VIRUSES IN WATER: THEIR DETECTION, SURVIVAL AND DISEASE POTENTIAL

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ABSTRACT

Present municipal wastewater treatment methods do not produce human enteric virus-free effluents and such contaminated wastewaters and raw sewage are currently being discharged into our aquatic environment. Among the many viruses discharged are such potential pathogens as poliovirus, coxsackie virus, echovirus, rotavirus and infectious hepatitis virus. A major problem facing environmental health officials in regard to water quality is related principally to the efficacy, reliability and economy of current methods to concentrate, detect and isolate extremely low concentrations of viruses. In this regard several methods were evaluated by this laboratory and several were found to be good candidates for assessing the occurrence of human enteric viruses in various types of water. The membrane-adsorption technique, adsorption to precipitable salts, and the aqueous polymer two-phase separation technique, all have been used successfully in the recovery of natural viruses in all kinds of water including the ocean environment. Preliminary studies of the bivalve filter feeder, Pinna atrina, indicated that this indigenous macromollusk of Hawaii would be useful as a biological monitoring system for human viruses in the Hawaiian ocean environment.

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Until the early years of the 70's, data on human viruses in public water supplies, rivers, streams, lakes and also in our ocean environment have been limited. Nevertheless, the accumulated findings of human pathogenic viruses in various kinds of water have resulted in greater public concern over the hazards that these viruses present. Currently, several agencies, at the Federal, State and local levels are attempting to develop guidelines to prevent unnecessary exposure of the public to virus infection from aquatic and terrestrial sources. A problem found in many parts of the world, including the U.S., is the increased demand on available water resources caused by the concurrent expansion of the population and of industry. This has made recycling of domestic wastewater almost inevitable in the future. One of several major problems to be overcome is the development of adequate methods to ensure that viruses pathogenic for man are eliminated from reclaimed water. This problem is further compounded by the fact that present water treatment procedures are not always adequate in preventing viruses from reaching recreational and mariculture water resources and community water supplies.

Another environmental health problem of our water resources has been the lack of standardized methodologies for detecting viruses in water. While several different methods for determining the occurrence of animal viruses in all kinds of waters have evolved during recent years, there is still unequivocal need for more sensitive and improved methodologies. Before elaborating further on some

of the current methodologies used to recover viruses from water, a brief background regarding this unique microorganism may be helpful.

Table 1 shows some of the essential properties that differentiate viruses from all other microorganisms including some intracellular-requiring microorganisms. Figure 1 shows the various sizes and structural complexity of several representative animal, bacterial and plant viruses.

More than 100 different enteric viruses are known to be excreted in human feces (Table 2). These viruses have been found in raw sewage or are known to be present in the feces of infected persons, including healthy carriers. It has been estimated that greater than 10^6 virus particles may be excreted per gram of fecal material and concentrations as high as 500,000 infectious units per litre have been detected in raw sewage in some parts of the world. The average enteric virus density in domestic sewage in the U.S. has been estimated to be about 7000 infectious units per liter (at Mililani STP-27 to 19,000 PFU/ℓ). The amount of virus present in raw sewage is highly variable, depending on such factors as the level of sanitation in the population, the incidence of disease in the community, the socioeconomic level, and the time of the year. Human enteric viruses survive the customary secondary sewage treatment and chlorination, as routinely practiced, in sufficient numbers to be isolated easily by today's concentration procedures throughout the year. It is not surprising, therefore, that these viruses have been detected in all kinds of water including the ocean environment.

Figure 2 shows the many potential routes of virus transmission from water back to man. Under the proper circumstances, it has been reported that as little as one virus infectious unit in water is capable of producing infection in man and this may thus pose a potential disease hazard. Documented waterborne outbreaks of virus disease have largely been limited to the agent of infectious hepatitis,

TABLE 1. Comparison of Some Biological and Chemical Properties of Viruses and Other Microorganisms

Organism	Obligate Intracellular Formation	Visibility by light microscopy	Growth on Artificial Media	Metabolic Activity	Binary Fission	Eclipse Period	DNA + RNA	Ribosomes	Coat Muramic Acid	Sensitivity to: Antibiotics	Sensitivity to: Interferon
Bacteria	-	+	+	+	+	-	+	+	+ sometimes	+	-
Mycoplasma	-	+	+	+	+	-	+	+	-	+	-
Rickettsiae	+	+	-	+	+	-	+	+	- diamino-pimelic acid	+	-
Chlamydiae	+	+	-	-?	+	-?	+	+*	+	+	+
Viruses	+	-	-	-	-	+	-**	-	-	-	+

*Although Chlamydiae contains ribosomes they also utilize host cell ribosomes.

**Viruses contain either RNA or DNA only.

CLASSIFICATION OF VIRUSES

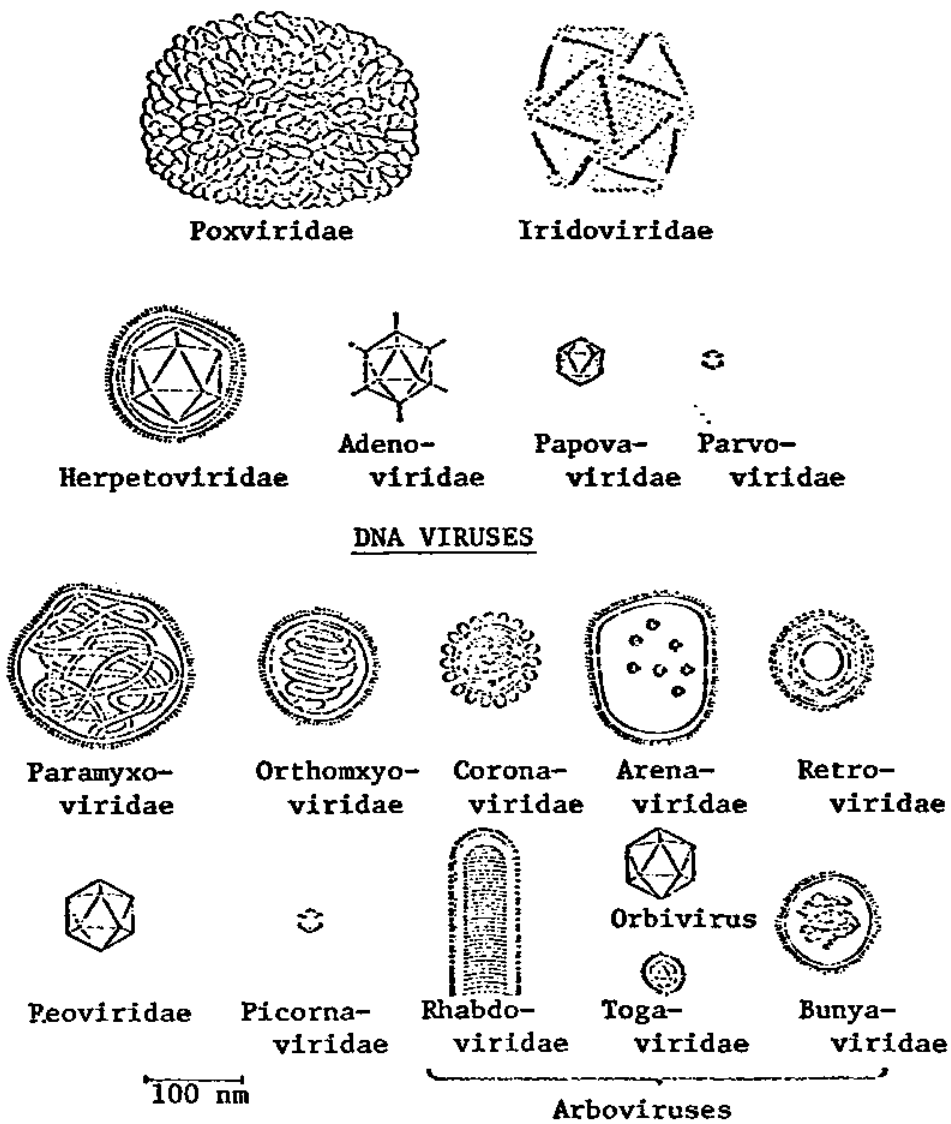


Fig. 1. Diagram illustrating the shapes and relative sizes of animal viruses of the major families (bar = 100 nm). (Source: The Biological of Animal Viruses, 1974. Academic Press).

TABLE 2. Human Enteric Viruses That May Be Present in Water

Virus Group	Number of types	Disease or Sign Caused
Enteroviruses		
Poliovirus	3	Paralysis, meningitis, fever
Echovirus	34	Meningitis, respiratory disease, rash, diarrhoea, fever
Coxsackievirus A	24	Herpangina, respiratory disease, meningitis, fever
Coxsackievirus B	6	Myocarditis, congenital heart anomalies, rash, fever, meningitis, respiratory disease, pleurodynia
New enteroviruses	4	Meningitis, encephalitis, respiratory disease, acute haemorrhagic conjunctivitis, fever
Hepatitis type A (probably an enterovirus)	1	Infectious hepatitis
Gastroeneteritis type A (probably an enterovirus)	2	Epidemic vomiting and diarrhoea, fever
Rotavirus (reovirus family) (gastroenteritis type B)	?	Epidemic vomiting and diarrhoea, chiefly of children
Reovirus	3	Not clearly established
Adenovirus	>30	Respiratory disease, eye infections
Parvovirus		
Adeno-associated virus	3	Associated with respiratory disease of children, but etiology not clearly established

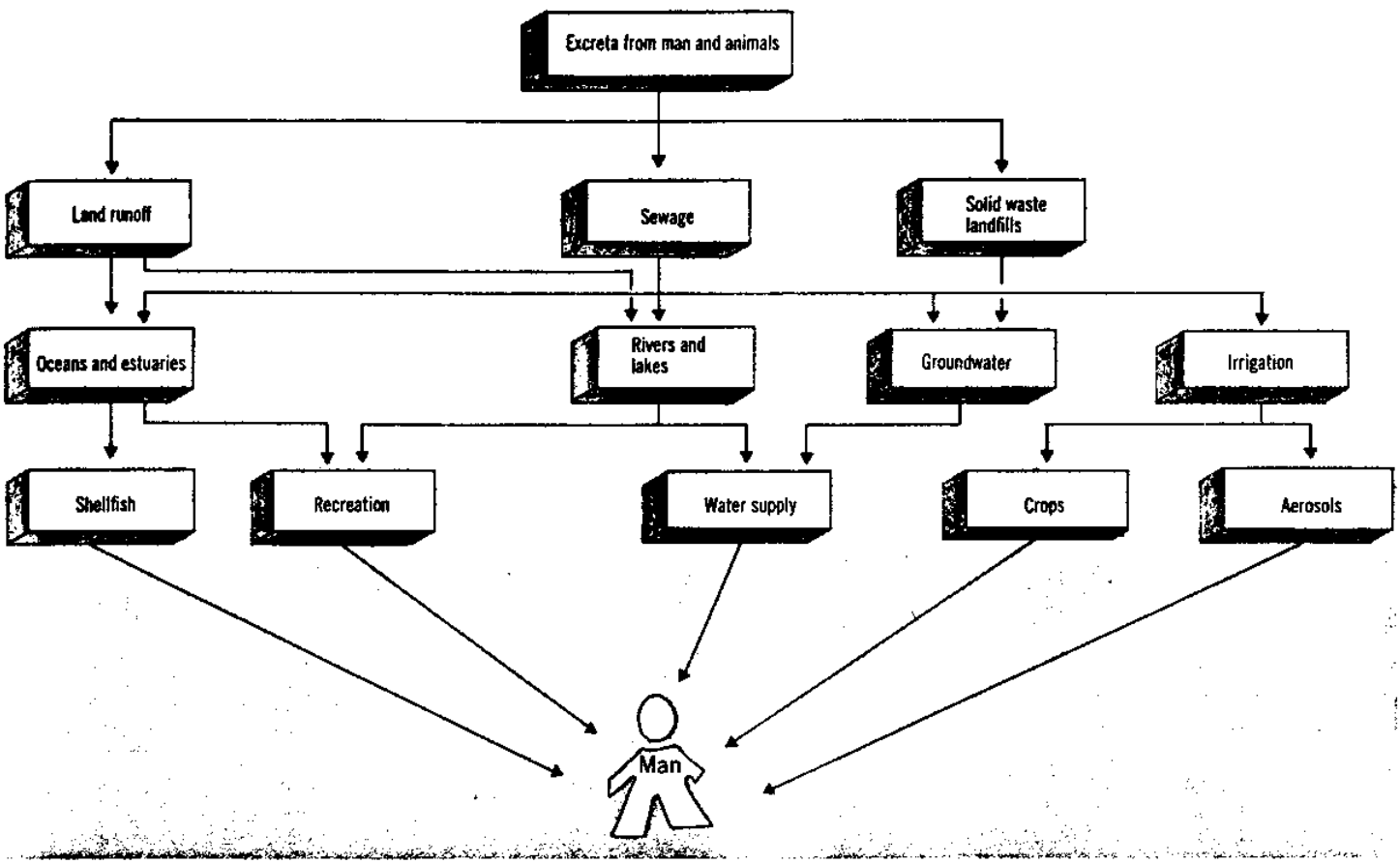


FIGURE 2. POSSIBLE MODES OF ENTERIC VIRUS TRANSMISSION (SOURCE: C.P. GERBA ET AL. ENVIRON. SCIENCE & TECH. 9:1122-1126, 1975).

mainly because of the explosive nature of these outbreaks and their characteristic symptomatology. Other waterborne enteric virus outbreaks are not so easily recognized. Well-documented outbreaks attributable to specific viruses are lacking. There are two principal reasons for this:

1. Many of these viruses cause subclinical or inapparent infections that are difficult to recognize as being water-borne. A person may acquire a viral infection by coming in contact with contaminated water. However, the infection remains subclinical and the individual may act as an effective carrier and transmit the virus to others who may then develop the clinical disease.
2. Current epidemiological techniques are not sufficiently sensitive to detect low-level transmission of viral diseases through water. Most enteric virus infections cause such a broad spectrum of disease syndromes that scattered cases of acute illness would probably have such a wide variety of symptoms to be attributed to a single etiological agent.

These reasons are probably the basis why almost 60% of all documented cases of disease attributable to drinking water in the U.S. are caused by unrecognized unknown agents. Furthermore, there are currently no routine methods available for the detection of the infectious hepatitis and nonbacterial gastroenteritis, such as human rotaviruses. A very recent publication reported on the propagation of human hepatitis A virus in cell cultures. Several sensitive serological techniques, such as the ELISA or enzyme-linked immunosorbent assay procedure are now available to detect the presence of these viruses and the human rotaviruses. It should be mentioned that the occurrence of hepatitis and gastroenteritis epidemics as the result of consuming raw or inadequately cooked shellfish from polluted waters has been well documented.

Shellfish can take up viruses during feeding and accumulate them in their digestive tract tissues, but there is no evidence that the viruses replicate. However, there is evidence to indicate that the enteric virus can survive for relatively long periods of time in oysters (>2 mos). Poliovirus has been isolated

from oysters harvested from a shellfish growing area that met accepted bacteriological criteria for shellfish harvesting. Serum hepatitis type B antigens have been detected through serological means (radioimmunoassay) in shellfish growing in waters receiving sewage discharge from a hospital.

A major factor governing the recovery and distribution of infectious enteric viruses in various kinds of water is their relative stability in the water environment. This ability to survive promotes the dissemination of the virus and may represent a potential route of viral disease transmission, particularly when disseminated to populated areas, recreational facilities, and the seafood-producing coastal waters. Human enteric viruses have been reported to vary in their survival periods in the different water environments. Thus, survival periods of 2 to 168 days in tap water, 2-130 days in sea water and up to 90 days in oysters have been reported. However, generalizations on virus survival can be dangerous. Factors, such as temperature and purity of the water play a role in survival time of viruses. Enteric virus survival times are prolonged at low temperatures and also in the presence of gross pollution by domestic sewage. The influence of other factors is at present inadequately understood and the amount and combinations of these factors in nature are numerous. The few studies accomplished on the survival of animal viruses in ocean water strongly suggest that three major factors appear to influence survival: 1) inactivating microorganisms, 2) inactivating chemicals, and 3) protective organic matter. It should be noted that there is increasing evidence to show that enteric viruses are often associated with the sediments in the various aqueous environments. This association of viruses with solids does not result in inactivation; in fact, virus survival appears to be prolonged. Human enteric viruses adsorbed to clays and soil particles have been shown to be infectious for both animals and cell cultures. In the detection of viruses in water these observations emphasize the necessity of examining the sediments and other solids. In field studies of coastal waters,

10- to 10,000-fold higher concentrations of enteroviruses have been found in the sediment than in the overlaying water.

Numerous problems are encountered in the elimination of viruses from all kinds of water. It is not within the purview of this talk to deal with this aspect in depth. Suffice to say that there are shortcomings in all of the methods employed today. Processes available for virus removal from water and wastewater have been separated into 2 general categories: 1) those involving physical removal, and 2) those causing inactivation or destruction of the virus. With 1) these include sedimentation, adsorption, agglutination and precipitation, and filtration. With 2) these include high pH, chemical oxidation by disinfectants, such as halogens, ozone, UV-light and photo-oxidation by certain dyes in the presence of light. Of these processes, those that bring about virus inactivation are preferable, since with simple removal one is still faced with the disposal of potentially infectious material.

Thus, the monitoring of human enteric viruses in sewage-polluted water becomes imperative. Virus monitoring techniques, although not perfected for all types of water, have been developed to detect virus under ideal conditions when only a single infectious unit is present in samples as large as 4000 liters or 1000 gallons of drinking water. New and improved techniques have also been developed for the quantitative recovery of viruses from sewage, estuary and marine waters as well as from shellfish.

Before discussing some of the methods that are currently used for concentrating human enteroviruses, two other related aspects, the physico-chemical properties of the virus particle itself and the sample methods used, need further comment.

With regard to the physico-chemical properties, since viruses are essentially nucleoproteins and behave as colloidal hydrophilic particles in suspension, many of those properties are identified with the properties common to proteins. In this

regard, viruses manifest properties of solubility which decrease with increasing concentrations of very soluble salts, such as ammonium sulfate.

Since their outer coats or capsids are proteins, viruses behave as typical amphoteric protein particles. Under acidic conditions, at pH levels below their isoelectric point, they have a net positive charge, while above their isoelectric point they are negatively charged. Since viruses exhibit polarity, they are also immiscible in organic solvents, such as chloroform, ethyl ether and fluorocarbon. Because of their unique surface properties, they adsorb readily to a number of substrates, such as celite, magnetic iron oxide (Fe_3O_4) clays, aluminum hydroxide floc, ferric hydroxide floc, tricalcium phosphate, various resin and cellulose derivatives and certain synthetic fibers.

Fundamentally, there are only 2 methods available for sampling water for the presence of viruses. One method is an in situ entrapment technique and is called the gauze-pad or swab method. This method is strictly qualitative and consists of suspending for a period of time, usually 24 hr to several days, a gauze pad in the water to be examined. This pad is then treated with an alkaline solution at >pH 8 (1N NaOH) to enhance elution of any entrapped viruses and the fluid expressed from the pad. The second method is simply a water sampling technique. This method is quantitative and consists of taking a sample of water in a container and examining for virus. This method is also called the grab- or dip-sample method. Both methods have been used in the field with varying degrees of success for detecting the presence of viruses in various types of virus-contaminated waters. The efficacy of both methods is closely linked with the nature of the water source. While it is quantitative, the grab- or dip-sampling method is limited in the amount of sample volume that it can handle satisfactorily. It should be noted here that it is only the quantitative approach that will definitively assess the distribution and extent of virus contamination in our

aquatic environment, and thereby permit meaningful conclusions to be made. Value judgements based on qualitative data will not resolve the public health management of virus-in-water problems nor elucidate the potential health threat of waterborne viruses and the epidemiology of viral diseases transmitted by the water route. The choice of the sampling method eventually will be dependent upon the investigator's objectives.

During the last few years, several methods for concentrating viruses from water have been reported. Many of these methods show sufficient promise to permit the quantitative assessment of viruses in waters. In our laboratory we have evaluated several of these methods and have modified some of them for our use, particularly in the recovery of human enteric viruses from sea water. Only 3 of the methods that we have used will be briefly described here and the advantages and disadvantages of the methods in terms of efficiency and application will be discussed.

Before elaborating on the concentration methods, mention should be made of some of the unique problems encountered which are intrinsic to the recovery of enteric viruses from sea water. A major problem is the great dilution of the virus upon discharge of the virus-containing sewage into the ocean environment, consequently, only techniques capable of processing large volumes of water can be used inorder to obtain meaningful results. Another problem is the high salt content of seawater which prevents the use of virus concentration methods employing increasing salt concentrations to precipitate out the virus. On the other hand, if virus-membrane adsorption methods are used to concentrate the virus and since adsorption is basically an electrostatic phenomenon, virus adsorption would be enhanced by the high salt content of sea water.

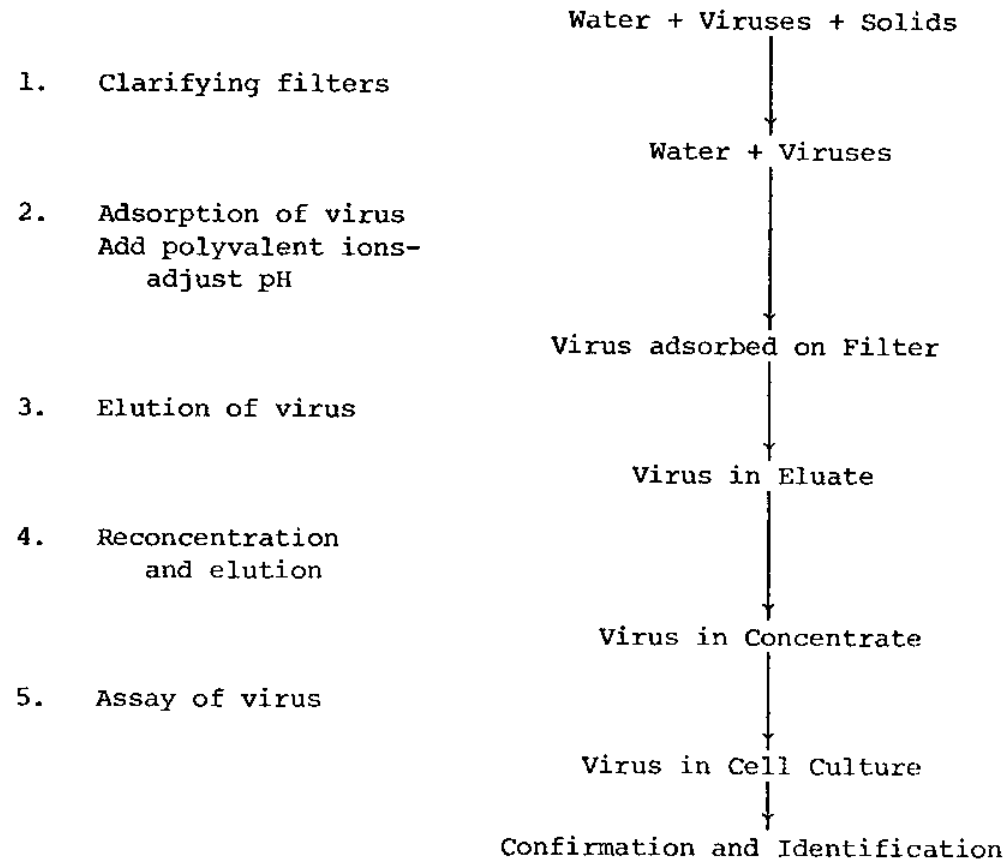
The first concentration method involves virus adsorption and elution. Because of their unique surface properties, viruses under specified conditions will efficiently adsorb to a variety of materials. Adsorption techniques are the only one applicable at present to studies of large-volume water samples (i.e. more

than 1000 liters). Adsorption materials can be divided into 2 categories: the fixed substrates, such as various membranes, filters and fiberglass; and the macroscopic chemical substrates, such as aluminum hydroxide, calcium phosphate, protamine sulfate, etc. With the former, they are made up of different chemical compositions and consequently, vary in their efficiencies to adsorbing virus. Thus, filters made up of fiberglass, epoxy-fiberglass, dynel, cellulose acetate or nitrocellulose adsorb virus very well. In contrast, filters made up of orlon, polyester and polypropylene adsorb virus poorly and are used primarily for the initial clarification of "dirty" water to remove particulates that might otherwise interfere with the virus-adsorbing filters. The adsorbing filters come in two forms: the standard disk and the cartridge type if filters. The latter, with its greater surface area, can handle large volumes of water and at increased flow rates. The recently introduced pleated type of cartridge filters have not only increased the efficiency and flow rates of the cartridge filters but also allowed their reuse which makes it economical.

In practice, if conditions warrant, the water sample may be initially clarified through non-adsorbing filters to remove particulates that might otherwise block the virus-adsorbing filters. The filtered water is then adjusted chemically to optimize virus retention and pass through the virus-adsorbing filters (Table 3).

Conditions used for virus adsorption will vary depending on the virus types being sought and the quality of the water sample. In clean water, only a slight acidification is needed, but in more highly contaminated waters, the optimal conditions for adsorption of human enteric viruses are a pH of 3.5 to 4.5 and an Al^{+++} concentration of 0.0005 M to 0.001 M. Polyvalent ions, such as Al^{+++} and Mg^{++} have been found to enhance virus adsorption. In sea water where adsorption is basically an electrostatic phenomenon, virus adsorption is enhanced by the high salt content of the water.

TABLE 3. Sequence of Steps to Recover Viruses



If clarifying filters are used; two problems should not be overlooked. First, the composition of the clarifying filters must be such that viruses themselves will not adsorb. Second, the loss of viruses associated with particulates which are retained by the clarifying filters.

After adsorption, the viruses are now slowly eluted from the filters. The elution conditions will vary depending on the virus. Routinely, an alkaline solution (pH 9.5 to 11.5) of a buffer 0.05 M glycine, or a proteinaceous suspension of 3% beef extract is passed through the adsorbing filters. Because of their amphoteric properties, desorption or elution of the adsorbed virus is achieved at this alkaline pH which renders the virus negatively charged. The proteinaceous composition of the eluting fluid essentially competes with the virus for adsorption sites and prevents readsorption of virus to the filters. The eluate is immediately neutralized (pH 2, 0.05 M glycine) to prevent inacativation of the virus and is further reconcentrated. Instead of beef extract, animal serum can be used. However, animal sera is expensive.

If clarifying filters are used, it may also be desirable to elute the surface-associated viruses from them so as to have a more quantitative recovery of virus.

Viruses in the neutralized eluates are further concentrated by either of the following methods: 1) reacidification (pH4, Al^{+++} 0.0005M) and readsorption to membrane filters; 2) adsorption to aluminum hydroxide floc; 3) acid precipitation of the protein eluate (e.g. beef extract); or 4) by two-phase extraction.

The first is simply a repeat of the initial concentration by membrane adsorption on a smaller scale. However, certain substances present in many waters will concentrate in parallel with the viruses. Many of these substances behave like viruses and may either block membrane filters and entrap viruses or some of them may even successfully compete with viruses for adsorption sites and cause loss of viruses into the filtrate. These substances are called membrane coating compounds or MCC. Recently, it was determined that MCC had a greater effect on the virus

adsorbing quality of cellulose acetate but not fiberglass filters.

The second method of reconcentration involves the formation of aluminum hydroxide floc, to which viruses adsorb and are recovered by centrifugation. Here $AlCl_3$ is added to a final concentration of 0.003 M, followed by the addition of 1 M Na_2CO_3 and the pH adjusted to 7.0. After thorough mixing for 30 mins and allowing to stand for 30-45 mins, the floc is then recovered by centrifugation (10,000 rpm x 10 min). The floc is then dissolved in a small volume of either 0.05 M glycine or borate buffer at pH 10.5 plus 10% calf serum. After brief centrifugation, the supernatant is immediately neutralized with pH 2 glycine and assayed for virus in cell cultures.

The third method of reconcentration involves lowering of the pH of the virus-containing protein (e.g. 3% beef extract) eluate to 3.5 which produces a flocculation of proteins. The virus-adsorbed floc is then recovered by centrifugation (3,000 rpm x 10 min) and is solubilized in a small volume of phosphate buffer at pH 9.0.

The last method -- the two-phase method -- has been successfully used either for the concentration and recovery of human enteric viruses directly from water or as a reconcentration method following virus adsorption procedures. The mechanism of two-phase separation is liquid-liquid partitioning which occurred as a result of differences in particle surface properties and their distribution between two liquid phases. Basically, the aqueous polymer two-phase separation system consists of dissolving two different polymers, such as dextran and polyethylene glycol in water under specified conditions of salt, pH, and polymer concentrations. Following a holding period, usually 18 to 24 hrs in the cold, two immiscible phases are produced. One phase, the bottom dextran phase, smaller in volume contains the virus. Additional salt (NaCl) may then be added and after overnight incubation in the cold, two immiscible phases reform. The viruses are finally recovered from the upper phase, dialysed and assayed in cell cultures.

There is, at present, no single method of virus concentration applicable to all types of viruses or to all waters. Each case presents its own problems. A method that works well under one set of circumstances may not be applicable to another.

The preceding methods mentioned can be applied individually or in various combinations. The method of choice or combination of methods is dependent on the kind of waters, the volumes sampled or the types of viruses being sought. The 3 methods mentioned above have been found to be most generally suitable for our field and laboratory experiments in a variety of natural waters. In dealing with the recovery of human enteroviruses from large bodies of water, such as the ocean water of Mamala Bay, there was a need for a system which would process large volumes of water rapidly at the sample site, and at the same time be sufficiently efficient to isolate the highly diluted viruses. To aid us here we adopted and later built a portable virus concentration system developed by Wallis and his colleagues at Baylor University and modified it for our purpose (Figures 3 and 4). The portable concentrator which can process 50 to 60 gallons or more of water per hour at the sampling site consisted of a series of 2 clarifying filters plus 2 to 3 virus adsorbing filters. After processing the water sample, the filters are taken back to the laboratory and the adsorbed virus eluted. The eluates are then further concentrated by the aluminum hydroxide floc method and the final reconcentrated eluates assayed for virus. While the efficiency of recovery of marker virus in laboratory tests is not high, approximately 20-30%, it should be noted that numerous natural viral isolates were obtained from sewage-contaminated ocean waters by this procedure. Furthermore, it should be emphasized that the system is portable. However, there are some disadvantages to the use of the portable virus concentrator operating in the ocean. These are the need of a fairly large boat, extra personnel and a power source to operate the concentrator. While the initial cost of putting together such a portable concentrator is high, once assembled, such an apparatus can be used in a wide variety of waters with little maintenance.

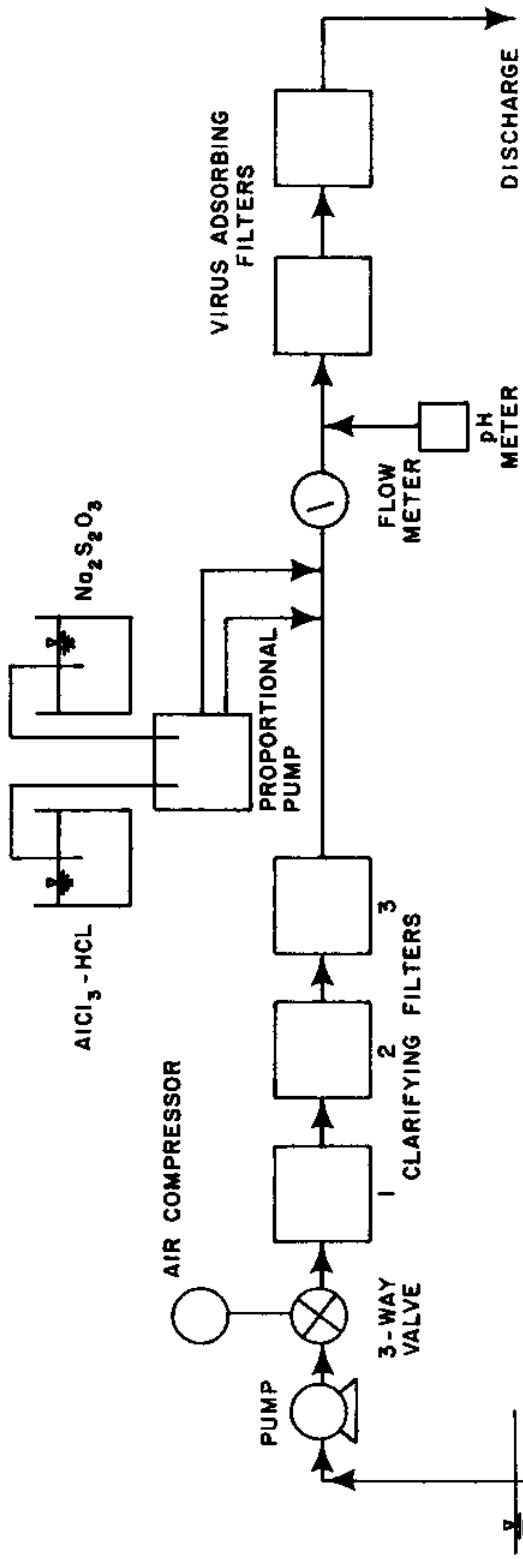


FIGURE 3. OPERATIONAL DIAGRAM OF AUTOMATIC VIRUS CONCENTRATOR.

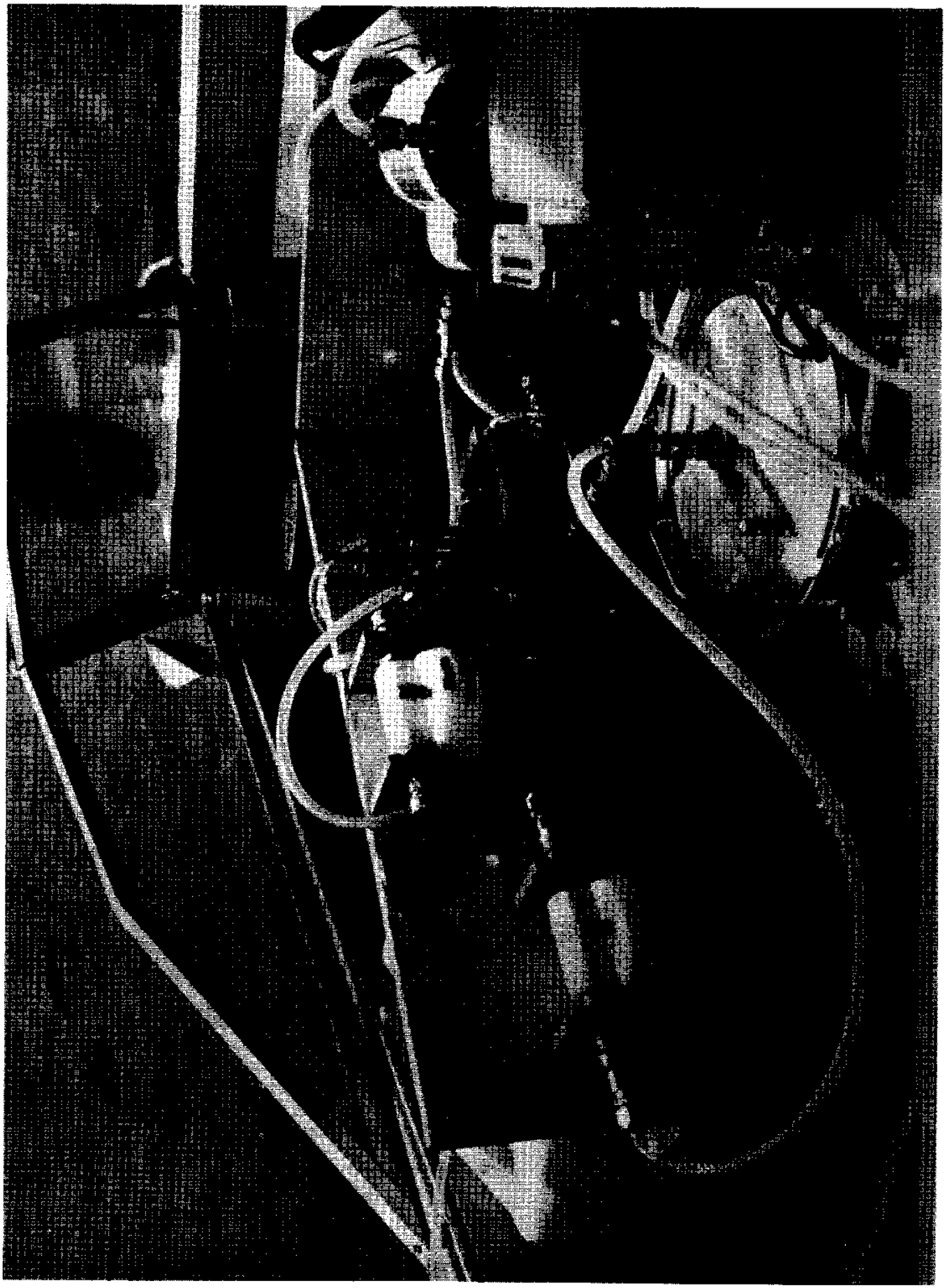


FIGURE 4. VIRUS CONCENTRATOR MOUNTED ON BOAT AND SAMPLING WATER IN MAMALA BAY.

Employing the portable virus concentrator and the supplementary methods mentioned, the problem of sewage-borne human enteric viruses, contaminating the ocean water of Mamala Bay off Oahu was investigated. Previous studies have shown that both treated and untreated sewages from Oahu contain a variety of human enteropathogenic viruses and that these agents are being discharged daily into the ocean environment including Mamala Bay via the Sand Island outfall. The raw sewage discharged into Mamala Bay represents by far the largest single source of sewage in the State and is approximately 60-65 MGD of the total sewage from the City of Honolulu.

The first part of the study was made when sewage was discharged through the old outfall pipe which extended some 3000 feet and at a depth of about 40 feet into the bay from Sand Island (Fig. 5). Human enteric viruses, such as poliovirus types 1 and 2, Coxsackieviruses B4 and B5, Echovirus type 7, were consistently isolated from the sewage outfall area and on occasion even at sampling stations 2 miles from the outfall pipe. However, in order to comply with the new sewage discharge standards advocated by the Environmental Protection Agency (EPA) for the disposal of sewage wastes, a new sewage outfall extending approximately 9,000 feet from shore inclusive of a multiport hole diffuser some 3,000 feet long and 240 feet deep was constructed and completed in December 1976 (Fig. 6). The new outfall was designed (a) to provide a high and rapid dilution of the sewage (200:1), (b) to cause a rapid and efficient dispersion of the sewage, (c) to be minimally affected by winds, tides and currents, and (d) to take advantage of the normal thermocline of the ocean water at the depths involved (240 feet), causing the sewage to remain submerged.

Although the entire Sand Island Sewage Treatment Plant is yet to be fully operative, sewage from the old outfall was diverted to the new outfall in December 1976. Surveillance for the presence of the human enteropathogenic

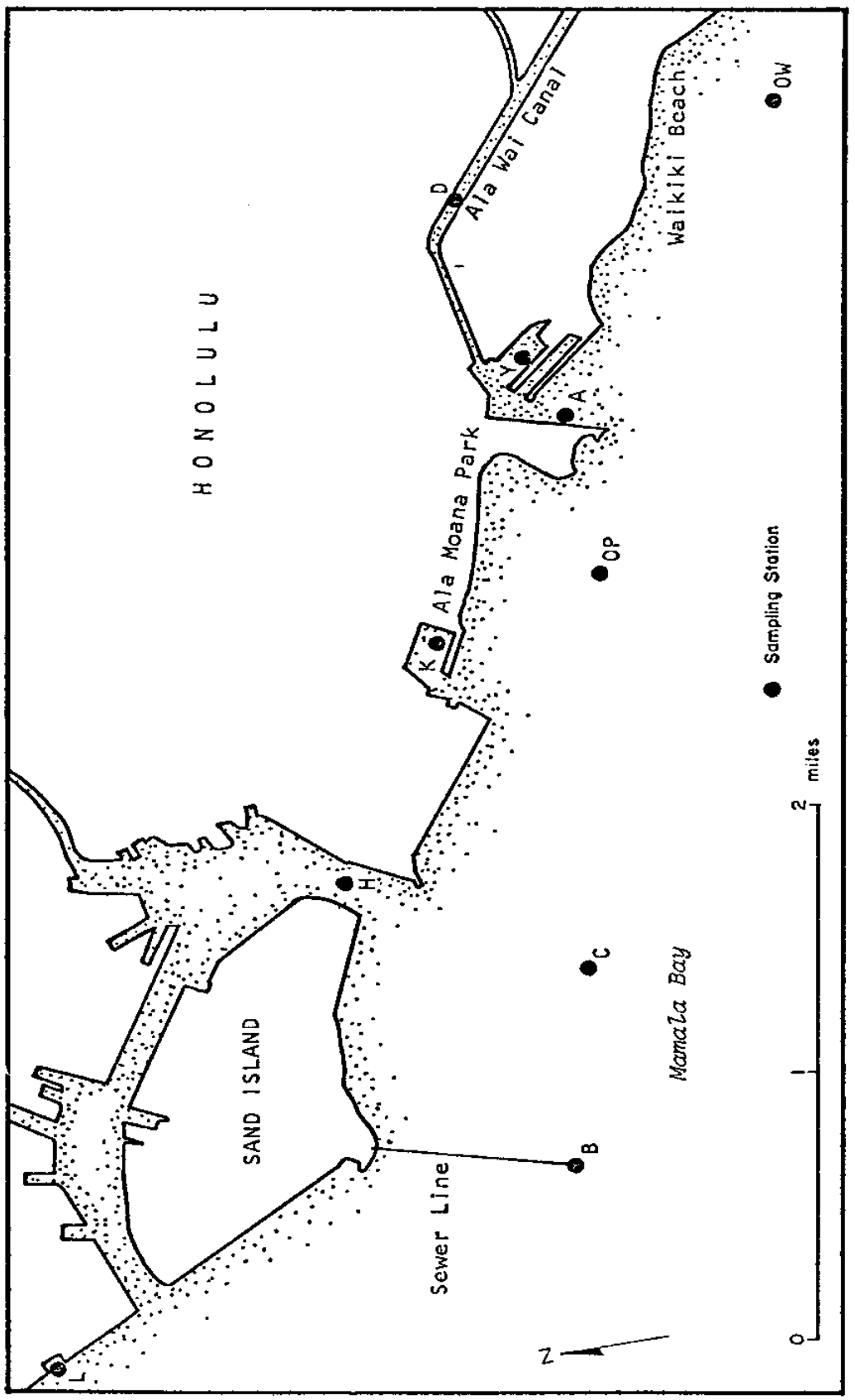


FIGURE 5. VIRUS SAMPLING STATIONS IN AND OFF MAMALA BAY.

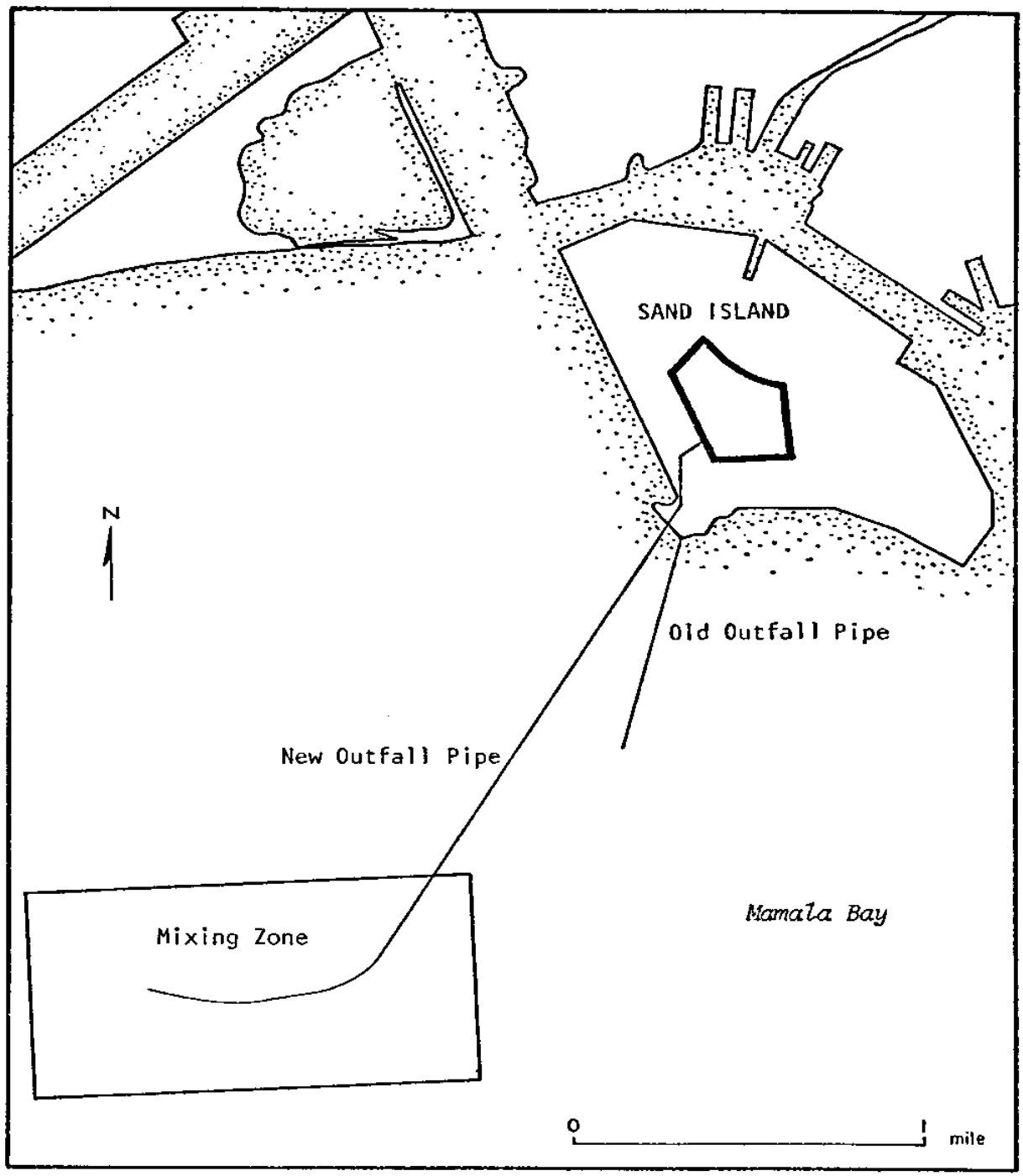


FIGURE 6. LOCATION OF NEW SEWAGE OUTFALL PIPE IN RELATION TO OLD OUTFALL PIPE IN MAMALA BAY.

viruses was conducted at both the old and new sewage outfall areas beginning in February 1977. Sampling conditions which regularly isolated virus in the old sewage outfall area were employed (volumes of 50 to 75 gallons) in both areas, but no virus was detected in either the old or new outfall areas. In regard to the new outfall, no virus was detected even in the area called the zone of mixing (1,500 feet on either side of the diffuser and 6,750 feet long), an area designated by the State Department of Health. However, when sampling volumes were doubled (100 to 200 gallons), virus could then be isolated only in the zone of mixing, but not outside the area. Thus, the new sewage outfall has not only markedly reduced the concentration of human enteropathogenic viruses present in the discharge area (by at least two to tenfold), but it has also confined the virus to the zone of mixing.

The above study, in conjunction with other related projects, has provided the necessary baseline data to the Department of Public Works of the City and County of Honolulu to apply to EPA for a waiver of secondary and tertiary treatments of the municipal wastewater for ocean disposal in Oahu. Virus isolation studies employing essentially the preceding methods have been successfully used in the following locations on Oahu, the old Kaneohe Bay outfall, the new Mokapu outfall, Pearl Harbor, boat marinas (Ala Wai, Keehi), etc.

Some additional remarks of caution should be made with regard to the concentration methods described here. It should be remembered that membrane reconcentration methods, although simple, can be hindered by the presence of humic acid, or by the presence of organic substances (MCC) that prevent virus adsorption and cause a loss of viruses into the filtrate. In addition, if the initial elution is accomplished with either beef extract or serum, both of which are MCC, then membrane reconcentration cannot be used. Under such circumstances, viruses may be reconcentrated by either the aluminum hydroxide floc method or the beef extract

protein precipitation method. Floc reconcentration at near-neutral pH's will not cause the humic acid to reprecipitate. It should also be remembered that some viruses do not coprecipitate with the floc.

In regard to two-phase separation, the method is slow, requires large refrigerated facilities and is not suitable for all viruses. It relies on a preferred partitioning of viruses between two aqueous phases. Not only do certain viruses not partition in the desired way, the reactants used (sodium dextran sulfate and polyethylene glycol) have been found to inactivate certain viruses.

In conclusion, it can be said that there are a number of methods available to monitor for viruses in a variety of waters. Since each water system presents different problems in the monitoring for viruses, no single standard method has been found yet that is applicable in all cases. The adsorption procedures have been generally acceptable for the isolation of enteric viruses in potable as well as a variety of surface waters.

The state of the art of virus detection is progressing to a point that investigators are now able to detect extremely small numbers of viruses in large volumes of water under, of course, ideal conditions (5 infectious units per 500 gallon). However, it should be emphasized that the recovery efficiencies of viruses from natural waters under field conditions is far different from that obtained under carefully controlled laboratory conditions. Under field conditions, a major common deficiency among several of these methods is the turbidity or "dirtiness" of the water, a common feature of many surface waters. The need to clarify turbid waters places an ill-defined limitation on a particular method since clarification may result in significant losses of virus in the water sample. If low concentrations of virus are present in the water sample, then their presence may go undetected. This problem could be overcome by use of the aqueous polymer two-phase separation technique which is best suited for quantitatively

detecting viruses from moderately turbid waters. The major disadvantage of this method is related to the limited volume of water that can be processed at a given time (2-4 liters). With clean water and the problem of pre-filtration or clarification would, of course, be nil.

While the use of "marker" viruses in so-called seeding experiments has presented many advantages in the comparison of various procedures and apparatuses, not too much emphasis should be placed on the results obtained. They should serve as a guide, and the results should not be accepted blindly. Among several limiting factors that can render seeding experiments suspect is the obvious fact that seeded viruses, free or adsorbed to the surface of particles will not behave in exactly the same way as naturally occurring viruses. Furthermore, laboratory strains of viruses frequently behave differently from natural viruses. Lastly, the use of one strain of one type of virus to demonstrate the behaviour, hopefully, of all enteroviruses presents a potential pitfall.

A good criterion to show that virus-in-water studies are being conducted successfully is the recovery of more than one kind of viruses from a variety of waters. Recoveries approximately in the proportions expected, based on the quality of the waters examined, provide additional assurance. With these facts available, one should feel confident in the field procedures employed.

It should be emphasized that the failure to recover natural viruses is not necessarily an indication that a method is ineffective. Many waters including some wastewaters have been found to yield no natural virus isolates.

Lastly, a few comments regarding the use of a marine animal, a macromollusk, for the detection of human enteric viruses in Hawaiian ocean waters. This study is presently being conducted in collaboration with Dr. Alison Kay. Experimental studies from our laboratory and others have indicated that shellfishes, such as mussels, clams and oysters, because of their unique filter-feeding system are

able to concentrate human viruses in their digestive system from which the viruses can then be recovered. In collaboration with Dr. Kay, we are currently evaluating the use of the bivalve filter feeder Pinna atrina as a biological monitoring system for human viruses in the Hawaiian ocean environment. Pinna, unlike the Eastern oysters (Crassostrea virginica and gigas) which are found in the shallow waters in Pearl Harbor, are indigenous to Hawaiian waters and are commonly found off the coast of Oahu. Although naturally found at relatively greater depths (30 m or 100 ft deep) than the Eastern oysters, this macromollusk should be useful in the detection of virus contamination in deep water areas, such as the new Sand Island outfall (>240 ft). Initial virus marker and field studies have determined that the Pinna is able to take up and concentrate human enteric viruses. Furthermore, the mollusk survives well in shallow waters and in laboratory tanks. Plans are underway to set these Hawaiian macromollusks in sewage-contaminated seawaters and to retrieve them periodically for examination of human viruses and also bacteria in their digestive system. Use of a natural biological system, such as the Pinna for the monitoring of human viruses in the ocean environment would not only facilitate surveillance but would also be highly efficient and economical.

Acknowledgments

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SAMPLING AND TRANSECTING TECHNIQUES ON TROPICAL REEF SUBSTRATES

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ABSTRACT

Details for constructing transecting equipment are described, including underwater writing slates, quadrats, transect lines. Qualitative, semiquantitative and quantitative survey techniques are compared. Methods for quantitative quadrat data collection are described: quadrate, quadrat-intersect, quadrat-weight methods; photographic transect method; line transect method. Techniques for collecting and counting benthic organisms other than substrate are described. Fish survey techniques are included.

SAMPLING AND TRANSECTING TECHNIQUES ON TROPICAL REEF SUBSTRATES

S. Arthur Reed[1]

Data on abundance and distribution of reef substrate and organisms can be gathered at three levels: (1) qualitative information, what kinds of organisms are present; (2) quantitative information, abundance of each species, what species are most common; (3) distribution of organisms, where the organisms are living, zonation of organisms. Various sampling and transecting techniques can be used to collect these types of data.

TRANSECTING EQUIPMENT

Underwater Writing Slate

Thin sheets of white plastic, about the thickness and pliability of thin cardboard with a matte surface on both sides, can be purchased at a local plastic supply company, and cut into smaller sheets of 8 1/2 x 11 in. The plastic can be written on with pencil and pencil eraser used to erase small errors even when completely submerged. The entire sheet is easily cleaned by scrubbing with a powdered cleanser and a wet sponge. The plastic sheets can be ruled off in chart form and check lists of organisms to be seen in a survey can be prepared in advance.

A plastic impregnated paper, trademark name polypaper, has recently become available. It is completely waterproof and resists ripping, stretching or shrinking. It can be written on and erased under water. Chart outlines can be printed on the paper by offset, mimeo, and spirit duplicator machines. The paper is available from Nalgene Labware Division, Rochester, N.Y.

The plastic sheets can be held on a clipboard with a good quality hard-

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board base. A rubber band around the bottom of the clipboard can be used to prevent the plastic from flopping (Fig. 1). The clipboard should be able to withstand many seawater submersions and as with all equipment, should be thoroughly rinsed with fresh water after use to prevent corrosion and rusting.

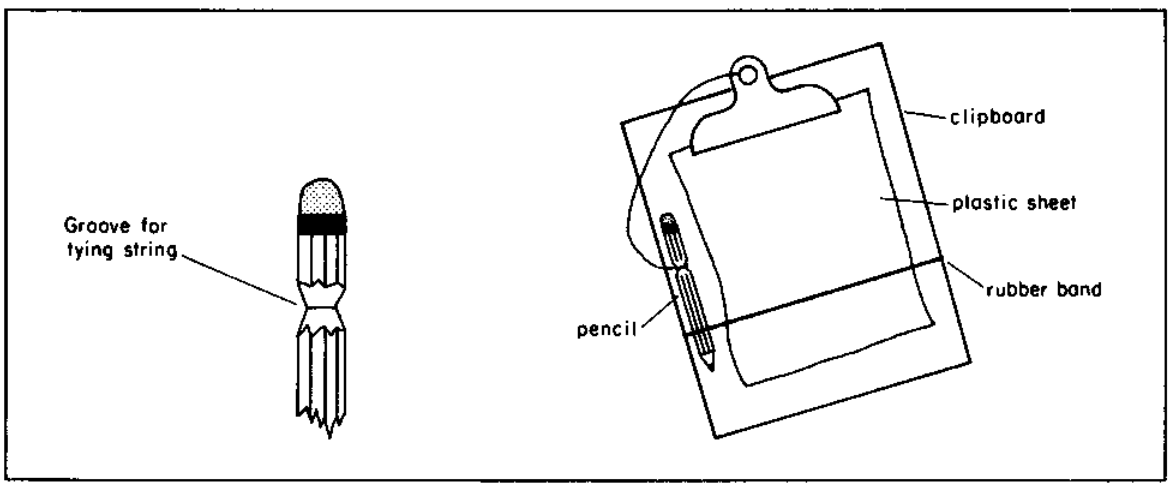

FIGURE 1. PLASTIC AND CLIPBOARD DATA RECORDER, WATERPROOF

A small groove should be cut around a wooden pencil just below the eraser and tied with a string with a square knot. The other end of the string is then attached to the clipboard. It can be held under the rubber band when not in use.

Quadrats

A quadrat is used to delimit or confine a known area of the bottom so that organisms can be counted or collected within that area. Quadrats are usually square, although they may be rectangular or circular in shape. The area of the square or rectangle can be calculated by: length x width = area; and the circle, $A = \pi r^2$.

Inexpensive, rugged, durable quadrats can be made from 1/4" or 3/8" reinforcing bar (rebar) used in cement construction (Fig. 2). A bar 4 m long is

bent at a 90° angle at each meter to form a square. The two ends are welded together. If a welding unit is not available, the two ends can be cut slightly longer, overlapped and wired together. String or thin nylon line or bailing

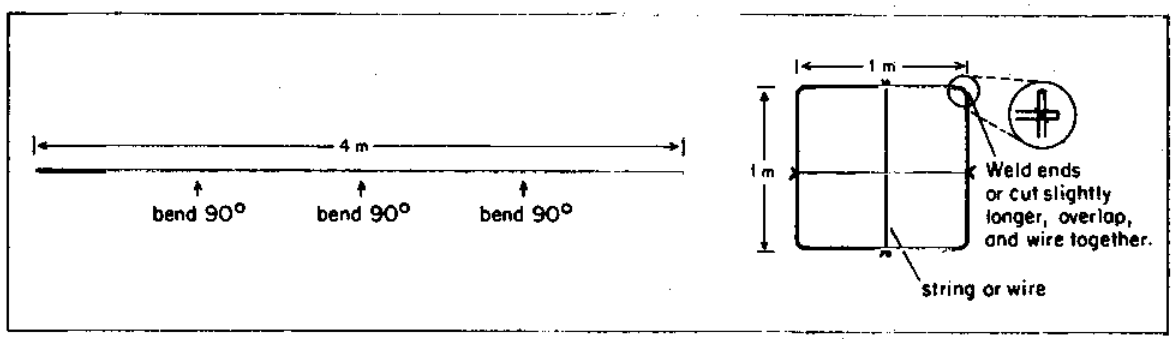

FIGURE 2. CONSTRUCTION OF A 1-m^2 QUADRAT

wire can be tied across the square to subdivide the 1-m^2 quadrat into any number of subunits. Any size smaller or larger quadrats can be constructed in a similar manner.

A light weight and durable quadrat can be constructed of 1/2" aluminum tubing fastened at the corners by copper elbow fittings. Quick setting epoxy glue is used to join the pipe and fittings. Holes should be drilled at the outer corner of each elbow to permit the pipe to fill with water when submerged.

A small 1/16-m^2 or 1/4 m on a side quadrat is very useful for measuring small, densely clustered organisms (Fig. 3).

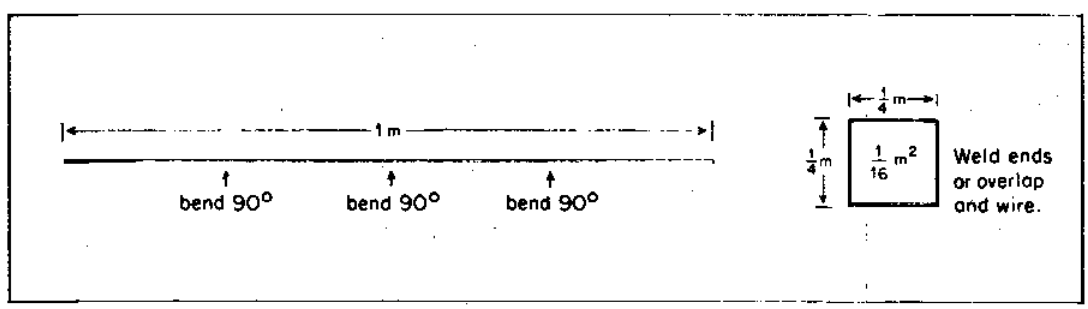

FIGURE 3. CONSTRUCTION OF A SMALL RECTANGULAR QUADRAT

A collapsible wooden quadrat can be made from 4-m sticks bolted together at the ends. Two strings the length of the hypotenuse of a triangle 1 m on a side (1.414 m) will hold the square rigid when in use (Fig. 4). This quadrat has the disadvantage of floating up off the bottom. It can be held down with lead weights or rocks. Alternatively, the sides could be made of flat metal bar. Cross wires can be used as a guide to mark off a 1/4-m^2 area.

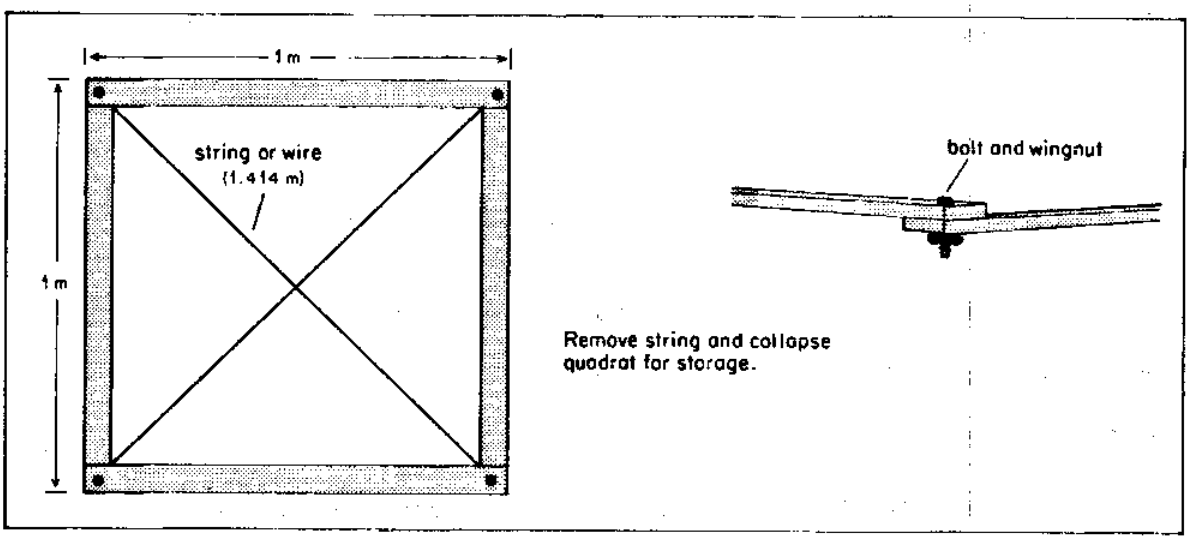

FIGURE 4. COLLAPSIBLE QUADRAT USING METER STICKS

Circular quadrats of various sizes can be made of hula-hoops, old basketball net rings, rim sealers or hoops on barrels. The area of each of these should be calculated.

A 1/2-sq dekameter (dk^2) quadrat for measuring larger areas can be constructed of nylon line (Fig. 5). Cut a 1/4-in. nylon line 34.14 m long and mark the line at 10 and 20 m. Tie the line at its ends to form a loop. At the 10- and 20-m mark and at the end of the loop, tie on small pieces of line or wire. When stretched out and tied down at the three corners the line will form a right triangle whose area will be 1/2 dk^2. This quadrat works well for counting organisms or small coral heads sparsely distributed over a large area. To

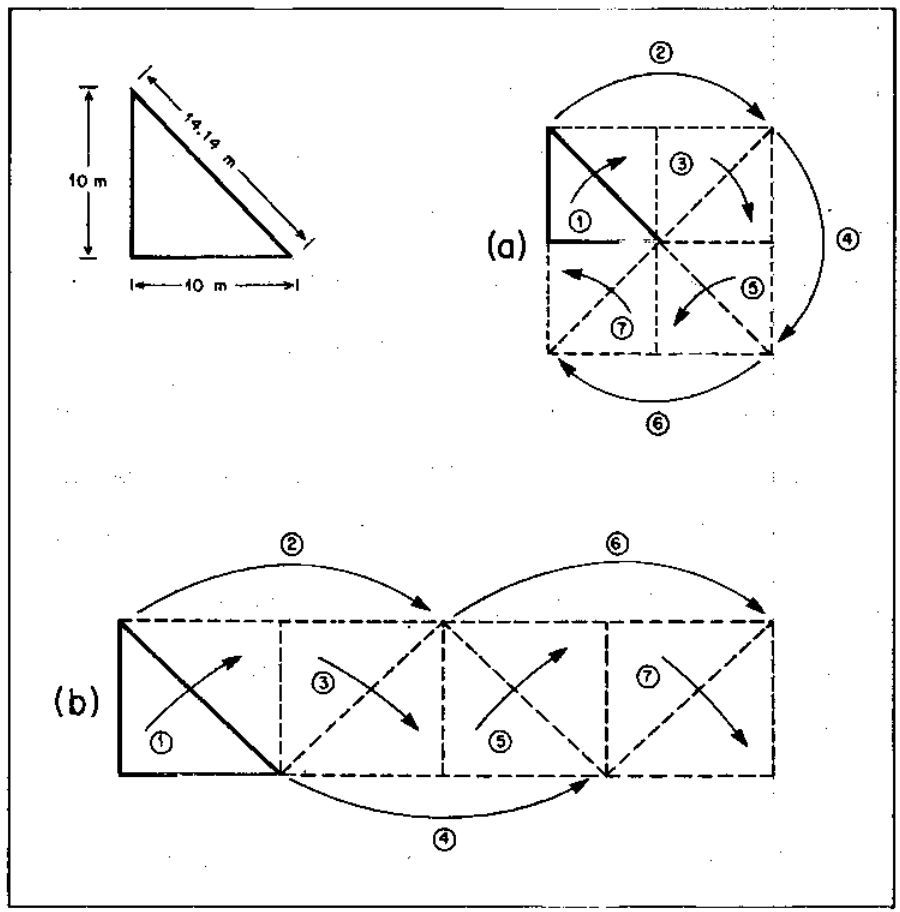

FIGURE 5. ONE-HALF SQUARE DEKAMETER QUADRAT

continue the survey, the triangle can be flip-flopped by releasing any one corner and pulling the line until all sides again become tight.

Transect Line

A transect line is used to identify a specific length along the reef. It permits the precise positioning of a survey site or a quadrat location. The exact location of the beginning and end of the transect line should be determined if that same region will be surveyed sometime in the future. The meter marks along the line are used as locations for placing a quadrat. Depending on the intensity of the survey every meter can be measured, or every other meter, or a random selection of meter points, or the first, middle and final meter. The surveyor must decide which interval is to be used.

Construction of a Transect Line

For durability, transect lines should be made of nylon. A polypropylene line is unsatisfactory because it floats. A line of 3/16 to 1/4 in. diameter is adequate. Lines can be of any length, but the most useful are 10 or 20 m long. Lay out the nylon line on the ground and tie one end to a post or tree. Pull the line hard several times to untwist the kinks and stretch it to its maximum working length. Using a meter stick, mark off the line every meter using an indelible felt marking pen. The ink mark should be about 2 to 3 cm wide and should completely encircle the line. Later, wrap a short length of black plastic electrician's tape tightly around the line at each mark. Some code to identify the 5, 10, 15 m marks can be used, e.g., a circle on either side of the 5-m mark, 2 circles on either side of the 10-m mark, and 3 circles at 15 m).

Allow about 1 m extra length at each end of the line to be used for tying the transect line to a coral head, small rock, concrete block or other weighted object to hold it in place on the bottom.

Fish Transect Line. A standard fish transect line used in many surveys in Hawaiian waters has been developed (Fig. 11). The line is 50 m long with 1-m lines tied on every 5 m. The short lines are attached to small floats so that they extend vertically up from the long transect line.

TYPES OF SURVEYS

Qualitative Survey

In a qualitative survey the surveyor merely records which organisms are present in the area being studied. This results in a list of organisms, commonly called a presence-absence list. If the surveyor has some idea of the kinds of organisms that might be seen, a checklist can be prepared on the underwater writing slate ahead of time. This reduces the amount of writing necessary

during the survey, which is sometimes difficult, especially if snorkeling in heavy surge conditions. Fish are recorded usually by random swims in the general area using snorkeling equipment. When a new species of fish is encountered, it is recorded. Algae and benthic invertebrates can be located either by wading with lookboxes or by snorkeling, even in water as shallow as 20 to 30 cm. If the surveyor's belly is not scraping the bottom, it is often easier and less fatiguing to snorkel rather than wade. Snorkelers, however, can become rapidly chilled, even in warm water as high as 27°C (80°F). A wet suit jacket or tight-fitting, long-sleeved sweat shirt should be worn to reduce heat loss. Many invertebrates are hidden and can only be found by carefully turning over rocks and rubble or digging through the sand. It is important that all rocks be replaced in their original position after searching, so that attached organisms are not killed. If a rock is turned upside down, all organisms on the (former) top and bottom will die because they are no longer in their suitable growing environment.

Qualitative surveys can be randomly done over a large expanse of a reef or within a more restricted area bounded by transect lines. If time is limited, a very narrow strip, say one meter on either side of a 20-m transect line might be intensively surveyed. If information is recorded on <u>where</u> along the transect line the organisms are found, a crude zonation study will result, i.e., some kinds of organisms might be found close to shore but not on the outer reef flat.

Substrate upon which organisms are living might also be recorded in a survey. General correlations of substrate preference for many species will emerge from these data, e.g., black brittle stars are always found under rocks in sandy depressions; rock-boring sea urchins are generally found in shallow waters in deep pits or pukas in the rock; large black sea cucumbers live on sand.

Semiquantitative Surveys

Relative abundance of organisms can be recorded without resorting to a complete count of every organism seen. General ranges of abundance can be estimated using terms such as abundant, common, uncommon, rare. Usually, some definition of these various terms should be agreed upon. For instance, abundant—over 50 organisms seen in an area, common—50 to 10 organisms, uncommon—10-5 organisms, rare—5 to 1 organisms. If a transect is used as a guide and a quadrat used to systematically study an area, these terms can be used to indicate what percent of the quadrats contained these organisms. In this case, abundant—100 to 50% of quadrats contained organisms; common—50 to 25%; uncommon—25 to 5%; rare—less than 5%. Of course, any word to indicate abundance can be used by the survey team. It is necessary, however, to specify the words and their quantitative definition. Once a set of terms is agreed upon or chosen, they should become standard terms and not altered, at least for the duration of that survey.

Semiquantitative Fish Survey

This method requires 5 to 10 trained surveyors who are able to easily identify a large number of fish species on sight. The surveyors simultaneously enter the water in the region to be surveyed. Each surveyor records the name of the fish species seen in the order of sighting—first, second, third. Once a species is recorded, it is thereafter ignored. Numbers of individuals are not recorded, only names. The survey is continued for a definite length of time (10-20 min.). On each list, the first fish seen is given a "score" or one, the second a two, and so on. The "scores" of all species are then summed. Thus, a single species may have the following scores on 5 different lists: 1, 2, 3, 1, 5 = 12 total score. The species with the lowest total score is the most common in that area. The others are ranked accordingly. Although the techniques does not measure actual abundance, it does give information on relative commoness of

the various species. Obviously, since many fish prefer a definite type of substrate, i.e., goatfish on sand, butterflys on live coral, all surveyors must remain in a region of common substrate type.

Quantitative Survey. Quantitative surveys are intended to gather as accurate information as possible on both the kinds (species) of organisms and their abundance within a precisely defined area. If the number of organisms and the total area are known, then the number per unit area, i.e., sea urchins m^2, can be calculated. Species can then be ranked according to their commoness in that area. The quantitative data can be used to compare different sites or compared with information gathered in the same area in future field trip visits to study any changes that might occur during different seasons or that may be associated with environmental changes, such as greater freshwater runoff, increased siltation, and increase in water temperature.

The first set of survey data collected in a field trip is the basis for which all future surveys are compared. This initial survey therefore is often referred to as the baseline survey. The more accurate and complete it is, the better the comparison in all future studies. It is very important therefore to decide in advance exactly why the survey is to be done; the various kinds of measurements, both physical and biological, that are to be made; the degree of accuracy and completeness that will be required; and the techniques and equipment that will be needed to collect the data. Several practice sessions are usually necessary before surveyors attain the ability to carry out the surveys in an efficient and controlled manner to insure good results for quantitative data collection. Even in surveys with experienced and trained technicians, the first survey results using a new or untried technique are often worthless and the effort is usually chalked up as a learning experience. Conditions on the reef are difficult to cope with and may include such frustrations as heavy

surge or water current movement, limited visibility due to water turbidity, movement of the transect line or quadrat during the counting session, inability to determine species of organisms because of unfamiliarity, loss of some specimens during counting or collecting, loss of writing slates, breakage of pencil points, flooding of face mask, and injury of worker. All of these problems have occurred in past surveys and should be expected. They can be very discouraging for an untrained survey team.

There are a number of different quantitative survey techniques that are available. Each one has advantages for measuring abundance of certain kinds of organisms. In some cases, you may find that none of these are adequate for your needs and you may want to invent a new piece of equipment or technique.

SUBSTRATE SURVEY TECHNIQUES

The substrate which is the bottom of the ocean can be made up of a number of materials, e.g., sand, silt, mud, rock, rubble, live coral, and algae. The surveyor is interested in knowing the amount of the bottom covered by each type of material.

Quadrat Method. A quadrat of convenient size (usually 1 m^2) is laid on the bottom (Fig. 6). The square should be subdivided into many smaller squares using string or wire. The amount of coverage of all substrate types in each subsquare is estimated. The data are then summed to determine total cover for each substrate type.

Quadrat Intersect Method

The quadrat is divided by string or wire into subunits (Fig. 6). At each intersect of the dividing lines, the underlying type of substrate is recorded. The most common organisms appear at a larger number of intersects with less common ones recorded at fewer intersects. This technique has an advantage of rapid recording data. Its disadvantages are that only a percent abundance

value is obtained, not an absolute value. Also, very rare substrate types may be unrecorded if they are not located under an intersect.

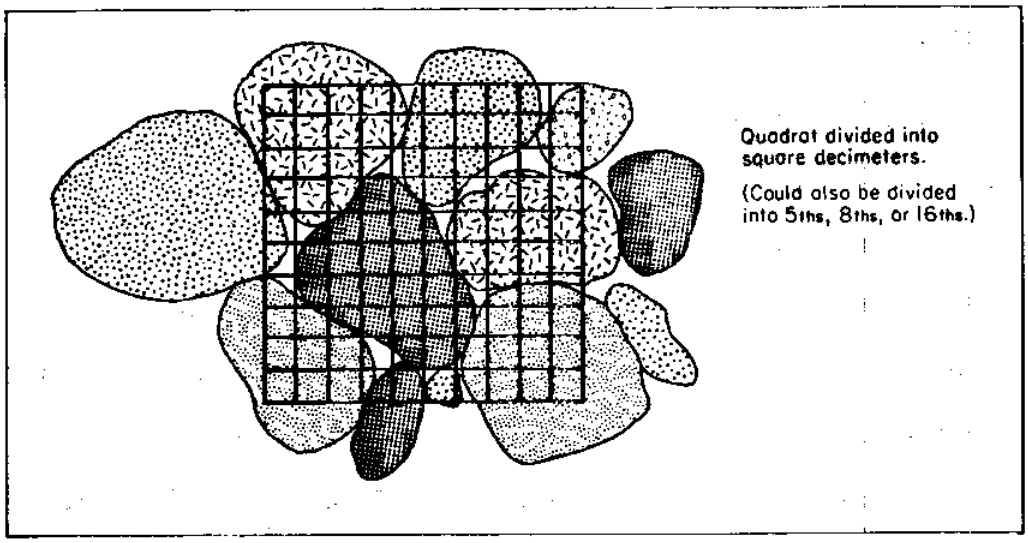

FIGURE 6. A QUADRAT OVERLYING THE BOTTOM MADE UP OF A VARIETY OF SUBSTRATES

Quadrat-Weight Method

It is often difficult to determine substrate coverage of algae because many species grow in long fronds which move back and forth in the water with wave action. Estimates of coverage may vary depending on the position of the algae fronds at any given instant. An alternative method is to gather all the algae within a quadrat (1/16 or 1 m^2 depending on abundance and uniformity of distribution of algae). The sample is placed in a plastic bag as it is collected and brought to shore. Species of algae are separated into piles and placed into smaller plastic bags to prevent drying and blowing away. Each species is then weighed on a balance. To minimize corrosion and rusting, great care should be taken to keep the balance free of saltwater droplets. The weight of the plastic bag should be subtracted from the total weight of each species.

Some algae are thick and robust or even contain a calcium skeleton, i.e. *Padina* sp. and *Halimeda* sp., while other species are thin and delicate. The weighing method tends to overemphasize the abundance of some algae; therefore, dry weight rather than wet might be a more useful technique.

The weighing technique also does not usually include measurements of coralline algae. These species encrust the surface of rocks and dead coral and are not collected.

Photographic Transect Method

A Nikonos II (or similar) camera is mounted on a support frame 1.25 m above a 1 x 0.66-m quadrat (Fig. 7). The support frame and quadrat are constructed of 1/2-in. aluminum tubing and copper pipe fittings. A plastic sheet supports the camera. Details of construction of this support frame may be obtained from the author. The frame precisely positions the camera over the rectangular quadrat at the correct distance to include the aluminum tubing just inside the photograph. An electronic flash unit may also be mounted on the frame next to the camera to provide even illumination at all depths. To record the substrate, the camera and frame are placed on the bottom and a color photograph (using slide film) taken of the quadrate. The frame is then moved to the next adjacent space where a second photo is taken. This procedure is repeated along a parallel line until all 36 photos are taken. Depending on which direction the rectangle is moved either an area 1 m x 24 m or 0.66 m x 36 m can be photographed. Transect locations and other pertinent information can be written in large letters on an underwater slate and photographed in the first frame. Because small patches of substrate, especially corals, may not show up clearly on the photo, a diver with a species check list can accompany the transect photographer

FIGURE 7. PHOTOGRAPHIC SUPPORT FRAME AND CAMERA IN USE

to record the presence of all coral species in each frame.

The developed photo slides are then projected onto a grided screen of white cardboard the same size as the original quadrat. Abundance of substrate types can then be calculated. This method is more efficient with respect to time spent underwater and area surveyed. A permanent photographic record of the substrate is also obtained for future reference and comparison.

Line Transect Method

This method is a rapid technique for estimating substrate cover. A transect line is laid on the bottom and pulled taut so that it does not move. The length of line overlying the various kinds of substrates is then measured to the nearest centimeter (Fig. 8).

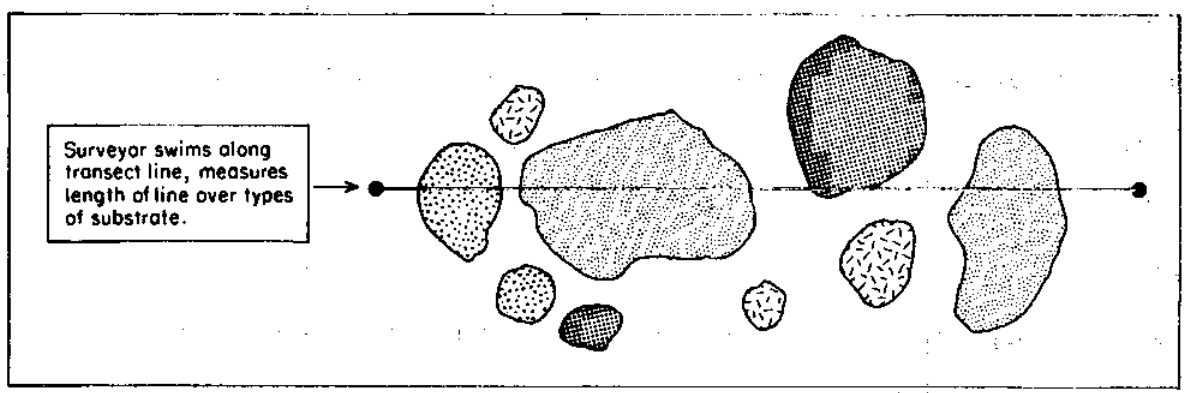

FIGURE 8. LINE TRANSECT METHOD FOR ESTIMATING SUBSTRATE

A disadvantage of this procedure is that small and uncommon patches tend to be completely excluded from the data. A second diver can accompany the surveyor to record all corals and other substrates in the vicinity not recorded in the transect.

BENTHIC ORGANISMS

These are organisms, usually invertebrate, that live on or within the sub-

strate. The surveyor is interested in numbers of organisms per unit area. Since the size is constant within a species and varies considerably between species, the bottom coverage of these organisms is unimportant and not considered. An exception to this rule is the occurrence of certain colonies that are tightly packed over a large area such as certain tube worms, bryozoa, or zoanthid sea anemones. In these cases, both area of coverage and numbers may be important.

Quadrat Method

A quadrat of appropriate size is placed on the bottom and all organisms of interest are counted and recorded. The most common size is 1 m^2. Some organisms, however, are sparsely spread over a large area and require quadrats of larger size such as the 1/2-dk^2 quadrat described earlier.

Many invertebrates are cryptic, that is they are hidden from view, either in holes, under rocks or buried in the sand. For a complete survey, these special hiding places should be thoroughly searched.

Many sand-dwelling organisms produce burrows or holes that are characteristic of that species. Once these are identified it is sufficient to count holes, assuming that one knows the number and kind of organisms in each hole type. However, in some cases two or three species live symbiotically within the same burrow, e.g., a gobie and a snapping shrimp in sand holes on shallow sandy reef flats. Careful examination of each type of hole is therefore necessary prior to the general survey.

Many worms live at various depths in the sand on many reef flats with no evidence or indication of their presence. To sample these areas, a tin can with both ends removed can be sunk into the sand to a given depth. The sand within the can is then scopped out into a mesh sieve. Water flushed through the sieve will separate organisms from sand (Fig. 9). Proper mesh size to

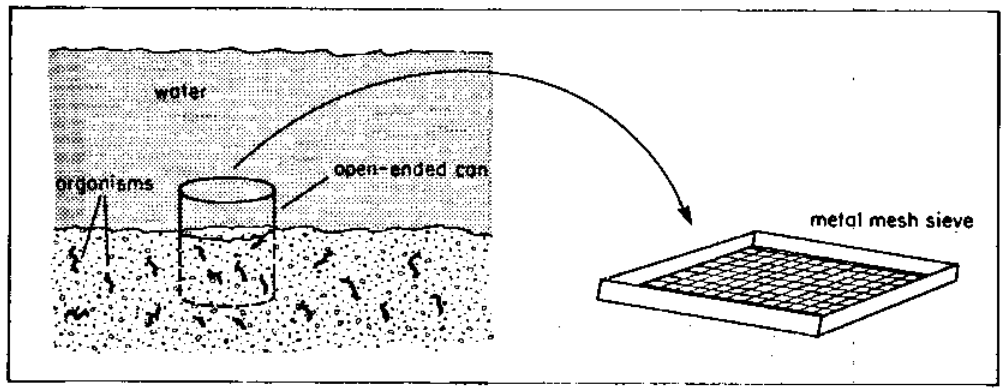

FIGURE 9. METHOD OF SAMPLING SAND-DWELLING ORGANISMS

retain various sized animals will need to be determined.

Nearest Neighbor Method

When organisms are sparsely spread over a large area, e.g., some species of sea urchins on a reef flat, the nearest neighbor technique (Batchelor 1971) can be effectively used. Lay out a transect line somewhere in the area to be surveyed. The precise location is not important. Starting at one end of the line, count off a random number of meter marks. A sequence of random numbers can be obtained from a table of random numbers. From this meter mark, measure the distance, in centimeters, to the nearest organism. This distance represents the radius of a circle, the area of which contains only a single specimen (Fig. 10). Next, measure the distance from the organism to its nearest neighbor. Relocate the transect line within the general area as many times as necessary and continue to take measurements. The second transect location may criss-cross over the first location. Continue until a minimum of 25 pairs of measurements are taken (nearest organism from line and nearest neighbor constitute a pair of measurements). Calculate the sum of all organisms and the sum of all areas. Divide total area into number of organisms to determine number of organisms per unit area, i.e., number of organisms per m^2.

Note that in the quadrat method the area is held constant and number of

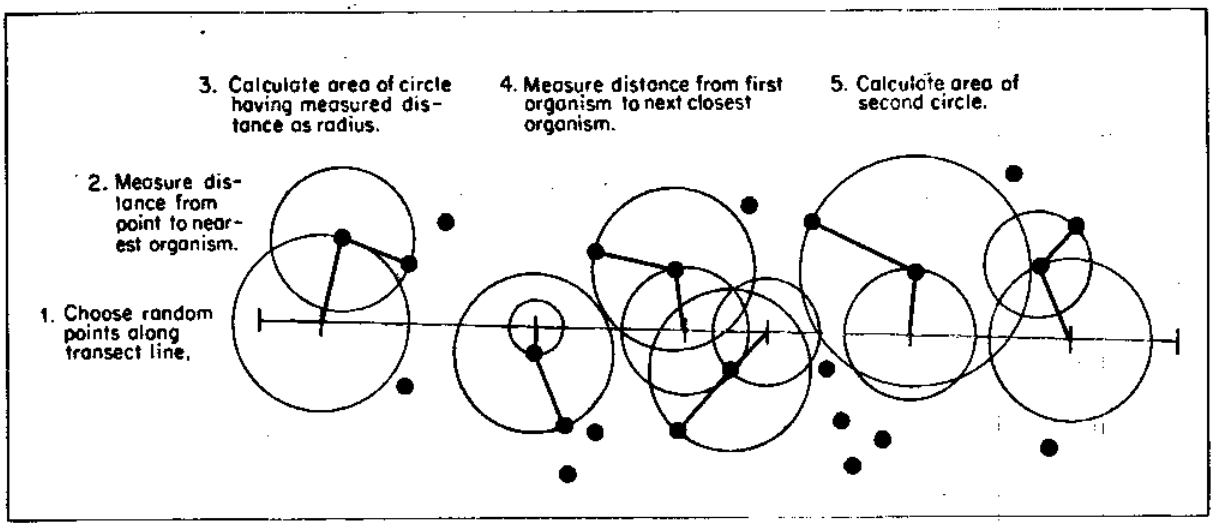

FIGURE 10. NEAREST NEIGHBOR METHOD

organisms is determined by counting. In the nearest neighbor method, the number of organisms is held constant and total area is determined. In either method, the final result is expressed as number of organisms/unit area.

Further calculations using the collected data can be made to determine whether the aggregation of organisms is clumped, evenly or randomly distributed.

Fish Survey

In recent years, a number of refinements have been made on techniques used to survey fish on the deeper coral reefs of Hawaii. One technique is to place a fish transect line (Fig. 11) on the bottom. Wait an appropriate length of time to allow frightened fish to resume their normal positions and activity (about 1/2 hr). The surveyor is stationed at the first buoyed marker along the line. While remaining still the surveyor records and counts the number of all species of fish seen in the water column 5 m along the line and 5 m to the

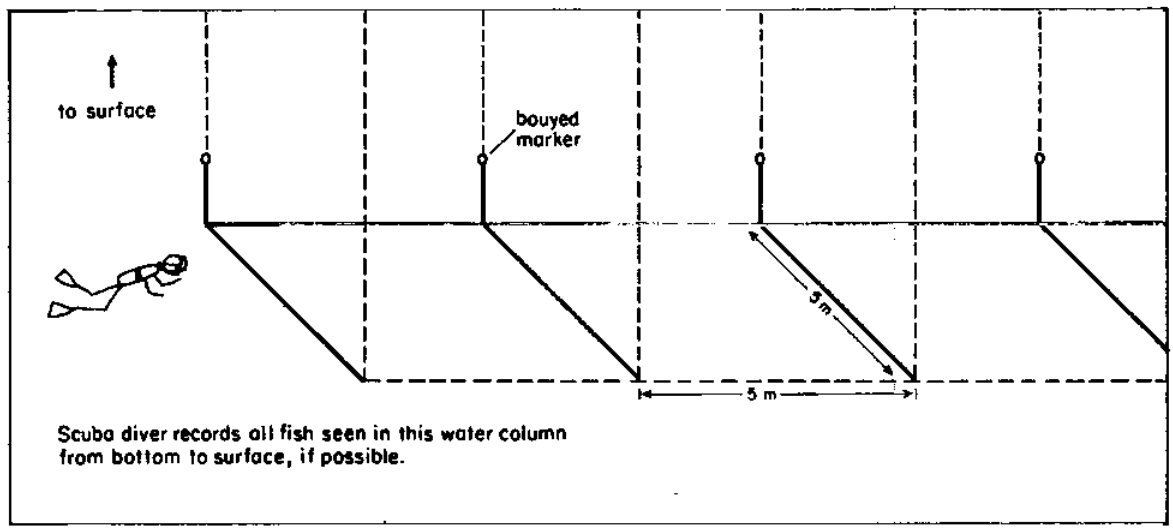

FIGURE 11. FISH SURVEY

right of the line. The surveyor then proceeds to the second 5-m marker and records another count. The count is repeated to the end of the transect line. For comparison, the surveyor turns around and repeats the survey on the other side of the transect line. With well-trained divers this method will record up to 80% of the fish in the transect area.

In all of these survey methods for measuring invertebrates and fish (those organisms not attached to the bottom and therefore able to move about) there will be some variability in results depending on such environmental factors as time of day (or night), tidal level, wave action, and turbidity. No two surveys will therefore include exactly the same number and kinds of organisms.

REFERENCE

Batchelor, C.L. 1971. Estimation of density from a sample of joint point and nearest-neighbor distances. *Ecology* 52:703-9.