver himself or his gear.

When an environment is altered by man's activities, there is a detectible, concomitant microbial response. Even the surface waters of the Puerto Rico Trench, into which pharmaceutical wastes, collected in barges and towed to the Trench surface waters, are dumped, will be impacted if the dumping is heavy enough and is practiced for many years. Changes in the microbial flora, in fact, have been recorded during and after such dumping,[9] with a dramatic change recorded in numbers of organisms growing on fresh water media, compared to salt water media, for water samples collected in the open ocean area receiving the pharmaceutical wastes. Most disturbing is the fact that potentially pathogenic organisms, predominantly the gram-positive organisms, including *Staphylococcus aureus,* a notorious pathogen for man, especially in hospital environments, can be isolated in large numbers from Puerto Rico Trench surface waters—proving that microbial community alterations can have health conseqences.[10] Thus, alteration of the microbial populations of river, harbor, coastal or ocean waters can occur upon dumping of chemicals, wastes, heavy metals, dredge spoil, etc. These materials can create a new hazard for human health if contact with such water occurs and it is the professional diver, in most cases, for whom the risk must be assessed.

In summary, only a few of the many conditions under which the aquatic environment becomes a human health risk have been touched upon. The extreme case, of course, is that of a sewer outfall releasing raw sewage into which working divers must enter for maintenance work or repairs. The health risk of these divers absolutely must be determined.

It is axiomatic that the human body can withstand a great deal of stress, including exposure to many microorganisms under normal conditions. When the abnormal situation arises, as when a diver must enter an outfall of raw sewage, the best knowledge available must be applied to protect that diver as completely and successfully as possible. This workshop is intended to achieve that goal.

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NOAA's Requirements and Capabilities for Diving in Polluted Waters

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Most of the mainline components of NOAA, such as the National Marine Fisheries Service (NMFS), the Environmental Research Laboratories (ERL), the NOAA Commissioned Corps, and the National Ocean Survey (NOS) in order to fulfill their mandated responsibilities, must send divers, at least occasionally, into waters that are considered polluted. These dives may be conducted for research purposes, including *in situ* experimentation, collecting and photographing substrate and associated fauna and flora, setting and retrieving equipment for hydrographic measurements, and placing arrays of animals in contaminated areas to determine rates of bioaccumulation. Much of the nonresearch diving, including identification of contracts made by sonar and wire-drag survey ships, installation and maintenance of current meters and tidal gauges, and ship husbandry, also takes place in polluted environments. The majority of NOAA dives are made close to the bottom or sometimes actually in the very soft sediments; because they accumulate contaminants and are usually environments conducive to microbial proliferation, the sediments provide divers the most exposure to these hazards. The exposure of divers to water-borne pathogens is probably the greater hazard, however. Exposure to solvents, herbicides, heavy metals, PCB's, petroleum hydrocarbons, and other organic and inorganic poisons, as well as cooling water from nuclear reactors, should also be considered.

The New York Bight and associated estuaries shown in Figure 1 are atypical of most estuarine/coastal systems in that they contain each of the aforementioned pollutants. These are among the most used, most impacted, and probably most studied systems in the country and therefore have served as test areas for equipment designed to protect NOAA divers from these pollutants.

Aside from the approximately 480 million gallons of raw sewage entering the estuary daily from the New York/New Jersey metropolitan area, three distinct dump sites exist within the New York Bight apex (Figure 1): the sewage sludge dump site receives 4.3 × 10^6 m^3/yr of sewage sludge, much of which is undigested or poorly treated; the dredge spoil dump site receives 4.6 × 10^6 m^3/yr, with spoils remarkably similar to the sewage

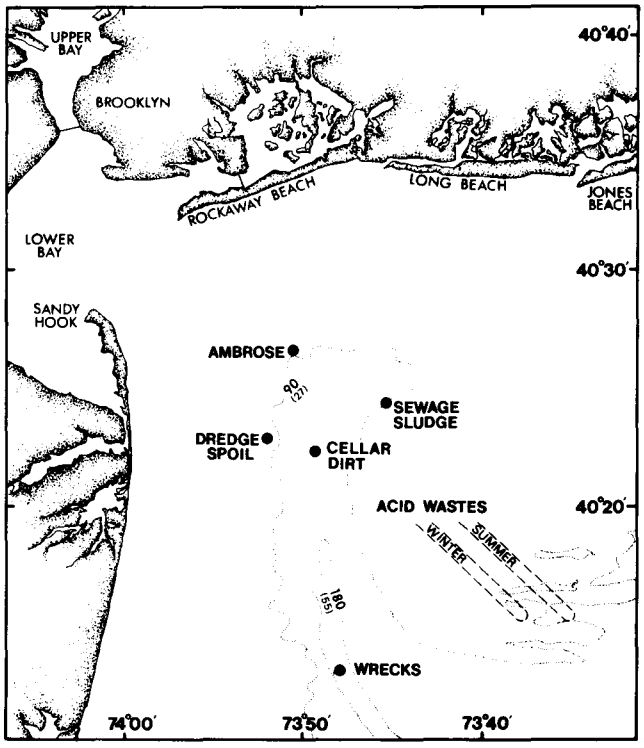

Figure 1. New York Bight apex and associated estuaries.

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sludge in composition; and the industrial acid waste area receives 2.7 × 10^6 m^3/yr.[1,2]

Correspondence in 1973 with the Food and Drug Administration of the Department of Health, Education, and Welfare reported heavy microbial loading, including pathogenic bacteria, human enteric viruses, and parasitic protozoa, at the sewage sludge dump site (G. Meyer, personal communication). This information prompted the Sandy Hook Laboratory, Northeast Fisheries Center, NMFS, which anticipated continuing an earlier research station at this site, to investigate ways of isolating divers from contaminated water. The criteria established for selecting helmets or masks for isolating divers were that they be capable of: 1) providing complete head isolation from polluted water; 2) connecting to a suit by water-tight connection; 3) demand and free-flow modes; 4) SCUBA or surface supply use; 5) providing good communications; and 6) that they be available for purchase "off-the-shelf." Suit selection criteria were established as 1) completely dry; 2) preferably with variable volume; 3) capable of mating dry to the demand/free-flow masks or hats; 4) swimmable; 5) easily washed and sanitized; and 6) commercially available "off-the-shelf."

The first system used by NOAA specifically for diving in polluted waters, i.e., the sewage sludge and dredge spoil dump sites in the New York Bight apex, was the Kirby-Morgan Band Mask (KMB) mated to a Viking variable volume dry suit as shown in the first photograph. (See Figure 2.) The skirt of the band mask is connected to the neck ring of the Viking suit by a clamp, resulting in a completely watertight seal. The free-flow mode prevents water from entering the mask through the second stage of the regulator by maintaining a higher than ambient pressure within the mask. This system has worked extremely well and is still NOAA's mainstay for diving in polluted waters.

The investigators at the Sandy Hook Laboratory, whose research would require them to dive in polluted areas, although experienced, NOAA-qualified SCUBA divers, had no experience with surface-supplied diving systems and techniques. To permit these investigators to use the KMB/Viking suit system safely and efficiently in support of their underwater research, I, as Laboratory Diving Officer, initiated a training program based upon my Navy and commercial diving experience.

The KMB/Viking suit system used to isolate the diver from microbial hazards is also effective in protecting the diver from hazards such as chemical and petrochemical contaminants.

The chemical industry and its associated hazardous waste disposal companies are highly concentrated in New Jersey around Bayonne Bay, Arthur Kill, and Raritan Bay. The chemical discharges of PCB's, chlorates, peroxides, acids, toluene, benzene, and other pollutants into these waters are transported to lower New York Harbor, Raritan Bay, and the New York Bight

Figure 2. Kirby-Morgan band mask and Viking variable volume suit.

apex. These contaminants are usually incorporated into the sediments on their route seaward, especially in areas where water energy is low.

A chemical waste storage site in Elizabeth, New Jersey, exploded on 21 April 1980; ironically, on "Earth Day." Of the approximately 60,000 55-gallon drums of acids, solvents, pesticides, explosives, and known carcinogens stored at the site, 20,000 drums were destroyed. The contents entered the atmosphere and the waters of Bayonne Bay through the run-off from water used to fight the fire. Toxicity was so high that a third of the firefighters reported symptoms of illness for up to 10 days afterwards—nose and throat irritation, coughs, shortness of breath, skin rashes, wheezing, nausea, and tingling sensations of the limbs. The New Jersey Department of Environmental Protection temporarily banned commercial fishing in the Hudson River from south of the George Washington Bridge to a line connecting Sandy Hook, New Jersey, and Rockaway Point, New York, and including the Navesink and Shrewsbury rivers, Sandy Hook and Raritan bays, and the Kills around Staten Island.[4]

The common assumption is that pollutants entering a water system are rapidly dispersed and diluted, posing little or no danger to the system and even less danger to

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divers who spend relatively little time exposed to these pollutants. This concept may be generally correct, especially in regard to open ocean dumping; however, hydrographic research into the water movements of estuarine systems indicates that pulses of discrete water parcels are common. These parcels may be quite different in temperature, salinity, nutrient levels, and levels of pollutants than the surrounding or receiving waters. It is conceivable that a diver working in an area receiving hazardous wastes might contact a parcel of water with relatively high pollutant levels.

The Sandy Hook Laboratory used the dry, surface-supplied diving system to conduct *in situ* experiments during July 1980 at an established research station in Raritan Bay, about 22 kilometers downstream of the chemical dump site explosion, with no ill-effects. At the same time, a few kilometers away, two sport divers using only wet suits and SCUBA were reported by the press to have surfaced with burning sensations around the neck and mouth, to the extent that they required hospitalization. Unfortunately, no additional information concerning this incident was available, but regardless of the cause of the burning sensations, the isolation of the fisheries diver from the water permitted a safe, efficient operation. Objectively, it should be stated that academic, state, and some federal researchers have used, and still use, only wet suits and SCUBA in some of these areas with no reported ill-effects.

Scientific interest in spills of petroleum products has increased greatly in the past decade and the requirement for research in relation to these spills has increased accordingly. The investigations range from those regarding small but almost continuous releases common to coastal areas with petroleum industries, to large well-publicized spills from tanker groundings and oil platform blowouts. The forms a petroleum spill may take are many, depending on the degree of refinement, environmental conditions, and how long the oil has been weathered. Sheen, pancakes, tar balls, and mousse (a mixture of oil, water, and air, named after the dessert, chocolate mousse, which it resembles) are the usual results of oil spills. At times all of these forms will occur together. Oil spills are difficult to dive and work in as the neoprene and rubber used in diving equipment are rapidly deteriorated by the oil, and most solvents used to remove the oil or tar are equally harmful to diving equipment.

Attempts to isolate divers from an oil-water environment by the use of a dry suit may prove extremely difficult in high temperatures because of the likely onset of hyperthermia. This was the case during diving investigations into the effects of oil during the IXTOC I blowout, conducted on the beaches and inlets of Padre Island, Texas, where air temperatures exceeded 38 °C. Coveralls were used to prevent the oil from reaching the diver's body, but a thin film of oil on the water's surface, called sheen, and at times globules of sticky tar, managed to move under the coveralls, coating and sticking to the diver's skin.

Toxicity is usually related directly to the degree of refinement of the spilled petroleum product. This should be considered when determining which diving equipment to use and whether the dive should even be made. In 1967, three U.S. Navy salvage divers required hospital treatment for third degree chemical burns when they surfaced through a small spill of either aviation gasoline or jet fuel. The divers were using shallow water surface-supplied equipment and coveralls for chaffing protection, both sufficient for the anticipated working conditions. An unnoticed overflow pipe from aviation gasoline and jet fuel storage tanks was the source of the spill.

Natural events and some of man's activities at times produce conditions of questionable environmental quality but high scientific interest. In 1976 a fish kill off New Jersey involved 8,600 sq. kilometers of water and claimed at least 100,000 metric tons of surf clams. With other dead benthic animals such as crabs, moon snails, razor clams, and tunicates, the amount of organic material on the seabed was enormous. A strong pycnocline isolated the bottom waters and the microbes (after removing the last traces of oxygen from the water) turned to sulfate reduction to sustain their metabolism.[5] We do not know whether the resulting very high concentrations of hydrogen-sulfide (H_2S) in the bottom water would have been toxic to divers. Later in the hypoxic event, when SCUBA was used for observations and photography, the concentrations were much lower. The maximum concentration of H_2S in clean water is .01 to .1 micrograms per liter ($\mu g/l$)—several orders of magnitude lower (A.F.J. Draxler, personal communication).

Finally, diving investigations into specific chemical spills such as Kepone in Chesapeake Bay or fish kills caused by thermal shock in the outfalls of nuclear power plants—previously accomplished using wet suits and SCUBA—may be more safely accomplished by isolating the diver from these environments.

At the conclusion of a dive, the diver is washed off, while still fully dressed, with fresh water from the ship's supply, or if fresh water is not available, with sea water from the ship's salt water system. A scrub down with a detergent and hot fresh water makes this procedure even more efficient. The diver, after undressing, should scrub his hands, which have been unprotected except for gloves, with an antibacterial cleanser and then shower. The suit is machine washed in a 50% sodium hypochlorite (bleach) and hot fresh water solution for 20 minutes, rinsed in fresh water, and hung up to dry at room temperature.

Divers who expect to conduct research in polluted areas are urged to maintain current immunizations for diptheria and tetanus, smallpox, and typhoid fever. If in spite of these precautions a diver exhibits signs of illness within a week of his last dive in polluted water, a physician should be alerted to the diver's possible exposure to pathogens or other pollutants.

The NOAA Diving Office is in the process of testing and evaluating various combinations of diving hats, masks, and suits for possible inclusion into the NOAA Diving Inventory. These include:

1) The Desco hat and Viking suit combination, also used efficiently in completely dry dives. Figure 3 illustrates how the neck dam of the hat mates to the Viking suit via a clamp, in the same manner as the KMB mask. The hat is equipped for use only in the free-flow mode and therefore has no regulator to leak water. This lack of a demand regulator is a drawback, however, when diving in water which is not polluted, as the air supply requirements of the free-flow mode are very much greater than the demand mode. The hat also tends to lessen the diver's mobility.

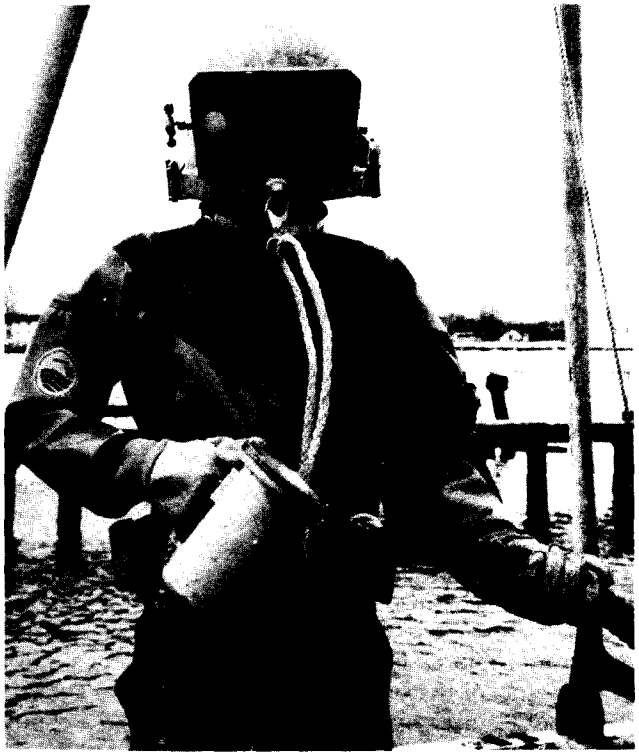

Figure 3. Desco diving hat and Viking variable volume suit.

2) The AGA Divator full face mask (Figure 4) is a demand mask that can be adapted for surface-supplied use. It is, however, usually used in the SCUBA mode where it vastly outperforms the face mask and mouthpiece combination of conventional SCUBA in preventing water from reaching the diver's face. This is accomplished by separate air intake and exhaust ports and a setting on the second state of the regulator which maintains a slightly higher than ambient pressure within the mask.

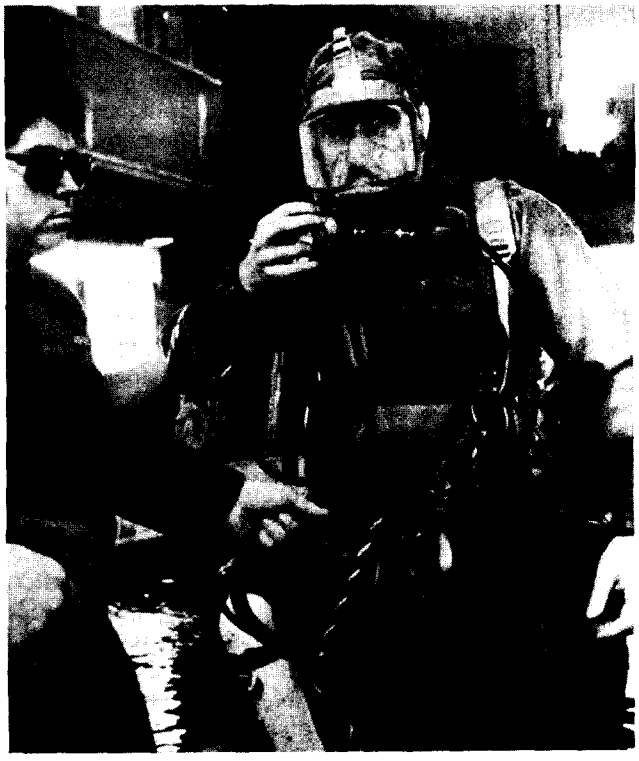

Figure 4. AGA full face mask and Aquala variable volume suit.

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3) The Superlight 17 mask (Figure 5) can be used in the demand or free-flow modes from a surface breathing gas supply. NOAA has not used this mask extensively, but is presently testing it for its usefulness in polluted waters.

Figure 5. Superlight 17 mask and Unisuit variable volume suit.

4) The Draeger constant volume suit (Figure 6) can be used with SCUBA or surface-supplied breathing gas, but only in a demand mode. The rubber hood, with a removable glass faceplate, over-pressurization relief valve, regulator mouthpiece and communications, is mated to the rubber suit by clamping the hood and suit together over a metal neck ring. The suit lacks the buoyancy control available with a variable volume suit and therefore requires some effort to swim when used with SCUBA. This suit is extremely watertight; however, as it may be used only in a demand mode, water does occasionally enter the mouthpiece. The suit has been used by commercial divers in very hazardous environments, including sewer-pipe inspections and repair, with no reported ill-effects.

Figure 6. Draeger constant volume suit and hood.

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DISCUSSION

Colwell: The concentration of hydrogen sulfide (H_2S) that you report is significant. Are there pockets of such concentrations in areas where you dive?

Phoel: The epicenter of this fish kill, where there was no oxygen, was located off Atlantic City, New Jersey. Moving out from the center of the area of the fish kill, we found small amounts of oxygen; at the same time we found H_2S. It was a month before we returned to dive in this area, which was good since we were not aware of a possible H_2S hazard! We brought up dead surf clams in fishing trawls, which indicated that these animals had moved out of the sediment seeking oxygen and, finding none, had died. Live healthy clams are never caught in fishing trawls. The bottom was littered with organic matter, and when the trawl came up, there was a stench of H_2S.

Gottlieb: Have you noticed any incidence of increased illness among divers who are diving in these environments —any type of enteric problems, gastroenteritis, eye infections, or respiratory or skin problems?

Phoel: One of the observations we have made, not only with respect to the divers, but with benthic people who have worked with sewage sludge for several years, is that they say they have a higher incidence of warts than before they worked with sludge. We have also noticed significantly higher incidences of cuts on the hands of divers becoming infected after diving in polluted sites.

Gottlieb: You take excellent precautions with your divers, in terms of the kinds of suits they wear for protection and cleaning gear after they emerge from these environments. But is it possible that we may not be speaking about a significant *microbial* hazard? If you do not see marked increases in infectious diseases, what kind of hazard are we talking about? How would you assess the hazard, based on your own experience? There is probably no place that can match the New York and New Jersey areas.

Phoel: If I consider the local SCUBA population as a control, then our precautions are not necessary, because they claim they do not become ill by diving with SCUBA in these areas.

Gottlieb: SCUBA divers and snorklers have the least protection, compared to the deep divers. Do you have any information about occurrences of illness in these divers? They say they are not getting sick, but there is a latency period, i.e., an incubation period. Has anyone attempted to correlate illnesses that may occur a week to ten days after diving, or is it that these people don't record such illnesses?

Phoel: Nobody is documenting such correlations and most of the SCUBA divers don't record their illnesses. If they become ill a week later, they have forgotten all about the possibility that diving may have been the cause of the illness.

Gottlieb: I don't know what kind of medical profile you have on your divers, but do you, or does anyone, do serological studies of these people? Do you know what their immunoglobulin makeup is, for example, to be able to correlate any increased susceptibility to infectious disease with their immunologic tolerance?

Phoel: The only medical profile we have, is what is required by the annual NOAA diver physical exam. Much of the evidence of microbial hazards is reported by anecdote. In the Baltic Sea, Morgan Wells and I recorded the rate of oxygen consumption by sewage sludge and both of us became ill during that study. I came down with 103° fever and was ill for several weeks afterwards. Morgan never became that sick, but he felt feverish too. This is the only kind of anecdote we have.

Williscroft: Basic training for divers in the NOAA program includes spending a couple of weeks in Miami and then traveling to the Norfolk area. Norfolk Harbor is relatively polluted and the divers from the Gulf area tend to come down with respiratory ailments when they are in Norfolk. We have experienced this for two years in a row—those who are from Norfolk and have dived repeatedly have perhaps built up some sort of immunity. They don't seem to develop the same respiratory problems.

Gottlieb: What about the move itself and the stress of the situation? Could that be a cause, or is it the actual diving?

Williscroft: That is a good question. We have no way to answer that now, but at least we have a theory that the polluted water is the cause.

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Wells: There are cases where we can clearly identify almost all of the items you mentioned as being associated with diving in such areas. There are very obvious cases, such as the one in which Bill and I were involved. In another incident, we shipped quantities of sewage sludge from various sources from Miami to the Bahamas. While we were working with the sludge underwater, using traditional SCUBA some of the time, it was blowing back into our faces. Several members of the party in the Bahamas became quite ill during these experimental studies, and, also, after collection and conducting experiments at sewage outfalls. I'm not sure sport divers are a good control group, because the areas we are talking about are obviously ones they don't want to dive in. Because they clearly are not diving in the same area, I don't consider them a valid control.

Gottlieb: There is no question that SCUBA divers are not exposed to the same hazards as working divers, or that they obviously do not choose to go into polluted areas. Furthermore, there are the ethical issues involved in doing a double blind study—sending SCUBA divers down into known polluted areas. What does one use as a valid control? I think the best control, under the circumstances, would be SCUBA divers.

Bachrach: Perhaps we could comment on the Anacostia experience.

Denham: Navy divers who, until just recently, were attending the U.S. Navy School of Diving and Salvage on the Anacostia River, as well as the EOD training on the Indian Head River, can provide some relevant information. I think there is no objective documentation of incidence of infection from experiences of divers, supervisors and instructors. It is readily documented that cases of ear infections are *not* related to diving in clean water. I think we are talking about two different kinds of polluted areas—an area of generalized pollution and those areas with specific, highly concentrated pollutants which affect professional working divers.

Greene: I think I'm going to take the opposite tack, one similar to Dr. Gottlieb's. The laboratory at the Anacostia River has a significant problem which has not been discussed. Navy divers perform two weeks of SCUBA training with mouthpieces and face masks in the Anacostia River where, not only the city of Washington is responsible for pollution of the river but also the diving barge itself, which is placed directly over where the divers work. The divers, therefore, have complete exposure. The diving school's Medical Department tried to do a study to see what the statistics were. But the Department found no dependable data, and the treatment records were not conspicious regarding the presence of gastrointestinal and other enteric problems. Because of an anecdotal report, at one time they tried to culture the cause of gastroenteritis, but most of the time they recovered no bacteria.

Sphar: If you look at the divers' medical situation from the Surgeon General's view and examine the statistics, you really won't find any significant increase in illness. Some of the minor conditions, like otitis exertna, are simply not reportable; there are no statistics available from the Navy Surgeon General's Office regarding conditions attributable to microbial and most other hazards to divers in the U.S. Navy. However, if you start gathering anecdotal information, of the sort Denham and Greene have mentioned, and approach people who have served at the Navy's diving school, they will immediately cite the Anacostia River. Specifically, I spoke yesterday with Dr. Zwingleberg, the Senior Medical Officer at the diving school in Panama City, Florida, who had been with the diving school when it was located in Washington, and he informed me that he has observed a definite increase in gastroenteric conditions during the SCUBA part of the training. He attributes this to daily exposure, sometimes twice daily, to the Anacostia River over a period of about two weeks, and reports that probably 40% of the SCUBA classes do have some type of gastroenteric condition, usually diarrhea. However, in terms of being an operational or training problem, only about 5% of the class loses any time from training as a result, and only very occasionally, perhaps once a year, has he seen a really serious condition requiring hospitalization.

Gottlieb: What is the diet of the people undergoing diver training? Do many of them eat a lot of seafood or specific items on a regular basis?

Sphar: No, not that we are aware of.

Phoel: I was a student in the course taught at Anacostia and as it's a long course of instruction, the students usually eat at the facilities. It's a normal diet. Also, because of the students' "can do" spirit, I think sick call is looked upon as the last resort. The student really has to be sick before he or she goes to sick call.

Williscroft: I also was a student in the course. The macho image of the diver is such that he will drag himself to *every* class.

Greene: Even more important is that if a student misses *any* of the classes, he has to return and repeat the *entire* course of instruction.

Williscroft: Speaking for myself, I picked up some kind of skin problem on my chest from the Anacostia ten years ago and have had it ever since.

Gottlieb: Are you dealing with a microbial infection or an allergic reaction to chemicals?

Williscroft: I don't know but I doubt that it is a chemical reaction.

Colwell: One of the kinds of pathogens we haven't considered yet are protozoa and related intestinal parasites. These may be a source of infection. We have absolutely no data regarding such illnesses.

Daggett: Someone mentioned the splashing of polluted water back into the face. Does that ever get up into the nose?

Wells: Yes.

Williscroft: In Norfolk, we are in the process of testing a suit that has been accepted by NASA. It combines the AGA full face mask Bill showed us today and isolates the diver completely from the environment, including his hands. Scientists from NMRI and the University of Maryland are testing this suit, taking cultures from the divers before and after using the suit, to see if there are any changes in the microbial types and abundances. Scrubbing the gear down, after the divers come out of the water, with various kinds of disinfectants, is also being looked at as a means of combatting infection. We will have more information on this in the next few weeks, but it is the first

time, to my knowledge, that we have done SCUBA diving with total isolation, including the hands.

Gottlieb: What you are saying then is that microbial hazards may exist, but they are not so great that they cannot be protected against. Although we are focusing on microbial hazards today, one of the important items we should pay attention to is protection. With full suits, full face masks, and isolation of the diver completely from the environment, there may not be a problem. In the cases that Bill and Morgan were talking about—when you had splash back—you did not have a protected diver, and any time you put people into that environment where they are going to be exposed to a heavy concentration of organisms, even into the nose, it's inevitable that they are going to get sick—irrespective of whether the agent is viral, bacterial, fungal, or protozoan in origin. It is like putting yourself into a test situation and saying, "What is the minimum microbial count that I can tolerate before I get sick?" How much of a threat is the microbial hazard to a fully protected diver? And how much of a hazard is it to a recreational diver?

Joseph: That doesn't preclude the necessity for being aware or knowing how to be protected.

Gottlieb: I don't mean to imply that at all. You are dealing with several kinds of hazards, not only microbial. In Bill's presentation, he talked about the protection against chemicals that is required, and, with chemical exposure as well, there is a potential for complete breakdown of resistance to microbial infections.

Colwell: Only under certain circumstances, that is, spills, will chemicals such as solvents be involved.

Gottlieb: Yes.

Wells: I agree, but the equipment we are talking about is expensive and requires significant training before it can be used. Also, it is quite often resisted by the diver. The equipment is unfamiliar and, in general, uncomfortable. So it is very important for us to identify those conditions under which the use of this equipment is necessary or should be required.

Williscroft: The more equipment you put on the diver, the less mobility the diver has, and the less capable he is of carrying out his task. This is a major operational consideration.

Wells: We are dealing with a situation where we are increasing the complexity of the operation and probably decreasing efficiency.

Weiss: How often does this equipment leak or become damaged?

Wells: It is not uncommon.

Denham: I think what has not been asked is, "What constitutes a protected diver?" We have methods for protecting the diver and most of them, based on microbial evidence, are pretty good. But just how good are they for protection against specific solvents?

Coolbaugh: People who are on the ships or docks who get splashed or breathe aerosols that come up with the divers when the divers emerge from these environments may, in fact, be more at risk that the divers themselves. The divers are at least shielded and washed down afterwards, but the support people are handling the lines and are essentially unprotected from anything that may come up from the bottom.

Gottlieb: An obvious question is whether there is an increase in illness amongst support personnel?

Coolbaugh: I haven't heard of or seen any documentation on this.

Gottlieb: What about anecdotal information?

Phoel: The only anecdote of this nature that I can report concerns handlers of sewage sludge. These scientists have reported an increase in warts on their hands and faces from handling sediment. I think, however, that the major impact is on the diver because he is working in and on the sediment, whereas the surface attendants, basically, only come in contact with the surface waters.

Gottlieb: The surface technicians and scientists could protect themselves with gloves.

Sphar: Dr. Zwingleberg says that since the move from the Anacostia to Panama City, gastroenteric infections have virtually disappeared. He pointed out that the waters in which the SCUBA training is now carried out conform to swimming waters for the general public. He reported that the ear infection problem has also decreased dramatically. He had previously thought the warmer water might create more ear infections, but this has not been the case.

Colwell: It would be very useful to determine the concentrations of microorganisms in the Panama City waters because, compared to the Anacostia River where there is nutrient enrichment, particularly from sewage treatment effluent, the *Aeromonas* and coliform counts are significantly high. Such bacteria may be present in very low numbers, if at all, in Panama City.

Bachrach: Is this caused, in part, by salinity as opposed to freshwater?

Coolbaugh: Williscroft reports the salinity to be 33 $^{o}/_{oo}$. I would estimate that it's around 30 $^{o}/_{oo}$.

Colwell: The Anacostia would be considered to be freshwater, in comparison to Panama City.

Joseph: When we initiated our diver project and discussions were held with medical officers associated with SCUBA diving in the Anacostia River, Panama City, and various other places, it soon became very obvious to us that there were a number of ill individuals who were coming to sick call and that perhaps there were a large number who, for various reasons, were not coming. But the common denominator in each of these locations was that we didn't have a great deal of recorded medical information. The medical officers are busy; in addition to their medical responsibilities, they have other responsibilities associated with their organizations. They are absolutely required to report certain illnesses, as mentioned. At the same time, there are certain infections that they do not report, simply because the requirement for reporting them has not been established. If it's not required, it's not done—unless someone takes an interest in doing it. Some of those to whom we talked are interested in the problem and are very curious about it; but as I said, nothing has been done to date in an organized manner on this problem. More than anecdotal information should be collected because there is a real need for the information. We have a responsibility to protect the health of the individuals required to dive in polluted environments. As taxpayers we should be interested in saving money and should determine the cost effectiveness in keeping a person on

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the job. The geographical distribution of government divers is extensive. Where are government divers being located and where are they operating? What types of environments are the civilian companies operating in? And are the areas in which they dive going to be increased in number?

Joseph: Jim Oliver has pointed out that there are problems in terms of aquatic pathogens, which aren't necessarily related to polluted waters.

Oliver: Are the protective devices that were described for total protection always worn or are they only worn in polluted waters? If so, how is it determined if the waters are, in fact, polluted?

Phoel: Usually by the seat of our pants. For example by testing the current: If there is an incoming tide and we will not be directly in the dump site and not in the bottom sediment, we will dive with conventional SCUBA.

Oliver: There are no chemical or biological assays of any kind done before diving in the water?

Phoel: No such assays are done just before a dive, but usually we know, historically, that a site is, or has been, polluted. Also, if it looks and smells bad, we'll dive protected.

Williscroft: On NOAA ships, when our divers are diving in areas that might be polluted, usually I get on the phone and try to determine whether the water in the diving area is polluted. In other words, we go to available sources of such information. If there is any question at all that the water might be polluted, we contact individuals who might have the information we need. If the water may be polluted, the divers are required to dive using protective equipment.

Daggett: What about thermally polluted waters, like those which could come out of a power plant. How are people geared up for that kind of situation?

Wells: To answer that indirectly, we have had problems with excessive heat in using our protective equipment. An example was the oil spill Bill Phoel had to work in. We have had problems of hyperthermia already, because our equipment was originally designed to protect divers against cold water, so the equipment is designed to be quite warm for the diver. NOAA has very little experience in diving in water warmer than ambient, but we are to start working on developing appropriate gear now. In the past, we tried pouring cool water inside the suit, a temporary and rather crude solution to the problem.

Gottlieb: In terms of thermally polluted waters, for example, hot effluent from a power plant, what would the microbial hazards be? You're not really going to have a heavy concentrations of microbes there.

Daggett: I beg to differ.

Gottlieb: What do you have?

Daggett: *Naegleric* fowleri perhaps, the causative agent of meningitis. There has been extensive sampling of thermally polluted waters from power plants and if your know how to collect these microbes, they are found fairly frequently in muddy sediment. That is why I raised the question.

Williscroft: The suit I mentioned that is being tested appears, at first glance, to have the greatest thermal conductivity. This indicates that it might be suitable for diving in thermally polluted water, but we don't know that yet for sure.

Colwell: The commonly accepted pollution indicators are not always going to help because there are situations where microorganisms that are naturally abundant in the environment are potentially pathogenic, such as *Vibrio cholerae*. This organism and related vibrios are part of the natural flora. There could arise the situation where clean water, with no or very few coliforms, would look like a good place to dive, but in fact would present a risk of getting a *Vibrio* infection. It's important to understand the microbial ecology of waters in which diving is done. Under certain circumstances, there will be present the naturally occurring, potential pathogens. It is very important for us to be able to describe these organisms and situations for physicians, particularly the potential for infections from parasites and protozoans, about which little is known. The physicians would have at least a starting point and could focus quickly on potentially infectious organisms. It's time to collect data in clinics and to develop an awareness in the medical community of possible infections, especially in the case where a diver develops a nonspecific high fever. As a result of this workshop, we have outlined the problems and, in the future, we will need to recommend solutions.

Shilling: In 1929, in Guantanamo Bay, Cuba, a fleet exercise was completely disrupted by bacillary dysentery. It was a very difficult situation where hundreds of men were affected. We took care of it on our ship by having big tubs of disinfectant in which, before they ate, the men had to put their arms in the tubs and hold them under water for 30 seconds at the minimum. These outbreaks were not simple problems. Very difficult situations can arise without diving into the water.

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U.S. Navy Diving in Polluted Waters

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U.S. Navy divers dive in many situations, including diving in polluted rivers, harbors and ocean environments. Today, I will describe the equipment and techniques currently being used by divers when diving in polluted waters, discuss some of the medical problems which the diver faces, and present the Navy's efforts to reduce and ultimately eliminate the problems of diving in polluted waters.

The specific equipment the Navy recommends for diving in polluted waters is shown in Figure 1. As can be

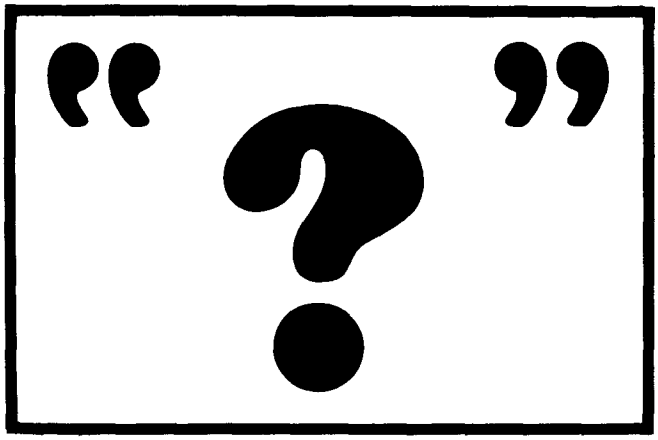

Figure 1

seen, there is no equipment specifically designated for the task. This, of course, doesn't mean that Navy divers don't dive in polluted waters. As a matter of fact, for many years the Navy diving school was at the Navy Yard in Washington, D.C., and training was done in the Anacostia River which is, at best, highly polluted.

The conventional procedures for diving in polluted water are:

1. Personnel with open cuts are not allowed to dive. Once injured in an operation, the diver is not allowed to dive for up to four weeks, until the infection clears.

2. Prior to a dive, divers are fully immunized against diphtheria, tetanus, smallpox, and typhoid.

3. Before and after a dive, divers irrigate each ear canal with a 2% acetic acid in aluminum acetate solution (Domeboro Solution).

4. If at all possible, divers use the full face mask system such as the lightweight diving outfit shown in Figure 2 or the Mk 12 Surface Supplied Diving System shown in Figure 3, or, of course,

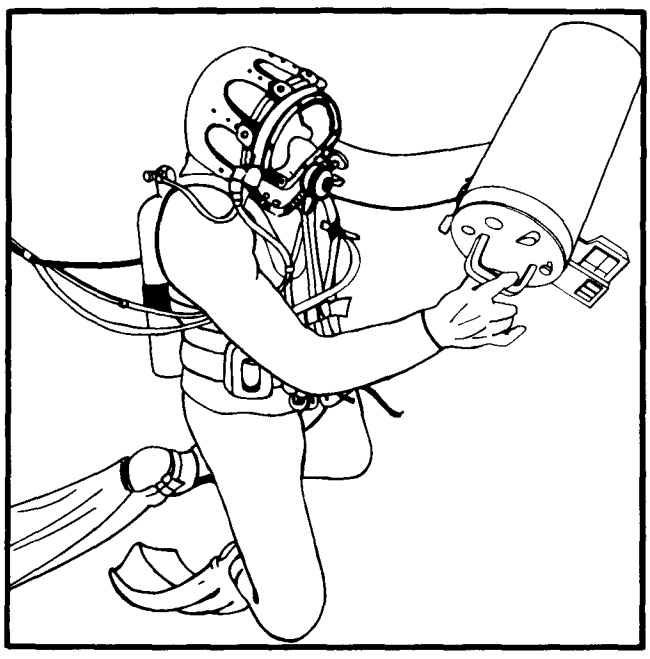

Figure 2

the old standby Mk 5 diving system shown in Figure 4. If SCUBA equipment must be used, divers should be suited in a wet suit with hood to keep the polluted water away from face and ears.

Figure 3

5. Finally, as a post dive measure, divers and equipment (suits, regulators, etc.) are completely washed down with freshwater and then disinfected with a disinfectant such as betadine.

The types of medical problems we are facing are:

(1) eye and ear infections;

(2) infected cuts;

(3) gastrointestinal infections.

One of the ways the Navy is attempting to eliminate these problems is to provide the diver with equipment which protects him from the environment. In this regard, the Naval Sea Systems Command (OOC) (LCDR S.A. Denham) has just funded the Naval Surface Weapons Center, Dahlgren Laboratory, to investigate the overall problems of diving in hazardous and contaminated waters. Current efforts in this area are to:

(1) Define the threats to the diver when diving in hazardous and contaminated waters.

(2) Determine the protection offered by both current Navy operational diving equipment and commercial equipment.

(3) Finally, outline modifications needed to update equipment to provide protection when diving in hazardous and contaminated waters.

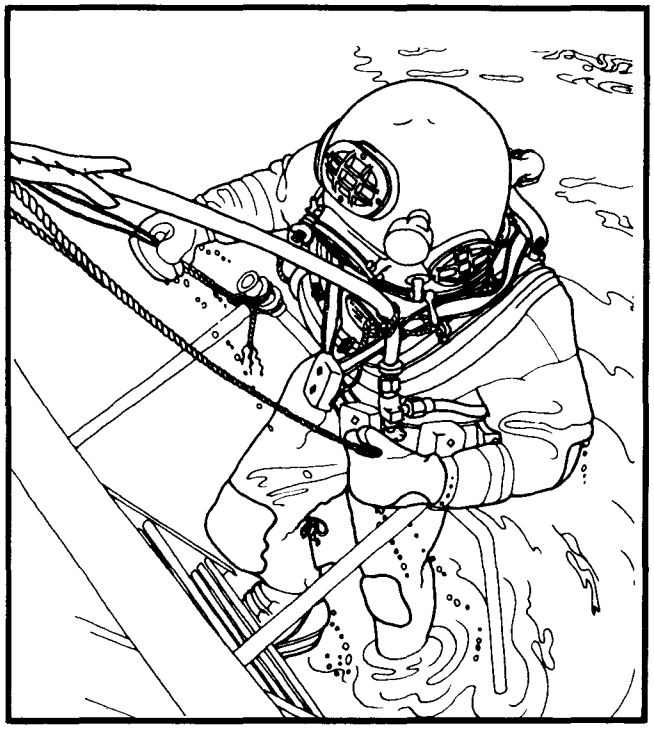

Figure 4

Diving in polluted water is indeed a serious problem. To date the operational diver has been ill-equipped to work in this environment. The Navy is now pursuing efforts to upgrade the operational diver's capability in this area.

DISCUSSION

Gottlieb: Have you noticed injuries, other than open cuts, getting infected? What kind of infectious disease problems have you noticed?
Pennella: At the EOD school located on the Potomac River, as well as the Diving School on the Anacostia River, we have noticed ear infections and eye infections. We have not done any comparative studies between the general population and our diver population.
Gottlieb: What organisms have you isolated from these infections?
Pennella: We have not done any studies to isolate an organism.

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Denham: We are undertaking to fund a study of the problem at NAVSEA. The first part of the task will be to define the problem or threat. We are looking at the scope of the Navy diver overall; he may perform salvage diving, ship husbandry diving, as well as specific military diving. Given this wide range, part of the effort will be to define exactly what the problem is.

Colwell: Some of the areas where we have actually obtained documentation of infections will be discussed later in this workshop. It has only been in the last few years that intensive studies of pathogens have been made.

Pennella: The observation made by the NOAA divers is basically the same as ours: divers don't like to go to sick call. They don't like not being able to dive. If they are unable to dive for a long period of time because of what they consider to be a minor infection, they may not be able to complete their diver training.

Kaper: Is the Navy concerned about germ warfare?

Pennella: We have a specific type of diver called the EOD diver who is assigned they responsibility.

Sphar: I think that this workshop will be very useful in helping us identify such problems in our diver population and to anticipate required changes in procedures. I do think that if we are about to embark upon any change in our system of recording illnesses of this type, it would be better done through a research protocol than by attempting to have the preventive medicine people change their reporting procedures at this time. I think we are going to see some possible areas for Navy divers to participate in research, as a result of this workshop.

Colwell: What we are attempting to do is to take a set of data we are really not yet able to analyze statistically and project into the future to predict what should be done in a diving operation to protect against a potential health hazard. Can we define the immediate problem and what the experienced divers see as a serious problem? Are these problems mainly equipment or the increasingly complicated tasks required to be performed in hazardous aeas where divers work, or both?

Wells: I have a partial answer to your question and a general comment on equipment. Historically, the University of Maryland and the Navy Medical group, who have been using our divers as subjects, have seen an evolution in the kind of equipment being used. This has made it somewhat difficult, I believe, to come up with nice, clean-cut results, because we are always changing equipment. This has caused me to be very concerned about the operational characteristics of our equipment. We are preoccupied with protecting divers against polluted waters, but are we actually going to kill one of them with the equipment we are using to protect them? We strap them pretty tightly into their equipment. We are putting neckrings on them and sealing these fellows up. We will conduct a study in March, 1981, on the operational characteristics of this equipment. For example, we have modified parts of the MK 1, and made some changes. We have had cases where the suit we were depending on to protect the diver against the water was over-inflated and actually exploded and flooded the diver. This is a serious, potential hazard and one that I am now becoming more concerned about.

Greene: With regard to chemical hazards, a few years ago, a chemical freighter sank in the Mediterranean Sea, while it was carrying a load of tetramethyl and tetraethyl in steel drums. The cargo drums were inspected about a year after the incident and were found to be rusting very rapidly and leaking chemicals. No one wanted to take on the job of retrieving the drums, but finally an Italian diving company accepted the clean-up task. I don't remember the exact results in terms of safety, but the problems of evaluating the nature of the hazard, of protecting the divers, and also of finding a way to survey the health of divers while the work was going on had to be addressed. It was going to take weeks. Blood samples were collected daily from the divers. The results were published, in *Underwater Physiology.* The information should be obtained, as it is pertinent to the objectives of this workshop. The potential problems we may face in the future were addressed earlier in the workshop. Diving in hydrocarbon-polluted waters and the problem of the equipment needed for that kind of diving are serious topics requiring discussion. There is no information, right now, on the deterioration of equipment used in such diving operations over an extended period of time. We might be safe for the first three or four days of diving and, then, unknown to the operational people, have serious equipment deterioration. Another issue relates to the availability of air in such diving operations. Where you have a large oil spill, hydrocarbon-contaminated air occurs. Just the problem of getting air to the diver, whether decompressed on the surface or not, has not been looked into and presents serious potential problems.

Joseph: It has been stated that diving equipment is in the testing stage. Is it tested in a controlled experimental setting or simply in a working mode where evaluation from day to day is under different situations?

Williscroft: Today it's been much more the second approach. However, there are some controls used and some scientific evaluation in progress.

Wells: We must consider the economics of diving. Economics have had a significant impact on the equipment presently being evaluated. Money being a problem, we started with the simplest and least expensive equipment. Now we are getting some additional funds, permitting slightly more sophisticated gear for NOAA divers. Economics does play an important role. We are talking about a jump in the cost per diver of a few hundred dollars, perhaps as much as three or four hundred dollars. Some pretty high figures can result—$20,000 for a complete dive team with the most advanced gear, such as the MK 1 system and related modifications for suits and support equipment.

Joseph: How do you relate those dollars invested in advanced equipment to dollars for simple evaluation? I could see your going overboard and developing elaborate equipment, but not having proper evaluation.

Wells: Once again, "gut feelings" are the initial criteria. If equipment offers potentially greater isolation of the diver and maintains an adequate degree of operational effectiveness, we invest in it. Then the researchers come along and tell us how good the equipment is under actual diving conditions.

Bachrach: The diver in full protective gear is immobilized

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to a large extent. There has been very little done to analyze the effect of the equipment on the performance of the diver. Some years ago, when we were doing human engineering work on the MK 12 to compare it with the MK 5, we went back to the literature and found that the MK 5 had never been studied from the standpoint of what it did to the diver. In San Diego, some years ago, it was found that the noise level in the MK 5 was around 110 decibels. You can imagine what this can do to a diver over a period of time in the water. The other problem is that of the diver himself. In a survey we did some years ago, we found that, in a large number of cases, although the Navy diver should have worn lightweight gear, he would take the "least effort" route and put on only a scuba tank. Unfortunately, the diver is effectively immobilized in a lot of situations by equipment which adequately protects but is poorly engineered from a human factors standpoint.

Gottlieb: Regarding Williscroft's comment about gear, especially in hydrocarbon-polluted water—in providing air, do you find any increase in molds?

Phoel: If the mask is put away with any moisture present, when we take it out a few weeks later there will be mold on it. Not much in the suits, because they are turned inside-out and dried.

Gottlieb: Do you find greater growth of molds in different types of polluted environments?

Phoel: I haven't looked into it. We don't as yet have such information.

Williscroft: You are dealing with divers out there working, many of whom don't have a high education level. You have to keep diving operations simple. If you give the diver a very complicated piece of equipment, the diver will not dismantle it completely to clean it. If you give the diver something that is simple to take apart and clean and a good reason for doing it, he or she usually will do it. We found that if we have something that is almost as good as the sophisticated equipment—that is 1/10th of the sophistication level—and we offer the divers a choice, they inevitably select the simpler gear.

Pannella: Also involved is confidence in the equipment.

Bachrach: Familiarity, too, which is, of course, part of confidence. When doing the MK 12 to MK 5 gear comparisons, the divers said they would like to use the old, familiar MK 5 helmet with the new MK 12 suit.

Phoel: All my divers have education to at least a bachelor's degree or more. But the problem of taking care of the equipment does exist. They like to leave maintenance to the technician. Another factor is that experiments have been designed to avoid areas where this equipment has to be worn. If principal investigators don't really like to wear the equipment or they are unfamiliar with it, they design experiments in such a way that they don't have to go into a polluted area. It's a real problem. If we come up with something that they would use, and use with confidence, I am sure that better science would be accomplished.

Bachrach: One of our toxicologists did a study of the air supply for a group of Navy divers and found high concentrations of ether in the compressed air. We couldn't figure out where the ether came from until someone pointed out that it's used to start compressors.

Joseph: I heard a comment the other day that there is increasing concern about the Russians using chemicals in Afghanistan. The Army is concerned about this and, in trying to build and evaluated protective equipment, has produced a pretty good suit. There's just one problem. Once the people are all suited up, they can't identify each other, can't communicate with each other, and therefore are protected but unable to function.

Colwell: If we could define hazards in given environments, then we could optimize the equipment for the environment. The ideal would be to keep costs down for the operation and minimize the risks.

Denham: Without prejudicing the study we are doing in the Navy, we would like to see a series of suits—using existing equipment, available equipment, as well as equipment yet to be developed—that the divers can pick from, based on the threat facing the diver. I hope we won't go overboard to give the diver maximum protection, but rather, develop equipment that provides the diver with *reasonable* protection and *minimized* risk.

Colwell: I hope we will be able to make some statements during this workshop on what the microbial hazards are in a specific environment, as far as can be determined. We will also need to outline the research that is required.

Joseph: I would second that. What should come out of this workshop, in addition to the presentations, is a list of problems, or needs, and possible solutions and approaches to solving the problems. As we progress in the workshop, I hope each of you will keep this objective in mind.

Greene: Some of the needs are: 1) to develop surveillance which will determine the magnitude of the problem; 2) to develop procedures to screen the environment we are diving in (Considering that the coliform count is not necessarily indicative of all microbial risks, what indicator would be appropriate to determine whether it was free of risk?); 3) to determine what pollutants are likely to be encountered; and 4) to establish the guidelines for when diving should and should not be allowed, as well as what risk is involved if a dive is made. I would put the risks in three categories: a) microorganisms, b) petroleum, and c) other chemical pollutants.

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Assessing the Potential of Microbial Hazards of Diving in Polluted Waters

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MICROBIAL HAZARDS OF DIVING

A review of the literature reveals a paucity of information on the microbial hazards of diving. I have been following the morbidity and mortality reports of the Communicable Disease Center for the past fifteen years, and I do not recall any reports pertaining especially to this subject. There may have been a report or two concerning otitis externa (swimmer's ear).

When talking about microbial hazards to divers in polluted waters, we must ask several very basic but obvious questions: (1) What kinds of pollution are involved? (2) What is the direct threat from the external environment? As a corollary, we then ask, how long do microbes (bacteria, molds, fungi, algae, viruses, and protozoa) survive in various types of polluted waters? How is a diver's resistance affected by a polluted environment? (3) With which type of diving are we concerned? Recreational?, e.g., SCUBA, wet suit. Professional/military? Saturation diving? Closed environmental systems? We must look at this problem broadly.

In 1965, the Environmental Pollution Panel defined environmental pollution as the "unfavorable alteration of our surroundings wholly or largely as a by-product of mans' actions, through direct or indirect effects of changes in energy patterns, radiation levels, chemical and physical constitution, and abundancies of organisms." Since we are concerned with diving, we will confine our remarks to water pollution.

In answer to our first question, let us review some of the sources of water pollution. The major pollutants are sewage and wastes from cities, industries, pleasure boats, commercial ships, and marinas. There is also agricultural runoff into rivers. The discharge of untreated wastes from these sources presents several biological hazards:

1. The contamination of drinking water (or even recreational water) by microbes, especially enteric microbes.

2. The contamination of seafood by pathogenic microbes.

3. The depletion of dissolved O_2 as a result of microbial degradation of the organic matter in the wastes. The results of this high biological oxygen demand are a decreased PO^2 level, a concomitant death or absence of aerobic life, and the possible proliferation of anaerobic organisms.

4. The increase in nutrient concentrations, such as phosphates and nitrates from sewage and also from agricultural runoff, which increases the potential for altering the survival and growth of microbes; enriched environments depress coliforms but increase *Pseudomonas, Klebsiella,* and *Aeromonas.*

5. Chemicals, which include detergents, pesticides, herbicides, and industrial wastes such as: (1) oil—ships, off-shore drilling, crankcase oils; (2) acids from strip or surface mines, as well as underground; (3) salts—from combatting ice, field irrigation, or industrial processes; (4) silt—sand, etc., from construction (houses, highways, erosion, channel dredging); (5) any combination of the preceding plus any other pollutant, i.e., radiation.

The nature of the chemical pollution could change the ambient microbial environment. One may see the survival of acid-resistant or salt-tolerant organisms. The nature of the chemical and physical pollution could alter the longevity

of the microbes in the water. In addition, these environments in contact with the skin could alter the skin's resistance to infection, especially if the skin has been damaged in any way. Also, these environments may alter the resistance of the eyes and the body orifices that are easily accessible to the pollutants.

Further, chemical pollution could cause allergic reactions. I know that we must confine our discussion to microbial hazards, but we are discussing pollution and I believe we must not blind ourselves to the potential hazards of allergic phenomena. Many people are allergic to detergents, perfumes, acids, etc., etc. Allergic reactions could vary from minor but annoying itching to anaphylaxis.

Currently we are faced with a situation where some of our rivers are now so loaded with vast amounts of wastes of all types, including human body wastes full of enteropathogens, that by the time they reach the sea almost all of the flow has been pumped out for municipal or industrial use at least once and returned to the river. This means that many of our recreational (let alone potable) fresh water sources are in effect partially diluted wastewaters which have undergone varying degrees of treatment and therefore represent a potential hazard to divers.

Thus, when sampling water sources for the presence of potential pathogens, one must know the depth profile of the sample as well as specific chemical composition and physical characteristics of the water at each depth. One must learn what the longevity of microbes in various types of pollution is.

We have briefly looked at the external environment and how that may influence the composition of the local microbial flora as well as exerting possible influences on host resistance. Let us now consider the diver. Pressure differentials may drive organisms and fluid somewhat deeper into tissues and cavities. Melnick [3] reported that swimmers in recreational waters ingest 10-15 ml of water per bathing period and will swallow the microbes, if present, in that water. Therefore, as stated above, there is the need to know, qualitatively and quantitatively, the microbes in a given environment and how their longevity is affected by the nature of the pollution. SCUBA divers and snorkelers may be prone to infection simply based on exposure because of the nature of their equipment (e.g., type of mouth piece). There may be fungal spores or mold on improperly cared for equipment, or in the air tanks and ancillary equipment, which may be inhaled and cause either allergic reactions or infectious processes.

Berg [1] has concluded that "any amount of virus in drinking or recreational water that is detectable in appropriate cell cultures constitutes a hazard to those drinking the water." In view of Bill Phoel's, Morgan Wells', and Ray Spahr's comments earlier today, such information may explain in part the enteritis seen in divers.

In view of the sparseness of reliable data concerning the microbial hazards of diving, one may conclude that either there is no high incidence of infectious disease related to diving, or that no serious attempt was made to correlate infectious diseases of divers with their diving. I am referring to infectious diseases other than ear and eye infections.

Aside from divers involved with chemical, biological, and radiological warfare and divers who dive into waters heavily polluted with microbes—these are individuals who are obviously under high risk—one has also to look at the multiple thousands of recreational divers. These divers will dive almost anywhere there is a body of water. Yet in view of the ethical constraints of doing valid double-blind studies, SCUBA divers represent the closest group to a control. Thus one asks, has there been any indication of increased incidence of infectious diseases in these divers? What about increased incidence of infection among diving trainees? (Ray Spahr gave some undocumented information on this earlier.) It would seem that the epidemiology, i.e., cause-effect relationships, would be hard to establish. Yet they should or must be established. One of the difficulties is the incubation period before the disease becomes manifested. This time delay makes correlation of disease to diving experience difficult.

Perhaps one outcome of this workshop will be the establishment of a retrospective as well as a prospective epidemiological study. It would be desirable to do serological studies as well as immunoglobulin profiles of divers and control groups.

In view of the apparent low incidence of reported infectious disease in recreational divers, and perhaps even professional divers, may we ask whether the possibility exists that the morbidity rate is too small to be concerned with? Can the microbial hazards be protected against relatively easily? [3, 4] Yet we must not forget the anecdotal stories that indicate that there is a potentially serious microbial hazard in diving.

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DISCUSSION

Williscroft: In your opinion, does the greatest hazard lie in the divers ingesting microorganisms or in inhaling an aerosol into the lungs?

Gottlieb: I wouldn't want it either way. If you ingest a pathogen, you may get enteritis, and, depending on the amounts ingested, it could be quite incapacitating. Inhaling a pathogen could result in a respiratory infection. Also, a diver could manifest anaphylaxis. Then a medical emergency exists.

Joseph: With respect to ear infections as a recognized problem, I recall that the Navy did a study on ear infections a few years back. I though the problem had been solved, yet we are still hearing about it. Why is that?

Greene: The solution Sam is referring to is the use of otic Domeburo drops several times a day. That is very effective in preventing ear infections, which are a fairly specific and unique problem involving *Pseudomonas* and a resulting transformation of flora probably related to hyperhydration of the skin which exists in a high pressure or helium environment. Unlike exposure via swimming, when the ear is immersed in cold or lukewarm water and exposed to polluting organisms, we think the hazard in hyperbaric chambers comes from the flora of the diver himself. The problem of actual inoculation of foreign organisms from aquatic environments is a different subject. The effective treatment in saturation diving is for the diver to be forced to stay in a position to treat the ear for five minutes; then he turns to treat the other ear; and repeats this at least twice a day. It's difficult to make anyone do that. In saturation diving, ear infections can be incapacitating.

Shilling: I don't think that there is any question that Kris is right. If you do the things you are supposed to do, ear infection is no problem. But there are many SCUBA divers who don't even see a doctor; or, if they do see a doctor, he hasn't a foggy idea what diving is about. So you have an educational problem, at least, in this connection.

In talking about the exchange of microflora in a confined space, during World War II, I did a very careful study of a submarine patrol. For the first week or ten days, everybody had everybody else's colds, sore throats, etc. Then it leveled off and everybody was fine until they picked up a prisoner. So divers are not the only ones subject to infection when held in a confined space.

Daggett: I wonder if positive data about infections, etc., reported for divers stems, not from a low incidence, but, perhaps, simply from not recognizing the source of the infection. Some protozoans that we are now aware of as pathogens were only recently discovered although they have been around for some time. After discovery, people in the clinical laboratory become aware of their existence, and you start picking up cases.

Wells: Some years ago we also experienced significant problems during saturation diving, in this case, hydrogen-oxygen. Looking at the diving records, I discovered we were losing over 15% of our diving time to ear infections. We undertook a study of skin infections as well, but ear infections were causing us to lose diving time. With behavior modification, using the techniques Kris talked about, we virtually eliminated the problem in our saturation diving program. I am now also changing the sanitary habits of divers at depth. Our problem now is simply a lack of discipline—divers not following prescribed methods. You pay for relaxing your standards in ear care. Where we have made sure that these ear care procedures have been put into effect, we have had very little problem; where it's relaxed, even in presumably clear water, we have significant problems.

Williscroft: There are divers who never have ear problems and there are divers who have ear problems every time they go into the water. This area might be a subject for a future study—to find out why some people don't follow the procedures.

Gottlieb: It may not be what you do or don't do; it may be the nature of the secretions in the ear.

Colwell: There are data now being gathered which suggest that lipophilic organisms are particularly attracted to the ear canal, because of secretions in the outer ear. In addition, the ear canal lipids provide attachment for the bacteria. There are some bacteria that, when attached to foreign surfaces or structures, anchor firmly. Such surfaces can act selectively for *Aeromonas*. These become the ear infection or skin infection organisms, or if you are unlucky enough to swallow some, gastro-intestinal infection. The chemical structure of ear secretions has been studied and the data show significant variation among individuals.

Gottlieb: I think a sensible path to take is to look at the ear secretions of divers not prone to infection, to find out what the microbiostatic components are, compared with the ear secretions of divers who seem to be very prone to ear infections.

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Bacterial Contamination of Divers During Training Exercises in Coastal Waters

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INTRODUCTION

A common situation faced in the diving profession is the necessity for divers to operate in harbor waters that are polluted with biological and chemical substances. Biological pollutants include overtly and potentially pathogenic microorganisms. These organisms pose serious hazards to the health of divers if they are ingested or enter breaks in the skin. Infection of persons in contact with polluted water is well documented [1, 2, 3] as are the numerous bacteria that cause these infections. We have found that *Aeromonas,* a potential pathogen emerging as a cause of wound infection[1] and enteric disease,[4] is prevalent in many harbor waters[5], along with such other pathogens as *Vibrio parahaemolyticus, Escherichia coli, Klebsiella,* and *Salmonella.*[6] Until recently, little was known about microbial hazards to divers; however, we are now assessing the extent of these hazards and the ability of various types of diving equipment to protect the divers from them. This study reports results obtained from the assessment of bacterial contamination of divers during exercises in several areas of the United States, and was conducted under the auspices of the Naval Medical Research and Development Command and the National Oceanic and Atmospheric Administration (NOAA).

Study Areas and Sampling Procedures

Harbor areas in which these studies were conducted were sites of NOAA diver operations and training exercises in Norfolk, VA, Seattle, WA, and New York, NY. In each area, water was measured (Figure 1) for temperature, dissolved oxygen concentration, transparency, and salinity. In addition, water and sediment samples were collected during the operations for determination of viable bacteria present and for identification of pathogenic bacteria that could represent a hazard to the divers. Bacteriologic analyses were carried out within 12-24 hr of sample collection.

Figure 1. Measurement of water conditions.

Before and after each dive, the nasal passage (Figure 2a), throat (Figure 2b), ear canal (Figure 2c), and mask (nasal area, Figure 2d) of each diver were swabbed with sterile, cotton-tipped swabs. These swabs (Figure 3) were placed into 5 ml of a liquid holding medium (Cary-Blair transport broth) which maintained the bacteria in a living but nonproducing state and at ambient temperature for transport to the laboratory. In most cases, samples were transferred to appropriate culture media within 24 hr. Assessments of bacterial contamination of divers were based on an approximate quantification of viable organisms present at each body site as well as on identification of specific potential pathogens that were not present before the dive.

Water Conditions

The variations in the physical and bacteriological condi-

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Figure 2a. Nasal swab.

Figure 2b. Throat swab.

Figure 2c. Ear swab.

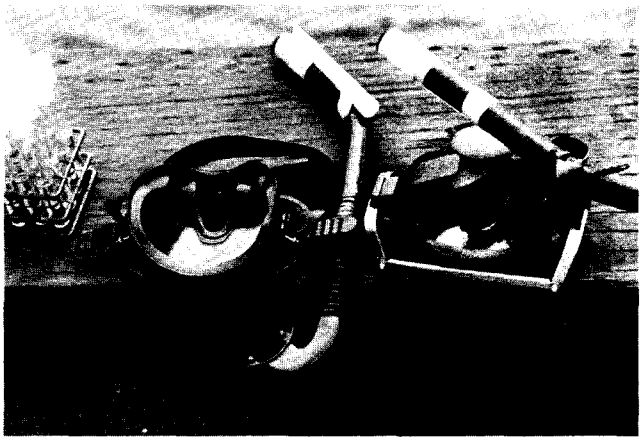

Figure 2d. Inside of face masks.

tions of the water at the different dive sites are apparent from the data in Table 1. Water temperatures ranged from a low of 4 °C in Norfolk to 18 °C in New York Harbor at Governor's Island pier. Water temperatures in Seattle fell between these extremes. Other measured parameters of dissolved oxygen ($D\text{-}O_2$), transparency, and salinity varied as well, as did the number of organisms, although the latter showed only small fluctuations. Interestingly, even when the water was relatively clear (e.g. 5-8 meter transparency), organism counts were high, which illustrates the fallacy of assuming that clear water is free of contamination by potentially harmful bacteria. This parameter can only be judged by identification and quantification of the bacteria present in the water.

Figure 3. Swab is placed into transport broth.

Table 1. Water conditions at dive site.*

Site (date)	Temp. (°C)	$D\text{-}O_2$ (ppm)†	Trans-par. (meters)	Salin-ity (ppt)†	Organ-isms, $X10^5$‡
Norfolk, VA (2/80)	4	8.6	1.0	12	14.0
Seattle, WA					
Lake Union (7/15)	15.6	12.5	3.0	0	7.2
Puget Sound (7/17)	9.5	9.8	5.5	29	7.8
Puget (7/17)	11.8	11.6	5.5	28	14.0
Lake Union (7/22)	17.3	11.3	3.0	0	9.6
New York, NY					
Pier (7/80)	18.0	6.4	2.0	25	12.0
Bight (8/80)	17.0	14.3	8.5	30	7.8
Bight (11/80)	9.0	9.0	4.75	30	14.0

*All parameters measured 1 meter below surface.
†ppm, parts per million; ppt, parts per thousand.
‡Direct count by epifluorescence, no. of organisms per ml.

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Figure 4a. Diver in wet suit.

Figure 4b. Unisuit with AGA mask.

Figure 4c. Superlight-17 hood/mask.

Figure 4d. Kirby-Morgan (Mark-1, Mod-0).

Diver Sampling

At the NOAA Atlantic Marine Center, Norfolk, VA, we conducted sampling procedures on 16 divers who wore four different combinations of diving gear. These were: (a) the standard neoprene foam wet suit with standard mask and backpack-supplied second stage regulator ("SCUBA"), shown in Figure 4a; (b) the Unisuit^{R} with AGA^{R} full-face mask, Figure 4b; (c) the Unisuit with the Superlight-17 hood/mask, Figure 4c; (d) the Unisuit with the Kirby-Morgan (Mark-I, Mod 0) helmet, Figure 4d. The distribution of equipment among divers participating in

the study is shown in Table 2. Identification of the predominant, potentially pathogenic bacteria isolated from the divers is presented in Table 3.

Table 2. Mask use by divers participating in the study.

Mask Type	Diver No. (Total No.)
SCUBA	3, 4, 5 (3)
AGA	2, 7, 9, 10, 14, 16 (6)
Superlight-17	1, 11, 12 (3)
Kirby-Morgan Mk1	6, 8, 13, 15 (4)

Table 3. Sampling of divers at the Atlantic Marine Center.

Diver No.	Mask Type	Sample site*	Organism(s) isolated
3	SCUBA	A-N	*Klebsiella oxytoca*
		A-N	*Aeromonas hydrophila*
		A-T	*A. hydrophila*
		A-E	*A. sobria*
		A-M	*A. sobria*
4†	SCUBA	B-N, A-T	*Enterobacter aerogenes*
		A-E	*K. pneumoniae*
		B-M, A-N, A-T	*A. sobria*
		A-M	*A. hydrophila*
5	SCUBA	A-E, A-M	*A. hydrophila*
9	AGA	A-E	*A. hydrophila*
		A-E	*A. hydrophila*
12†‡	SL-17	B-N, A-M, A-N	*Ent. aerogenes*

*Abbreviations: A = After dive; B = Before dive; N = Nose; T = Throat; E = Ear; M = Mask.
†Divers 4 and 12 reported sore throats on the day of the dive.
‡Diver 12 was the third user of this mask.

These data give an indication of qualitative contamination of divers, i.e., the identity of bacteria that were present on the sampling sites after, and thus as a result of, the dive. Divers not included in this Table 3 (Numbers 1, 2, 6, 7, 8, 10, 13-16) showed no detectable differences in bacterial flora before and after the dive. Three of the divers who wore wet suits were contaminated after the dive by bacteria from the water, with 10 separate isolates from 12 different sites. In contrast, of the six divers in the Unisuit/AGA combination, only one showed post-dive contamination of the ear, a possible result of leakage that occurred around the face seal. The isolate of *E. aerogenes* recovered from diver no. 12 was present in the nasal passage before the dive and probably was responsible for contamination of the mask. This finding is indicative of the potential for spread of bacteria from diver to equipment and underscores the need for proper cleaning of equipment between users.

Similar sampling procedures were conducted at the NOAA Pacific Marine Center, Seattle, WA, where we followed four divers through ten days of diver training exercises in Lake Union (fresh water) and in Puget Sound (salt water). In contrast to the waters in Norfolk, Seattle area waters were relatively clear; however, counts of organisms were only slightly lower (Table 1). Assessments that represent relative bacterial contamination levels of the divers are presented in Tables 4, 5, and 6.

Table 4. Relative bacterial contamination of divers in Lake Union, 15 July.*

Diver no.	Swab Site[b]	Morning		Afternoon	
		Before	After	Before	After
1	Ear	+	4+	3+	4+
	Nose	2+	+	+	2+
	Throat	–	+	+	+
	Mask	3+	3+	4+	3+
2	E	3+	4+	+	4+
	N	4+	3+	2+	3+
	T	+	–	+	2+
	M	4+	2+	4+	3+
3	E	4+	4+	4+	4+
	N	+	2+	+	+
	T	+	+	+	–
	M	+	+	2+	+
4	E	+	4+	+	4+
	N	4+	4+	4+	2+
	T	+	2+	+	2+
	M	3+	+	2+	4+

*Based on number of colonies on primary cultures of swabs taken from the respective sites. 4+ = confluent growth; 3+ = 100 – 300 colonies, 2+ = 50 – 100 colonies, + = 1 – 50 colonies, – = no growth.

When wet suits (with hoods) were used, especially in fresh water (Table 4), heavy post-dive ear contamination was common, with sporadic increase in numbers of bacteria in the nose and throat. Masks became contaminated after the first dive and remained so throughout the day. This contamination appeared to be heaviest during salt water diving (Table 5), a possible reflection of the lack of fresh water with which to wash equipment between dives, and another indication of the importance of such cleaning, which was standard procedure after dock-side dives.

In most instances when divers wore the Unisuit (Table 6), bacterial levels were high, but relative levels changed little over the day's diving period. We have postulated that, while the dry suit affords excellent protection from the polluted water environment, increased humidity and heat inside the suit could promote rapid growth of indigenous bacteria. This possibility currently is under more detailed investigation.

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Table 5. Relative bacterial contamination of divers in Puget Sound, 17 July.*

Diver no.	Swab Site[b]	Morning		Afternoon	
		Before	After	Before	After
1	E	4+	4+	+	4+
	N	3+	3+	4+	4+
	T	+	+	2+	3+
	M	–	4+	3+	4+
2	E	2+	4+	3+	4+
	N	2+	3+	4+	3+
	T	2+	3+	3+	3+
	M	+	4+	4+	4+
3	E	4+	4+	3+	4+
	N	3+	4+	4+	3+
	T	2+	2+	4+	4+
	M	+	4+	+	4+
4	E	+	4+	+	4+
	N	4+	4+	4+	4+
	T	3+	3+	3+	4+
	M	2+	4+	4+	4+

*Key to Table 4 applies.

In Tables 4 and 5, the data indicate that the bacterial levels in the ear of diver no. 3 consistently were high. This diver in fact developed a severe case of right and left external ear canal infection on 18 July which forced him to stop diving. On reviewing the bacterial cultures from samplings of his ears, we noticed that there were striking qualitative changes in the bacterial flora present between the morning and afternoon dives of 17 July. The flora was heavy and mixed after the morning dive, but before the afternoon dive (about 4 hours had elapsed), his ear contained a nearly-pure culture of *Pseudomonas aeruginosa,* a pathogen that commonly causes "swimmers' ear." The flora again was mixed after the dive, with the infection progressing to the point where the diver experienced discomfort and treatment became necessary. Bacteriological findings dictated that effective treatment would be acetic acid-glycerol ear drops, which rapidly cleared the infection. This case illustrates the importance of using prophylactic ear drops even when diving in apparently clear waters.

Sampling of divers in the New York Bight was done aboard the NOAA ship *George B. Kelez,* during operations in which divers were monitoring an experiment to cap the dredge spoils site with sand. The water in the Bight is relatively clean, with mild temperature, high D-O_2, and good visibility (Table 1). We again sampled the divers' ears, noses, throats, and masks, and also sites on the boot and suit. We found that the divers became contaminated by several organisms during the dive (Table 7), a probable result of suit leakage since all wore the Unisuit/AGA combination. After the dives, we attempted a disinfection procedure that involved spraying the fully-suited divers with Betadine^{R} Surgical Scrub

Table 6. Relative bacterial contamination of divers in Lake Union, 22 July.*

Diver no.	Swab Site	Morning†			Afternoon‡	
		Before	After		Before	After
5**	E	+	4+	*	4+	4+
	N	4+	3+	*	3+	3+
	T	2+	3+	*	1+	3+
	M	3+	3+	*	3+	3+
6**	E	3+	4+	*	3+	4+
	N	4+	3+	*	2+	2+
	T	+	–	*	+	+
	M	3+	+	*	+	+
7	E	+	3+		4+	4+
	N	–	+		+	+
	T	–	+		+	–
	M	+	+		2+	3+
8	E	3+	3+		4+	4+
	N	3+	3+		3+	4+
	T	3+	2+		4+	+
	M	–	2+		+	+

*Key to Table 4 applies.
**Different divers in morning and afternoon.
†Unisuit with standard mask.
‡Unisuit with AGA mask.

Solution. As shown by the data in Table 7, no organisms were recovered from the suit exterior following the Betadine spray. More detailed studies on suit disinfection currently are in progress.

Table 7. Sampling of divers during New York Bight capping operation, November 1980.

Diver No.	Sampling Site	Organisms Isolated* Before Dive	After Dive	Betadine Spray†
1	Ear	—	—	ND
	Nose	—	*Klebsiella pneumoniae* *Pseudomonas* sp.	ND
	Mask	—	*K. pneumoniae* *Pseudomonas* sp.	ND
	Throat	—	*Pseudomonas*	ND
	Boot	GPR	*Enterobacter cloacae*	—
	Suit	—	GNC	—
2	Ear	—	—	ND
	Nose	—	—	ND
	Mask	GPR	*Pseudomonas* sp.	ND
	Throat	—	—	ND
	Boot	*Ent. agglomerans*	*Pseudomonas* sp. *Ent. cloacae*	—
	Suit		*Pseudomonas* sp.	—

*Abbreviations: GPR = Gram Positive Rod; GNC = Gram Negative Coccus; ND = Not Done.
†Thorough spray with 100% Betadine Surgical Scrub solution followed by thorough rinse with fresh water.

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Table 8. Disinfection of divers in Norfolk, VA, May 1981.

Disinfectant	Ranking of relative no. organisms present*								
	Suit P			Suit V			Suit A		
	Pre†	Post	Dis	Pre	Post	Dis	Pre	Post	Dis
None	10	10	10	10	10	8	2	3	6
Water	8	10	10	2	10	10	2	9	2
Betadine	9	10	5	2	6	2	2	10	3
Amway	7	10	7	5	10	3	7	7	1
Zepamine	8	10	2	1	10	2	9	9	5
Formula 100	7	10	10	6	9	2	10	10	10

*Abbreviations: Pre, pre-dive; Post, post-dive; Dis, after disinfection. Sampling site was between knee and ankle.

†Rank of 1 = 10 or less CFU/10cm² suit area; rank of 10 = greater than 10^6 CFU/10cm².

Correlations may exist between the ability of an organism to adhere to a diver or to diving equipment and its ability to initiate an infection. Our studies on *Aeromonas* show that its adherence capability is related, along with other factors, to its ability to cause human disease.[7] Associations of this type emphasize the importance of being able to protect divers from microbial hazards present in polluted environments. Our work is progressing toward this goal by evaluation of suits that physically separate the diver from the hazard and by study of means to remove the contaminating organisms as the diver leaves the water.

ACKNOWLEDGEMENTS

This work was supported by National Oceanic and Atmospheric Administration Contract 01-8-M01-2027 and by Naval Medical Research and Development Command work unit MR04105.001.0057. We are grateful for helpful discussions with Dr. R. I. Walker, and acknowledge the very valuable contributions to this study made by D. M. Rollins, J. D. Gillmore, A. N. Green, and C. L. Williams of NMRI, and by C. Caravoulias and E. Elliot of the University of Maryland. We also thank Ms. D. Boyle for preparation of the manuscript.

The use of trade names is for identification purposes only and does not constitute an endorsement by the Department of the Navy or the National Oceanic and Atmospheric Administration.

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DISCUSSION

Phoel: What was the site and depth of the measurements taken in the New York Bight?

Coolbaugh: Measurements were made from samples collected at stations over the dredge spoils site. Samples were collected at a depth of one meter.

Phoel: Regarding the data you presented for the Kirby-Morgan and the Superlight-17—were those dives conducted in a demand or free-flow mode?

Wells: In a demand mode.

Phoel: You might consider a free-flow mode and see if you get the same results.

Gottlieb: When you isolated specific organisms, did you merely identify them or did you also attempt to determine concentration, that is, the number of organisms per unit volume or number of viable cells per unit area?

Coolbaugh: Total viable counts were measured for the water samples. In most of our studies over the past year, specific organisms were isolated without quantification of each species. Quantification was limited to determining the number of coliforms and *Aeromonas,* as well as total viable count.

Gottlieb: How do you account for the marked change in ear flora between the first and second dive, when the diver entered the water with a mixed flora and came out with a pure culture of *Pseudomonas*?

Coolbaugh: The apparently pure culture of *Pseudomonas*

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came from the diver's ear before his second dive, and 3 to 4 hours out of the water after his first dive.

Gottlieb: How do you account for such a rapid change?

Coolbaugh: When we sampled after the first dive, we probably absorbed some of the water from his ear canal onto the swab, so the culture reflected the flora from the water. As the water drained out, the *Pseudomonas* became the predominant organism since we assumed that by this time the external otitis was already established.

Gottlieb: When you swabbed the ear and recovered *Pseudomonas,* do you think that was a result of not going in far enough to recover any other organisms, or do you think others were washed out as a result of the dive?

Coolbaugh: When a man dives in a wet suit, his ears generally become flooded. When that water drains there must be a slight flushing action, with only those organisms remaining that are able to adhere, or that have already colonized the ear *(Pseudomonas* in this case).

Greene: Does *Pseudomonas* actively suppress viability of other organisms present?

Coolbaugh: I think it can.

Colwell: May I provide some data that is ancillary? It may explain what might happen. One of our students has observed that when rates of attachment are measured using a *Vibrio* sp. in the presence of another *Vibrio* sp. or mixed flora, inhibition of attachment was observed. But observed also was that there is preferential attachment when two cultures are mixed by laterally flagellated organisms. The evidence points to a selection of flora attaching to surfaces, including the skin.

Coolbaugh: Pathogens will often have a selective advantage, based on an ability to adhere preferentially and enhanced virulence, compared with other, nonpathogenic, water-borne organisms.

Colwell: While Dr. Coolbaugh and Ms. Caravoulias did not estimate the number of organisms *per se,* the water sample itself was analyzed and the data used to develop an index; that is, we wanted to know, when *Aeromonas* concentrations reach a certain level, whether there would be an increase in the number of positive swabs. It might well be that it would be appropriate to try to enumerate these bacteria within the ear.

Coolbaugh: It seems that even when we have a hard time finding *Aeromonas* in the water samples, the divers often will pick up this organism after diving.

Williscroft: I was with Dr. Coolbaugh on most of these projects, and he and his people were operating under what you might call extremely difficult circumstances. They were continuously inserting their sampling process into an ongoing, operational situation. You can imagine how the divers loved that.

Coolbaugh: One problem we face in designing experiments to accomplish our goals is what you might call an ethical one. That involves sending divers in wet suits into water we know is heavily polluted, just for the purpose of seeing what they will pick up. On our current trip to Norfolk, we decided that all divers should be in dry suits, and that the safety of the men came first. In New York, the water is, bacteriologically at least, relatively clean, although we can't comment on possible toxic chemicals that may be present. The low bacterial levels in the Bight may be a result of the almost-completed capping operations.

Phoel: The sand capping is completed, and we will be diving there in 2 weeks.

Joseph: In general, when discussing a relatively clean area, you have to remember that not all organisms survive well in a highly saline environment, especially if they are from an external source.

Oliver: There have been some interesting studies on coliforms in marine waters and their die-off. In cold water with a high saline content, these bacteria may not be killed, but merely debilitated. The combination of salts and cold damages the membranes, and when the organisms are placed onto a selective medium, inhibitory dyes will kill the bacteria. Instead, a recovery stage is necessary where the organisms are put into a nonselective medium at 37°C for a half-hour or so to allow the bacteria to regenerate or repair their cell envelopes

Joseph: I would refer to the comment Dr. Coolbaugh made that, in some instances, organisms were not isolated from the water, but were recovered from the divers. The ear or throat may provide a more suitable revitalization environment for the organisms, as opposed to direct plating on artificial media.

Coolbaugh: The procedures certainly are different. Swabs from divers are placed into a transport medium to keep the bacterial cells viable until we can put them on appropriate media in the laboratory, while water samples are plated directly onto selective media. This difference could explain the apparent disparity.

Phoel: Just a quick remark on the dredge spoils site itself—the dumping is sporadic, unlike the sewage sludge dump site, which has a daily input. The influx of material is variable and I would imagine that a result would be a variation in bacteriological findings as well.

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The *Legionella* Group of Bacteria in the Environment and in Human Disease

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CLASSIFICATION

Although *Legionella pneumophila* was first described as an important human pathogen in 1977,[1] there is good evidence that this organism and related species have been in the environment and have played a role in human disease for at least several decades. Thus far five species have been recognized in the genus *Legionella* (Table 1).

L. pneumophila is the type species which was responsible for the outbreak of severe pneumonia among the legionnaires attending a convention in Philadelphia in 1976 and outbreaks before and after 1976 in other locations.[2*] This species is also responsible for a large number of nosocomial pneumonias, and Pontiac fever, a mild acute disease characterized by febrile myalgia not involving the lungs. At the present count, there are six distinct serotypes[3,4], all proven or suspected human pathogens.

L. micdadei[5] was named in honor of Joseph E. McDade who was chiefly responsible for establishing that legionellosis is an infectious disease. It was first isolated in 1943 by Tatlock[6] by inoculating defibrinated blood from a patient into guinea pigs. The disease involved 40 soldiers housed in the same barracks at Fort Bragg, NC, in 1942, smaller numbers in the summers of 1943 and 1944, and civilian populations elsewhere. The disease was characterized by 3 to 5 days of fever, moderate prostration, splenomegaly, a papular eruption typically limited to the legs, and rapid return to normal health. The disease was called pretibial fever (because of the location of the papular eruption) or Fort Bragg fever. It was recently shown that the Tatlock agent is virtually identical to the HEBA agent isolated by Bozeman et al.[7] in 1959 from a suspected case of *pityriasis rosea,* and to the Pittsburgh pneumonia agent isolated in 1979 from two immunosuppressed renal transplant recipients.[8] There were a total of 13 similar cases in Pittsburgh and Virginia, with 7 fatalities.

L. bozemanii,[9] the WIGA agent, was isolated by Bozeman et al.[7] from a Navy diver who died of acute bronchopneumonia following a diving exercise. A similar agent was isolated in 1978[10] from a fatal case of pneumonia. The patient apparently acquired the infection when he was thrown from his boat and swam in murky, swampy water in South Carolina for 15-20 minutes before he was rescued. He was on antileukemic and immunosuppressive therapy because of stable chronic lymphocytic leukemia. *L. bozemanii* infection has tentatively been attributed to several other pneumonia cases.

L. dumoffii[9] was named in honor of the late Morris Dumoff, probably the first to cultivate *Legionella* on bacteriologic media. It includes strain NY 23 isolated from a sample of water from a cooling tower in New York, New York, and Tex-KL isolated from a postmortem lung specimen obtained in Houston, Texas.

L. gormanii was recently proposed as a new species for a strain isolated by George W. Gorman from a creek bank at a country club in Atlanta, Georgia. Although this is the only isolate, there is good evidence that this species has been involved in human disease.

The classification in the genus *Legionella* is based primarily on DNA-DNA hybridization. Strains of the same species hybridize to at least 70 percent, or 60 percent in the case of two strains of *L. bozemanii.* Interspecies relatedness varies from 4-25 percent, which in some cases may not be significant. However, the five

*Reference for most of the early work, not otherwise quoted. The review of the literature was completed in December, 1980.

Table 1. The genus *Legionella:* Disease association and distinguishing characteristics.*

Species	Isolation		Disease association**
	Strain	Source	
L. pneumophila	Olda (47)†	Blood	Mild fever
	Pontiac (68)	Sentinel guinea pig	Pontiac Fever (mild)
	Philadelphia plus at least 30 strains, 6 serotypes	Worldwide, from clinical, autopsy, and environmental specimens	Usually severe pneumonia, often nosocomial, some cases of Pontiac fever
L. micdadei	Tatlock (43)	Blood	Ft. Bragg fever (mild)
	Heba (59)	Blood	Not known
	Pittsburgh	Lung	Severe pneumonia
L. bozemanii	Wiga (59)	Lung (diver)	Severe pneumonia
	MI-15	Lung (fisherman)	Severe pneumonia
L. dumoffii	NY 23	Cooling tower	No direct association
	Tex-KL	Lung	Severe pneumonia
L. gormanii	LS-13	Creek bank, Atlanta	Severe pneumonia (indirect evidence)

Species	DNA-DNA hybridization		Predominant fatty acids	Fluorescence (long wavelength UV excitation)	Oxidase	β-Lactamase
	Intraspecies (%)	With type species (%)				
L. pneumophila	80-71‡	—	i-16:0	Dull yellow	±	+
L. micdadei	90-76	5-7	a-15:0 a-17:0	Dull yellow	+	–
L. bozemanii	56-64	13-6	a-15:0 i-16:0	Blue white	–	+
L. dumoffii	90-98	16-8	a-15:0 a-17:0 i-16:0	Blue white	–	+
L. gormanii	—	20-0	a-15:0	Blue white	–	+

*From cited references.

**Some isolations were not directly associated with outbreaks or sporadic cases, but involvement in previously undiagnosed cases was indicated by immunofluorescence in autopsy lung specimens or rising antibody titers in surviving patients.

†Year of isolation, if prior to 1976.

‡The two numbers refer to tests performed at 60 °C and 75 °C, respectively.

species closely resemble each other phenotypically. As Brenner et al.[9] put it, "Ideally, a genus should contain a group of genetically and phenotypically related species. When both criteria cannot be met, phenotypic relatedness should take precedence to ensure that the genus designation is of practical use at the bench."

EPIDEMIOLOGY AND ECOLOGY

From an anthropocentric point of view, the chief common phenotypic characteristic is the potential human pathogenicity of all five species. The disease most commonly encountered is described as a severe progressive pneumonia with high fever and abscess formation and is often associated with neurological symptoms, diarrhea, and evidence of kidney and liver involvement. The presence of numerous bacteria can be demonstrated by fluorescent antibody in the lungs, but seldom in the other organs.[12]

The five species probably also share the same habitat in waters and soil. The infections with *L. bozemanii* of a diver and of a fisherman ejected from his boat have already been noted. In many cases outbreaks of legionellosis have been attributed to the fine sprays produced by cooling towers and evaporating condensers. These devices are widely used to reject unwanted heat into the atmosphere and depend for their operation on intimate contact between the circulating water and the ambient air. Contamination is derived either from the water or air. During the Bloomington epidemic, *L. pneumophila* was isolated from a cooling tower, from two water and two soil specimens from the nearby river and its banks. A cooling tower was also believed to have been the source of a major outbreak at the Medical Center Hospital in Burlington, Vermont. This outbreak was described in the News and Comment section of *Science.*[13] There have been numerous other outbreaks involving air-conditioning systems. For example, an outbreak involv-

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ing six members of a country club in suburban Atlanta, Georgia, was traced to the exhaust of an evaporating condenser blowing directly on a nearby practice green and two tees.[14] One strain was isolated from the evaporating condenser and one from the moist sandy soil near the creek. In the Bloomington and Atlanta outbreaks the environmental isolates were not always of the same serotype as those obtained from patients. Thus, environmental isolations only indicate prevalence of *Legionella* but do not establish causal relationships. Most instructive was the investigation of Dondero et al.[15] of an outbreak in a hospital in Memphis, Tennessee, involving 44 cases with 7 deaths. On the assumption of an incubation period of two to ten days, the cases coincided with the operation, following an emergency, of an auxiliary air conditioner which had not been used for nearly two years and had not received chemical treatment of its water. In the St. Elizabeth outbreak of 1965, cases were epidemiologically associated with proximity to excavations.

L. pneumophila has also been isolated from a variety of aquatic habitats not related to epidemics or sporadic cases. Fliermans et al.[16] examined 200 specimens obtained from 23 lakes in Georgia and South Carolina and found that 90 percent were positive by the fluorescent antibody test. His observations were confirmed by four isolations. *L. pneumophila* was also isolated from an algal-bacterial mat community growing at 45 °C in association with cyanobacteria *(Fischerella* sp.).[17] *Legionella* was also found to be quite stable in tap water and dilute solutions of common disinfectants. It thus appears that *Legionella* can multiply and survive in a great variety of environments.

PROPERTIES OF THE MICROORGANISMS

Legionella are rather typical Gram-negative microorganisms. They are short blunt rods, measuring 0.3-0.9 by 2-5 μm, dividing by nonseptate binary fission. They have two unit membranes separated by a peptidoglycan layer. Long forms >10 μm are often seen when the organisms are not actively multiplying.[18,19] Fresh cells growing on a highly satisfactory medium often have flagella and sometimes pili.[20] Flagella, in contrast to somatic antigens, cross-react with antisera prepared against cells of different serotype or species.[5] *Legionella* do not stain intensely with safranin, but do stain well with basic fuchsin in the Giménez procedure, or by the silver impregnation method of Dieterle. These are not unusual properties and *Legionella* have no morphologic features that distinguish them from other Gram-negative bacteria.

The medium of choice and the only medium that supports the growth of all strains during primary isolation is charcoal yeast extract agar.[21] This medium contains 1% yeast extract, activated charcoal, cysteine, soluble ferric pyrophosphate, and agar. Best results were obtained when NaCl was not added. Ristroph et al.[22] showed that charcoal can be omitted if the yeast extract is filtered instead of being autoclaved. Smalley et al.[23] showed that a small amount of sodium selenate greatly enhances growth. The recommended pH is 6.9. *Legionella* are slow-growing bacteria, requiring 2-10 days to form colonies, depending on the strain and passage level, and under optimal conditions have a generation time of 100 minutes.

Legionella have an active amino acid metabolism, and serine, and in some cases proline, appear to be the chief sources of energy.[24] Some of the strains are powerful pigment producers, a reaction enhanced by tyrosine. It is often stated that *Legionella* do not produce acid from common carbohydrates. Weiss et al.[25] have shown, however, that these organisms do metabolize glucose to a moderate extent. Much of the glucose carbon is incorporated and glucose-1-phosphate is utilized quite well, which suggests that glucose is chiefly utilized for the synthesis of glycoproteins rather than for energy. *Legionella* have a rather unusual fatty acid composition.[26] All species have large amounts of branched-chain fatty acids, but each species has a characteristic pattern; for example, i-16:0 is most abundant in *L. pneumophila,* a-15:0 in *L. bozemanii,* and a-15:0 and a-17:0 in *L. micdadei.*[5]

Differential characteristics have been studied in some detail. The most obvious differences are seen in the oxidase reaction, positive in *L. pneumophila* and *L. micdadei* but not in other species; in the blue-white fluorescence elicited by 366 μm UV in *L. bozemanii, L. dumoffii,* and *L. gormanii,* but not in the other species; and, most importantly, in the β-lactamase reaction which is negative in *L. micdadei,* but positive in the other species.[11] *L. pneumophila* is highly susceptible to rifampin and erythromycin. Erythromycin greatly increases the chances of survival of patients in uncomplicated cases and is the drug of choice. However, the other species are not quite as susceptible to erythromycin as *L. pneumophila. L. micdadei* is highly susceptible to penicillin, but *L. bozemanii* is not.[5] With this type of infection, rifampin might be the only recourse for treatment, provided the infecting agent is promptly identified.

HAZARD OF INFECTION

Attempts to find objective similarities, no matter how tenuous, between *Legionella* and other genera have not been successful.[27] However, we might wish to compare briefly *Legionella* with *Pseudomonas.* Both groups are aerobic Gram-negative bacteria commonly found in waters and soil. Both groups are heavily involved in infection of immunosuppressed patients. *Pseudomonas* produces powerful exotoxins[28] and the same may be true of *Legionella.*[29] However, the comparison between *Pseudomonas and Legionella* cannot be pushed much further. *Pseudomonas* are fast-growing organisms with moderate nutritional requirements. Intracellular growth in the patient or in the experimental animal is not a prominent feature. *Legionella,* on the other hand, is a slow-growing fastidious microorganism

which is often seen in the phagocytic vacuoles or phagolysosomes of macrophages. Horwitz and Silverstein[30] showed that *Legionella* grow quite well in adherent human monocyte cultures. Growth was strictly intracellular, however, since the medium did not support the growth of *Legionella.* Plasmids play an important role in the physiology of *Pseudomonas,* coding for antibiotic resistance and other properties. Plasmids have been described in *Legionella,*[31] but they are lost upon passage on bacteriologic media.

Some of the properties of *Legionella* that are closely associated with virulence may be lost upon repeated passage. It was shown by McDade and Shepard[32] and Ormsbee et al.[33] that *Legionella* lose virulence for the guinea pig and chicken embryo and lose cytotoxicity upon repeated passage on bacteriologic media. Whether or not *Legionella* acquire virulence through animal passage is not known. All isolations thus far have involved man or animal passages, which introduces a bias in favor of virulence. Isolations directly from patients or animals on bacteriologic media have been made, but environmental specimens are too contaminated for such a procedure. The method most commonly used for such isolations is the one described by Morris et al.,[34] which in its initial steps is identical to the one used for rickettsiae.[1] Guinea pigs are inoculated intraperitoneally with the specimens and, on the second day of fever, splenic tissue is passed into the yolk sacs of chicken embryos. Heavily infected yolk sacs are passed onto bacteriologic media. Two techniques have been described that might be valuable in the isolation of *Legionella.* One is the negative enrichment technique of Thorpe and Miller.[35] The specimens are inoculated into tryptic soy broth to which are added CNV antibiotics (colistimethate, nystatin, and vancomycin). During incubation for 8 h at 22 °C there is a great reduction in the number of most bacteria, while the viability of the more fastidious *Legionella* remains constant. Highly imaginative is the method of Hranitzky et al.[36] by which 01 serovars of *Vibrio cholerae* were isolated. The specimens were passed through small columns of polystyrene beads coated with specific antibodies. After a thorough wash the beads were used for the cultivation of the desired microorganisms. Both techniques need to be applied to *Legionella* to avoid the bias introduced by animal passage.

In conclusion, what is the hazard of legionellosis? We have an infectious disease discovered almost exactly four years ago (January, 1977), which we now know has involved tens and, perhaps, hundreds of thousands of people throughout the world and which has a mortality rate of over 10 percent. Some of the immediate hazards can probably be eliminated: there is evidence that the biocides commonly used in cooling towers can be effective,[37] and a small survey has indicated that air-conditioning maintenance personnel are not at increased risk of legionellosis.[38] However, the broader risk of legionellosis cannot be assessed, because we still know too little of the biological properties and ecology of *Legionella.* More specifically, we need to investigate the following problems, preferably with low passage level strains derived both from patients and from the environment: (1) Physiology of the organisms, with special emphasis on those characteristics that are most commonly associated with the acquisition of virulence; (2) extracellular products, since many of the clinical and pathological findings suggest that *Legionella* elaborate powerful exotoxin; (3) development of specific antigens with low lipopolysaccharide content for use in surveys of humoral and cell-mediated immunity, since the serological studies done thus far provide only incomplete information on the extent of human involvement.

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DISCUSSION

Cavari: What is the cell density of *Legionella* in the environment?
Weiss: Sufficiently high for Fliermans to find them without difficulty on slides stained with fluorescent antibody, prepared with sediment obtained from large volumes of water.
Cavari: How can *Legionella* compete with other microorganism when it has such a slow growth rate?
Weiss: This is the reason why I suggested two methods of isolation. One method is to add antibiotics to a medium that allows the growth of fast-growing bacteria, but not *Legionella.* Under these conditions the fast-growing bacteria are preferentially killed. The other method consists of using antibodies for concentrating *Legionella* by affinity chromatography.
Cavari: What caused the epidemic outbreaks of *Legionella*?
Weiss: There is some evidence that an air-conditioner was involved in the Philadelphia outbreak, but this was not well documented. The best evidence that an air-conditioner was involved was obtained in the Memphis,

Tennessee outbreak. A flood knocked out the air-conditioners. Because of the heat, a reserve air-conditioner which had not been used for two years and which had not been serviced, was quickly put into operation. The result was an outbreak involving 44 cases.

Kaper: If a Navy diver came in and complained of a respiratory illness, would you suggest culturing specimens in an attempt to isolate *Legionella*?

Weiss: Yes. However, before culturing I would examine smears of fluorescent antibody. But I think culturing would be very important.

Gottlieb: If this organism is so slow-growing and so fastidious, how does it survive for such long periods of time, for example, in an air-conditioner that has not been used in two years?

Weiss: Perhaps this is the mechanism of survival. A microorganism which cannot grow unless conditions are just right is also protected from the unbalanced growth which can lead to cell death.

Colwell: May I offer another suggestion? *Legionella* lives in association with blue green bacteria which provides vitamins and other nutrients. *Legionella* is an organism that naturally occurs and exists in the aquatic environment. In the water reservoirs of the air-conditioners, algae also grow and could support growth of *Legionella.*

Weiss: This is an important point.

Williscroft: Speaking from an operational point of view, is *Legionella* a particular hazard to me as a diver?

Weiss: This is what we need to find out.

Denham: Have any attempts been made to isolate *Legionella* from marine environments?

Joseph: There has been a report of Pontiac fever among operators of steam turbines and evaporating condensers who cleaned their equipment with sea water.

Weiss: One outbreak occurred on the Potomac River and the other on the James River, but I do not know the exact location or salinity concentrations.

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Protozoa from Polluted Waters; Potential Human Pathogens

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The health risk to divers working in polluted waters to potential protozoan pathogens has not been adequately assessed. The most likely pathogens to be encountered are amphizoic protozoa rather than obligate parasites. Recent isolation of small free-living pathogenic amebae from estuarine and oceanic waters suggests these organisms may pose a low level threat to the health of divers.

INTRODUCTION

Although the nature and diversity of protozoa in variously polluted waters has and is being studied, the existence of potential human pathogens in these waters has not been adequately assessed. There currently does not exist a sufficient basis for determining the degree of exposure to protozoan pathogens by individuals working in polluted waters. Some exposure to potential protozoan pathogens in polluted waters is likely, but the risk is probably low for divers using an air supply and wearing a diving suit.

On the basis of current knowledge, the most likely health risk to divers is from amphizoic protozoa. Amphizoic protozoa are those that are capable of being either endozoic (symbiotic or parasitic) or exozoic (free-living).[1] But exposure to obligate parasitic species in polluted waters cannot be dismissed.

Amphizoic amoebae have been isolated from polluted waters and are known to be causative agents of human disease, or on the basis of virulence testing mice, potentially hazardous to man. Thus far, these pathogenic organisms have been identified as small free-living amoebae[2] and specifically two genera, *Naegleria* and *Acanthamoeba.*

BACKGROUND

There have been several recent reviews concerning these two genera.[2,3,4,5] It is necessary to provide some background for those not familiar with these organisms before addressing the potential health risk posed.

Free-living Pathogens

The life cycle of these amoebae is relatively simple. There is an active amoeboid form (vegetative trophozoite) which feeds primarily upon bacteria, but also upon fungi. Both types of amoebae form a cyst which is resistant to dessication. *Naegleria* trophozoites will also transform to a nonfeeding, swimming, biflagellated form under certain conditions. The only known pathogenic *Naegleria* species, *N. fowleri,* cannot be distinguished on a morphological basis from some nonpathogenic *Naegleria* species. Either the trophozoite or the flagellate form of *N. fowleri* is infective.

The rapidly fulminating human disease caused by *N. fowleri,* primary amoebic encephalitis (PAM), is contracted by the entry of the amoebae through the nose. It has been speculated that the depletion of mucous in the nasopharynx subsequent to long periods in water permits entry to the olfactory nerve which the amoebae follows to the brain. With the exception of a single human case having systemic involvement, infections have been restricted to the central nervous system. A recent environmental isolate of *N. fowleri* from a cooling lake receiving thermal waters produced both brain and lung infections in mice.[6] Based on this observation Willaert and Stevens[6] suggest that *N. fowleri* may cause subacute and acute respiratory infections in humans. Only a few human cases have been successfully treated with antibiotics.[3,5,7]

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Reported infections of *Acanthamoeba* have not been restricted to the central nervous system.[7] There are reported cases of eye infections and involvement of the pulmonary system. In a case of transient diarrhea, *Acanthamoeba* were isolated from stools, but it is unclear whether the amoeba was the causative agent. Infection of the central nervous system by *Acanthamoeba* is pathologically different from PAM and is clinically designated as granulomatous amebic encephalitis (GAE).[8] Invasion of the body may be through the nasal route, by inhalation, ingestion, or from a wound. Rather than a single species being involved as is the appartent case with PAM, several different species of *Acanthamoeba* have been implicated in reported infections.

Human Cases and Water Activity

There have been other reports of infections by other genera of amphizoic amoebae, but these have been of animals other than man.[1] The total number of known human cases involving *Naegleria* and *Acanthamoeba* is not large, totaling about 124.[3,5,7,8] Most cases have been associated with activity in water. When PAM was first recognized, in Australia, there appeared to be a specific association with saltwater tidal pools, but since these first cases, the vast majority have been contracted in warm or thermally polluted freshwater. The apparent association with saltwater does not appear to be typical.[5] There have been environmental isolates of *N. fowleri* from polluted waters (other than thermally polluted), but cases of PAM have not been reported from the isolation area.[9] Only from Australia, again, has an apparent association of some cases of GAE with saltwater been reported.[3] As with *Naegleria* infections, the majority of cases have not been associated with saltwater.

The methods of entry and the number of reported cases imply that these organisms pose a low-level hazard to divers using an air supply and wearing a suit. The risk increases with prolonged periods in the water, with splash back of polluted water in the face mask, or with the exposure of a wound. The greatest risk would be for divers not protected by a suit or using an air supply.

Presence in Polluted Waters

Another factor affecting the level of risk, would be the degree of exposure to amphizoic amoebae. There is recent evidence that several pathogenic *Acanthamoeba* species not yet implicated in human cases are associated with polluted estuarine and marine environments. Sawyer, *et al.*,[10] have described *A. hatchetti,* pathogenic when tested experimentally, collected from sediment from the Baltimore harbor. During the course of an investigation of the use of starch gel electrophoretic analysis of enzymes for the identification of small amoebae, additional pathogenic *Acanthamoeba* were discovered by this laboratory[11] (and unpublished observations).

Prior to investigating the application of this technique to *Acanthamoeba* species, the method was found effective for separation of pathogenic and nonpathogenic *Naegleria* strains.[12] Since the study of *Acanthamoeba* isolates included strains known to kill experimentally infected mice, strains nonpathogenic to mice, and strains which had not been tested for virulence in mice, all strains used were tested for virulence by intranasal instillation in CFW mice. Six previously not tested environmental isolates collected by Dr. Tom Sawyer (1-1Δ , 2-1Δ , NYB5a, OC3-A, OC3-2) killed experimentally infected mice. Two of these (1-1Δ , and NYB5a) were isolated from a New York dump site 7-8 miles offshore from Long Island, New York, and closed to shellfishing because of high coliform counts. One isolate was from a Philadelphia dump site located 40 miles offshore from the Maryland-Delaware area and also closed to shellfishing because of high coliform counts. The zymograms of propionyl esterase, acid phosphatase, and alkaline phosphatase proved identical to each other for 1-1 Δ and 31-B. Strains 2-1Δ and NYB5a also formed a matching pair. Zymograms of remaining two strains did not match each other or those of the matching pairs. These zymograms are also unique from the other 26 *Acanthamoeba* strains thus far examined, which include both pathogenic and nonpathogenic isolates. These results suggest that the six newly discovered pathogenic strains may include several new species, with two being from dump sites.

Dr. Tom Sawyer has found that *Acanthamoeba* can be readily isolated from active and inactive dump sites, as well as from polluted waters[13] (and personal communication). *Acanthamoeba* are frequently isolated from unpolluted estuarine and oceanic waters. The sites from which *Acanthamoeba* are readily isolated often have high coliform bacterial populations. Because pathogenticy testing of *Acanthamoeba* from acquatic isolations is not routinely performed in mice, the frequency of isolation of the virulent strains is not known. It is also not known whether strains pathogenic to mice are necessarily pathogenic to men.

Obligate Parasites

Two obligate parasites which pose a possible hazard to divers are another amoeba, *Entamoeba histolytica,* and flagellate, *Giardia lamblia.* Both cause intestinal disease and can be distributed by the improper disposal of human feces.[13,15,16] For both organisms, cysts in water or in contaminated food must be ingested to contract the disease. Active trophozoites will not survive the acidity of the stomach. Cysts of *E. histolytica* will survive typical municipal chlorine levels, temperatures of 0 C for several weeks, and temperatures of 30 C for up to 3 days.[15] High bacterial densities will adversely affect the viability of cysts.[14] How well cysts of either organism survive in seawater is not known. It is probable that these two organisms could not tolerate high salinities (Dr. L.S. Diamond, personal communication).

Risk to Divers

Potential protozoan pathgens apparently present a low-

level health risk factor for divers in polluted waters. Invasion by these protozoa would probably be effectively blocked by using a mask, an air supply and a suit. Unprotected divers would be at greater risk depending upon the frequency of exposure to these protozoa. The population densities of potential protozoan pathogens in polluted waters has not been assessed. The current sparse data implies that amphizoic amoebae are the potentially pathogenic protozoans most likely to be encountered by divers. Eight different *Acanthamoeba* strains belonging to several different species, isolated from estuarine and oceanic waters, are pathogenic to experimentally infected mice[9,10] (and unpublished observations). Whether these organisms infect humans is unknown, but it would not be surprising. There may be other unknown amphizoic protozoa in polluted waters potentially pathogenic to man. The risk from obligate parasites is probably even lower. Exposure to *E. histolytica* and *G. lamblia probably would be restricted to areas of sewage effluent.*

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DISCUSSION

Colwell: Would you like to comment about other kinds of parasites?

Daggett: There has been research done on this subject, but I am not current with all of the literature. It is necessary to consider, in this case, how the organisms are transmitted. There would be low risk of direct infection from most trematodes (flukes) and cestodes (tapeworms), since many require ingestion of an intermediate host. There are others which do not require an intermediate host. There has been some investigation into survivability to these parasites in treated sewage. Apparently tapeworm eggs survive the sewage treatment process fairly well. Eggs of *Ascaris,* a nematode which will infect humans, also survive the treatment process. It is a question of how much exposure will occur.

Colwell: There are silent infections as well as those manifesting mild symptoms

Daggett: Yes. Some of these infections, initially, are symptomatically mild. As I have noted, there is one case in which the presence of an *Acanthamoeba* was associated with diarrhea. Symptoms of what might be a mild, common ailment could be one of these pathogens. You get a headache. You have a fever. You think you have a cold. Then some hours, or a day or two after you have been in the water, you are rushed to the hospital comatose. Treatment is for viral or bacterial meningitis and proves to be ineffective. You just died of a *N. fowleri* infection. This scenerio is not uncommon in these cases. Medical personnel are not usually aware of the disease. Clinic laboratory personnel often mistake the amoebae for odd white

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blood cells. There are only three documented cases in which recovery occurred after antibiotic treatment. In some cases, in spite of the correct identification of the illness and the administration of what are thought to be effective antibiotics, the infection is fatal.

Joseph: Could you explain how these organisms are isolated when there actually may be only a few of the organisms swimming around in a large body of water?

Daggett: The acanthamoebas which were found to be pathogenic were isolated by Sawyer from estuarine and oceanic sediment. Frankly, it does not take very much sediment to isolate the organism. The original plates on which they were isolated were inoculated with less than a cubic millimeter of sediment. There was selection for acanthamoebas by the type of medium used, and potential pathogens were favored by culturing at 40°C. Amoebae can be isolated from the water column by filtering large volumes of water, for example 10 liters at a time, through an appropriately sized filter. The filter is placed on a plate of medium. Isolation should be on a non-nutrient agar, because bacteria will inhibit or kill the amoebae if the bacteria are too dense. If you are isolating bacteria, it is not probable that these amoebae will be cultured.

Joseph: By using zymograms can you distinguish between pathogens and non-pathogens?

Daggett: For *N. fowleri,* yes, and for *Acanthamoeba,* yes—if the isolated strain is one for which the pattern is already known. There are about 30 recognized *Acanthamoeba* species. We have examined over 30 strains, but not representatives of all the species. The differences in the patterns imply that there may be at least another 30 species. With acanthamoebas, it is probably necessary to do pathogenicity testing in animals.

Joseph: Are different species isolated from different geographical areas?

Daggett: Apparently not. For example, a nonpathogenic *Acanthamoeba* was isolated in 1965 from a nasal swab of a human in Indiana. In 1974, another strain was isolated from a fish in Alabama. The fish had lesions, but whether the lesions were caused by the amoeba is not known. Neither of the strains were infective for mice, but they were isoenzymatically identical in our analysis. They have an identical maximum temperature growth. Isolated almost 10 years apart, in different states, in different kinds of circumstances, they are, nevertheless, the same species. It is possible to find the same species in both fresh and sea water samples. Strains of *Acanthamoeba* are relatively tolerant of salinity changes, if gradual. *Naegleria fowleri* is apparently broadly distributed. In our studies, strains isolated in various geographical areas of the United States, as well as Australia, New Zealand, Belgium, and Czechoslovakia had the same zymograms.

Phoel: We dive in nuclear power plant outfalls, which are freshwater and very warm. What do you suggest we do to avoid this hazard?

Daggett: Use a nose plug. *Naegleria fowleri* has been isolated in the water effluent of power plants. In most cases it appears that entry is through the nose. As long as entry into the nose is prevented, it should be safe to dive. For reported cases caused by *Acanthamoeba,* there have been apparently several different routes of infection, including through wounds. The preventative measures to be taken are not always as simple to suggest, since the course of these infections is not as well understood as those of *N. fowleri.*

Crosa: Have any DNA studies been done for pathogenic and nonpathogenic strains?

Daggett: No. The reason there have not been any hybridization studies is that it is very difficult to extract the DNA from these organisms and keep it intact.

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Use of Heterotrophic Activity Measurements for Studying the Temperature Effect on the Survival of *Aeromonas* spp. and for Rapid Screening of Water for Pollution

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Concentrations of *Aeromonas* spp. and of total viable, aerobic, heterotrophic bacteria have been determined over a two-year period in water and sediment from the Anacostia River at the site of a diver training school. During the winter months, total bacterial counts in the water column and in the sediment were slightly higher than at other times of the year, whereas *Aeromonas* spp. decreased in number by several orders of magnitude. The significant decrease in number in the Anacostia River during the cold months of the year is explained by the low metabolic activity of Aeromonas at low temperatures evidenced by V_{max} of glucose uptake which was *ca.* 10 times lower than that of the mixed bacterial population present in Anacostia River water samples incubated at 4 °C. Heterotrophic activity measurement is suggested for rapid screening of waters for pollution.

INTRODUCTION

Aeromonas spp. are frequently isolated from soil, water and human feces.[1] The ecology of *Aeromonas* in the aquatic environment has been the subject of several studies recently published[2,3] and reported (O. P. Daily, S. W. Joseph, R. I. Walker, C. R. Lissner, and R. J. Seidler, Abstr. Annu. Meet. Am. Soc. Microbiol. p. 177). A unique occurrence of primary soft-tissue infection caused by two species of *Aeromonas, A. hydrophila* and *A. sobria,* in a diver conducting operation in the Anacostia River has recently been reported.[4] The Anacostia River site at which a diver training school was located has been monitored for *Aeromonas* spp. as well as for total, viable, heterotrophic bacterial populations in the water column and sediment. The incidence and concentration of *Aeromonas* spp. recovered from divers and their gear was found to be related to the number of *Aeromonas* spp. in the water column.[5]

The objective of the investigation reported here was to examine factors influencing the survival of *Aeromonas* spp. in the Anacostia River, in order to determine which control the seasonal incidence of *Aeromonas* spp. in the natural environment.

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MATERIALS AND METHODS

Sampling

Water samples were collected with a sterile Niskin sampler (General Oceanics, Miami, Fla.) 1 m below the surface and 1 m above the sediment. Sediment was collected using a small, hand-operated grab sampler (Wildlife Supply Co., Mackinow, Mich.). Water and sediment samples were stored on ice and returned to the laboratory for examination immediately after the collection, with less than 2 hours lapsing before bacteriological analyses were carried out.

Enumeration and Isolation Procedure

Total viable, aerobic, heterotrophic counts were made by spread-plating on plate count agar.[6] For direct counts the acridine orange staining procedure of Hobbie, Daley and Jasper was employed[7] using epifluorescence microscopy. Total coliforms were enumerated by membrane filtration as described elsewhere[8].

Isolation of *Aeromonas* spp.

Volumes of 2000, 200, 20, and 2 ml water samples were filtered using 0.2 μ pore size Millipore filters. After filtration, the filters were incubated for 24 hours in Rimler-shotts broth[9] at 37 °C. Positive cultures, i.e., those yielding a yellow reaction in the broth, were streaked on

MacConkey agar and incubated at 37°C for 24 hours. Nonlactose-positive colonies were transferred to gelatin agar and incubated at 37°C for 24 hours. Gelatin-liquefying and oxidase-positive colonies were confirmed as *Aeromonas* spp. by subsequent testing.[5]

Preparation of Bacterial Cultures for Glucose and Acetate Uptake Experiments

An *Aeromonas* sp. isolated from Anacostia River water was incubated overnight on a rotatory shaker in Tryptic Soy Broth medium (Difco Laboratories, Detroit, Mich.) at 37°C. Cells were harvested, washed twice with 0.2 μ freshly filtered river water, and suspended in the filtered river water.

Glucose and Acetate Uptake

Radioactively labelled substrates were obtained from the Radiochemical Centre (Amersham, England). All uptake experiments were done in duplicate and, in general, no more than a 25 percent difference in results obtained was ever observed between duplicate flasks. Glucose and acetate uptake were determined by the method of Vaccaro and Jannasch (10). D-(U-^{14}C) glucose, 51 mCi/m mole, and (U-^{14}C) acetic acid, sodium salt, 57.8 mCi/m mole were used, and a constant amount of labelled substrate (0.1 uCi/10 ml) was mixed with known concentrations of pure, unlabelled glucose or acetate.

Glucose or acetate, at concentrations of 7-100 and 14-170 μg/1, respectively, were added to 10 ml of bacterial suspensions in 0.2μ filtered river water and the mixtures were placed in 125 ml sterilized flasks. The flasks were sealed with serum stoppers through which a plastic cap containing a precombusted (450°C) folded glass fibre filter had been inserted. Following incubation of the closed system at the indicated temperatures for 1 hour, the reaction was stopped by injection of 0.5 ml of 6% trichloracetic acid and 0.5% HgCl in 40% formalin. *Ca.* 0.2 ml phenethylamine was injected onto the filter wick to absorb the $^{14}CO_2$ evolved. After further incubation for 1 hour, with shaking to enhance entrapment of the released $^{14}CO_2$, the wicks were removed and placed in liquid scintillation vials. The bacterial suspensions were filtered onto 0.2μ Millipore filters, washed with 10 ml filtered river water and placed in scintillation vials. Ten ml of scintillation fluid was added and radioactivity counted in a Beckman scintillation spectrometer (Beckman model LS-3100).

ATP concentration was determined by the luciferin-luciferase method as described by Cavari.[11]

RESULTS AND DISCUSSION

Enumeration of Bacteria

Water and sediment samples were collected over a two-year period at the Anacostia River diver training site. Both direct enumeration of total bacteria and viable count of the aerobic, heterotrophic bacteria were made, as well as counts of *Aeromonas* spp. (Table 1). Comparison of the total numbers of bacteria, obtained by direct and viable count measurements, found in the warm summer months with results obtained for the colder months in winter revealed some increase in the winter (2-8 fold) (Table 1). At the same time, the *Aeromonas* spp. counts decreased by several orders of magnitude, both in the water column and sediment. A similar effect of temperature on *Aeromonas* has been reported by Hazen (12) and Warens (C. E. Warens, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, p. 177). The number of *Aeromonas* spp. in the Anacostia River in the summer months was very large compared to the concentrations of other bacterial species found in natural

Table 1. Number of bacteria in water column and sediment samples collected from Anacostia River at the Navy Yard, Washington, D.C.

Date of sampling	Jul. 18, 1978	Aug. 9, 1978	Feb. 9, 1979	Nov. 26, 1979	Feb. 27, 1980
			TOP WATER		
Water temperature	28°	28°	0.5°	14°	4°
Direct count/ml	3.0×10^6	———	7.4×10^6	1.4×10^7	1.1×10^7
Total viable count/ml	2.0×10^4	3.5×10^4	1.6×10^5	2.8×10^4	1.3×10^5
Aeromonas/ml	>100	>100	0.02	0.1	0.003
			BOTTOM WATER		
Water temperature	28°	27°	0.5°	11.5°	4°
Direct count/ml	2.5×10^6	———	7.2×10^6	8.0×10^6	8.3×10^6
Total viable count/ml	2.0×10^4	3.6×10^4	1.2×10^5	1.2×10^4	1.1×10^5
Aeromonas/ml	>10	>100	>0.8	N.D.	0.001
			SEDIMENT		
Direct count/ml	3.0×10^8	———	3.5×10^7	2.1×10^9	3.7×10^8
Total viable count/ml	1.6×10^7	9.6×10^7	2.7×10^7	4.3×10^7	5.5×10^7
Aeromonas/ml	$>10^3$	$>10^4$	0.25	0.14	0.013

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waters. Interestingly, several other sites in the U.S.A. have been shown to harbor high numbers of *Aeromonas*.[3]

Effect of River Water on Viability

Experiments performed to determine the cause of the observed decrease in counts of *Aeromonas* spp. during the cold months of the year employed pure cultures of an *Aeromonas* sp. which had originally been isolated from the Anacostia River. A mixed culture obtained from Anacostia River water was also employed. Both the pure culture and the mixed culture were grown in Tryptic Soy Broth medium and the cells harvested and washed twice in river water passed through a 0.2μ Millipore filter, then suspended in freshly filtered river water to a concentration of approximately 5×10^5 cells/ml. The cells were incubated at 4 °C and 25 °C. At zero time, after 1 hour, and at 48 hours, aliquots were taken for enumeration of viable cells. As can be seen from Table 2, transfer of the Anacostia River mixed bacterial culture from a rich grown medium to filtered river water did not result in a significant change in viable count within the 1-hour period of incubation at 4 °C and 25 °C. At 25 °C, the viable count increased *ca.* 10-fold after incubation for 48 hours.

The same treatment applied to the *Aeromonas* sp. resulted in an immediate decrease in the cell count of about 2 logs; however, incubation at 25 °C for 48 hours resulted in an increase in the viable count of about 2 logs. But when incubation was continued at 4 °C for 48 hours, no significant change in cell number was observed.

Heterotrophic Potential

The heterotrophic activity of the *Aeromonas* sp. and of the mixed culture of river bacteria was determined at 4 °C and at 25 °C by measuring the rate of uptake of ^{14}C-labelled glucose and acetate.

As shown in Table 3, the rate of glucose uptake by the mixed culture of river bacteria was 3.7 times higher at 25 °C compared with that observed at 4 °C. The rate of glucose uptake by the *Aeromonas* sp. incubated at 4 °C was *ca.* 10 times lower compared with the rate observed for the river bacteria; i.e., 4.1×10^{-6} and 4.23×10^{-5} μg hr^{-1} cell^{-1}, respectively.

The effect of temperature on the rate of glucose uptake by the *Aeromonas* sp. was greater than that of the river bacteria, i.e., 8.4 times higher at 25 °C compared with the rate measured at 4 °C. Acetate uptake by the *Aeromonas* sp. was not observed when the culture was incubated at 4 °C, whereas at 25 °C, both the river bacteria and the *Aeromonas* sp. yielded approximately the same V_{max}(Table 4).

The effect of temperature on the rate of glucose uptake by the *Aeromonas* sp. and river bacteria was further studied, employing 5 different temperatures. (See Figure 1.) The rates of glucose uptake presented were

Table 2. Total viable counts of Anacostia River bacteria and of *Aeromonas* sp., after transfer from a rich medium to filtered river water.

	River bacteria			*Aeromonas* sp.		
INCUBATION	Time of incubation (hrs.)			Time of incubation (hrs.)		
TEMPERATURE	0	1	48	0	1	48
4 °	4.0×10^5	3.2×10^5	1.05×10^5	4.65×10^5	1.0×10^3	5.5×10^2
25 °	4.0×10^5	1.05×10^5	1.26×10^6	4.65×10^5	1.5×10^3	2.3×10^5

Table 3. Comparison of rates of glucose uptake by river bacteria and by *Aeromonas* sp. incubated at two different temperatures.

	Incubation temperature	Viable count $\times 10^{-5}$	V_{max} g.1^{-1}.hr^{-1}	$\frac{V_{max}}{TVC} \times 10^{-5}$
River bacteria	4 °	5.2	22.0	4.23
	25 °	5.1	80.0	15.69
Aeromonas sp.	4 °	0.9	0.37	0.41
	25 °	0.9	3.10	3.44

Table 4. Comparison of rate of acetate uptake by river bacteria and by an *Aeromonas* sp. incubated at two different temperatures.

	Incubation temperature	Viable count $\times 10^{-5}$	V_{max} g.1^{-1}.hr^{-1}	$\frac{V_{max}}{TVC} \times 10^{-5}$
River bacteria	4 °	2.9	0.79	0.27
	25 °	2.9	15.9	5.48
Aeromonas sp.	4 °	4.65	0	0
	25 °	4.70	25.6	5.45

obtained by dividing the rates by the number of cells determined by direct count.

As shown in Figure 1, a doubling of the rate of glucose uptake by river bacteria was observed when the temperature of incubation was elevated from 10 °C to 15 °C, compared with the increasing rate observed when temperature was increased from 4 °C to 10 °C. Doubling of the rate was again obtained when the temperature was elevated from 15 °C to 20 °C. Further elevation of the temperature to 25 °C resulted in a small increase in the rate of uptake. The most profound increase observed in the rate of glucose uptake by the *Aeromonas* sp. was obtained when the temperature was elevated from 20 °C to 25 °C.

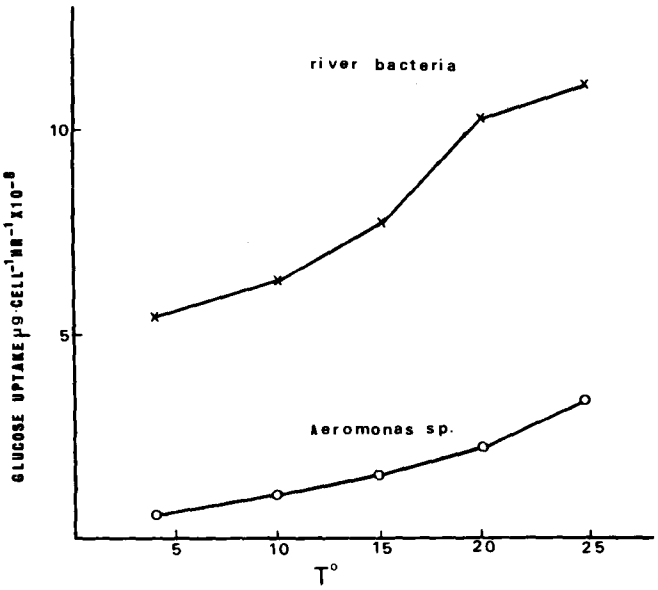

Figure 1. Effect of Temperature on Rate of Glucose Uptake by River Bacteria and by *Aeromonas* sp.
x ——— x **river bacteria**
o ——— o ***Aeromonas* sp.**

It is well known that the total number of fecal bacteria can be reduced significantly by a variety of factors including predation and parasitism and, to a lesser extent, the physical environment itself.[16,17] Results obtained in the study reported here revealed a dramatic effect of cold temperature on the number of *Aeromonas* found in the Anacostia River. It is interesting to note that transferring a mixed culture of river bacteria from a rich medium to filtered river water did not cause a significant change in total viable count, whereas when a pure culture of an *Aeromonas* sp. isolated from the Anacostia River water was treated similarly, the viable count decreased approximately two orders of magnitude. This decrease in viable count could not be explained by predation, parasitism, or effect of selected environmental parameters, such as salinity or E_h, because transfer was made directly to river water from which parasites or predators were excluded by filtration. It is possible that the drop in viable count of *Aeromonas* occurred because the strain, originally isolated from the Anacostia River, is not an autochthonous species. By exposing these cells to unfavorable nutrient conditions such as those found in natural waters, sublethal stress occurred and the *Aeromonas* cells failed to develop in the medium, as has been reported by *E. coli.*[18,19,20,21] Another possibility is that the cells were killed during this transfer. Also, the treatment of the bacteria for the enumeration process may have been responsible for the apparent die-off of allochthonous bacteria introduced into natural waters.[22]

The decrease observed in the number of *Aeromonas* would not be recognized if cultures were incubated at 25 °C for 48 hours. That is, after incubation for 48 hours, an increase in viable count of *ca.* two logs compared to the count at 1 hour was observed, a larger increase for the time interval between 1 hour and 48 hours than the mixed culture of river bacteria, which increased only *ca.* one log. The maximum yield of *Aeromonas* obtained during the first 48 hours of incubation most probably arose from the effects of the limited substrate available in the filtered river water, since the river bacteria achieved a maximum yield of 1.26×10^6 cells/ml on the substrate available in the filtered river water whereas the *Aeromonas* sp. achieved a maximum yield of only 2.3×10^5 cells/m. after incubation for 48 hours. It is important to note that in the interval between 1 hour and 48 hours, when incubation was at 4 °C, there was no significant change in viable count of either the river bacteria or the *Aeromonas* sp., with the river bacteria maintaining a high total viable number of cells and *Aeromonas* sp. demonstrating a much reduced population size.

CONCLUSIONS

In summary, the increase in total numbers of aerobic, heterotrophic bacteria observed for Anacostia River water samples collected during the cold months of the year can be explained by a combination of effects including surface runoff from storms and decreased activity of predators at low temperatures,[23] and increases in numbers of psychrotolerant bacteria as well.[24] Whereas the decrease in number of *Aeromonas* in the Anacostia River during the cold months most probably is caused by the physiological inability of *Aeromonas* to compete with other, autochthonous microorganisms. The V_{max} of glucose uptake of the Aeromonas sp. at 4 °C was *ca.* 10 times lower than that of the mixed population of river bacteria, but only 5 times lower at 25 °C. In the case of acetate, no uptake was observed at 4 °C by the *Aeromonas* sp., whereas at 25 °C, the V_{max} for both river bacteria and the *Aeromonas* sp. was the same.

Thus it can be concluded that when *Aeromonas* spp. enter bodies of water in temperate environments via sewage effluent or other sources, a significant decrease in the number of viable cells can occur when the water temperature is low. Based on heterotrophic activity measurements, the rate of nutrient uptake by *Aeromonas* will be too low at temperatures below *ca.* 15 °C to survive.

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It is suggested that measurement of heterotrophic activity can serve as a rapid screening for pollution in bodies of water. With this method, a reliable answer with respect to the level of pollution can be achieved within 3-4 hours. In Table 5, several parameters of water quality, collected during the last year at different locations in the United States, are presented. Good correlation between total coliforms and glucose or amino acid uptake rate supports this suggestion.

ACKNOWLEDGEMENTS

This research was sponsored by NOAA Grants 04-8-M01-71 and 04-8-M01-73.

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Table 5. Summary of different parameters measured at different sites.

Sampling site	Direct count (in ml)	Total viable count (in ml)	Total coliforms (in ml)	ATP µg/1	Glucose uptake rate µg/1/hr	Glucose turnover time (hr.)	Amino acids uptake rate µg/1/hr	Amino acids turnover time (hr.)
Anacostia River	1.1×10^7	1.3×10^5	345	29.0	10.6	3.7	39.9	2.2
Norfolk Harbor		8.3×10^3	124	21.4	5.3	5.8	34.9	2.4
Yankee Pier, N.Y.	1.2×10^6	1×10^5	90	5.3	3.1	6.4	25.9	1.9
New York Bay		2.5×10^6	100	3.9	1.5	4.7	20.0	4.7
Chesapeake Bay		2.5×10^4		7.5			5.6	5.2
Lake Union, Washington	9.6×10^5	3.1×10^3	5		0.25	18.2	1.0	23.4
Puget Sound	7.8×10^5		0.3	3.0	0.10	41.7	1.1	16.4
Puerto Rico	2.5×10^5	1.0×10^1		0.14			0.04	1105.5

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DISCUSSION

Weiss: I am curious as to whether you determined if your heterotrophic parameter is influenced by other ions, apart from Na^+.

Cavari: No, but work will continue in the laboratory at the University of Maryland to determine the answer to your question.

Attwell: One thing we have found is that the nutrient concentration is quite critical in determining the way in which organisms react to salt. If you reduce the nutrient concentration to below 1000 micrograms per liter, the organism becomes more sensitive to salinity than it would otherwise be. Therefore, one can envision a situation where the organism may, or may not, be active in a given body of saline water, depending upon the nutrient concentration.

Joseph: Does that correspond to a saturation of energy source? Or a sub-saturation point?

Attwell: At the concentrations which we have employed, we find that a saturation is reached at concentrations above 1000 micrograms per liter.

Cavari: If we try to grow *Vibrio cholerae* at salt concentrations of 0.5, 1, and 5%, we can consider that the growth also depends on the concentration of the substrate, which is much higher in the growth medium than in the natural environment. Therefore, survival at high salt concentrations can be achieved if the concentration of the substrate is high. Such conditions can be met in sediment, where high substrate concentrations can be found.

Williscroft: Is there a simple method that can be used by an average diver in the field to determine glucose uptake and acetate uptake? Is it possible for somebody to develop a litmus paper type of a test that would show some qualitative differences?

Colwell: Actually, instruments are available for relatively rapid analyses of this type.

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Actinomycetes, a Possible Hazard Encountered in Diving Operations

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Counts of viable mesophilic and thermophilic actinomycetes were made at a number of estuarine and marine stations. The total viable counts ranged from 1.5×10^5 to 0/g sediment and from 2.5×10^2 to 0/100 ml water. *Thermoactinomyces* species were used as indicators of terrestrial input into aquatic systems and changes in their viable count paralleled those of other actinomycetes. Viable counts diminished with distance from estuarine and coastal areas. Material from a location on the Demerara Abyssal Plain remote from estuarine influence did not yield actinomycetes. Members of eight genera, some of which contain pathogens, were isolated in this study. Divers operating in waters likely to be contaminated with actinomycetes, most of which are apparently allochthonous, may encounter pathogenic forms. Infections acquired in such situations could therefore be of an uncommon type caused by actinomycete bacteria.

INTRODUCTION

Actinomycetes are Gram-positive bacteria which grow typically in the form of branched elongated cells or branched filaments. Most genera form an extensive mycelium which in some cases fragments into coccoid or rod shaped elements. Certain genera occur as individual branched cells and seldom produce a mycelium.[1] A common feature of actinomycetes is the ability to produce spores. These range from the sensitive mobile spores produced by members of the genus *Actinoplanes* through a variety of structures to the resistant endospores of *Thermoactinomyces,* which can survive for many decades.[2,3]

A number of actinomycete species are human pathogens. They may be obligate pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium leprae* or may exploit opportunistic infection as in the case of microaerophilic *Arachnia* and *Actinomyces* species, normally present in the oral cavity of adults. Actinomycete infections acquired from natural waters are, however, most likely to arise from exogenous species, particularly members of the genera *Nocardia, Actinomadura* and *Streptomyces.*[4]

Nocardia enter the body through wounds or by inhalation to give rise to systemic or pulmonary nocardiosis. Superficial nocardiosis may produce cutaneous or subcutaneous abscessing infections of the eye or mucous membranes and may, on some occasions, produce actinomycetoma. Infection with *Actinomadura* or *Streptomyces somaliensis* is prevailingly percutaneous, through skin lesions, giving rise to actinomycetoma. Cutaneous lesions can also be caused by certain nontuberculous mycobacteria. Infection may occur when abraded skin contacts water in a swimming pool[5] or an aquarium harboring the organism.[6]

Actinomycetes have a worldwide distribution particularly in the upper layers of soil[7,8] which are probably their primary reservoir. They are also found in decaying organic material[9] and in sewage.[10] These bacteria can also be isolated from freshwater,[11] estuarine,[12] and marine[13] situations. Our early work is directed to establishing the source and distribution of actinomycete bacteria, particularly pathogenic forms, in saline natural waters. The eventual aim is to predict situations in which such pathogens may be concentrated and assess the danger of infection from such contaminated waters.

MATERIALS AND METHODS

Collection of Samples

Samples studied were collected between July and

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December 1980. The stations, in order of increasing salinity, were the Anacostia River at the U.S. Naval Yard, Washington, D.C. (salinity 0 to 2 °/00); the Chesapeake Bay, Eastern Bay (depth 12.8M, salinity 17.8 °/00), and Bloody Point (depth 31.1 M, salinity 22.8 °/00); New York Harbor, Yankee Pier (salinity 25 °/00); and the New York Bight samples, "A", longitude 73 °52 'W, latitude 40 °24 'N (depth 19.8M, salinity 30 °/00) and "B", longitude 73 °49 'W, latitude 40 °22 'N (depth 28M, salinity 30 °/00), obtained 11 weeks after sample A. Both samples A and B were from the region of a dump site for dredge spoils in New York Harbor. Sample B included core material from a 3-4 inch layer of sand deposited by the Army Corps of Engineers during operations to cap the dump site. Sediment from below the sand layer appeared to consist of the original dredge spoil and was also sampled.

Sediment samples were obtained from two regions of the Demerara Abyssal Plain. Station "A", longitude 45 °48 'W, altitude 10 °24 'N (depth 4805M, salinity 35 °/00), is considered distant enough from land to be unaffected by effluent from the Amazon River. Station "B", longitude 49 °6 'W, altitude 8 °6 'N (depth 4430M, salinity 35 °/00), is within the ocean floor area thought to be influenced by Amazon River effluent.

Water samples were collected from 1 M below the surface of the water column and 1 M above the bottom by means of a Niskin aseptic sampler (General Oceanics, Inc., Miami, Florida). Sediment was collected by means of a Ponar grab sampler except in the case of the Demerara and New York Bight "B" material which was collected by type U.S.N.E.L. box corer and diver hand corer, respectively.

Salinity was measured by using a salinity refractometer (American Optical, Buffalo, New York).

Bacterial Enumeration

To isolate actinomycetes, water samples were inoculated directly onto agar plates[14] and triplicate 10 cm^3 and 100 cm^3 volumes were passed through 0.45 μm membrane filters (Schleicher and Schuell; Keene, H.H.). Membranes were incubated grid surface uppermost for the isolation of *Thermoactinomyces* species,[15] but an imprint technique gave greater recoveries of other isolates.[16] Sediment samples were suspended in phosphate-buffered saline (7.2 g NaCl, 1.48 g Na_2HPO_4, 0.43 g K_2HPO_4, distilled water 1 dm^3, pH 7.2) before inoculation onto agar plates.

Mesophilic actinomycetes were isolated on M3 agar[14] incubated at 30 °C and at 15 °C; *Thermoactinomyces* species were isolated on C.Y.C. medium[17] incubated at 50 °C. Microaerophilic actinomycetes were cultured on Brain Heart infusion agar (Difco Laboratories, Detroit, Mich.) in Gaspak Jars with H_2 and CO_2 generator at 37 °C (G.M. Schofield, Pers. Comm.).

Viable aerobic heterotrophic marine eubacteria were enumerated by membrane filtration using Marine Agar 2216 (Difco) and incubation at 25 °C.

Thermoactinomycete isolates were assigned to species by means of characteristics suggested by Cross and Unsworth.[18] Mesophilic isolates were grouped according to criteria set out by Cross and Goodfellow[9] prior to more precise identification.[19]

RESULTS AND DISCUSSION

Our preliminary findings (Tables 1 and 2) confirm previous observations showing that actinomycetes can be isolated from estuarine and marine sediments and waters.[12,13,20]

The numbers recovered ranged from 1.5×10^5 to 0/g in sediments and from 2.5×10^2 to 0/100ml in waters examined. The viable counts presented above indicate that actinomycete species are particularly numerous in sediments. For eubacteria isolated on marine agar, the ratio of the number recovered per gram of sediment to the number per milliliter of water ranged from 1.3:1 (N.Y. Bight B, sediment: top water) up to 3.6×10^2:1 (N.Y. Bight A, sediment: bottom water). The corresponding ratio for actinomycetes range from 1×10^2:1 (N.Y. Bight A, surface sediment: bottom water) up to 5.8×10^4:1 (Anacostia, sediment: bottom water) in waters carrying these organisms. This concentration of actinomycetes in sediment may be attributable to active proliferation of the bacteria, as there is evidence for activity of some species in aquatic sediments.[12,21] However, the majority of actinomycetes isolated from aquatic situations are considered to be allochthonous organisms washed in

Table 1. Numbers of actinomycete and heterotrophic marine bacteria colony forming units per gram of sediment.

Station	*Thermoactinomyces* sp.	*Actinomycetes* 30 °C	*Actinomycetes* 15 °C	Marine bacteria
Anacostia	2.5×10^4	2.8×10^4	5.2×10^3	4.0×10^7
Chesapeake Bay				
Eastern Bay	7.1×10^3	2.3×10^4	3.5×10^4	NT*
Bloody Point	1.8×10^4	1.0×10^5	3.2×10^4	NT
New York				
Yankee Pier	2.5×10^3	1.2×10^4	2.8×10^3	7.0×10^4
N.Y. Bight A	4.0×10^1	7.0×10^1	3.1×10^1	8.0×10^5
N.Y. Bight B Upper	2.0×10^1	1.7×10^2	1.2×10^2	8.0×10^3
N.Y. Bight B Lower	1.1×10^3	1.1×10^4	5.3×10^3	NT
Demerara Abyssal Plain				
A	0	0	0	1.0×10^2
B	1.5×10^1	0	0	1.5×10^2

*NT = Not tested.

Table 2. Numbers of actinomycete and heterotrophic marine bacteria colony-forming units per 100 milliliters of water.

	TOP WATER				BOTTOM WATER			
Station	*Thermoactinomyces* sp.	*Actinomycetes* 30 °C	*Actinomycetes* 15 °C	Marine bacteria	*Thermoactinomyces* sp.	*Actinomycetes* 30 °C	*Actinomycetes* 15 °C	Marine bacteria
Anacostia	7.2×10^1	1.8×10^1	2.5×10^1	2.0×10^8	6.0×10^1	2.5×10^1	1.3×10^1	$<1 \times 10^4$
New York								
Yankee Pier	1.0×10^1	8	NT*	8.5×10^3	NT	NT	NT	NT
N.Y. Bight A	0	0	0	1.0×10^6	1.0×10^2	1.2×10^1	3.0×10^1	2.2×10^5
N.Y. Bight B	0	1.2×10^1	0	6.3×10^5	0.7	9	2.5×10^2	4.2×10^5

*NT = Not tested.

from terrestrial sources[11,20,22] and which may accumulate in the form of spores produced by most species.

We have used members of the genus *Thermoactinomyces (Tha.)* as indicators of input from terrestrial sources. Growth and sporulation of *Tha.* species are restricted to decaying organic material at temperatures in the region 35-60 °C. The spores are common and often numerous in soil, from which they are washed into flowing surface waters and ultimately deposited in sediment.[23] They are unable to proliferate in the sediment because of low temperatures and the sparsity of nutrients and oxygen. They can, however, remain viable for many years and, therefore, accumulate.[17] Procedures which differentiate between spore and vegetative forms of thermoactinomycetes, to be reported elsewhere, provide further evidence that the organism exists as a spore in sediments. *Thermoactinomyces vulgaris, Tha. sacchari,* and *Tha. thalpophilus* were all found in excess of 1.0×10^3/g of Anacostia River sediment. Similar numbers of the former two species were also isolated from Chesapeake Bay sediments, but *Tha. thalpophilus* was absent. It therefore seems likely that the ratio in which various *Tha.* species occur may indicate something about the nature and source of the terrestrial input.

Actinomycetes are numerous in estuarine and coastal effluents. The resistance and longevity of *Tha.* spores accounts for their persistence in more remote locations, such as the Demerara Abyssal Plain station "B" at a depth of 4430M where other actinomycetes are less prevalent or absent. Members of the genera *Nocardia, Micromonospora, Microbispora,* and *Streptomyces* were discovered from marine sediments, including deep sediments off the East African coast.[13] Actinomycetes were not isolated from Demerara station "A," considered free of coastal and estuarine influence.

Isolates characteristic of eight genera and also a microaerophilic form have been encountered in this study so far (Table 3). Organisms identified as mycobacteria have been isolated from the Chesapeake Bay during earlier work carried out in our laboratory. Members of certain of the genera listed in Table 3 are human pathogens and specific identification is to be made and reported elsewhere.

Table 3. Actinomycetes isolated at each station examined.

	Thermoactinomyces	*Dactylosporangium*	*Microbispora*	*Micromonospora*	*Nocardia*	*Saccharomonospora*	*Streptomyces*	*Streptosporangium*	*Microphilic*
Anacostia	✓*	0†	✓	✓	✓	0	✓	✓	✓
Chesapeake									
Eastern Bay	✓	0	0	✓	0	0	✓	0	0
Bloody Point	✓	0	0	✓	0	0	✓	0	0
New York									
Yankee Pier	✓	✓	✓	✓	✓	0	✓	✓	✓
N.Y. Bight A	✓	0	✓	✓	✓	✓	✓	✓	✓
N.Y. Bight B	✓	0	0	✓	✓	0	✓	✓	0
Demerara Abyssal Plain									
A	0	0	0	0	0	0	0	0	0
B	✓	0	0	0	0	0	0	0	0

* ✓ = Isolated
† 0 = Not isolated

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It appears that actinomycetes, including genera containing potential pathogens, are prevalent in estuarine and coastal regions. These bacteria may be associated with terrestrial run-off, sewage effluent,[10] polluted waters,[12] and with material such as coastal dredging spoil dumped at sea. The latter is evident in the figures for N.Y. Bight B given in Table 1: 1.7×10^4 actinomycetes/g were recovered from the lower sediments, i.e., dumped dredging spoil, but only 3.2×10^2g were recovered from the upper capping material. Many of the organisms encountered produce spores which may remain viable for long periods, and accumulate.

In view of the above observations, it seems advisable for divers working in situations likely to be heavily contaminated to wear suits which minimize direct contact with the water. It appears wise to avoid disturbing sediment and to wash the diving suit in clean water before removal. It is perhaps most important to be aware that the possibility of actinomycete infection exists, because such infections are uncommon and in consequence often not diagnosed.[24,4] Wounds in particular provide a port of entry for organisms such as pathogenic *Nocardia* and *Mycobacterium* species. Correct and early diagnosis followed by appropriate therapy are vital in overcoming potentially serious actinomycete infections.

ACKNOWLEDGEMENTS

We thank J.W. Deming for the collection of material from the Demerara Abyssal Plain.

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DISCUSSION

Williscroft: Has anybody looked at how many of the organisms being discussed here and found also in sediment remain in the water column after mixing and resettling of the sediment?

Attwell: We have not done work specifically to answer that question.

Gottlieb: Did you do any tests to determine what proportion of your organisms were in the vegetive and spore forms? That could make a difference. What roughly was the proportion?

Attwell: Certain of our studies, to be reported elsewhere, suggest that some isolates are in the vegetive form, but the majority are present as spores.

Daggett: I am intrigued that you get higher counts after you sample in the New York Bight.

Attwell: The higher counts come from the material dredged from New York Harbor and dumped in the New York Bight.

Williscroft: The ointment we use is a mixture of bacitracin and neomycin. It has been our experience that the bacitracin and neomycin give a much more rapid elimination of infection than just the bacitracin alone when we have infections on the hands or face.

Attwell: The situation could be complicated by the fact that a number of *Nocardia* in superficial infections occur in association with other bacteria. If you remove the accompanying bacteria, the *Nocardia* infection will also be cleared up. There is no evidence of widespread actinomycete infections among divers. There are, however, reports recently of a number of swimmers picking up mixed bacterial infections which include *Nocardia*.

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Molecular Approaches to the Study of Infectious Diseases

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Most of the publicity surrounding the techniques of "genetic engineering," or recombinant DNA, has centered on the production of substances such as interferon, insulin, human growth hormone and other such therapeutic compounds. I will speak today about the application of techniques of molecular biology to basic research in infectious disease, including the pathogenesis, epidemiology, diagnosis and prevention of infectious disease.

Because many bacteria behave differently in the natural environment than they do in the human host, it is sometimes difficult to accurately assess their public health significance. For example, virulence factors such as toxins that are expressed and produced in human infections may not be produced in the acquatic environment. Bacteria can mutate spontaneously or in response to certain chemicals, light wavelengths or other factors, and may lose certain phenotypic properties not essential to their survival and growth outside the body. However, they may still retain the potential for producing these substances, and upon return to the body, begin to produce these enzymes, toxins, etc., in response to their new environment, thus causing disease. Because conventional methods for determining pathogenicity may not accurately assess such bacterial strains, a definitive answer to questions of public health significance may require the examination of the genetic material of these strains, a genetic rather than phenotypic examination. The latest techniques of molecular biology and recombinant DNA in particular are proving to be extremely useful in such situations.

An example of the usefulness of recombinant DNA in infectious disease research is the case of nonenterotoxigenic *Vibrio cholerae. V. cholerae,* The causative agent of cholera, produces a powerful toxin which appears to be the major virulence factor in the disease. Cholera toxin (CT) has been extensively studied and consists of an 82,000 dalton molecular weight protein composed of two subunits, designated A and B. All strains of *V. cholerae* implicated in epidemic disease produce CT, but many strains of nonenterotoxigenic *V. cholerae* have been isolated from environments such as sewage, oysters, and brackish water from such diverse areas as Louisiana, Maryland, Guam, Brazil and Great Britain.[1,2,5,10] The question that arises about these strains is, what is their significance in the epidemiology of cholera? Are they merely mutants of toxigenic strains which have the potential to revert to toxigenicity and then serve as a reservoir of disease? Furthermore, why are they nonenterotoxigenic? Perhaps they are not entirely nonenterotoxigenic, but produce a partial or defective toxin not detectable by conventional means. Another possibility could be a membrane defect whereby the toxin is not correctly produced or released. If they produce absolutely no toxin at all, then it is uncertain whether a functional structural or a regulatory gene is lacking. To answer the questions raised by these strains, the genetic basis for nonenterotoxigenicity was examined.[9]

MATERIALS AND METHODS

There are three basic bacterial genetic elements that contain the information necessary to direct the synthesis of enzymes, toxins, virulence factors and all other phenotypic characteristics of the organism. All three elements, viz., the chromosome, plasmids and transposons, are composed of deoxyribonucleic acid (DNA). All bacteria contain a single circular chromosome, a molecule with a molecular weight of $1 - 3 \times 10^9$ daltons in an average bacterium. In addition, many bacteria contain extrachromosomal elements called plasmids, which are small circules of

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DNA with molecular weights of $1 \times 10^6 - 3 \times 10^8$ daltons. Plasmids replicate independently of chromosomes and often encode resistance to antibiotics and heavy metals, among other properties. Transposons are molecules of DNA which are integrated into plasmids or chromosomes and cannot exist independently of the other genetic elements. Transposons often encode for antibiotic resistance and can excise themselves from a plasmid and integrate into a chromosome or vice versa. All three genetic elements can be responsible for virulence factors of pathogenic bacteria. For example, cholera toxin is encoded by chromosomal genes. The bacterium responsible for the majority of cases of travelers diarrhea, enterotoxigenic *Escherichia coli,* produces two types of toxins, a heat labile (LT) and a heat stable toxin (ST). The LT is encoded on a plasmid, while a transposon residing on a plasmid is responsible for the ST.

These genetic elements were considered in investigating the cause of nonenterotoxigenic *V. cholerae.* An intitial hypothesis was that the nontoxigenicity was due to a repressor molecule encoded by a plasmid. Yet when DNA from these was extracted and examined, no correlation between the presence of plasmids and nontoxigenicity was seen (Figure 1). The next approach utilized the similarity of *E. coli* heat labile toxin (LT) to cholera toxin. Both toxins share structural, enzymatic activity and antigenic similarities. LT has been cloned and characterized by Dallas and coworkers;[6,7] and the amino acid sequence for the protein was found to be very similar to CT. Because of the high amino acid sequence homology present between these two proteins, the genetic information encoding LT and CT were presumed to be highly homologous also. Related DNA sequences can be studied by the use of DNA/DNA hybridization techniques whereby the two different DNAs are denatured by heat or high pH so that the two strands of the double-stranded DNA molecule

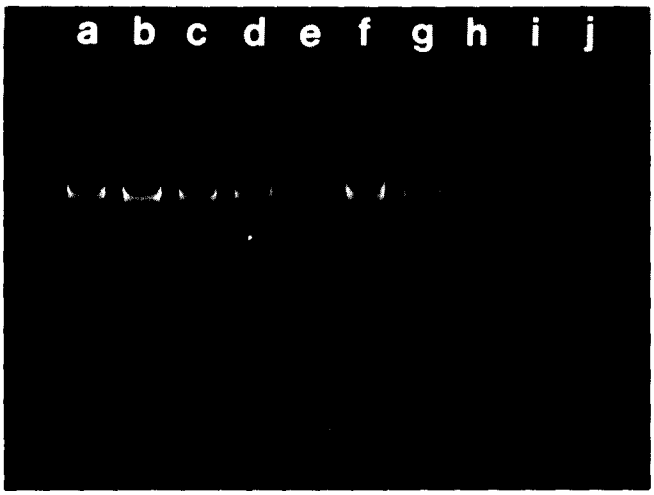

Figure 1. Screening for plasmids in *V. cholerae* by agarose gel electrophoresis. The bright diffuse band present for each strain represents chromosomal DNA while fainter bands below the chromosome represent plasmid DNA. Most strains were found not to possess plasmids.

are separated. The denatured strands from each sample are mixed and conditions adjusted so that the strands can reanneal, i.e., seek out similar DNA sequences from the other sample and join together to become an intact, double-stranded molecule. By radioactively labeling a specific DNA sequence, a highly specific probe for that sequence is obtained.

Such a probe for LT was obtained by purifying DNA from the cloned LT genes, which are carried on the recombinant plasmid pEWD299. The purified DNA was then labeled *in vitro* with ^{32}P. Total DNA from nonenterotoxigenic *V. cholerae* strains was prepared, cut with restriction endonucleases, and the resulting fragments separated by agarose gel electrophoresis. The cut DNA was then denatured and tranferred to nitrocellulose filter paper by the method of Southern.[12] The paper containing the *V. cholerae* DNA was then incubated overnight in a solution containing the labeled LT DNA probe. The labeled DNA hybridizes to homologous sequences on the filter paper and, after washing and drying of the paper, can be visualized by autoradiography. Thus, any DNA sample containing sequences similar to LT will be seen as a darkening of the X-ray film after the autoradiograph is developed (Figure 2).

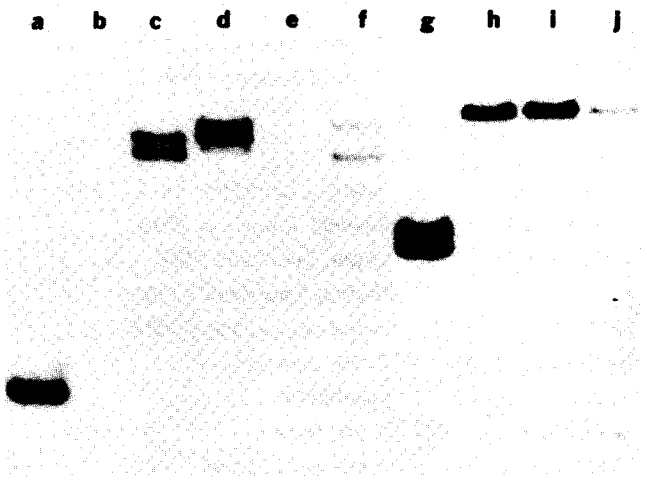

Figure 2. Autoradiograph showing hybridization of the *E. coli* LT Probe to *Hind* III restriction fragments of *V. cholerae* strains. Chromosomal DNA was extracted from each strain, digested with *Hind* III restriction endonuclease, separated on agarose gels and transfered to nitrocellulose paper. The LT probe was labeled with ^{32}P and where it has hybridized to DNA from *V. cholerae* is indicated by darkening of the X-ray film. Lanes a and b are *E. coli* controls and c through j are strains of *V. cholerae* 0-1. Lanes: a) P307, b) C600, c) 569B, d) ATCC 14035, e) 1074-78, f) 395, g) 4808, h) 30167, i) 62746, j) 1944.

All toxigenic strains of *V. cholerae* hybridized with the LT probe, owing to the homology between the CT and LT genes. No nonenterotoxigenic strains isolated from throughout the world showed such a homology (Table 1). The reason for their nonenterotoxigenicity, then, is due to the lack of structural genes encoding toxin. They are not simple mutants that were once enterotoxigenic;

rather, they lack the entire gene for toxin production and therefore cannot revert to enterotoxigenicity. Thus, the nonenterotoxigenic strains of *V. cholerae* found in areas such as England, Brazil or Maryland cannot serve as a reservoir of cholera because they lack the genes for cholera toxin. Moreover, they lack other necessary virulence factors such as colonization factors, and have been shown to be avirulent in human volunteers (M. Levine, personal communication).

Table 1. Strains of *Vibrio cholerae* examined for homology with *Escherichia coli* LT DNA*

Strain number	Source	Homology with LT
V. cholerae 0-1: 3784, 3786, X725, X392, X316,	Environmental, Guam	–
1196-74, 1074-78, 2634-78, 2633-78	Sewage, Brazil	–
MP19812	Water, Bangladesh	–
VL5962, VL6007, VL6085	Water, England	–
V69	Water, Maryland	–
692-79, 1094-79	Sewage, Louisiana, 1979	–
K5	Water, Louisiana, 1980	–
1528-79, 1727-79, 1742-79, 1954-79, 1955-79, 1956-79, 2074-79, 2075-79	Oysters, Louisiana, 1979	–
N-20, N-32, N-44	Sewage, Louisiana, 1978	+
E7708, E8500	Water, Louisiana, 1978	+
E7626	Shrimp, Louisiana, 1978	+
1077-79	Leg ulcer, Louisiana, 1979	–
E7323, E7657, 4808	Stool, Louisiana, 1978	+
1165-77	Gall bladder, Alabama	–
569B, 395, ATCC 14035	Stool, India	+
30167, 62746, 1944	Stool, Bangladesh	+

*Taken from Kaper, et al. (8)

RESULTS AND DISCUSSION

This technique can be simplified and used as a diagnostic tool. It is unnecessary to extract pure DNA from strains to be tested for the presence of toxin genes. Instead, the DNA present in a bacterial colony can be tested for hybridization to LT genes. The cultures can be grown on filter paper placed on a nutrient medium and then lysed and denatured by transfer to a pad saturated with sodium hydroxide. Then the filter paper can be hybridized as above and the colonies that possess toxin genes will appear as dark spots on the autoradiograph (Figure 3). This can be simplified even further by simply spotting stool samples onto nitrocellulose papers placed on selective media and hybridizing as above.[11] Thus, pathogenic bacteria that are difficult and expensive to detect by conventional methods can be detected simply and inexpensively once specific DNA probes are prepared. This technique is not limited to enteric pathogens and stool cultures. Blood samples have been tested in a similar manner and Epstein Bar Virus (EBV)[3] can be detected through the use of a DNA probe specific for EBV. This technique represents a great advance in the diagnosis and epidemiology of infectious disease and will undoubtedly be extensively utilized in the future.

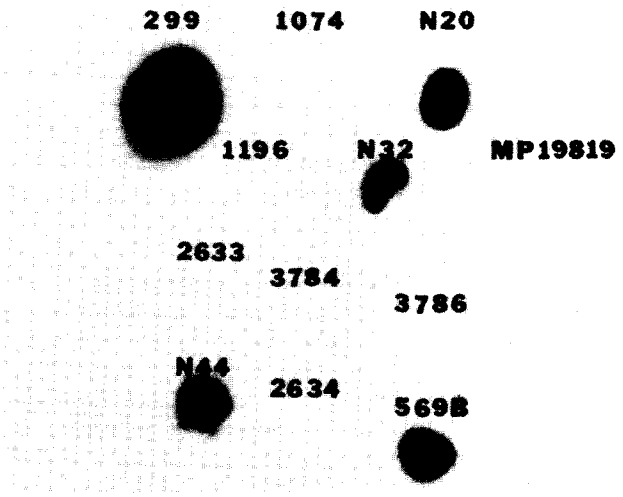

Figure 3. Colony hybridization of *V. cholerae* 0-1 strains on nitrocellulose filters after hybridization with ^{32}P labeled LT probe. Only those colonies possessing DNA sequences homologous to LT bind the probe, subsequently darkening the film. Colonies of *E. coli* C600 (pEWD299), *V. cholerae* N-20, N-32, N-44, and 569B are positive while *V. cholerae* 1074-78, 1196-74, MP19819, 2633-78, 3784, 3786, and 2634-78 are negative.

Another technique which should prove extremely useful was recently employed by Cohen and Falkow[4] to illuminate the newly described Toxic-Shock Syndrome (TSS). Little was known about the causative agent of this disease or its virulence properties. The bacterium *Staphylococcus aureus* was implicated in TSS, but this species also colonized healthy women and no useful distinction was known between case and control isolates of *S. aureus*. Cohen and Falkow analyzed case and control isolates of *S. aureus* by separating total cell protein by polyacrylamide gel electrophoresis. When stained, the gel showed many proteins for each strain, separated by electrophoresis on the basis of molecular weight. The separated proteins were transferred to nitrocellulose paper (NCP) by electrophoresis and the NCP was soaked overnight in a solution of serum drawn from patients of TSS. If patients have been exposed to protein antigens produced by the *S. aureus*, antibodies in their serum will bind to the proteins contained in the NCP. The bound antibodies are then detected by adding radioactively labeled staphylococcal protein A, which binds specifically to human IgG antibodies. Where the ^{125}I labeled protein A, and therefore the serum antibodies, have bound is visualized by autoradiography. Using this technique, these investigators found that *S. aureus* isolated from TSS patients had two antigenic

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proteins visible on the autoradiograph that were absent from *S. aureus* isolates from healthy individuals. Thus, these proteins may represent the toxin (or toxins) or toxic subunits that cause the clinical manifestations of the disease.

This technique has also been used to illuminate other diseases such as syphilis.[9] It could also be usefully applied to the study of microbial hazards to divers in polluted waters. Pre- and post-exposure serum samples could be tested with protein profiles of a variety of pathogenic bacteria isolated from the dive areas. Differences between pre- and post-exposure serum samples would indicate that the divers sustained an infection due to the bacterial species analyzed, even if no clinical manifestations were evident. Thus, this technique could detect asymptomatic infections or symptomatic infections that, for whatever reason, go unreported. Valuable information as to the exact nature of microbial hazards associated with diving in polluted waters would then be available to the physicians responsible for the health of the divers.

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DISCUSSION

Joseph: What that signifies then is that if an antibody is produced against an organism, there is a possibility that the organism has caused either an overt or subclinical infection. Also, in developing the test for a wide range of organisms, the protein factor is then associated with the nuclear material, which codes for that particular factor.
Kaper: Correct.
Colwell: The beauty of the experiment is that you examine antibody-associated protein, that is, the total protein of the component of a given species.
Kaper: This can help isolate the pathogens that are important. As in the case of toxic shock syndrome, we found two proteins that are apparently present in strains associated with clinical cases but not in those of controls.
Weiss: You labelled the DNA extrinsically?
Kaper: Yes. The purified DNA probe is nick-translated, a procedure in which the four bases of the DNA are radioactively labelled. You can buy these reagents commercially. Simply mix them together with probe DNA, add appropriate enzyme, and allow the reaction to proceed for 90 minutes. The result is labelled DNA.
Coolbaugh: Why is it that the positive bands do not represent total proteins, not just toxins? A large number of positive bands would result that would represent all the proteins recognized by the antibodies.
Kaper: That's true. A lot of proteins would be observed, but by comparing pre- and post-exposure, one can distinguish the important ones; then, one can do specific preparations. For example, you can just do membrane preparations of the cell, and look at membranes to locate where the proteins are.
Williscroft: Can these techniques be used in the field under the kinds of conditions we are talking about?
Kaper: Some could be. All the radioactive probes could be prepared commercially, and I suspect they probably will be. You could take the reagents out on the ship and start with stool specimens placed on a filter, take the radioactive probe, and mix all the solutions under field conditions. A problem might arise in that you need a darkroom for placing X-ray film next to the filters and to develop the film. Research is underway to eliminate the need for radioactivity by use of a colorimetric assay.

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Lactose–Positive Vibrios in the Marine Environment

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INTRODUCTION

I would like to describe a relatively new bacterium that has been known only for about five years. This is a rather unusual and extremely virulent bacterium, a *Vibrio,* which can cause death through ingestion or by entry into cuts. The Center for Disease Control (CDC) in Atlanta started in 1964 to receive occasional bacterial isolates from sources other than intestinal tracts, which were first thought to be *Vibrio parahaemolyticus,* but were subsequently differentiated from that organism by a relatively few number of taxonomic traits.[1] Most notable of these is the fermentation of the sugar lactose. Hence, it has been called the "lactose positive *Vibrio*," but now bears the scientific name, *Vibrio vulnificus.* It is a salt-requiring bacterium and has been shown by DNA reassociation studies to be a distinct species from *V. parahaemolyticus, V. cholerae,* and a variety of other vibrios. I should also point out that, unlike *V. parahaemolyticus,* which is rarely if ever fatal, infections by *V. vulnificus* frequently result in death. The clinical characteristics are best illustrated by a study the CDC performed in 1979 on 39 cases of the disease[2] (Table 1). Of the 39, 24 acquired the bacterium by ingestion. The incubation period here is very fast, with an average time until the symptoms appear of about 16 hours. Chills and low blood pressure (hypotension) are typical, and 3/4 patients develop skin lesions on the extremeties. The extremeties subsequently become very fluid-filled (edematous). Of significance was that 75 percent had pre-existing liver diseases and 83 percent had consumed raw oysters. Eleven of these 24 patients died within two weeks of hospitalization.

Unlike the infections acquired through ingestion, those people who acquired the disease after cutaneous inoculation generally were relatively healthy to start with. These infections are always seawater associated. Typically, there is a pre-existing open wound or cut, or one is received while the person is in seawater. Quite commonly, the wound is acquired during cleaning of shellfish or while trying to harvest oysters or crabs. Only about 7 percent result in death this way, but cutaneous infections frequently result in limb amputation.

Table 1. Symptoms of *Vibrio vulnificus* infections.*

Ingestion (24 Cases)	Wounds (15 Cases)
Median incub. pd. = 16 hr	Median incub. pd. = 12 hr
Chills	Localized edema
Hypotension	Erythema
75% cutaneous lesions	Pain
Edematous extremeties	Rapidly spreading cellulitis
Necrotic ulcers	Necrotic ulcers
75% pre-existing hepatic disease	Generally healthy individuals
83%—raw oysters	Seawater associated
46%—death	7%—death

*Adapted from Blake *et al.* (reference 2).

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One of the real problems in treating this disease involves the time factor. It takes only 12-16 hours on the average for the syptoms to develop, and, most significantly, the average time from onset of symptoms to the time of death is about 24 hours. A quote from the 1979 CDC paper[1] I think best illustrates this aspect: "Summaries of the histories of five persons from whom blood isolates were obtained are as follows. The first was a patient, age 83, who died within 12 hours after coming to the emergency room where cellulitis of the leg was noted. The second case was a 45-year old male who entered the hospital with skin lesions and died suddenly. The third case was a 69-year old male who had ulcers on one leg. He died four hours after admission. The fourth case was an adult male who developed lesions on his leg while on a fishing trip and died within 48 hours. The fifth case was an elderly man. The organism was cultured from both blood and spinal fluid four hours before death. He was found unconscious in his room 12 hours before death. He had been seen the previous day and did not seem ill." So it is a very rapidly occurring infection. Also, as illustrated by these five cases, 90 percent are males and 95 percent are age 40 or more. There have been about 75 cases of infection caused by this bacterium now reported in the United States.

LABORATORY INVESTIGATIONS

I'd like to discuss two major aspects of the findings we've obtained on this bacterium over the last four years. The first involves its pathogenicity in animals and how it correlates to man, why only certain people come down with the disease, and something about the epidemiology of the disease. The other is the ecology and distribution of the bacterium in the marine environment.

In order to study an infection, one needs a suitable animal model. As shown in Table 2, we examined a number of animals—mice, rats, hamsters, rabbits, and guinea pigs, and found all to be killed by injections of the bacterium.[3] Mice were selected for further study, since they gave approximately the same symptoms as seen in man, notably edema, blood pressure decreases and wound necrosis (tissue death). Also, the time to death in the mice averaged only 2½-5 hours. Most significantly, it is evident from Table 2 that *V. vulnificus* kills animals when injected subcutaneously. This is unique among vibrios, and does not occur even with *V. cholerae.*

One way of comparing the pathogenicity of different bacteria is to look at the "LD_{50}", which is the number of bacteria that must be injected to kill half of the animals being tested. When this was done, we found an LD_{50} for *V. vulnificus* of 10^5 to 10^6 cells (Table 3). These numbers are not especially high or low, but the unusual thing again is that the bacterium is able to kill subcutaneously. This has a relevance to the several references made in this meeting about infection of cuts in the skin, and, of course, to one of the two major ways a person can acquire *V. vulnificus* infections.

Looking first at the infection obtained after cutaneous entry of the bacterium, Figure 1 illustrates what happens in laboratory animals if you inject this organism just under the skin. Figure 1a shows normal mouse subcutaneous tissue with its neatly oriented muscle fascicles. Figure 1b shows what develops when the skin infection occurs. The muscle tissue is greatly disoriented, and large amounts of edema fluid are present. When sublethal injections are administered subcutaneously, the animals develop necrotic ulcers at the injection site.

The reason for the great amount of fluid accumulation turns out to be a very unique and fascinating aspect of this infection. If a blood sample is taken from the infected mouse, and the blood cells are centrifuged down, one obtains the "hematocrit" value, which is the percent of the total blood volume which is made up of red blood cells. In the case of a mouse the hematocrit is nor-

Table 2. Experimental mortality.*

Infecting organism†	*Mice*			*Rabbits*	*Hamsters*
	i.p.	s.c.	i.v.	i.p.	i.p.
Vibrio vulnificus (CDC strain C7184)	62/62‡ (100)§	34/34 (100)	8/8 (100)	10/13 (77)	9/9 (100)
V. parahaemolyticus (ATCC strain 27519)	19/24 (79)	0/24 (0)	6/6 (100)	NT**	NT

* Mice were challenged i.p., s.c., and i.v. with *V. vulnificus* and *V. parahaemolyticus.* Rats and hamsters were injected i.p. with *V. vulnificus* only.
† Inocula consisted of 0.1 ml of saline containing about 2 x 10^8 cells.
‡ Number of deaths out of total number of animals tested.
§ Number in parentheses indicates percent mortality.
**NT, Not tested.

Adapted from Poole and Oliver (reference 3).

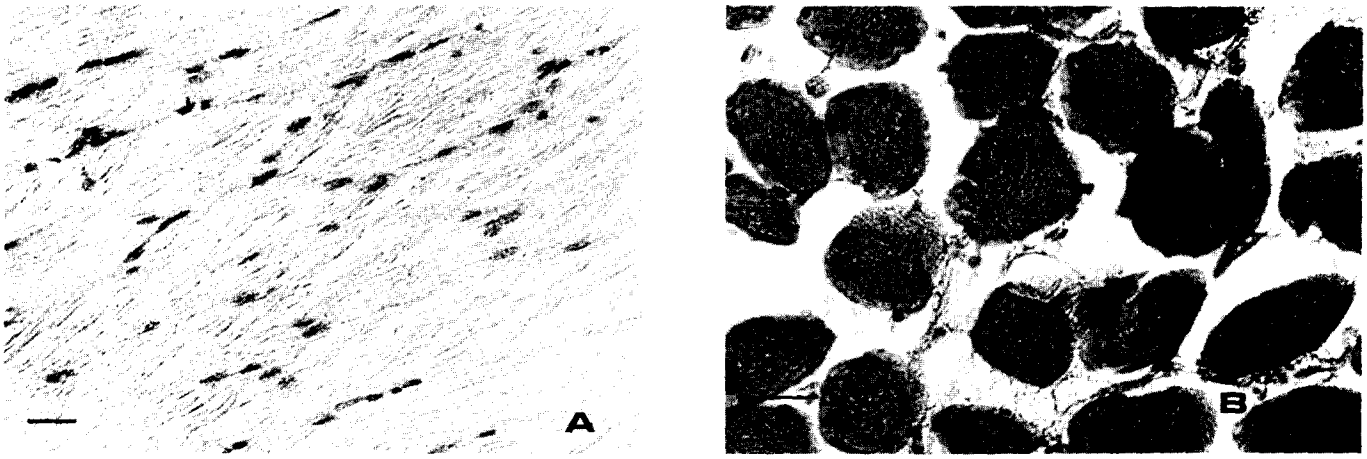

Figure 1. Histology of cutaneous edema.

A. Unaffected skeletal muscle fascicle. Bar = 20μ.

B. An adjacent area of the same strand of striated muscle in which individual muscle fibers are separated by the edema fluid in the area of a subcutaneous injection of 2 x 10^7 *V. vulnificus* cells. Bar = 20μ.

Table 3. Estimated LD_{50} as determined by s.c. and i.p. injections of live cells into mice.

Injection	*V. vulnificus**		*V. parahaemolyticus*
	C7184	B3547	27519
s.c.	2 x 10^5	9 x 10^5	†
i.p.	8 x 10^5	2 x 10^6	8 x 10^7

*Two CDC strains of *V. vulnificus.*

†No observed mortaility with an inoculum of 10^9 cells.

Adapted from Poole and Oliver (reference 3).

mally about 45 percent. As seen in Figure 2, when *V. vulnificus* is injected subcutaneously (or by any route, for that matter), we rapidly see a hematocrit of 65-70 percent develop.[4] This results in blood similar in consistency to molasses. You can cut the tail off a mouse and it doesn't bleed. The reason for this is not because the animals make more blood cells, but because the blood fluid is leaving the blood and accumulating in the adjacent tissues. The loss is not just of water from the blood, but also of blood proteins. This is due to very marked damage to the blood vessels caused by the bacterium.

Let me turn now to the infection as acquired on ingestion of the bacterium. To examine this, we used a very common technique termed the "ligated ileal loop." This technique involves surgically tieing off sections (loops) of the intestine, injecting the bacterium into the loops, then waiting 18 hours. The loops are then examined for accumulation of fluids, which is an indication of the bacterium's ability to produce intestinal infections. The problem was that every time we did this the animals

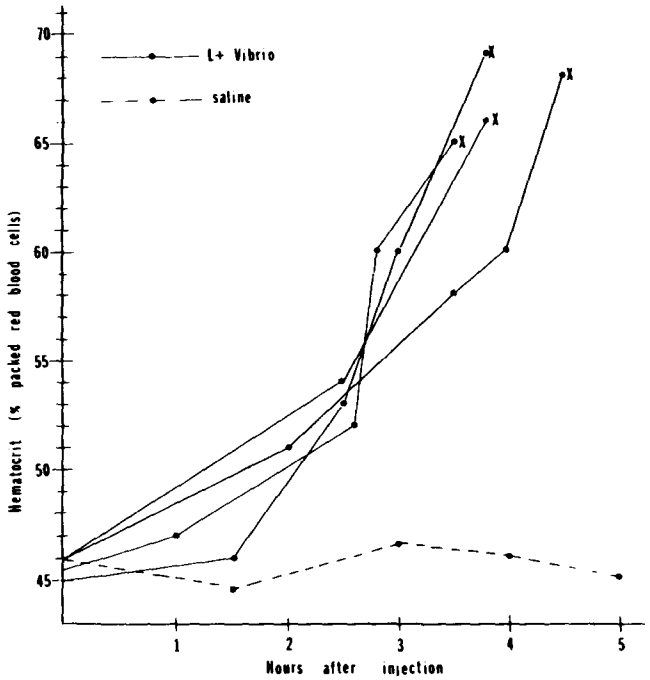

Figure 2. Effect of *V. vulnificus* infection on hematocrit values in mice. (Solid lines, hematocrit values for mice injected i.p. with 2 × 10^7 *V. vulnificus* cells; dashed line, control mouse injected i.p. with saline; X, time of death.)

Table 4. Observed mortality in ligated ileal loop studies.

Ileal loop inoculum	*Mortality**	
	Rats	*Rabbits*
Vibrio vulnificus	12/12	5/6
V. parahaemolyticus	0/6	1/4
Saline	0/3	0/2

*Number of animals dead before 18 h out of the total number tested.

Adapted from Poole and Oliver (reference 3).

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receiving *V. vulnificus* cells would die within a few hours (Table 4). The data suggested how a person might acquire the disease on eating *V. vulnificus* contaminated oysters: once the bacterium got into the intestines it could penetrate (rapidly) through the intestinal wall to the blood. The result would be a massive septic shock resulting in death, typically due to heart failure. To further examine the invasion through the intestine, we performed some histological studies on the intestinal tissues.[5] Figure 3a is a scanning electron micrograph of normal rabbit intestinal tissue, showing the villi. After the bacterium is present for one hour, the surface degradation is readily apparent (Figure 3b). If a single villus is examined by light microscopy, one gets a picture as shown in Figure 4a. This is a typical villus in cross section magnified about 500 times, showing the uniform "brush border" surface. After nine hours in the presence of the bacterium, the villus appeared as seen in Figure 4b. Figure 5a is a transmission electron micrograph of the underlying submucosa of normal rabbit intestine, showing neatly ordered collagen bundles, red and white blood cells, and the endothelium (membrane wall) of a blood vessel. Figure 5b is the submucosa seven hours into the infection. Gram negative bacteria are seen throughout the submucosa, the collagen bundles are very much disoriented, and extensive damage to the blood vessel wall has occurred. This blood vessel damage is what allows all the fluid loss from the blood which I referred to earlier.

The next question was why, if this bacterium is so virulent, weren't more people dying from the disease? We suspected the answer might be revealed in the data presented on the 39 cases reported by the CDC. Of the 24 people who acquired the infection after ingestion of the bacterium (presumably from eating raw oysters), 75 percent had a pre-existing liver disease, 4 had hemochromatosis (an extremely rare blood disease), 10 had cirrhosis of the liver, and 2 had hepatitis. Of those who did not have a pre-existing blood or liver disease, 3 did have a history of alcoholism or alcohol abuse and one had another blood disease. The common aspect of all of these diseases is a defect in iron metabolism. Iron is a substance frequently essential for bacterial disease production, and it seemed plausible that if people had an unusually high level of iron in their blood, this might predispose them to the infection. So we injected mice with low levels of iron, and then *V. vulnificus,* to see if elevating the blood iron did anything to affect the rate of disease production.[6] Table 5 shows that when only *V. vulnificus* was injected into mice, at numbers from 10^8 down to theoretically less than 1 cell, a rather typical

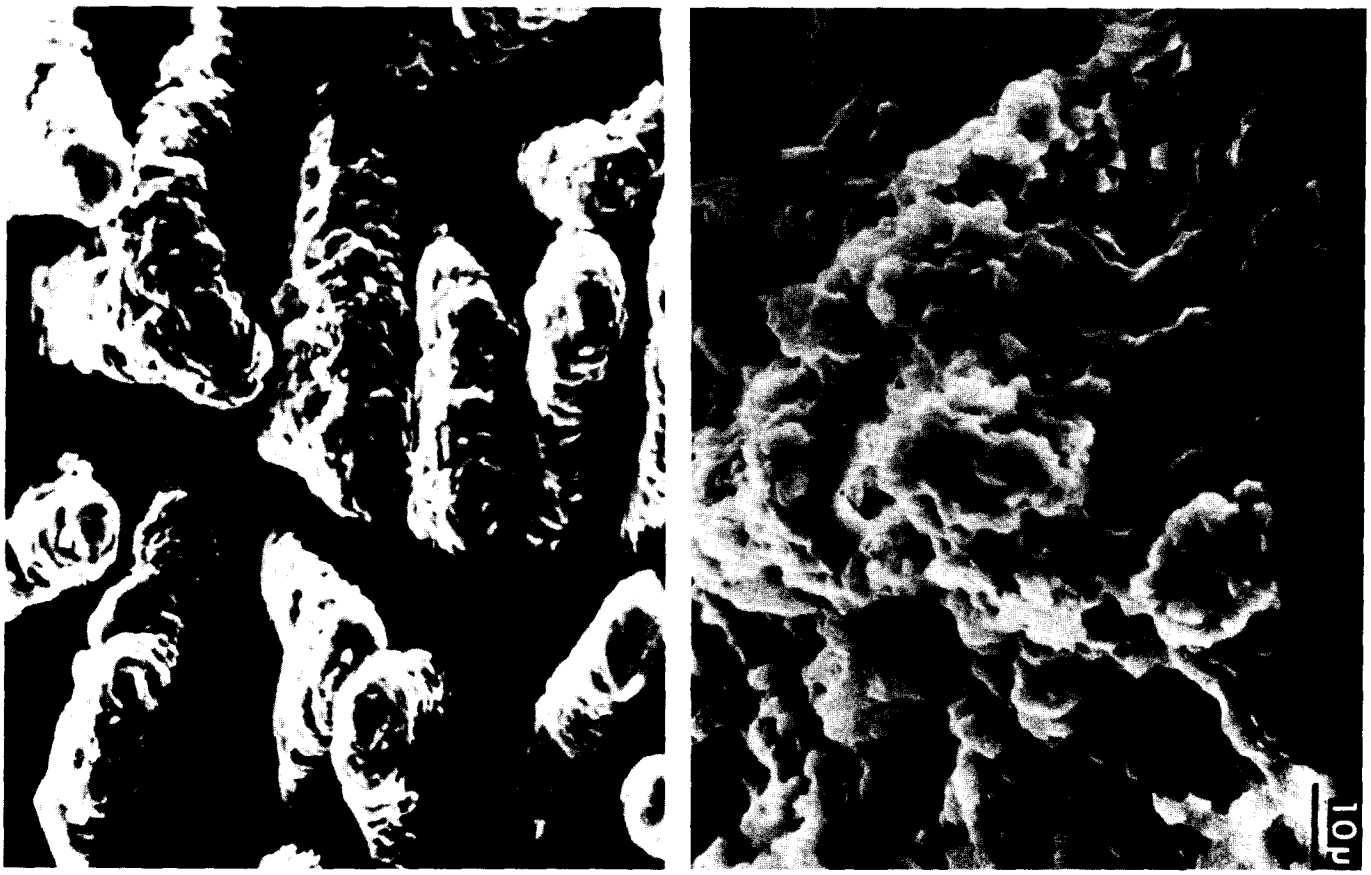

Figure 3. Scanning electron micrograph of villi from rabbit intestine (ileum).

A. Control tissue (x280).

B. Experimental tissue, after 8 hours in presence of *V. vulnificus.* Large areas of surfaces are not recognizable as intestinal tissue. Degeneration of the tissue is extensive and the surface appears to be covered with cells in the process of blebbing. (x1400).

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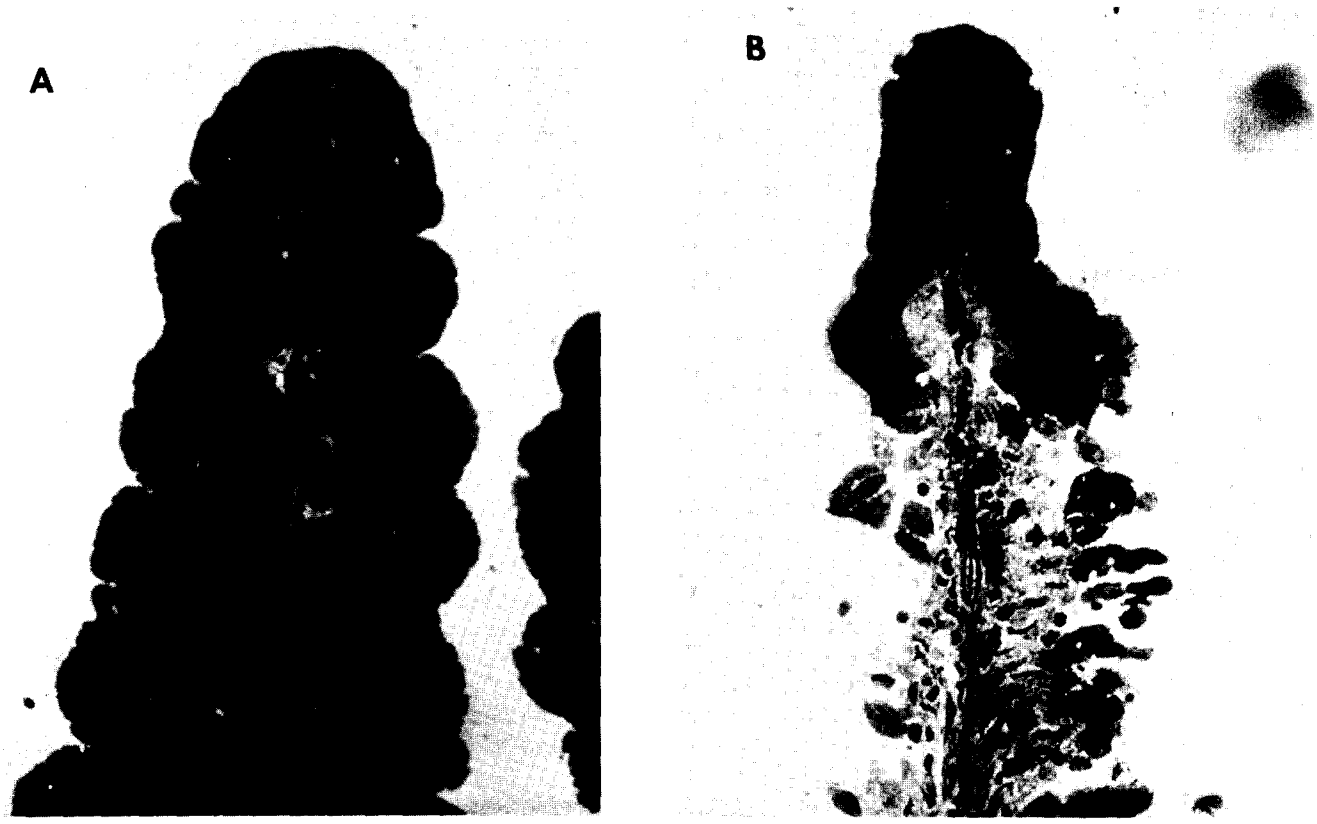

Figure 4. Light micrograph of villus from rabbit intestine (ileum).

A. Control (x460).
B. Experimental, after 9 hours in presence of *V. vulnificus.* Note extensive degradation of the villus. (x230).

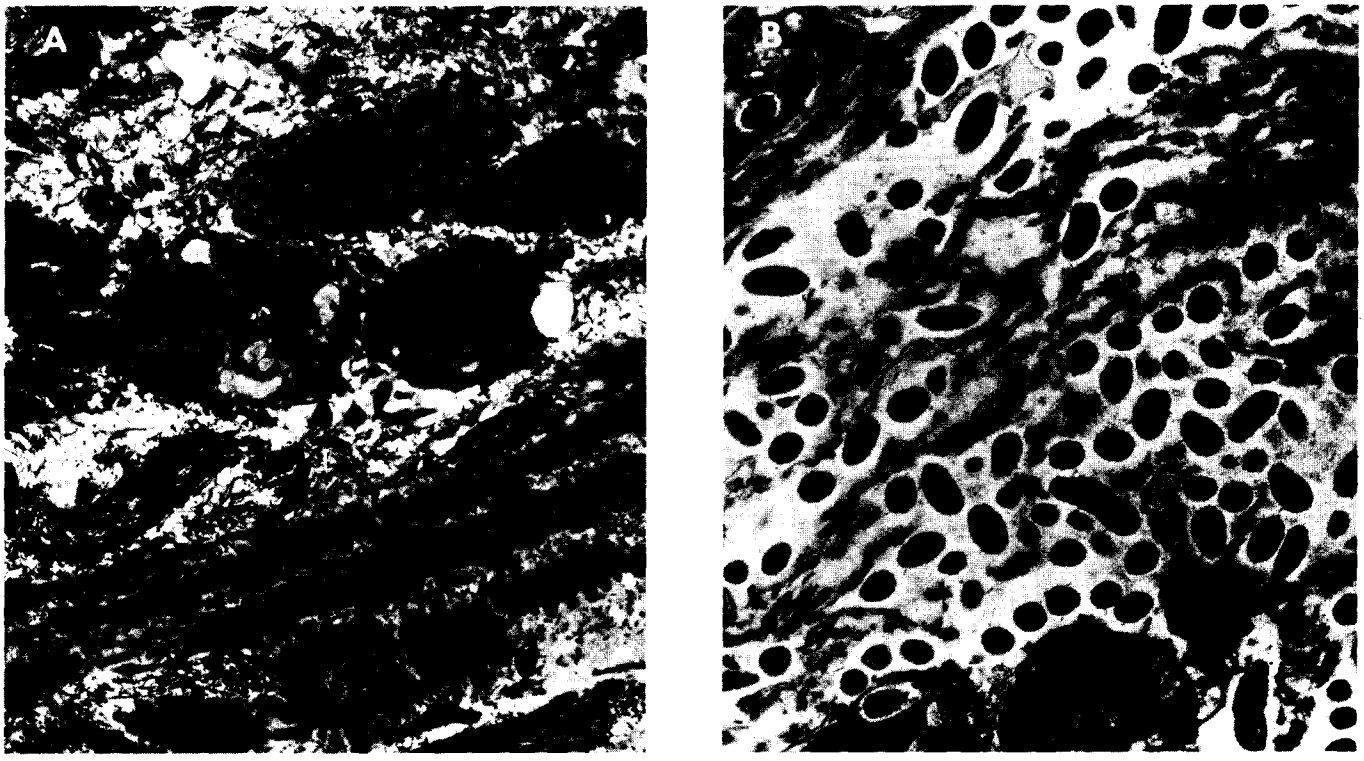

Figure 5. Transmission electron micrograph of submucosa of rabbit intestine (ileum).

A. Control. Note compact, orderly arrays of collagen bundles. (x3500).
B. Experimental, after 7 hours in presence of *V. vulnificus.* Note presence of bacteria in submucosa, and marked disruption of collagen bundles. (x3500).

LD_{50} of 6 x 10^6 was obtained. The animals did not die if they received less than about a million bacterial cells. If iron was injected first, at only 4 micrograms per gram of body weight, 4 out of 5 mice receiving only a single bacterial cell died! This gave an LD_{50}, after iron was injected, of 1 cell. Since injecting iron into an animal is not the same as having a liver disease, however, we took these studies one step further and caused cirrhosis of the liver by injections of carbon tetrachloride (CCl_4). The CCl_4 injections were at a level which produces some liver damage, but does not kill the animals. In fact, the liver regenerates itself in a few days and there is no permanent damage whatsoever. Using CCl_4 to damage the liver, and thereby to release iron to the blood (the liver is an iron "storehouse"), resulted in essentially the same results as seen with iron injections (Table 6). Finally, we have been able to correlate this ability of *V. vulnificus* to kill animals at low inoculum levels with the actual increases in serum iron. Figure 6 shows that 24 hours after the liver is damaged by CCl_4 the serum iron peaks (dotted line). With this peak in iron level, and only when the serum iron is elevated, is *V. vulnificus* able to cause mortality when injected at low numbers. When the liver repairs itself and iron is no longer released, greater numbers of bacterial cells are required to kill. So, it appears from these data that people who are likely candidates for the disease are those who have chronic diseases that involve the liver, or some other dysfunction that increases serum iron levels.

Table 5. Effect of iron on LD_{50} determinations.

Log Inoculum	Number Dead (n = 5) PBS*	Fe†
8	5	—§
7	4	—
6	1	—
5	0	5
4	0	5
3	0	5
2	—	5
1	—	4
0	—	4
− 1	—	0
	LD_{50} = 6 x 10^6	LD_{50} = 1 x 10^0

*PBS, phosphate buffered saline control.
†Fe, ferrous ammonium citrate at 4 μg/g body weight.
§Inoculum not tested.

Data from Wright *et al.* (reference 6).

Table 6. Effect of CCl_4 treatment on mortality in mice.

V. vulnificus inoculum*	Route	Mortality Untreated	After CCl_4
2.1 x 10^9	i.p.	4/4	4/4
2.1 x 10^5	i.p.	2/8	9/10
2.1 x 10^3	i.p.	0/6	9/11
2.7 x 10^9	Oro-gastric	4/7	9/9
2.7 x 10^3	Oro-gastric	0/8	10/10

*0.1 ml CCl_4 (20% in olive oil) administered i.p. 24 hours before challenge.

Data from Wright *et al.* (reference 6).

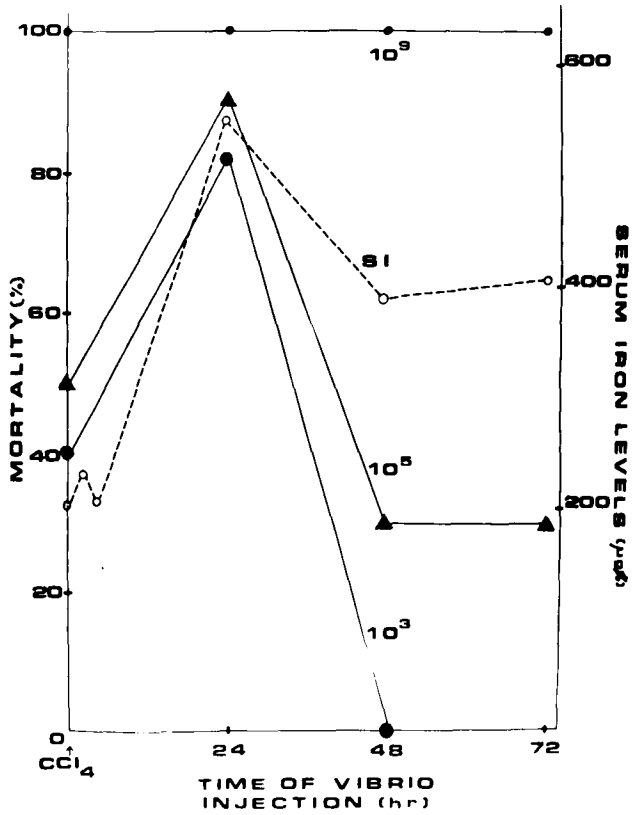

Figure 6. The effect of elevated serum iron levels on *Vibrio vulnificus* infection in mice after CCl_4 treatment. Inocula of *V. vulnificus* (10^3, 10^5, and 10^9) were injected at 0, 24, 48, and 72 hours post injection of CCl_4 (at arrow). Increased mortality for inocula of 10^3 and 10^5 correlated directly with increased iron levels (SI) which were monitored over the same time period. Inocula of 10^9 always produced 100% mortality.

Let me turn to the question of the correlation between the infection and the consumption of raw oysters. The correlation is certainly a strong one, in that 83 percent of the people who died in the CDC study had eaten raw oysters prior to acquiring the disease.[2] To examine this we inoculated fresh, raw oysters with the bacterium and kept the oysters on ice.[7] After various periods of time the oysters were assayed for the continued presence of the bacterium. Figure 7 shows that a fairly rapid die-off occurred which closely paralleled that observed when the bacteria were incubated in cold seawater alone. So,

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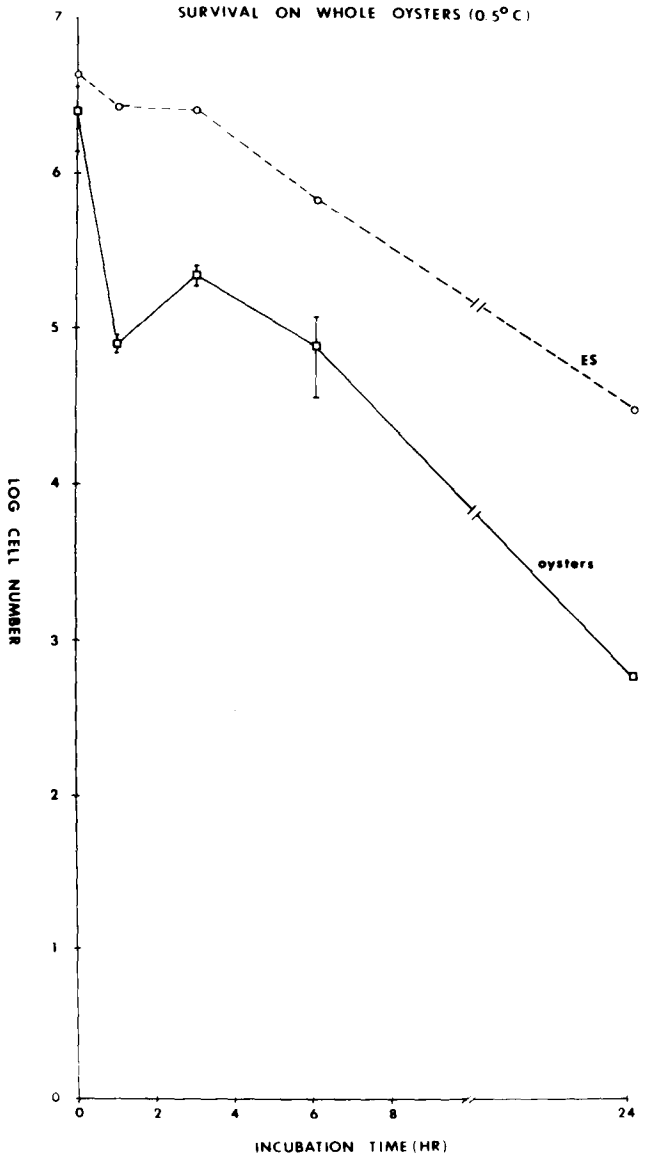

Figure 7. Survival of *V. vulnificus* (strain C7184) on whole oysters maintained at 0.5°C (on ice). ES = estuarine salt solution. Data points are average of bacterial counts of 2 oysters sampled at each time. Vertical bars on data points represent individual counts.

it would appear that properly chilled oysters, such as would normally be served at a restaurant, would not pose a problem in the epidemiology of this disease. The die-off of *V. vulnificus* in oysters on ice should be fast enough to reduce the bacterial numbers to quite low levels. Still, however, those persons with underlying chronic liver disease would seem to be at a risk in eating raw seafood, since only a few *V. vulnificus* cells would be required to initiate the infection. It should also be noted that the bacterium grows quite rapidly in unchilled raw oysters, and this could pose a significant health problem if they were eaten before being chilled for some time.

DISTRIBUTION OF THE BACTERIA

Finally, I come to the final aspect of this paper, that of the distribution and ecology of the bacterium in the marine environment. The disease has been reported from all eastern and southeastern states around to Texas, and to a lesser extent on the West Coast. Most cases occur in the warmer months of May through October. Since June of 1980, we have been conducting a quite extensive and systematic study that will ultimately involve sampling 16 sites from Portland, Maine, down to Miami Beach, Florida. At each site we are examining the water, sediment, plankton, and shellfish in an attempt to find *V. vulnificus* in the environment. We have now examined over 2,100 vibrio isolates from 5 Southeastern sites (Table 7) and have found 44 percent to be lactose

Table 7. Lactose-fermenting Vibrio spp: distribution in the environment.*

SEAWATER	365/678†	53.8%
SEDIMENT	190/518	36.7%
PLANKTON	298/721	41.3%
ANIMALS	97/240	40.4%

*June 1980—Dec. 1980; Cape Hatteras to Savannah, Georgia (6 sites, 11 samplings).

†Figure represents number of ONPG (+) isolates over number of sucrose (−) presumptive vibrios (VPLO).

positive (as indicated by the ONPG assay). Not only does this type of bacterium occur in virtually every marine environment we have examined, but they occur in very high numbers.[8] What we are seeing, however, turns out not to be just *V. vulnificus,* but a great number of other species of lactose-fermenting vibrios. They include a great many physiological types, and although several of them that we have isolated are at least 90 percent similar to *V. vulnificus,* they are not, in fact, *V. vulnificus.* We have randomly injected some of these isolates into animals and find about 30 percent of the cultures are capable of causing death. Thus, while there are many species of (presently undescribed) bacteria which are similar to, but not the same as, *V. vulnificus,* there do appear to be some which are potentially pathogenic to man. Much work remains to be done on these, and on characterizing the distribution and ecology of *V. vulnificus.*

CONCLUSIONS

To summarize, we are dealing with a newly described marine bacterium. While the number of cases that are reported every year is not high, the fatality rate is quite high. The disease can be acquired through ingestion, probably through eating raw seafood, or through a wound that was pre-existing or which is acquired while in seawater. In either case the bacterium has a remarkable ability to penetrate through tissue and reach the blood and cause death. It seems likely that, at least in those cases of the infection which are acquired by ingestion, the people at greatest risk are those with

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underlying chronic liver disease or other dysfunctions which may increase the serum iron levels. Finally, it is evident that there are numerous species of lactose-fermenting vibrios in the marine environment, only one of which is *V. vulnificus.* Many, however, are lethal to laboratory animals, and probably are pathogenic for man. At this point in our studies, we are not able to define the ecology of *V. vulnificus* specifically, but hope to be able to do so shortly.

ACKNOWLEDGEMENTS

I am indebted to the following people, whose studies in my laboratory are presented in this paper: M. Poole, J. Dellinger, A. Wright, L. Simpson, R. Warner, and D. Cleland. These studies have been supported by grants from the Charlotte-Mecklenburg Heart Association, the United Way of North Carolina, Sigma Xi, and Public Health grant AI 1705901 from the National Institute of Allergy and Infectious Disease.

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Virulence Mechanisms of an Aquatic Pathogen

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Strains of the marine fish pathogen *Vibrio anguillarum,* isolated in the Pacific Northwest, harbor a plasmid associated with virulence. These strains can grow unaffected in the presence of iron-binding compounds such as transferrin due to a more rapid and efficient iron uptake mediated by the virulence plasmid in conditions of iron limitation which mimic those found in the vertebrate fish system. In addition, *V. anguillarum* growing under conditions of iron limitation show at least two novel outer membrane proteins. One of them, an 86,000 dalton protein we called OM2, is inducible only in those cells in which the virulence plasmid is present.

INTRODUCTION

Plasmids, Iron and Infection

Worldwide, vibriosis caused by *Vibrio anguillarum* and related vibrios is one of the most serious diseases of salmonids and other fishes in mariculture. The disease is a hemorrhagic septicemia that affects the destruction of a variety of host cells and tissues types.[1,2] We have found that high virulence strains of *V. anguillarum* biotype 1 from America's Pacific Northwest harbored a specific plasmid class; the plasmid, however, was absent in strains of low virulence (Figure 1).[3] Plasmids are supernumerary chromosomes present in bacteria which can carry genetic information for a vareity of functions, such as antibiotic resistance and various virulence factors.

A plasmid encoded virulence determinant in *V. anguillarum* could be responsible for specific surface antigens which would permit attachment of the bacterium to fish target cells or organs, as a prelude to the invasion process. Alternatively, the plasmid could carry determinants involved in the synthesis of a toxin or of hydrolases which could act at some stage during the invasion process. However, no correlation of this sort was found (manuscript in preparation).

In general, all bacterial virulence determinants are in some way directed toward enabling bacteria to grow in spite of the antagonistic factors of the host's defense mechanisms. Because of the septicemic nature of infection by *V. anguillarum,* the putative virulence plasmid must be in some way associated with the ability of the bacterium to grow in the host. The ability to utilize essential components present in the host at lower con-

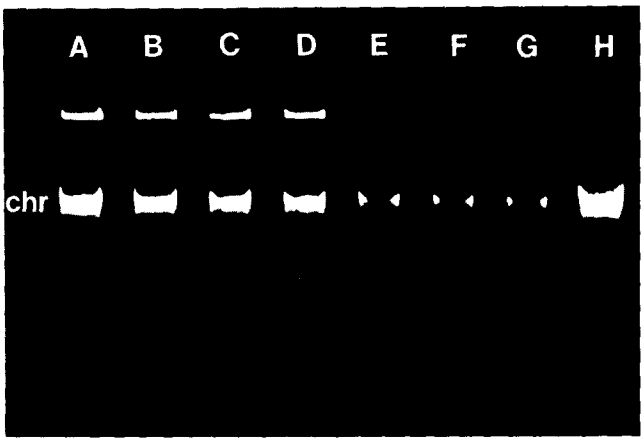

Figure 1. Agarose gel electrophoresis of deoxyribonucleic acid from strains of *Vibrio anguillarum* containing the virulence plasmid (labeled with the penicillin resistance transposon) and plasmidless isogenic derivatives. Lysates from high-virulence strains contain the plasmid (lanes A to D). Lysates from isogenic low-virulence derivatives do not contain the plasmid (lanes E to H). Deoxyribonucleic acid (DNA) was prepared and gels were prepared and run according to.[4] Reprinted by permit from *Infection and Immunity*, 27:897-902, 1980. Copyright © 1980 American Society for Microbiology.

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centrations than the physiological level for bacterial growth would be of the utmost importance, especially in the case of septicemic strains of bacteria. During recent years, the role of iron in host-bacteria interactions has generated a considerable amount of interest.[5] Because of its capacity to engage in electron transport and in other biological reactions, iron is essential for bacterial growth.[6] In vertebrates, iron is mainly present intracellularly and therefore not freely available to pathogens.[7] Iron that is in body fluids and for which organisms must compete is bound by the high affinity iron-binding proteins, transferrin and lactoferrin.[5] These proteins are normally unsaturated; consequently they limit the availability of iron in the blood and on mucosal surfaces, a condition that may result in the enhancement of nonspecific resistance to bacterial infections. In contrast, mechanisms whereby invading bacteria may selectively and successfully compete for the bound iron could become crucial in enabling them to establish infection.

I have recently reported[8] that the putative virulence plasmid specifies a very efficient iron-sequestering system that enables high-virulence plasmid-containing *V. anguillarum* to survive under conditions of iron limitation similar to those of normal vertebrate physiology. Under such conditions, growth of the low-virulence strains lacking the plasmid is inhibited.

MATERIALS AND METHODS

In order to determine whether a plasmid-mediated iron-sequestering system existed in *V. anguillarum,* the growth kinetics of the high virulence biotype 1 *V. anguillarum* 775 (pJM11) strain was compared with that of its cured low-virulence derivative H775-3. Cultures were grown in medium containing trace amounts of transferrin that binds iron and makes it unavailable for microbial growth. The presence of 180 μ g/ml of transferrin inhibited the growth of the low-virulence plasmidless strains. but the inhibition was reversible when excess iron (0.2 mM $FeCl_3$) was added to the medium (Figure 2a). Figure 2b shows that growth of the high-virulence parent strain 775 (pJM11), which possesses the virulence plasmid, was not affected by concentrations of transferrin that were inhibitory for cured strains. The results of infectivity studies strongly supported the contention that possession of a plasmid-mediated iron uptake system is a selective advantage for *V. anguillarum.* Experimental infection of fish with low-virulence cured strains of *V. anguillarum* showed (Table 1) that the mean lethal dose values (LD_{50}) were decreased about 300-fold if iron was included in the inoculum. In contrast, the presence of iron did not affect the LD_{50} of the plasmid-containing high-virulence strain 775 (pJM11). Similar effect of iron-decreased LD_{50} values can be obtained with other plasmidless low-virulence derivatives.[8] Thus, there was a selective advantage for the plasmid-containing strain only when iron was absent. Further support for this contention is shown in Table 2. In this case, experimental infections were carried out by using mixtures containing different proportions of the plasmid-containing high-virulence strain and its plasmidless low-virulence derivative, again in the presence or absence of iron in the inoculum. Independent of the original proportion, if iron is not included in the inoculum, the only bacteria that can be recovered are those which contain the virulence plasmid. If iron is included in the inoculum, analysis of the fish blood after the same interval of infection shows that the original proportion of the plasmid-containing and the plasmidless strains that were inoculated is approximately maintained. These results

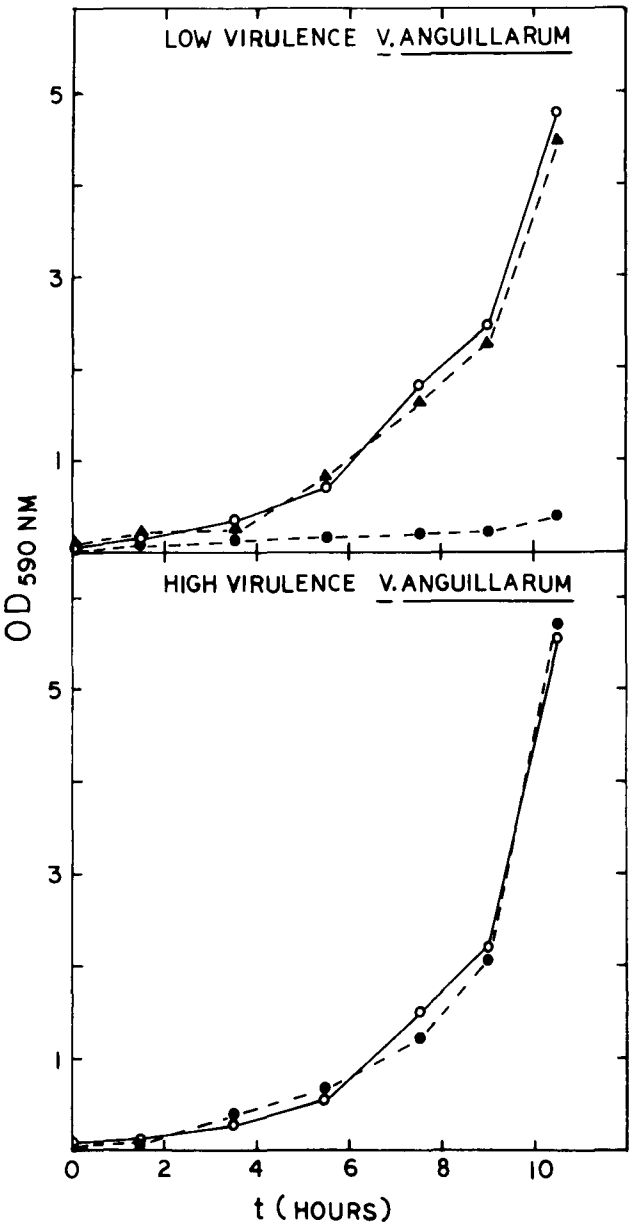

Figure 2. Effect of transferrin on the growth rate of low-virulence (a) and high-virulence (b) *V. anguillarum.* Growth curves were obtained with: o, minimal medium; •, minimal medium plus 2.3μM transferrin; ▲ minimal medium plus 2.3μM transferrin and 0.2μM$FeCl_3$. Conditions are described in[8]. Absorbance at 590 nm (A590) was measured using Gilford-vacuum micro-sample spectrophotometer. Reprinted by permit from *Nature,* 284:566-568, 1980. Copyright © Macmillan Journals Ltd. 1980.

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thus demonstrate that there is a greater selective advantage for the plasmid-containing strain when iron is not included in the inoculum.

Table 1. Effect of iron on experimental infections of fish with cured low virulence strains of *V. anguillarum*.

Strain	LD_{50} Control	LD_{50} with iron	Increase in virulence
H-775-7	3.2×10^6	8.7×10^3	368-fold
775 (pJM11)	4.3×10^3	3.3×10^3	1.3-fold

Control LD_{50} were determined as previously described.[4] LD_{50} values with iron were determined by including 86 g iron as ferric ammonium citrate in the bacterial inoculum. In all cases virulence was tested on juvenile coho salmon *(Oncorhynchus kisutch)* weighing about 14 g. Virulence is quantified as LD_{50} values (number of microorganisms required to kill 50% of the inoculated animals) as determined by the Reed-Muench method.[9] There were no deaths in the controls in which 86 g iron (as ferric ammonium citrate) in saline solution was injected. Reprinted in part with permission from *Nature*, 284: 566-588, 1980. Copyright © Macmillan Journals Ltd. 1980.

Table 2. Effect of iron on the course of mixed infections with a high virulence plasmid carrying strain and a low virulence plasmidless strain of *V. anguillarum*.

Proportion in inoculum (%)		Addition of iron (Fe^{3+})	Proportion in blood	
Low virulence	High virulence		Low virulence	High virulence
48	52	–	0	100
48	52	+	40	60
93	7	–	0	100
93	7	+	84	16
0	0	+	0	0

Bacterial mixtures were inoculated by injection behind the dorsal fin of juvenile coho salmon. After approximately 20 hours fish were sacrificed and bacteria in blood identified by using selective markers present in the high- and low-virulence derivatives.

Outer Membrane Proteins Induced by Iron-Limitation

We have now initiated the characterization of the plasmid-mediated iron uptake system in *V. anguillarum*. The results of these studies (published in reference 10) indicate that new outer-membrane proteins are induced under conditions of iron limitation and at least one of these proteins is produced only when the virulence plasmid is present. The high-virulence plasmid-containing strain of *V. anguillarum* 775 and its isogenic low-virulence derivative were grown in minimal medium to which iron as $FeCl_3$ was added to various concentrations. In some cases 3μM transferrin was added to the growth medium. Total cell envelopes as well as outer membranes were prepared from cells grown under these various conditions and were analyzed by SDS-polyacrylamide gel electrophoresis. In Figure 3, lanes A to H show total cell envelopes for both strains at different $FeCL_3$ concentrations. There are five major proteins which were later identified as outer membrane proteins,[10] OM1-5 present in the plasmid-containing strain grown at 2μM $FeCl_3$. One of these, OM2, is missing from the plasmidless derivative. The analysis of Figure 3 clearly shows that there is a threshold of $FeCl_3$ concentration (below 4 M) at which both OM2 and OM3 are inducible, whereas those proteins are not detectable at higher iron concentrations in plasmid-containing

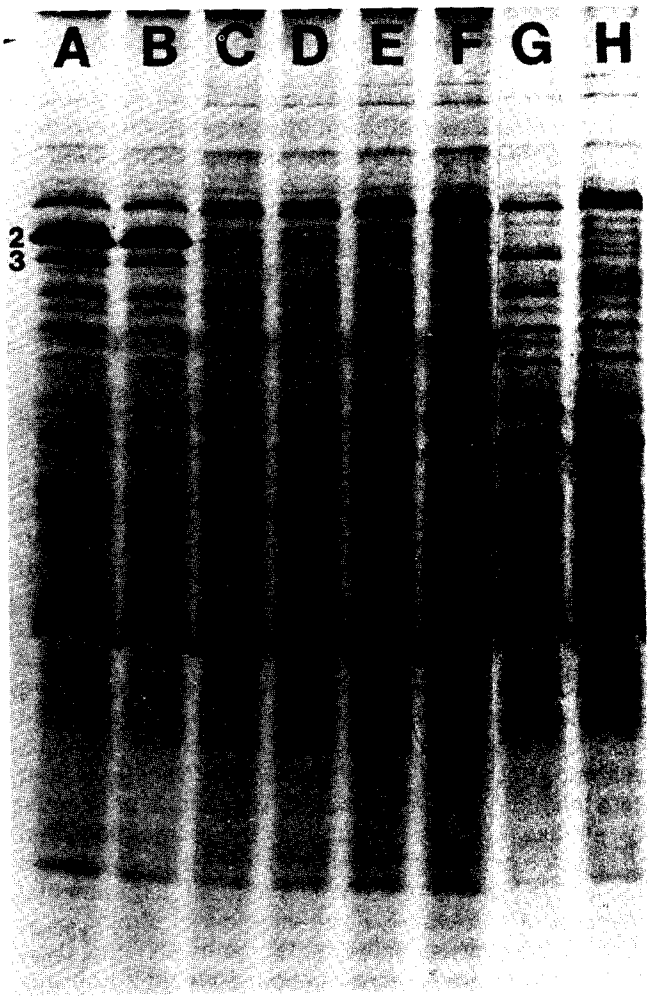

Figure 3. Polyacrylamide gel electrophoresis showing the range of $FeCl_3$ concentrations at which OM2 and OM3 proteins are induced. Lanes A to F: total cell envelopes of plasmid carrying *V. anguillarum*. A: cells grown in 3μM transferrin. B to F: cells grown in $FeCl_3$. B: 2μM; C: 4μM; D: 8μM; E: 12μM; F: 24μM $FeCl_3$. Total cell envelopes of the plasmidless derivative, G: 2μM $FeCl_3$; H: 8μM $FeCl_3$. Reprinted in part from *Infection and Immunity*, 31:223-227, 1981. Copyright © American Society for Microbiology 1981.

strains (lanes A-F). Lane A shows that, as expected, both OM2 and OM3 are present in the plasmid-containing strain grown in the presence of 3μM transferrin. In the case of the plasmidless strain, the OM3 protein is the only protein induced at 2μM $FeCl_3$ (lanes G and H). Thus iron limitation conditions which allow a very rapid uptake of radioactive iron by plasmid-containing strains of *V. anguillarum*[10] also induce the synthesis of two specific outer membrane proteins, OM2 and OM3 of 86,000 and 79,000 daltons respectively. The OM2 protein is associated with the presence of the virulence plasmid. (These results are published in reference 10). Although OM2 is present only in strains containing the virulence plasmid, it remains to be seen whether this protein is actually coded for by the plasmid and induced in response to a decrease in the iron concentration or is a chromosomal product that is regulated by a plasmid-specific substance. It is of interest that both OM2 and OM3 are induced in plasmid-carrying *V. anguillarum* obtained from experimentally infected fish (manuscript in preparation). The possible role of these two proteins in the iron metabolism and virulence of *V. anguillarum* is presently under investigation.

Iron Acquisition and Diving

With respect to the topic of hazards for divers, the question is whether we should be concerned that such a powerful mechanism of virulence mediated by a plasmid could be transferred from the fish pathogen to a human pathogen present in the aquatic environment. For instance, it was reported in this meeting by Dr. J. Oliver that infections by *Vibrio vulnificus* (lactose-postive vibrios) occur mainly in compromised patients who have higher than normal serum iron levels (e.g., liver cirrhosis). The reason for the higher incidence of infections in these patients is that iron in their serum is more available for bacterial utilization, whereas iron in the serum of normal patients is bound by transferrin and is not easily available. If the *V. anguillarum* plasmid which specifies an iron-sequestering system were transferred to these lactose-positive vibrios, the consequences could be disastrous. We should add here that during the summer months high densities of many *Vibrio* species including *V. parahaemolyticus, V. alginolyticus* and many undefined psychrophilic and mesophilic species coexist in the intestine of inshore marine animals. Consequently, it appears quite likely that these different *Vibrio* species and strains of each species could interact genetically. To stress this point we should add that recently Williams[11] has described another plasmid-mediated iron uptake system as being specified by the Col V plasmid of those invasive strains of *Escherichia coli* associated with cases of bacteraemia in man and domestic animals. From an evolutionary and epidemiological standpoint, it would be rather interesting to assess whether the *V. anguillarum* and *E. coli* plasmid-mediated iron uptake systems are related.

ACKNOWLEDGEMENTS

This research was supported by Washington Sea Grant Contract #04-7-158-44021 from the National Oceanic and Atmospheric Administration.

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DISCUSSION

Oliver: What is the saturation of the transferrin?

Crosa: About 30%.

Oliver: Are you familiar with Despheral?

Crosa: Yes.

Daggett: Do you find any difference in susceptibility between sexes?

Crosa: No. What we do is get a homogeneous size.

Colwell: Drugs that cure plasmids, if it is a drug of treatment for humans, has that been done?

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Crosa: No.
Colwell: To summarize briefly. We have progressed to the point of being able to document some real, as well as potential, problems. An important, immediate issue is to match the need with the equipment. Research work must be done over the next few years to develop the safest, most cost-effective equipment for divers.
Comment: One of the weak points in our discussion is the lack of documentation concerning patient-related information. We don't even document how much diving time is lost to infection.
Colwell: Fortunately, we are in the position to get such information via serological surveys. We have agreed that it is a problem in getting a diver to admit that he or she is, in fact, ill, especially during diver training. Divers won't report problems as they occur because they fear the reaction of their peers or the trainers. Perhaps, an anonymous questionnaire might be useful. Physical symptoms can be recorded and questions can be asked that don't single any one diver out.
Daggett: There is a real problem with a questionnaire, a sociological/psychological kind of problem. Many people will not fill out forms. My brother is a mining engineer. Recently, he was given a form to complete. The company was interested in data on safety hazards that are not reported, as well as work attitudes. Half of the employees filled the form out falsely; others refused to fill it out or were afraid that if they answered every question honestly it would be held against them. Useful epidemiological data may not be obtainable by a questionnaire.
Williscroft: Years ago, while I was serving as an enlisted man aboard a submarine, a young medical doctor, who was also a diver, came aboard. He was interested in doing a sociological study and provided forms for the crew to fill out. The crew got together and with the aid of every available book on psychology, wrote down all kinds of symptoms. The young medical doctor published a paper, including the responses!
Wells: We might be able to avoid these problems. For example, a better idea of what's going on could be gained from civilian NOAA divers. There is a definite advantage to working with civilian divers who won't be penalized, as perhaps, Navy divers might be.
Williscroft: In discussion with Jim Oliver about sampling the divers, we concluded that if we could come up with a convenient method for the divers to take swabs from wounds and throat swabs, and store them until the swabs could be mailed to a central point, if time would allow, it might be possible to build a meaningful data base.
Wells: We use the "eyeball" test of pollution in waters, but is there a generalization that is useful to determine whether a body of water represents a high risk area?
Daggett: Perhaps from our discussions we can add comments about classes of water people dive in. Based on what we know, probably the only threat from clean, thermally polluted waters would be amphizoic amebae. From what I know about protozoa, I would consider such diving a second degree risk.
Colwell: Unfortunately, *Aeromonas* also abound in thermal effluents, especially fresh water effluents from cooling towers.
Wells: It would be a valid statement to say that there is a greater risk of infection associated with diving in sewage-polluted water compared to warm waters free of sewage.
Daggett: Away from a dump site, a previous dump site, or from thermally polluted waters, with respect to protozoa, exposure risks should be minimal, based on our present knowledge.
Williscroft: What is the risk with increasing concentration of microorganisms? If an outbreak occurs and two or three people diving in the effluent become ill, they live in a community, and, therefore, will shed the organisms back into the sewage system. How long will this cycle keep regenerating before dying out?
Daggett: I don't have any data on the survivability of all protozoa, but some will survive for at least a week or so. They will die off once input into the system ceases. It's not a situation like that of the bacteria, where a relatively short period of time is involved. The organisms disappear, once input into the system ceases. I suspect that if one did attempt to correlate incidence of pathogenic protozoa with presence of certain kinds of bacteria, one might be able to predict that if "X" bacteria were present, a good chance existed that amphizoic, pathogenic amebae would also be present, because they feed on bacteria. Thus, a link, or indicator, might be developed.
Wells: Can we make a summary statement to the effect, if this is true, that there is an increased risk in diving in thermally polluted water from certain organisms or groups of organisms, even though such an area is considered clean?
Colwell: The usual statement that has been made in the past is that, if the fecal coliform concentration is very high, there will be a risk for divers from pathogens. The difficulty is that the fecal coliform count is not reliable for vibrios, aeromonads, *Legionella,* etc., which are the pathogens newly recognized as being more likely to be encountered by divers, both in polluted waters and also in relatively clean waters.
Daggett: I think we should emphasize that there is need for some kind of rapid method of identifying, based on some set of indicator organisms, the rating of a site that could give divers a clear assessment of what protective equipment they need. I would suspect, for example, in the case of what is inferred or known about potential protozoan pathogens, that probably you could determine their presence by the presence of the bacteria. So I think we could develop some fairly simple tests to indicate some level of how hazardous an area is going to be.
Colwell: What is interesting is that we are covering an area that is totally new. Environmental microbiology problems include *Legionella, Aeromonas,* etc. and these can become significant clinical problems under the right conditions.
Joseph: From Dr. Weiss' and subsequent discussions, we learned that we can be caught by surprise, even when we know what is out there, how it gets there, how we contract the disease, etc. Why is that a problem? Because there is not enough interaction between ecological, environmental, and medical microbiologists. This workshop represents one of the few occasions when a group of individuals from various specialties have gotten together to respond to the specific problem of infectious disease hazards to divers.

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Williscroft: As I see it, one of the things we are looking for, to come out of this conference is for the diver to be able to ask, "If I have to dive there, does that pose a problem? If it poses a problem, can I protect myself by immunization and/or do I have to protect myself, in addition, by isolating myself from the environment? What degree of isolation do I need to give myself?" Developing some guidelines that are easy to understand and easy to apply is at least one focus of this conference.

Colwell: I think some guidelines could be produced quite readily.

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Closing Remarks

Arthur J. Bachrach, Ph.D.
Naval Medical Research Institute
Bethesda, Maryland

It has been a pleasure and a privilege to attend this workshop. I have found the papers of great interest, the discussions most informative, and I congratulate you all on the meeting. I have been asked by Drs. Colwell and Joseph to offer a few comments on the workshop. All I am able to do is to comment in the light of my own experience, manifestly not in the field of microbiology but rather in diving research, particularly the psychophysiology of diving.

One of the areas in which I have been active is the analysis of stress as it relates to diving accidents. In our work on accident methodology, and in that of others here and abroad, the problems of microbiology and hazards of microorganisms do not appear to be a significant source of difficulty. The excellent presentations offered here in the last few days clearly demonstrate that, indeed, there is a potentially hazardous environment for the diver in polluted waters and where dangerous organisms flourish. Why has this not shown up in the diving casualty and accident literature as a problem? I can hazard a guess or, perhaps better stated, guess a hazard.

The typical environment in which divers work may provide the first piece of evidence. Navy divers are less likely to enter environments such as the New York Bight where, as we have seen in the presentations, waters are severely polluted. NOAA divers are more likely to operate in such waters because of the nature of their particular mission and, as we have seen, they are protected by suits and facegear to minimize contact with the pathogens. It appears that the microorganisms are a potential hazard, but become a real threat only when a cut or wound allows penetration and infection. Protective measures such as outer garments and careful rinsing after exposure appear to minimize the risk.

Protection itself appears to be something of a problem. As Robert Browning said in *Herakles,* "A man in armour is his armour's slave." Protection always involves a certain amount of immobility. From the accounts of NOAA divers here as well as from the experiences of Navy divers, protection against environmental hazards needs to be balanced against impairing divers' ability to perform their underwater work by saddling them with cumbersome protective gear. Divers themselves may elect not to don protective gear or the appropriate equipment because of a preference for the easiest mode of operation.

Some years ago Tom Berghage and a few of us published a report on Navy diving–who was doing it and under what conditions. We reviewed the records at the Navy Diving Safety Center in Norfolk of a total of over 120,000 dives over a two-year period. According to the records, divers often threw on a bottle to go over the side to do a particular task when standard operating procedure specified another mode, perhaps a lightweight surface supply. But the more cumbersome mode was "too much trouble" for the little task they had to perform. This is a very realistic circumstance and not necessarily a function of a diver's macho image. It is a simple fact that people tend to work in the simplest mode and would have to believe that a definite risk, one from which there is little chance of avoidance or escape, was involved before they would follow a routine of protection which costs more time and effort.

The macho image has been touched upon in a number of discussions. Machismo may contribute to a diver's failure to report a gastrointestinal disorder that might be attributable to microorganisms from his diving environment, but this isn't very likely. Experience at the Navy Experimental Diving Unit has shown that there are minimal incidences of physical disorders that might be related to a polluted environment, even the mighty mucky Anacostia. Although it is patent that minor problems will, in all likelihood, go unreported, serious disorders would probably be sufficiently disabling to draw the attention of the divemasters. It is likely that the protection afforded by even minimal diving gear helps, but it is more likely that the age range and physical con-

dition of the typical Navy or NOAA diver are in themselves a protective mechanism. The young, physically fit, active diver is probably less susceptible to the onslaught of pathogens than the typical individual.

I am not suggesting that there is no problem. My predilection would be to begin a systematic study of exposed divers. The fine work reported in the past few days demonstrates that the environment can be effectively studied if there is accurate reporting of microorganisms present in specific areas. Prophylaxis seems to be working under normal circumstances and cuts and wounds can be a focus of study that results, it is to be hoped, in improved prophylactic measures.

I might note at this point that wounds under normal diving conditions, free from microbiological hazards, provide a serious problem. A diver injured or wounded underwater and requiring decompression presents a major medical emergency problem in terms of recovery and repair. Knowledge about proper maintenance under these conditions is, to a degree, lacking. Accordingly, wound handling could be an important area of study—in polluted as well as relatively aseptic diving conditions. A serological study of divers during a series of dives could provide important data about exposures and hazards.

At the moment, the diving community would rate microbiological problems below those of hypothermia and decompression, but the efforts you are making to gather essential data will provide important data for further analysis.

ACKNOWLEDGEMENTS

The opinions and assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

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Richard Attwell, who received his doctorate from the University of Bradford in West Yorkshire, England, has returned to England as a Senior Lecturer at Manchester Polytechnic after a year at the University of Maryland as a Visiting Scientist. He is primarily interested in the taxonomy, physiology, and ecology of actinomycetes.

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After receiving a B.S. in Biology at C.W. Post College, he entered the U.S. Navy and served as Gunnery Officer and Legal Officer on the *USS Rushmore* (LSD-14). Upon graduation from the U.S. Navy Deep Sea Divers School as a Salvage and Diving Officer, he served on the *USS Hoist* (ARS-40) as Operations Officer. He participated in several search and salvage operations, including the recovery of the H-bomb lost off Palomares, Spain. While working towards an M.S. in Marine Sciences at Long Island University, Mr. Phoel gained additional construction diving experience on various projects including pipeline repair. After receiving his M.S., he was employed by the New York University Medical Center, operating the triple lock hyperbaric chamber and performing experiments with HOP toxicity, effects of HOP on senility, and routine HOP therapy.

Emilio Weiss, former chairman of the Department of Microbiology, Naval Medical Research Institute (NMRI), has served at NMRI in various capacities since 1954. In 1979, he was appointed to the first NMRI Chair of Science. He is also Professor in the Department of Preventive Medicine and Biometrics of the Uniformed Services University of the Health Sciences, and President of the American Society of Rickettsiology and Rickettsial Diseases. A graduate of the University of Chicago (Ph.D., 1948), he has been involved primarily in the study of the biological properties of chalamydiae, rickettsiae, and other microorganisms that are either obligate or facultative intracellular parasites. His interest in *Legionella* stems from the fact that these organisms are facultative intracellular parasites and were isolated by methods developed for rickettsiae.

In addition to the contributors, the following participated in the discussion.

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