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Indicator organisms for estuarine and marine waters

(Indicator organisms; pathogenic microorganisms in the aquatic environment; public health significance of estuarine indicator organisms)

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1. SUMMARY

The use of indicator organisms for estuarine and coastal waters has been reviewed. The natural flora of the environment must be considered in selecting an indicator organism, but, more importantly, recent work which shows a viable but non-recoverable stage of pathogens entering the marine environment demonstrates that the conventional detection of indicator microorganisms is misleading, if not inaccurate. Results suggest that the newly developed epifluorescent/immunofluorescent direct detection of pathogens in the environment may be the most reliable method for determining public health hazards in marine and estuarine waters.

2. INTRODUCTION

The microbiology of the world oceans and coastal waters has been under study since the late nineteenth century. Among the earliest investigations concerning the number of bacteria and their

distribution in seawater was that of Russell [1–3] who conducted a survey of the distribution of bacteria in the Gulf of Naples. The early workers established general distributional patterns for bacteria in the sea, using agar plate and dilution methods for the enumeration of bacteria. Bacteria were found in small numbers, uniformly distributed in water several miles from shore and at all depths. Early workers also found that the water of harbors and bays may contain large numbers of bacteria, but that the number of bacteria becomes very small at comparatively short distances from shore [4,5]. The work of Fischer is, of course, classic, since he investigated sea water over a wide area of the Atlantic Ocean and found bacteria throughout, the largest numbers being found near land or in the presence of floating seaweed [6,7].

Seasonal studies were done by Lloyd [8] in the Clyde Sea, where the waters are relatively shallow. The surface waters were richest in bacteria, the number decreasing with depth but usually increasing slightly at the bottom. A variety of studies was done in the early years of marine microbiology, summarized by Benecke [9] and ZoBell [10]. The observation that direct microscopic examination of seawater showed bacteria to be much more abundant in seawater than cultural methods would

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suggest, was made as early as 1929 by Cholodney [11].

Marine bottom deposits are known to contain much larger numbers of bacteria than the water column. Russell [1] showed that bacteria were distributed in sediments of the Bay of Naples at depths ranging from 50–1000 m below the surface of the water. ZoBell and Morita [12], in a study carried out about 25 years ago, showed that bacteria are indeed present in sediment cores taken in the Phillipines and Marianas trenches. Some 25 years earlier, Reuszer [13] had demonstrated that bacteria are much more numerous in bottom deposits than in overlying water, and that the number of bacteria decreases with depth into the sediment. Dale [14] showed a strong relationship between bacterial numbers and sediment properties, i.e., the number of bacteria is highly correlated with grain size and other granulometric properties.

Sampling methods for microbiological analyses of water include the J-Z sampler and the Niskin sampler [15]. Deep ocean environmental samplers have also been developed in recent years [16,17]. Sediment samples are unfortunately still obtained by core or grab [15]. Animals are collected by means of traps and trawls.

The methods for the enumeration of microorganisms, estimating their biomass, and measuring their activities in the ocean, were the basis of a recent symposium [18], and include the classic methods of plate count and filtration. More recently developed methods include ATP measurement, a biochemical technique for estimating total microbial biomass as a function of the luciferin-luciferase reaction, which is quantitatively dependent on the amount of ATP in the sample [19], and bacterial cell wall component analyses [20]. Several different approaches to microscopic bacterial counts are now available, all employing fluorochromes and epifluorescent microscopy. Some of these methods enumerate total (i.e., living and non-living) bacteria [21–23], whereas others purport to count only living bacteria [24–26]. Recently, Tabor and Neihof [27] compared 2 of the direct viable count methods with amino acid uptake, and found that amino acid uptake consistently measured a larger number of cells. The

epifluorescent-immunofluorescent method offers exciting new opportunities for determining directly the presence of pathogens in water, even when these organisms are viable but non-recoverable [28–29].

2.1. Generic distribution

The results of the work of pioneers in marine microbiology at the turn of the century have been firmly established. More recently, Sieburth [30] surveyed seawater from the Pacific, Caribbean, and Atlantic Oceans, culturing bacteria in agar roll tubes indicated within 4.5°C of the temperature of the water from which they were obtained. The isolates were keyed to genus, and a characteristic bacterial flora was reported in the 150 µm-thick surface film overlying 'low-count' waters off the west coast of Central America in an area known for upwelling; in all the samples, pseudomonads were dominant. However, very recently, the same areas have been re-examined and the dominant organisms, especially at the ocean dumping sites, have been identified as *Vibrio* spp. [31]. Murchelano and Brown [32] found that heterotrophic bacteria in the Long Island Sound water column showed bacterial concentrations ranging from 10^3 – 10^4 per ml, with variations in seasonal concentration mirroring that of the phytoplankton. Gram-negative rods constituted 99% of the isolates. 8 Genera were identified, but 92.3% were *Achromobacter*, *Flavobacterium*, and *Pseudomonas*, with *Pseudomonas* dominant in the summer and *Flavobacterium* in the spring. Again, more recent work suggests a far more prominent role for *Vibrio* [31].

There is a wide oceanic distribution of bacterial genera associated with geomicrobial cycling in nature. For example, nitrogen-fixing, nitrite- and nitrate-forming, nitrate-reducing, cellulose-decomposing, agar-decomposing, and chitin-decomposing bacteria have been isolated from seawater and sediment [33]. Denitrifying bacteria in shallow-water marine sediment were documented by Patriquin and Knowles [34]. Nitrogen-fixers have been isolated, including nitrogen-fixing vibrios ([35]; M.L. Guerinot and R.R. Colwell, in press). *Thiobacillus*-like bacteria, i.e., reduced sulfur-oxidizing bacteria (thiosulfate or elemental sulfur),

have been isolated from seawater and sediment in the Atlantic Ocean, Black Sea, and other areas [36–38].

Yeasts are readily isolated from coastal waters [39], especially in association with seaweeds [40]. Actinomycetes, mycelial bacteria, can also be isolated from bays, coastal and offshore waters [41]. However, more recent work [42] suggests that the actinomycetes are perhaps more commonly of terrestrial origin, especially at ocean dumping sites.

A very interesting group of bacteria is the 'filterable form' or 'mini-bacteria'. The occurrence of filterable bacteria in sea water was reported by Oppenheimer [43], who found up to 12 viable cells per ml of sea water filtrate after passage through a membrane with a pore diameter of approx. 0.4 μm . Brisou [44], using filtration procedures, showed that L-forms of bacteria can be isolated from marine molluscs. Anderson and Hefferman [45] used a process of double filtration of sea water, filtering first through a 0.45 μm membrane and then through a 0.22 μm pore diameter filter. A group of microorganisms, not usually encountered by the conventional techniques of pour-plates or one-stage filtration, was obtained. Many could not be identified, but the largest single group belonged to the genus *Spirillum*. Other isolates were placed in the genera *Leucothrix*, *Flavobacterium*, *Cytophaga*, and *Vibrio*.

A group of microorganisms also often overlooked is the oligotrophic bacteria. These include organisms which require prolonged incubation periods or low nutrient conditions. Such organisms have been studied in Chesapeake Bay and were found to include *Hyphomicrobium*, *Nocardia*, and sheathed bacteria [46]. Recent work of Martin and MacLeod [47] suggests that the oligotrophic bacteria might better be defined as organisms with narrowly defined patterns of nutrient utilization.

3. INDICATOR ORGANISMS

For centuries man has used the sea as both a dumping ground, with wastes dumped into rivers and streams or directly into the ocean, and as a source of fish for food. When populations in the settlements along coastal regions of the world were

small and sparse, and fishermen harvested offshore, the risk of contamination of fish by domestic and/or industrial wastes was low. Nevertheless, even in the earliest recorded times, disease was widespread and life expectancy short. With the world population increase, and concomitantly more densely inhabited cities, domestic and industrial waste problems have magnified. The spread of disease via fecally contaminated water and foods in many countries is guarded against by hygienic treatment of sewage wastes. Despite such efforts, outbreaks of disease still occur. As a result, standards have been proposed and employed to maintain water quality for specific use, i.e., drinking, agriculture, mariculture and recreation.

Standards are developed to ensure that water supplies are either free of disease-causing organisms, including pathogenic bacteria, viruses, and other microorganisms, or carry concentrations of pathogens at a level that does not pose an unacceptable risk to health. The list of potentially pathogenic organisms found in water is long, and even now, remains incomplete, since new pathogens are constantly being described, as for example, the *Legionella* spp. The task of assaying fresh and salt waters for all such organisms on a regular basis would be monumental. For this reason, those concerned with water potability, as well as food safety, including microbiologists, sanitary engineers, and medical personnel, have devised schemes using indicator organisms as a warning of the unsafe condition of waters and foods, the protocol specifying the use to which the water is to be put. Most indicator organisms were selected for fresh water systems, since river, lake, and ground waters are the most common sources of community water supplies. However, sewage wastes are dumped all too frequently into estuaries and oceans, as well as into rivers.

Although the oceans cover three-quarters of the earth's surface, the dilution of contaminated influent waters is rarely sufficient or rapid enough to rid estuarine, coastal and deep marine waters of microbial health hazards and the effect of pollutants carried in wastes. Thus, indicator organisms appropriate for measuring the quality of estuarine and marine waters are needed. Questions to be answered, with regard to estuarine and marine

water standards include: (i) for which activities are water quality standards needed; (ii) are corresponding fresh water indicators adequate and appropriate indicators of health hazards or increased risk of disease arising from the presence of pathogenic organisms of fecal origin; and (iii) are such indicators appropriate and satisfactory, even for fresh water? Of greatest importance is defining what the indicators will be used to indicate, i.e., fecal pollution or risk of disease. In addition to public health problems associated with infectious microorganisms transmitted by water, it has become evident that there are also risks of disease caused by organisms which occur naturally in the aquatic environment. Gastroenteritis or wound infections are caused by *Vibrio parahaemolyticus* and related *Vibrio* spp., as well as by *Pseudomonas* and *Aeromonas* spp., none of which are necessarily associated with fecal pollution. It is increasingly evident that indicators of sanitary quality are not appropriate markers for the presence of pathogens that are autochthonous to the aquatic or marine environment. Therefore, yet another indicator or set of indicators is required.

Historically, an appreciation of the spread of water-borne pathogens, e.g., *Salmonella* spp. and *Vibrio cholerae*, was developed in the late 1800s. A dramatic example is the 1854 cholera epidemic in London. In the late 1880s, *Klebsiella pneumoniae* and *Klebsiella rhinoscleromatis* were isolated from the feces of patients by Von Fritsch. Escherich [48] isolated *Bacillus coli*, now classified as *Escherichia coli*, and *Aerobacter aerogenes* (*Enterobacter aerogenes*) from feces. These bacteria and other coliforms were used in the past as indicators of water-borne pathogens, that is, the presence of fecal contamination being correlated with the occurrence of pathogens, for which direct detection methods were not available. Thus, 'indicator organisms' are markers of health hazards and/or water (or food) quality. Mossel [49], when discussing markers in foods, differentiates *indicators* from *index* organisms, the latter suggesting the risk of occurrence of pathogens, indicators being more general and suggesting inadequate bacteriological quality. Cabelli [50,51] also differentiates *fecal indicators* from *water quality indicators*. Fecal indicators would be inadequate as markers of pollution

by organisms from fecal wastes if these organisms could multiply in the environment. A water quality indicator would be necessary to indicate these organisms, in addition to those organisms which do not have a fecal source, but are health hazards and are present in the environment [50].

Indicators must fulfil basic requirements, including: (i) specificity to the source of pathogens or fecal contamination; (ii) sensitivity of detection; and (iii) resistance to disinfection and to the environment that either equals or slightly exceeds that of the pathogen [50,52–58]. Since an indicator of fecal pollution is usually considered to be an index of the risk of the presence of fecal pathogens, the indicator must be present when the pathogen is present, and its source should be feces. An indicator must be sufficiently abundant in feces that the probability of detection is high, and it should be present in higher concentration than the pathogens. Also, an indicator should be as resistant, or more resistant, than the most resistant fecal pathogens, and it should not multiply in the aquatic environment, whether freshwater or marine. In addition to these basic requirements, simple techniques must be available for detection and quantification of the indicator. The ideal indicator, therefore, is the pathogen itself [56]. In fact, *Salmonella* spp. has been suggested as an index of pollution in fresh water [59]. The major arguments against using pathogens directly as indicators have been that they are difficult to isolate and identify, which is in most cases no longer a problem, and that the absence of one pathogen does not rule out the presence of another, nor does it have predictive value for the concentration of the pathogen [58]. Indeed, testing for the presence of every possible pathogen would be very expensive in time, effort, and resources.

Indicators are needed for application in the marine environment to help protect the quality of shellfish beds and primary contact recreational waters, and to prevent the spread of enteric disease. Sewage treatment and subsequent dilution obtained upon entry into rivers or by direct disposal into coastal waters via marine outfalls and sludge dumping is monitored by bacteriological methods. Unfortunately, marine and estuarine waters present special problems, since the behavior

of pathogens and indicators differs from that in fresh water. Indicators used in freshwater environments are not always appropriate for salt water.

Estuaries are less saline than the ocean, and salinity, as one parameter, can range from 0‰ at the mouth of a river to 35‰ where the estuary meets the ocean. The waters may be stratified into a cold saline bottom layer and a less saline top layer in the typical salt-water wedge type of estuary [60]. Most ocean waters have a salinity of 32–35‰. The water temperature of estuaries is higher and more variable than that of the ocean. Tidal flushing is controlled by influent fresh water and ocean water, as well as by the physical shape of the estuary itself. Biologically, estuaries are more productive and densely populated than oceans, with the populations including microorganisms, plants, and animals. Freshwater organisms and other material entering estuaries and oceans are subject to competition with, and predation by, the autochthonous flora and fauna, in addition to effects of changes in the chemical constituents and physical structure of the surrounding waters. Clearly, any proposed indicator must be examined in the water in which it will be monitored and/or used as a regulatory aid.

Many groups of microorganisms have been proposed as indicators of water quality and fecal pollution. Indicators discussed separately, and in detail in the symposium proceedings edited by Hoadley and Dutka [61], include: coliforms, fecal coliforms, *Clostridium perfringens*, *Pseudomonas aeruginosa*, *Vibrio* spp., bifidobacteria, *Candida albicans*, *Salmonella* spp., fecal streptococci, and *Klebsiella* spp. Bacterial and biological indicators were also the subject of a symposium, the presentations of which were published by James and Evison in 1979 [62].

The coliform group, i.e., total coliforms, are aerobic and facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas formation within 48 h at 35°C [63]. Total coliforms are the most universally used indicator group, but include bacteria, in addition to *Escherichia coli*, that are not specifically associated with fecal pollution, i.e., *Klebsiella* spp., *Citrobacter* spp., and *Enterobacter* spp. [58]. Fecal coliforms are differentiated from other coliforms

by their ability to produce gas from lactose at 44.5°C within 24 h [63]. Several techniques are available for enumeration of total and fecal coliforms, including tube enrichment and membrane filter methods, which are continually being compared and modified.

Although a positive fecal coliform test has been equated with the presence of *E. coli* and the presence of fecal pollution, fecal coliform-positive *Klebsiella pneumoniae* [64–67] and *Enterobacter aerogenes* [68,69] have also been isolated from the environment. Hendricks [69] concluded that fecal coliform test-positive *E. aerogenes* were not indicative of fecal pollution, whereas fecal coliforms, identified as *E. coli*, were indicative of fecal pollution. Auban et al. [70] found *K. pneumoniae* to be the predominant fecal coliform in a fresh water lake at high temperatures, and considered it to be a fecal indicator under these conditions. Bagley and Seidler [66] distinguished the presence of *K. pneumoniae*, alone, as indicative of fecal pollution, and the concurrent isolation of fecal coliform positive *K. pneumoniae* and *E. coli* as indicative of recent fecal pollution. They concluded that the ability of bacteria to grow in EC broth at 44.5°C was not sufficient proof of a fecal source, and that whether or not *K. pneumoniae* was correlated with fecal pollution, it was indicative of a potential health hazard.

Enterococci have been proposed as indicators of fecal pollution for coastal bathing waters [71]. Enterococci are Gram-positive cocci which grow at temperatures between 10–45°C, and survive exposure to 60°C for at least 30 min, grow at pH 9.6 and also grow in 6.5% sodium chloride [72,73]. The enterococci are part of the genus *Streptococcus*, and include certain group Q streptococci (e.g., *Streptococcus avium*), *Streptococcus faecalis*, *Streptococcus faecium* and *Streptococcus durans* [72,73]. The enterococci group excludes *Streptococcus bovis* and *Streptococcus equinus*, which are not usually found in human feces [74]. Recently, Schleifer and Kilpper-Balz [75] proposed that *S. faecalis* and *S. faecium* be transferred to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. Fecal streptococci comprise all these organisms, as well as *Streptococcus mitis*, and *Streptococcus saliv-*

arius, and are, therefore, less indicative of human fecal pollution. In fact, Moore and Holdeman [76] found that *S. salivarius* ranked 38th on a list of human fecal bacteria, making it the most frequently encountered fecal streptococcus in human feces, and placing just behind *E. coli* which ranked 37th (the most abundant species was *Bacteroides vulgatus*).

Fecal streptococci are characterized by their ability to grow in the presence of azide and ethyl violet at 35°C, in Azide Dextrose Broth and Ethyl Violet Azide Broth, or to produce red or pink colonies on KF streptococcus agar, lack the catalase enzyme, and grow in bile broth at 35°C [63]. The enterococci have a fecal source, survive sewage treatment better than coliforms, and are present in concentrations of 10–100 times less than *E. coli* in treated sewage [77]. The survival of enterococci in sea water is longer than that of *E. coli* and total coliforms, but growth does not usually occur in sea water, regardless of the total salt concentration [78]. Enterococci have not been considered to be ubiquitous in the natural environment or to multiply in water [79], even though biotypes of *S. faecalis* have been isolated from insects and plant surfaces. The above cited qualities, in the view of some investigators, make enterococci useful indicators of fecal pollution.

The use of fecal coliform-fecal streptococcus ratios was proposed by Geldreich and Kenner [80] to differentiate between human and other animal fecal pollution. Since fecal streptococci survive longer than fecal coliforms in fresh water, the changing ratio of fecal coliform-fecal streptococcus makes the use of this ratio of limited and questionable significance [58,81].

3.1. 'Die-off' of indicator bacteria in sea water

The most widely used indicators for primary contact waters and shellfish waters are total and fecal coliform estimates. The concentration of *E. coli* is also used in shellfish guidelines. Although coliforms are used as indicators, their effectiveness is now being questioned for both fresh and salt water applications. One major problem with fecal coliforms is their low survival rate in sea water, compared to other indicators and pathogens. Dilution alone does not account for the rapid decrease

in coliform counts near sewage outfalls in marine waters [82]. Various mechanisms have been proposed for the 'die-off', including ultraviolet (UV) radiation [83], predation [84], competition [85], osmotic stress [86], antibiosis [82], and heavy metal toxicity [87,88].

Solar radiation is considered by some investigators to be a significant factor responsible for the bactericidal action of seawater. In field studies, mortality rates of coliform bacteria exposed to seawater are much greater in daylight than in the dark [83,89,90]. Gameson and Gould [91] report that half of the lethal effect of light is due to wavelengths below 370 nm, while the remainder results from light wavelengths of 370–400 nm. However, Fujioka et al. [92] stress the effect of visible light as more significant than that of UV light, with respect to the bactericidal action of light on both fecal coliforms and fecal streptococci in seawater. High turbidity, which blocks the penetration of sunlight, slows the rate of bacterial mortality [93]. Reduction in coliform counts has been shown to be correlated with intensity of sunlight and has been concluded to be related to diurnal and seasonal variations. These variations should be taken into consideration in designing ocean outfalls and sampling protocols for coastal waters [94].

The mechanism of bacterial 'die-off' caused by exposure to light may be related to the presence of sensitizers in cells, which absorb light and cause cell damage [95]. Another theory is that light energy reacts with oxides to form superoxides, which may damage cells [96]. Kapuscinski and Mitchell [97] demonstrated that, in *E. coli*, sunlight damages the catalase enzyme system, which is necessary for the degradation of peroxide. Long UV and visible wavelengths affect membrane permeability and damage active transport [98]. Inhibition of amino acid uptake, traced to the effects of UV and visible light, has also been demonstrated for estuarine bacteria [99].

Interestingly, the effect of sunlight is a function of the salinity of water [100]. Fecal coliforms and fecal streptococci are considerably more resistant to sunlight when diluted in fresh streams than in marine waters [92]. Chojnowski et al. [101] reported that the mortality rates of coliforms in

fresh water exposed to light are approximately half those obtained in experiments carried out using estuarine water, and one third of those when 100% sea water was employed.

Osmotic stress alone adds to the 'die-off' of coliforms in sea water. Sublethal stress has been recorded for *E. coli* after exposure in test media to sea water of various salinities and incubation for up to 30 days in the dark [102]. The time taken to reach 90% mortality for sewage coliforms in river water (0‰ salinity) was twice that for coliforms in sea water (> 30‰ salinity) incubated in the dark [103]. Studies of non-illuminated, highly saline water taken from Great Salt Lake (UT), which has an ionic composition similar to that of seawater, showed a higher death rate for *E. coli* in water from the more concentrated north arm of the lake (200 g/l chlorides) than in water from the southern portion (61 g/l chlorides) [104]. An inverse relationship was also found between salinity of estuarine water and survival of an *E. coli* strain isolated from the Rhode River estuary in Maryland, U.S.A. [105].

Temperature is directly related to coliform 'die-off' rates in both fresh and sea water [100,103]. A 10°C increase in temperature doubles the mortality rate of *E. coli* [105] and other coliform bacteria [103]. In a multiple linear regression analysis of the effects of temperature, dissolved oxygen and salinity on *E. coli* survival in diffusion chambers in the Rhode River estuary, temperature was associated with over 70% of the variation in numbers of viable *E. coli* [105]. In highly saline water collected from Great Salt Lake, low temperature had a sparing effect on mortality rates [104]. Cold temperatures allowed a lower death rate of *E. coli* in water collected from the more saline arm of the lake. However, Hussong et al. [106] suggest that numbers of false-positive total coliforms, i.e., presumptive total coliforms which are not confirmed at the completed step, are increased in sediment when the water temperature is < 10°C.

Parasitism by bacteriophage was suggested by Mitchell [107] as a factor responsible for the destruction of fecal microorganisms entering aquatic environments. Interestingly, Roper and Marshall [108] demonstrated that sorption of bacteria, and their specific phage, to saline sediment interfered

with viral infections, thereby allowing for persistence of bacteria in sediment. Destruction by bacteriophage is also thought to occur in sewage treatment plants, although predation may be more important in the removal of enteric pathogenic bacteria from the final clarified effluent [109].

Predation has been suggested as another mechanism contributing to the bactericidal effect of sea water on non-marine bacteria [84]. Ciliated protozoa were found to be responsible primarily in reducing the survival time of *E. coli* in activated sludge [110]. In estuarine waters, protozoan predators, rather than lytic bacteria, were associated with the decrease in numbers of *E. coli* [111,112]. As an extension of this hypothesis, McCambridge and McMeekin [113] reported that sunlight and microbial predators acted synergistically to reduce the numbers of enteric bacteria.

Fresh and stored sea water yield higher mortalities than artificial seawater. Thus, it appears that there are substances in seawater, in addition to the inorganic salts, which increase 'die-off' of *E. coli* [103]. These include antibiotics and other antagonistic chemicals produced by marine bacteria and plankton. Catalase-sensitive lipopolysaccharides, produced by some strains of *Pseudomonas* and *Chromobacterium*, are active against terrestrial bacteria [114]. Sunlight-induced injury to the catalase enzyme [97] would render terrestrial bacteria more susceptible to these antibiotics. Marine phytoplankton also secrete antagonistic substances during most of the year [115], which may affect the survival of bacterial pollutants. Acrylic acid, produced from the breakdown of dimethyl- β -propiethetin of some marine algae, has an antibacterial effect both in sea water and in the intestinal tract of penguins [116–119]. Oyster extracts and sea water containing acrylic acid produced by *Phaeodactylum tricornerutum* were shown to have an antibacterial effect against *E. coli* [120]. Thus, the presence of acrylic acid in oysters would have the potentially dangerous effect of masking a possible health hazard posed by the presence of human pathogens.

Reduction in counts of bacteria in seawater can also be caused by sedimentation, in addition to 'die-off'. In laboratory experiments, using natural seawater, the concentration of *E. coli* was shown

to decrease because of starvation. Interestingly, the addition of nutrients resulted in floc formation, followed by adsorption of the cells onto suspended particles, with concomitant precipitation [121]. Ogawa suggested that precipitation was a factor in the 'disappearance' of coliforms in coastal waters, and that, in sediment, these bacteria survive longer because of the higher concentrations of nutrients present in sediment. However, Xu et al. [28] reported that *E. coli* survived for at least 2 weeks in salt water microcosms, but were not culturable (see below). Sedimentation is considered to be the most important factor associated with reduction in numbers of bacteria in the water column in the deep ocean [122].

3.2. Indicator bacteria in sediments

The presence of indicator bacteria in sediments has been reported by investigators [123–126]. Bottom sediments were found to contain larger numbers of *E. coli* [127] and *Enterobacter aerogenes* [128] than the overlying seawater, because of a higher concentration of organic matter in sediment. In laboratory studies using autoclaved estuarine sediments and seawater, *E. coli* has been shown to increase in number [127], whereas when non-autoclaved sediments and water were employed, persistence of *E. coli* was noted, with limited growth of the organism. LaLiberté and Grimes [129] made similar observations on the in situ growth and persistence of *E. coli* inoculated into dialysis bag-contained sediment. Gerba and McLeod [127] suggest that the type of nutrient and microflora present in sediment may differ from those present in sea water, allowing for greater competition with *E. coli* in sediment. The particle size of sediment was found to be inversely proportional to the concentration of nutrient, and to survival of *Enterobacter aerogenes* [128]. Since the survival of fecal coliforms in sediment samples was prolonged at 4°C, the temperature of sediment in situ is also an important factor in their persistence [130].

Since the concentration of fecal indicators [127,128,131,132], microbial pathogens including *Salmonella* spp. [131,132], and enteric viruses [132,133] are higher in sediment than in the overlying waters, samples of sediment, rather than

water, should be taken for examination of the presence of indicator bacteria in the case of primary contact water and shellfishing areas. Although De Flora et al. [134] found greater numbers of viruses and bacteria in surface water than in bottom water or sediment samples collected from moderately or grossly polluted coastal sites, they also suggested that sediment was a transient reservoir for viruses and that viruses survived longer in some sediments than in sea water.

Wave action, tides, currents, storms, run-off, and human activities, including dredging and pleasure-boating, can disturb sediments, stirring up particulates to which organisms which persist in these sediments are adherent. Resuspension of sediment allows the sediment microorganisms to enter the water column which may previously have contained negligible numbers of indicator bacteria and pathogenic organisms. It has, in fact, been documented that dredging resulted in the release of sediment-bound fecal and total coliforms and fecal streptococci in the Mississippi River [135–137].

3.3. Survival of indicator bacteria in marine waters

Although the preceding section has focused on (i) die-off of *E. coli* and other indicator bacteria in seawater, and (ii) their persistence in sediment because of various factors associated with the particulate nature of the latter, it must be emphasized that recent evidence disputes claims that coliforms are rapidly killed by sea water. Coliforms appear to be injured, stressed, or debilitated to such an extent that special techniques may be required to recover them [138–140]. Methods for recovery of stressed coliforms have been proffered for fresh water [141] and for frozen or heated foods [142]. For stressed coliforms in marine waters, the methods presently available which allow 'repair' include filtration through membrane filters which are subsequently placed on two-layered plates [143], modification of the most probable number (MPN) method requiring transfer of all presumptive tubes showing growth whether or not gas is produced [140], use of special membranes that 'nestle' the bacteria [144], and pour-plating followed by overlaying with a selective medium [145]. The pour-plate method also has been used for

enumeration of fecal coliforms and enterococci in seafood samples.

Dawe and Penrose suspended dialysis tubes filled with bacterial suspensions or untreated samples of water in sea water (8–12°C), and found that, although the rate of ‘injury’ for coliforms was high, the rate of survival was also high, based on ATP content per cell [138]. It was suggested that sea water, rather than being bactericidal, acts to protect ‘injured’ bacteria from death.

Recently, work in our laboratory has shown that *E. coli* cells in saltwater microcosms remain viable, yet ‘non-culturable’ on laboratory media [28]. Viability of cells was determined using a direct viable microscopic technique [24]. Other microcosm experiments employing *V. cholerae* in salt water [28] and *Salmonella enteritidis* in fresh water showed the same result, i.e., all the cells remained viable, but were non-culturable on routine laboratory media, as had been observed for *E. coli* [146]. This result was documented for at least 60 days after inoculation. The apparent ‘dormancy’ of these enteric bacteria, possibly arising from depletion of available nutrients, compromises the use of *E. coli* and related coliforms as indicators of fecal pollution in fresh and salt waters, and renders highly questionable the phenomenon of ‘die-off’ or genuine mortality of these bacteria.

3.4. Current indicators for the presence of enteric viruses

Finding an appropriate indicator for the presence of enteric viruses, i.e., poliovirus, coxsackievirus A, coxsackievirus B, and echovirus [50,147], in sea water, is a vexing problem, especially for those responsible for regulating the use of sewage-contaminated sites, including fresh, estuarine, and marine water and sediment, and shellfish harvested from these waters. Fecal coliforms and total coliforms have been used as indicators of contamination by domestic sewage, and therefore of the risk associated with exposure to bacterial and viral pathogens. However, in fresh water and waste water effluents [148–154], marine waters [133,152,155–157], and in shellfish meats [152,158,159], the total coliform and fecal coliform counts cannot be used to indicate the presence of enteroviruses. The lack of association of these

indicators with enteric viruses is due to the fact that the latter survive longer in the ‘recoverable’ stage than fecal coliforms in fresh water [148,150] and marine water [160], resulting in continuously changing ratios of viruses to the fecal indicators. However, Goyal et al. [155] observed a positive correlation between the concentration of virus in sediment and total coliform concentration. Although De Flora et al. [134] found significantly larger numbers of viruses and bacteria in surface waters than in bottom water or sediment from polluted coastal waters, they also concluded that sediment was a transient reservoir for viruses and that survival of viruses was longer in some sediments than in sea water. In open ocean water, viruses persist longer when associated with, or attached to, solids, than when not associated with solids [161]. Viruses also survive longer in sediment than in overlying water [134], resulting in greater concentrations of enteric viruses recorded as being present in sediment than in water samples [132]. Since adsorbed viruses remain infectious [162], viruses in sediment and resuspended sediment are potential health hazards.

Most viruses also survive chlorination better than bacteria. Grabow et al. [153] reported that hepatitis A virus was more sensitive to chlorination than *Mycobacterium fortuitum*, coliphage V1, and poliovirus type 2, but more resistant than *E. coli*, *Streptococcus faecalis*, coliphage MS2, reovirus type 3, and rotavirus SA11. Total and fecal coliforms are also destroyed by chlorine more quickly than viruses [149]. Cold temperatures and factors associated with gross pollution appear to prolong virus survival in rivers [150]. Since fecal streptococci and enterococci survive longer than fecal coliforms, in both fresh water and sea water, fecal streptococci and enterococci have been suggested as better indicators of enterovirus contamination in river water [150,160], relatively less polluted waters [148,157], and coastal recreation water [71]. However, the viability in virus titers recorded for sediment and sea water samples is less than that of any of the bacterial indicators examined [134].

Isolation of enteric viruses when the number of indicator bacteria was extremely low has been reported for sediment samples [133], marine water

[155–157,159,160], and ground water [151]. It is important to point out the disturbing finding of Gerba et al. [156] that enteroviruses can be present about 40% of the time in approved recreational and shellfish harvesting waters, approval being based on total or fecal coliform standards. Although the number of total coliforms was reported to be correlated significantly with the number of viruses present in water samples, the amount of variation associated with the indicator was such that it could not be considered a good predictor. A positive correlation between coliforms and enteric viruses was found for ocean waters, but only for samples collected close to a sewage outfall. At further distances from the outfall, viruses could be isolated, even in the absence of fecal indicator bacteria [157]. Vaughn et al. [152] found little difference in the viral quality of open and closed shellfish waters. Furthermore, echovirus, the vaccine strain of poliovirus, and other, unidentified viruses, could be isolated from clams collected from both areas [152].

Contamination of shellfish with enteric viruses has been well documented, and the subject has been reviewed extensively by several investigators [163–165]. Enteric viruses have been isolated from shellfish collected from approved shellfishing waters. For example, Fugate et al. [158] reported the isolation of echovirus type 4, and poliovirus types 1 and 3, from oysters collected in approved waters off the Louisiana and Texas Coast. The coliform MPN of water and coliform MPN, *E. coli* MPN, and aerobic plate count of the oysters, thus, were not indicative of the presence of viruses [158]. No correlation was found between fecal coliforms in water and the number of viruses isolated from oysters collected from approved and prohibited oyster harvesting sites in coastal Mississippi [164,165]. Rather than using fecal coliforms as indicators, it was proposed that a virus, such as poliovirus type 1, could be helpful, and that concentrations of a viral or bacterial indicator in the sediment might also be valuable as a predictor of health risk from viral contamination. Similarly, Goyal et al. [159] found no significant correlation between virus concentration in oysters harvested from approved shellfish sites and the bacteriological quality of water and shellfish.

The relationship between risk of viral disease and presence of viruses in water is not well known. The fact that a small number of viruses constitutes an infective dose has prompted the recommendation of less than one viral infectious unit per 10 gallons (37.8 l) of water as acceptable [166–167]. Methods for the detection of viruses in water are still being improved, and recoveries are low. Therefore, the detection of one virion indicates the presence of many more viruses which go undetected. Sobsey and Glass [168] have developed filtration methods which have an average recovery efficiency for poliovirus from tap water of 30%, ranging between 17–65%. In a comparison of positively charged microporous filters with conventional standard methods, i.e., negatively charged filters, for the recovery of enteric viruses from tap water, conventional methods averaged less than 5% recovery efficiency, whereas the new methods averaged 64% and 22.5%, for 1- and 2-stage protocols [169]. Sobsey et al. [170] have also devised methods for recovery of viruses from shellfish, with virus recovery efficiencies averaging 46%. But, as has been noted, epidemiological studies are needed for primary contact and shellfish harvesting waters [44,50,156,171]. To date, the evidence available suggests that the presence of viruses in shellfish can be linked to viral gastroenteritis and hepatitis outbreaks.

3.5. Proposed indicators for the presence of viruses in water and shellfish

Enteric viral disease outbreaks, traced to swimming in polluted waters or eating contaminated shellfish, have made it clear that coliform standards are not sufficient for monitoring the health hazards posed by the presence of viruses in sewage or the presence of viral pathogens in freshwater, marine waters, or in shellfish. Viruses which may be transmitted by water, and are currently of interest, include enteroviruses, adenoviruses, reoviruses, hepatitis A virus, rotaviruses, coronaviruses, and the norwalkvirus [50,172]. Total and fecal coliforms, *E. coli*, enterococci, fecal streptococci and *S. faecalis* do not survive as long as the viruses employed in studies accomplished to date, and therefore cannot be relied upon to be present and recoverable when enteric viruses are

also present. Coliforms can be a useful indicator for very recent contamination, but clearly not for contamination after a prolonged period of time after occurrence. Fecal streptococci and enterococci, which survive longer in seawater than coliforms, but have not been studied as thoroughly as the coliforms, have been proposed as indicators for the presence of enteric viruses [71,173], but, as stated above, cannot be relied upon to indicate the presence of viruses. Viral indicators, suggested for waste-water, include Sabin vaccine poliovirus [164,174–176], cyanophage [177,178], and coliphage [179,180]. The difficulties associated with the use of a virus as an indicator are many, the most important of which are: no single human virus is always present in every sewage-impacted area; viruses are often present only in relatively small numbers; and better methods for virus concentration are badly needed [175]. Total virus count was recommended as a method preferable to enumeration of poliovirus for those countries with high enterovirus infection rates [175]. Katzenelson and Kedmi [181] found that poliovirus was absent in about 50% of waste-water samples tested, although the total virus concentrations were high. They also recommended that a system be developed to isolate and identify total viruses in water.

Smedberg and Cannon [177] proposed the use of LPP-cyanophages, i.e., viruses of blue-green algae of the general *Lyngbya*, *Phoridium* and *Plectonema* [182] as indicators of the presence of human enteric viruses and reliability of disinfection for treated wastewater. Cyanophages can be found throughout the year in wastewater. Furthermore, they are more resistant to chlorine than coliforms, and can be correlated with the number of fecal coliforms present in wastewater [177,178].

Coliphages have also been proposed as indicators [183,184]. Correlations between the number of fecal bacterial pathogens and indicator bacteria and their respective bacteriophages in fresh and marine water have been reported [185]. The concentration of coliphages was reported to be at least as high as the number of enteroviruses in waste-water and other waters [179]. In estuarine waters, the survival time of coliphages is comparable to that of enteric viruses [186]. Coliphages are more resistant to chlorine than enteroviruses and

survive longer in sand columns than poliovirus type 1 [179]. A rapid test for coliphages, as a water quality indicator for bacteria, was developed for fresh water [187]. Using this test, a linear relationship for the number of fecal or total coliforms with coliphages has been reported [188].

The value of coliphages has also been investigated, with respect to indication of enteric viruses in shellfish and shellfish-harvesting waters [186]. As a result of their studies, Vaughn and Metcalf [186] recommended the use of coliphages as an indicator for raw sewage and treated effluents, but not for estuarine shellfishing waters, because coliphages were found to increase in number by replication in estuarine waters. Furthermore, they were accumulated by oysters to a greater extent than enteric viruses, and showed shifts in dominant type over time [186]. Thus, the presence of coliphages was concluded not to be related to the presence of enteric viruses in estuarine water [186].

In freshly shucked oysters contaminated with poliovirus and stored at 5°C, the virus persisted longer than 28 days [189]. In fact, viruses in contaminated oysters can survive processing, refrigeration, and freezing [190].

In addition to bivalves, other marine invertebrates, including crabs, must be suspect agents in the transmission to man of enteric viruses present in polluted waters. Even crabs caught in unpolluted waters may be contaminated with viruses accumulated when the crabs were exposed to polluted waters, either by swimming and/or feeding on contaminated shellfish in polluted waters [191]. In laboratory studies, DiGirolamo et al. [192] found that crabs concentrated high titers of poliovirus from contaminated mussels and filtered sea water that had been artificially seeded. In similar experiments, polioviruses were isolated from all parts of crabs, being present in highest concentrations in the hemolymph and digestive tract of the animal [193]. Poliovirus survived for up to 6 days in crabs held at 15°C. Recovery of poliovirus type 1, simian rotavirus SA11, and echovirus type 1 was reduced by 99.9% in crabs after they had been boiled for 8 min [193].

3.6. Epidemiology and coastal bathing water quality

As the human populations located along urban

coastal areas have increased, so too has the amount of material discarded into the ocean. It is now well known that larger concentrations of bacteria are found near the mouths of rivers and bays in highly populated areas and other coastal regions than in the open ocean. To protect bathers from water-borne enteric diseases acquired by swimming in coastal waters, some indication of the extent and amount of fecal pollution is needed. Epidemiological studies are valuable for determining whether or not swimming in sewage-polluted waters poses a health risk, and, also, what organism or organisms are best suited to predict such health hazards. In addition to assessing the risk of enteric viral and bacterial diseases such as infectious hepatitis, typhoid fever, shigellosis, and cholera, the risk of contracting diseases affecting the eyes, ears, nose, throat and skin, as well as wound infections, is a matter of serious concern. Organisms which cause the latter group of diseases are not necessarily sewage-associated, and may not be signalled by the traditional drinking water indicator bacteria [194]. An important aspect of epidemiological studies is the determination of the infectious dose for a particular pathogen, and its virulence.

In a review of water-borne pathogens, Geldreich [195] focused on enteric pathogens, including *Salmonella*, *Shigella* and *Leptospira* (freshwater) species, enteropathogenic *E. coli*, *Francisella tularensis*, *Vibrio cholerae*, *Mycobacterium tuberculosis*, hepatitis A virus, poliovirus, other human enteric viruses and parasitic protozoa and worms. Not all of these organisms are necessarily of concern for the marine environment. In addition to the organisms listed by Geldreich [195], Moore [196] cites as being communicable, otitis media, sinusitis, and gastroenteritis of unknown etiology, but dismisses certain organisms as irrelevant for coastal bathing waters, including *Staphylococcus aureus*, clostridia, *Mycobacterium* spp. and *Candida* spp., based on their biology. However, staphylococci, especially *Staphylococcus hominis* and *Staphylococcus epidermidis*, and including *S. aureus*, have recently been isolated from coastal, offshore, and deep ocean sites [197,198]. Also in conflict with Moore [196] are the frequent isolations of *Clostridium botulinum* and *Clostridium*

perfringens from marine sediment [199–201]. According to Moore [196], typhoid fever is of concern at the present time only in areas of high enteric morbidity. Wound and enteric infections caused by bacteria of the Vibrionaceae and skin rashes caused by trematodes, acquired from swimming in estuarine and ocean waters, were also included in a review of recreational water use published by Cabelli [51].

Most of the epidemiological studies of bathing water quality were done in the 1950s in the U.S.A. and U.K. Notable of these are the reports of Stevenson [202] in the United States and Moore [203] in Britain. Indicator organisms used to compare to the incidence of disease in both the American and British studies were total coliforms. However, the authors of these reports reached different conclusions. Stevenson [202] reviewed results of surveys of swimming pools and beaches on the Ohio River, Lake Michigan, and Long Island Sound, and concluded that a positive correlation existed for fresh waters between high total coliform concentrations and illness in swimmers, i.e., more swimmers than non-swimmers became ill, with more than one-half of the illnesses being ear, eye, nose, and throat diseases, one-fifth gastrointestinal, and the remainder skin or other types of infections. On the other hand, Moore [203], who reported on an epidemiological study of bathing waters carried out on the coasts of England and Wales, found no evidence to support the conclusion that sewage-contaminated water was a significant cause of illness. It should be pointed out, however, that isolation of *Salmonella* spp. from seawater was interpreted as being evidence of the wide dissemination of this organism in the population at large, rather than a risk of infection for bathers. In a later publication, Moore [196] concluded that the environmental hazards of swimming are difficult to assess and require statistical proof of association, causation, and specificity of disease with cause of the disease. Two other factors important in epidemiological studies were cited, these being the incubation time of the pathogen, and dose response of morbidity related to frequency of swimming [196].

The most recent report of results of an epidemiological study of coastal bathing waters was of a

study conducted over a 5-year span in the 1970s at beaches in New York City, Lake Pontchartrain, LA and Boston, MA [71]. A direct linear relationship was observed between swimming-associated gastrointestinal disease and water quality. Other types of disease were not reported. Bacteria enumerated in the waters included enterococci, total and fecal coliforms, *E. coli*, *Klebsiella* sp., *Enterobacter* sp., *Citrobacter* sp., *Clostridium perfringens*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, and *Vibrio parahaemolyticus*. The concentration of enterococci was strongly correlated with gastrointestinal symptoms, while fecal and total coliforms were found to be poorly correlated. As reported in earlier studies in the U.S.A. by Stevenson [202], swimmers showed a higher rate of symptoms than non-swimmers [71], even in marginally polluted marine waters, the latter being indicated by the presence of enterococci.

Although total coliforms have been used as indicators of primary contact water quality, this approach has been discontinued in many areas in favor of the more fecal-specific fecal coliforms [204]. Enterococci have been suggested as better than either the fecal coliforms or *E. coli* as an indicator of fecal pollution for bathing waters since the correlation of enterococci with gastrointestinal symptoms was highest [71,204].

4. CONCLUSIONS

A wide array of indicator organisms has been proposed for brackish, estuarine, and coastal waters. It is clear that no single indicator, index or reference organism exists for titrating public health safety of these waters. With the development of immuno-epifluorescent microscopy methods for direct detection of specific human pathogens, and considering the 'viable but non-culturable' stage which allochthonous human pathogens undergo when released to natural bodies of water, it is concluded that the direct viable approach [24] to measuring the occurrence and probable pathogenic potential of microorganisms in waste-receiving waters warrants further investigation [29]. Biotechnology will also make significant contributions to assurance of public health safety of water.

Monoclonal antibodies will be used for direct detection of specific pathogens, and the advent of gene probes will make possible the direct detection of toxin-gene carrying microorganisms in water and other samples from aquatic environments. Prospects for the future will, most probably, be brightest for non-culture methods of direct detection of human pathogens in the environment.

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