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Enterococci in the Environment

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INTRODUCTION

Enterococci are important members of gut communities in many animals (e.g., see references 82, 86, 87, 101, 105, 144, 195, 202, and 240) and opportunistic pathogens that cause millions of infections annually (231, 233). Their abundance in human and animal feces, the ease with which they are cultured, and their correlation with human health outcomes in fresh and marine waters have led to their widespread use as tools for assessing recreational water quality worldwide (333, 335, 345–347). The enterococci are most frequently used as fecal indicator bacteria (FIB), or general indicators of fecal contamination, but they are also used as surrogates for pathogens and/or health effects in risk assessment and other modeling applications (61, 214, 285, 303, 329, 346). Research spanning more than 3 decades, however, has shown that these bacteria are widely distributed in a variety of environmental habitats, even when there is little or no input from human and/or animal fecal sources. These extraenteric habitats include soil and sediments, beach sand, aquatic and terrestrial vegetation, and ambient waters (rivers, streams, and creeks); they may also be considered heterothermic habitats, in which temperatures are variable, in contrast to the gastrointestinal tract of warm-blooded animals, where the temperature is relatively constant.

The overall goal of this review of enterococci is to present the reader with an understanding of (i) the taxonomy and phylogeny, (ii) the microbial ecology (occurrence, persistence, and survival in nonenteric habitats), and (iii) the use of these bacteria in protecting human health from waterborne illnesses. In this review, unless otherwise stated, we define "environmental enterococci" as those bacteria found in a variety of extraenteric habitats, such as ambient waters, aquatic and terrestrial vegetation, beach sand, soil, and sediments.

ENTEROCOCCI AND THE GENUS *ENTEROCOCCUS*

Previously classified in the genus *Streptococcus*, the enterococci were proposed to be a division comprised of bacteria that generally grow at temperatures of between 10°C and 45°C in 6.5% NaCl at pH 9.6 and to survive at 60°C for 30 min (66, 68, 218, 293). This classification scheme, proposed previously by Sherman (293), correlated with a serological scheme developed by Lancefield in the 1930s, wherein the enterococci reacted with group D antisera whereas nonenterococcal streptococci reacted with antiserum group A, B, C, E, F, or G (198). In 1984, *Enterococcus* was proposed as a unique genus, separate from *Streptococcus*, when DNA-DNA and DNA-rRNA hybridization revealed that species such as *Streptococcus faecalis* and *S. faecium* (now *Enterococcus faecalis* and *E. faecium*, respectively) were relatively distantly related to nonenterococcal streptococci such as *Streptococcus bovis* (67, 283). Pres-

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TABLE 1 Species of the genus *Enterococcus* and their currently known habitats

Group	Species	Known habitat(s)	Human pathogen	Reference(s)
E. faecalis	*E. faecalis*	Human, animal (multiple), plant, insect	Yes	70, 203, 235, 283
	E. haemoperoxidus	Surface water		320
	E. moraviensis	Surface water		320
	E. silesiacus	Drinking water		323
	E. termitis	Animal (termite)		323
	E. caccae	Human		54
E. faecium	*E. faecium*	Human, animal (multiple), plant, insect	Yes	70, 192, 203, 235, 283
	E. durans	Human, animal (multiple), insect	Yes	67, 70, 203
	E. hirae	Animal (multiple), plant		101, 203, 235
	E. mundtii	Soil, plant	Yes	66
	E. villorum	Animal (hog)		339
	E. canis	Animal (dog)		82
	E. ratti	Animal (rat)		324
	E. asini	Animal (donkey)		86
	E. phoeniculicola	Animal (bird)		202
	E. canintestini	Animal (dog)		243
	E. thailandicus	Human, animal (cattle)		55, 295, 316
E. avium	*E. avium*	Human, animal (multiple)	Yes	67, 121, 203, 257
	E. pseudoavium	Human		65
	E. malodoratus	Animal (cattle)		67
	E. raffinosus	Human	Yes	65, 241
	E. gilvus	Human		330
	E. pallens	Human		330
	E. hermanniensis	Animal (dog)		195
	E. devriesei	Animal (cattle)		322
	E. viikkiensis	Animal (broiler plant)		269
E. gallinarum	*E. gallinarum*	Human, animal (multiple), insect	Yes	67, 70, 203
	E. casseliflavus	Plant, soil, human, animal (multiple)	Yes	67, 203, 239, 241
E. cecorum	*E. cecorum*	Animal (chickens)		88, 360
	E. columbae	Animal (pigeon)		87
Ungrouped	*E. saccharolyticus*	Animal (cattle), sewage		203, 270
	E. aquimarinus	Seawater		321
	E. sulfureus	Plant		218
	E. dispar	Human		68
	E. italicus	Animal (cattle)		107
	E. camelliae	Plant		316

ently, there are 36 known *Enterococcus* species, classified into five groups (Table 1).

We have adopted the following conventions of nomenclature and terminology. When referring to a confirmed member of the genus *Enterococcus*, the technical genus and/or species (e.g., *E. faecalis*) or the inclusive equivalent (*Enterococcus* sp. or spp.) is used. When referring to organisms that are identified only by isolation and the correct phenotype on selective-differential medium, the generic term "enterococci" is employed. Finally, in older publications where designations such as "fecal streptococci" or previous species names (e.g., *S. faecalis* or *S. faecium*) were used, we employ that terminology with the understanding that the terms, especially fecal streptococci, are largely synonymous with enterococci. The term "intestinal enterococci," used by the European Union to describe the FIB group used for water quality assessments, is largely interchangeable with enterococci (363) but has been defined by biochemical characteristics set by the International Organization for Standardization (168).

It is noteworthy that the early classification system proposed by Sherman (293) is occasionally still used today to differentiate enterococci from nonfecal streptococci as well as to identify enterococci based upon reactions to group D and, in some cases, group Q antisera (241, 257). Enterococci are spherical or ovoid cells arranged in pairs or chains (144, 241). The enterococci are Gram positive, non-spore-forming, obligately fermentative chemoorganotrophs. They are catalase negative, although some species produce pseudocatalase, and they are usually homofermentative, producing lactic acid (144, 192, 241). Motility differs among species; e.g., *E. gallinarum* and *E. casseliflavus* are motile, and *E. asini* and *E. phoeniculicola* are not (66, 86, 202). Pigmentation also differs among species; i.e., yellow-pigmented species include *E. sulfureus*, *E. casseliflavus*, and *E. mundtii* (66, 218), and pigmented species are commonly found among plants (1). Enterococci are also found in the gut of insects (e.g., *Drosophila*) (70). The known habitats of various *Enterococcus* spp. are catalogued in Table 1, but it is important to note that as more environmental habitats are

TABLE 2 Environmental stressors that negatively impact survival of enterococci

Stressor	Type of stress	Source(s) of enterococci	References
Sunlight	Ambient and simulated sunlight	Environmental strains; sewage	30, 59, 60, 71, 73, 77–81, 94, 103, 112, 113, 180, 187, 215, 217, 255, 280, 286, 306–308, 344
Salinity	Estuarine and marine waters	Environmental strains; sewage	10, 53, 75, 94, 187, 229, 308
Disinfection	Chlorine/UV/peracetic acid	Sewage; pure cultures of *E. faecalis*	25, 52, 57, 58, 152, 156, 182, 194, 222, 272, 273, 328
Starvation	Oligotrophic conditions, glucose deficiency	Pure cultures of *E. faecium*, *E. durans*, *E. flavescens*, *E. avium*, *E. pseudoavium*, *E. malodoratus*, *E. raffinosus*, *E. mundtii*, *E. faecalis*, *E. hirae*, *E. gallinarum*, and *E. casseliflavus*	160, 210–212, 302
Predation	Bacterivorous protozoa	Environmental strains; pure cultures of *E. faecalis*	75, 126, 147, 166, 169, 170, 229, 317

explored, and as methods for the identification of organisms to the species level become less labor-intensive and more standardized, the list of known habitats for members of the genus *Enterococcus* will doubtless increase.

In general, enterococci are commensal bacteria, potentially helping in digestion and other gut metabolic pathways. Some *Enterococcus* spp., such as *E. faecium* and *E. faecalis*, are used in probiotics to treat diarrhea and improve host immunity (108). While most species of *Enterococcus* are commensal organisms, some species are opportunistic human pathogens. *E. faecalis* and *E. faecium* have become particularly important etiological agents of nosocomial infections (231, 233), including urinary tract infections, endocarditis, bacteremia, neonatal infections, central nervous system (CNS) infections, and abdominal and pelvic infections (231, 241). Of particular concern is the intrinsic antibiotic resistance among certain species, particularly resistance to aminoglycosides and cephalosporins, or acquired resistance to many others, most prominently vancomycin (233, 325). While *E. faecalis* is the species most commonly implicated in nosocomial infections, *E. faecium* has shown resistance to the widest array of antibiotics (233, 325), and *E. avium*, *E. casseliflavus*, *E. durans*, *E. gallinarum*, and *E. raffinosus* have been isolated from patients diagnosed with enterococcal infections (241). Because of their near-ubiquitous distribution in the feces of animals, including humans, they are commonly used as FIB, or surrogates for pathogens, in water quality analyses (see Use of Enterococci as Fecal Indicator Bacteria).

Several genotyping techniques have been employed with this group to obtain correct identification to the species level and further discrimination at the subspecies level for clinical, environmental, and food-related issues, including ribotyping (232), repetitive extragenic palindromic PCR (REP-PCR) or BOX-PCR (16, 244), pulsed-field gel electrophoresis (PFGE) (128, 326), 16S rRNA gene sequencing (62, 244), and multilocus sequence typing (MLST) (276). REP-PCR has been used to target repetitive genetic sequences and, based on the genome size and the location of the repetitive elements, to generate unique banding patterns (or "fingerprints") to differentiate strains. While one of the major criticisms of this technique has been the lack of a consensus on interpretations of the resulting fingerprints (216), horizontal, fluorophore-enhanced REP-PCR (HFERP) improves alignments between multiple gels and reduces within-gel groupings in the resulting dendrograms (185). HFERP can identify isolates with 77% agreement with 16S rRNA genetic sequencing and superior discrimination among environmental isolates (244).

The current standard in the clinical identification of *Enterococcus* spp. and strain typing is PFGE (163, 242). Whereas ribotyping techniques accurately discriminate among species (140) and are less expensive than PFGE, the latter method has better discrimination among closely related strains (128). MLST has proven to be useful in epidemiological studies of *E. faecalis* and *E. faecium* (56, 91, 276), and studies have shown an accuracy equivalent to that of PFGE for the identification of organisms to the subspecies level (163, 242).

ECOLOGY

Responses to Environmental Stressors

When enterococci are released from the gastrointestinal tract of warm-blooded animals into secondary habitats such as environmental waters, aquatic vegetation, or sediment, they are subjected to a host of biotic and abiotic stressors that generally lead to a decline in the population over time.

Sunlight. Sunlight has been a suspected stressor of bacteria since at least 1877 (95). Major mechanisms of sunlight damage to microorganisms include the direct absorption of UV light by DNA or the indirect effect of the formation of endogenous and exogenous reactive oxygen species. The ability of DNA to absorb UV light was discovered in 1929 (117), leading to studies of the mechanism of UV damage to DNA and microbial inactivation (33, 97, 189). Many mesocosm studies have noted the germicidal effect (defined as a loss of culturability) of sunlight on enterococci (77, 112, 187, 255, 286, 306–308) (Table 2); however, the reported time required to achieve a 90% reduction in the concentration (T_{90}) (equivalent to a 1-log reduction) varies widely according to geographic and seasonal factors and the experimental design (e.g., the source of the inoculum or physicochemical properties of the water). For example, in marine and estuarine waters inoculated with sewage, the reported T_{90} values range from 2 h (112) to 35 h (187). Generally, mesocosm studies of saline waters conducted in warmer climates (112, 306–308), during summer months (255, 306, 307), and in waters with relatively low turbidity (112, 187) reported lower T_{90} values (more rapid die-off) than those reported for colder climates (187), winter months (255, 306–308), and turbid waters (187). A similar trend was observed in freshwater mesocosm studies, where the reduction in levels of enterococci was enhanced at higher temperatures (i.e., during summer months and in warmer climates) (180, 255, 308). Mesocosm studies comparing sunlight inactivation in fresh versus marine waters

(112, 255, 308) have generally found inactivation to be more pronounced in the latter.

While sunlight inactivation of microorganisms is a natural, low-cost process for the treatment of contaminated water, its efficacy depends on numerous environmental factors, including the chemical composition of the water (e.g., dissolved oxygen and turbidity) and site characteristics (e.g., depth). The depth (77–79, 112, 180, 215) and turbidity (60, 78, 113, 187) of irradiated water are inversely proportional to the effectiveness of sunlight disinfection. Both factors are positively correlated with the absorbance, which is the difference between the amount of light energy (measured at a specific wavelength) that enters a sample and the amount that passes through it (338). Once absorbed, UV light loses its germicidal properties; thus, nonspecific absorption (by substances other than the intended target) hinders the efficiency of UV light disinfection. The sunlight-mediated inactivation of FIB in waste stabilization ponds (WSPs) has been shown to increase with dissolved oxygen (DO) concentrations in a wide variety of systems, including anaerobic, secondary facultative, and maturation systems and algal ponds (64, 67, 70–72, 267). The proposed mechanism for the observed synergistic action between DO concentrations and sunlight inactivation postulates that endogenous chemicals (e.g., porphyrin derivatives, flavins, and menaquinone) can act as "sensitizers" when they absorb light; reactions between excited sensitizer molecules and oxygen lead to the formation of reactive oxygen species (singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radicals), resulting in photooxidative damage to the organism (73, 80, 81).

Although mesocosm studies are helpful for isolating factors that may contribute to the variability in the survival of enterococci, they cannot reflect all of the complex biotic and abiotic interactions that occur in aquatic environments. Furthermore, the use of single laboratory-grown strains (as opposed to wastewater isolates) as a mesocosm inoculum may elevate inactivation rates for enterococci (103), thus further compounding the issue. In general, based on mesocosm studies, the time required to achieve a decrease in the concentration of enterococci of 3 orders of magnitude (i.e., 99.9%) ranges from 0.9 to 52 h (76, 83, 103, 255, 308). Sassoubre et al. (280) investigated field-relevant dark inactivation and photoinactivation rates by sampling two sites in San Pedro Creek, CA, hourly over 25 h. Between 6 a.m. and 6 p.m. (time points before and after sunlight exposure), concentrations of enterococci decreased by approximately 1 order of magnitude (280). Comparable results were observed in a study in Hawaii, where 22 streams were sampled during the time before the sun rose (a.m.) and at noon (p.m.); the concentrations of enterococci were approximately 0.5 logs lower in the afternoon (344). Boehm et al. (30) conducted a 72-h experiment to investigate the diurnal variation in microorganism concentrations at Avalon Beach, CA, and noted that concentrations of enterococci during the day were approximately 0.5 logs lower than those during the night (30). A similar study conducted under dry weather conditions in a tidally influenced salt marsh found a fairly wide range of concentrations of enterococci (spanning up to 3 orders of magnitude), with the early-morning concentrations generally being 1 to 2 orders of magnitude higher than those in the late afternoon (94). Hourly sampling in South Korea resulted in similar conclusions: under dry weather conditions, sunlight inactivation of enterococci was responsible for a reduction of 1 to 2 orders of magnitude (59). A recent study showed that exceedances of current regulatory standards for enterococci at marine beaches (i.e., 104 CFU/100 ml) are more frequent during the night and late-afternoon hours (from approximately 6 p.m. to 8 a.m.) than during the morning and early afternoon, suggesting that sampling at different times of the day can significantly influence beach management decisions (100). Interestingly, a recent study described a possible mechanism for the extended survival of some enterococcal species exposed to sunlight: carotenoid pigment quenching of reactive oxygen species in certain strains appears to confer a competitive advantage against sunlight-induced inactivation (over nonpigmented isolates) (217).

Salinity. The ability of enterococci to grow in the presence of salt (6.5% NaCl) is one of the distinguishing characteristics of the genus (see Enterococci and the Genus *Enterococcus*). The greater salt tolerance of enterococci than of fecal coliforms and *Escherichia coli* probably contributes to their better performance as indicators of human health risk in marine recreational waters than members of the coliform group (see Use of Enterococci as Fecal Indicator Bacteria).

Many field (53, 94, 344) and mesocosm (10, 75, 187, 308) studies reported an inverse relationship between salinity and the detection/survival of enterococci (Table 2). A field study conducted in tidally influenced Hawaiian streams (salinity range of 0.60‰ to 37.3‰) found a negative relationship between concentrations of enterococci and physicochemical water parameters (temperature, salinity, and dissolved oxygen content) (344). Similar findings were reported for coastal Mississippi waters (salinity range of 0.00‰ to 26.4‰) and a salt marsh in California (salinity range of 29.4‰ to 30.5‰) (53, 94).

In a mesocosm study conducted in England using raw sewage as a source of enterococci and ambient water of various salinities (6.00‰ to 40.3‰), researchers observed an inverse relationship between salinity and the time required to achieve a 90% reduction in concentrations of enterococci (i.e., less time was required at higher salinities) (187). In a New Zealand mesocosm study utilizing freshwater and marine waters inoculated with either raw sewage or an inoculum from a waste stabilization pond (WSP), enterococci persisted longer in freshwater than in marine waters; interestingly, enterococci from raw sewage were more sensitive than WSP enterococci to salinity (308). The decay of enterococci in freshwater and marine subtropical environments seems to follow the same pattern. Anderson et al. (10) investigated the persistence of FIB from dog feces, wastewater, and soil known to be contaminated with feces in mesocosms filled with either river or Gulf of Mexico waters and sediments (10). Overall, decay rates of enterococci in the marine mesocosms were at least 2-fold higher than in the freshwater mesocosms, regardless of the location (i.e., water column or sediments) (10). Similar results were recorded in a mesocosm study conducted in Australia, where researchers investigated the decay of indigenous sediment enterococci from freshwater (salinity range of 1.6‰ to 3.3‰) and marine (salinity range of 33.8‰ to 35.4‰) environments (75). Over a period of 60 days, concentrations of enterococci from marine sediments decreased by up to 2 orders of magnitude, while freshwater sediments maintained nearly intact concentrations for the duration of the experiment (75) (see also "Environmental Reservoirs and Extraenteric Habitats").

Conflicting results were reported in a field study conducted on the River Seine and the Belgian coast of the North Sea, where the differences in concentrations of enterococci between two different

water types were minimal (229). The authors of that study attributed the decrease in concentrations of enterococci to the actions of predatory protozoa and stipulated that salinity may play a more important role in culturability than in mortality rates, which were assessed by the loss of [^{3}H]thymidine-labeled strains (229).

Disinfection. Disinfection of wastewater is a barrier that is intended to prevent the contamination of receiving waters with FIB and pathogens. The abilities of microorganisms to survive disinfection vary both with the organism and with the disinfection method. Although fecal coliforms or total coliforms are generally used to assess the efficacy of disinfection (152), some studies suggested that the survival of enterococci against disinfection is a better predictor of the fate of viruses than are coliforms (84, 368). Because ineffective wastewater treatment can allow enterococci and pathogens to enter environmental waters, the responses of enterococci to disinfection methods are discussed here.

The most common disinfection strategy in the United States is the utilization of chlorine (or chlorine derivatives such as chloramines) followed by UV light irradiation. In a study examining the efficacy of chlorine disinfection by adding various concentrations of chlorine (11.8 mg/liter to 23.2 mg/liter in the form of sodium hypochlorite) to filter-sterilized wastewater effluent from the primary treatment stage, concentrations of enterococci decreased by more than 5 orders of magnitude after 15 min of contact time (25). Similar results were reported almost 3 decades later, when comparable concentrations of sodium hypochlorite (8.0 to 30.0 mg/liter) were added to wastewater effluent from the primary treatment stage seeded with pure cultures of selected organisms (including *E. faecalis*) (328). In both instances, enterococci exhibited first-order decay: a rapid decrease of culturable enterococci measuring approximately five orders of magnitude was observed after 5 to 15 minutes of contact time, depending on the chlorine concentration (328). Concurring results were reported in a more realistic scenario, where concentrations of enterococci in wastewater influent and disinfected effluent were compared for six wastewater reclamation facilities (five of which used chlorine disinfection) (152). Like fecal coliforms, enterococci were highly susceptible to disinfection, as both FIB were found in only 27% of disinfected effluent samples; however, the decrease in the concentration of fecal coliforms through wastewater treatment was higher than that of enterococci. A higher percentage of disinfected effluent samples contained other types of indicator organisms, including total coliforms, *Clostridium perfringens*, F-specific (F^+) coliphage, and somatic coliphage (152). While chlorine appears to be an effective disinfectant against enterococci (Table 2) (and other non-spore-forming bacteria), the potential for the formation of harmful by-products has led to the exploration of other modes of disinfection, specifically UV light and ozonation.

A review detailing the effectiveness of UV disinfection against enterococci (and other organisms) in wastewater and drinking water systems was recently reported (162). The reported reductions in levels of enterococci are somewhat variable (spanning approximately 2 to 5 orders of magnitude), depending on the treatment processes prior to UV exposure (e.g., sedimentation or coagulation) and the type and intensity of the UV source (155, 162, 174, 194, 301), with higher reductions observed with more extensive downstream processes and stronger UV dosages. Comparisons of UV disinfection efficacies on a seeded laboratory strain of *E. faecalis* versus environmental isolates indicated that the former is more resistant (57, 156, 222). Meta-analyses of the existing literature indicated that chlorine disinfection considerably outperforms UV radiation in reducing concentrations of enterococci by as much as 2 orders of magnitude (58, 152, 182, 272, 273) (Table 2).

Starvation. The transition from the animal gastrointestinal tract, a nutrient-rich environment, to oligotrophic environmental waters exposes enterococci to nutrient starvation, one of the abiotic factors detrimental to their survival. One of the first reports on the survival of enterococci under nutrient starvation conditions indicated that *E. faecalis* survived for extended periods in sterilized sewage (presumably due to the availability of organic nutrients) but declined rapidly in sterile lake water and phosphate buffer, indicating that oligotrophic conditions (exemplified by the sterile lake water and phosphate buffer) were deleterious to the survival of enterococci (302).

Previous studies have identified at least 42 proteins in *E. faecalis* that are induced under starvation conditions (e.g., glucose depletion or incubation under oligotrophic conditions) (122, 124, 146). Furthermore, carbohydrate starvation can enhance resistance to multiple stressors, including heat, oxidative stress, acid, ethanol, and sodium hypochlorite (122, 124, 146, 199). One protein in particular (gls24), belonging to the class A starvation proteins in *E. faecalis* (synthesized in both growing and resting cells but differentially expressed during starvation) (123), was overexpressed under both starvation conditions mentioned above (122, 123). An additional analysis of the protein (and the corresponding *gls24* gene) revealed that the gene is under the control of a stress-inducible operon and that a mutation in *gls24* has a pleiotropic effect on cellular morphology (the formation of shorter chains of cocci), stress sensitivity (reduced growth in the presence of bile salts), and the expressions of several genes involved in pyruvate metabolism (124).

Sigma factors are regulatory proteins that modify stress responses in bacteria by controlling the initiation of transcription and are well characterized for *E. coli* (σ^S) and *Bacillus subtilis* (σ^B) (158, 159, 161); however, their counterpart in the genome of *Enterococcus* spp. has not been fully described. Benachour et al. (24) identified two genes (*sigV* and *rsiV*) in *E. faecalis* that are under the control of the same operon and are predicted to encode sigma and anti-sigma factors. Further analysis indicated the differential expression of the operon in response to exposure to various stresses; notably, it was overexpressed under conditions of glucose starvation and complete starvation, suggesting that it plays an important role in the response of enterococci to nutrient depletion (24). Another *E. faecium* regulatory protein (σ^{54}) was suggested to be a potential virulence factor capable of influencing the rate of autolysis (and, by extension, the nature and composition of the biofilm matrix [173]) and governing sensitivity to certain bacteriocins (51, 74).

The viable-but-nonculturable (VBNC) phenomenon describes a state in which bacteria that can normally be cultured under a defined set of conditions lose that ability while retaining viability, as assessed by measurements of membrane potential, infectivity, mRNA expression, the ability to reproduce, or cell envelope integrity (160, 188, 259, 341). A series of studies explored the starvation-induced existence of the VBNC state in *Enterococcus* spp. (160, 210–212), in which viability assessments included the presence of mRNA, cell envelope integrity, and the ability to reproduce (assessed by Kogure direct viable counts). The authors of those studies found marked differences in the time to the loss of

culturability for various *Enterococcus* spp. and evidence supporting the existence of the VBNC state for *E. faecalis* and *E. hirae*. It is particularly important to improve our understanding of the existence and nature of the VBNC state in enterococci, because VBNC cells would be recognized by molecular methods, such as quantitative PCR (qPCR), but not counted by culture methods, which could well contribute to the large differences in quantities estimated by conventional and emerging methods (318, 341).

Predation. Grazing by bacterivorous protozoa, bacteriophage infection followed by virus-mediated lysis, and predation by some bacteria are among the biotic effects that control the abundance of prokaryotic organisms in the environment. Predation by bacteria has been well described for *Vibrio* spp., most notably *Vibrio parahaemolyticus*, where infection by predatory *Bdellovibrio* spp. plays a role in the population dynamics of these species (230, 319). Bacteriophage infection affects a much wider range of bacteria, and viral infection was suggested to be a mechanism responsible for the elimination of up to 50% of autochthonous bacteria from aquatic habitats (109, 266, 327). Bacteriophages that infect various *Enterococcus* spp. ("enterophage") from different sources (i.e., raw sewage, cow manure, and environmental waters) were recently described (31, 223, 268, 279). However, the effect of enterophage on bacterial survival was not tested directly, since the main objective of these works was to examine the utility of enterophage as a microbial source tracking marker. Nonetheless, the relatively high concentrations of enterophage that specifically infects *E. casseliflavus*, *E. mundtii*, or *E. gallinarum* from cow fecal slurry (10^4 to 10^5 PFU/100 ml) and *E. faecalis* or *E. faecium* ($\sim 10^3$ PFU/100 ml) from raw sewage (268) indicate that, at least in these instances, lysis by enterophage can be a predatory factor on populations of enterococci.

Protozoan grazing is an important top-down control of bacterial populations in aquatic environments (e.g., see references 18, 75, 125, 224, and 229), including allochthonous bacteria such as enterococci (20, 263, 289). Some estimates suggest that protozoan grazing is responsible for up to 90% of the overall mortality of both autochthonous and allochthonous microorganisms from freshwater and marine environments (8, 229). Factors that affect predation rates include temperature and characteristics of prey populations. Digestion rates of both flagellated and ciliated protozoa increased exponentially at temperatures between 12°C and 22°C (294), and a direct correlation between rates of predation and temperature was found in a variety of environments, with more vigorous grazing and an increase in protozoan concentrations at higher temperatures (7, 9, 19, 225, 294). Prey characteristics such as cell wall morphology and the physiological state may also influence the magnitude and efficiency of protozoan grazing (23, 127, 220, 300, 343). Notably, lower rates of grazing were observed for Gram-positive organisms (including *E. faecalis*) than for *E. coli* (75, 126, 169, 170, 253). Nonetheless, several experiments conducted in mesocosms and environmental chambers documented decreases in concentrations of enterococci in marine (29, 75, 146, 229) and freshwater (75, 229, 317) environments in the presence of protozoa (Table 2).

The apparent predilection of protozoa for Gram-negative organisms may be explained by the physiological state of the enterococci and the preferences of different types of protozoa for particular prey. Hartke et al. (147) showed a more active grazing of zooflagellate protozoa on *E. faecalis* cells harvested from the exponential growth phase than on glucose-starved cells, while nanoflagellates did not appear to exhibit a preference (147). Similarly, it was shown that while *E. faecalis* concentrations decreased by more than an order of magnitude over 72 h in coculture with *Acanthamoeba polyphaga*, amoeba levels were ~80% lower than those of the negative controls that were grown in the absence of enterococci (166). That same study found increases in amoeba numbers when cocultured with *E. coli*, *Bacillus cereus*, and *Salmonella enterica* serovar Typhimurium, indicating that enterococci are not a good food source for *A. polyphaga* (166).

Environmental Reservoirs and Extraenteric Habitats

In contrast to the initial conception of *Enterococcus* spp. as inhabitants of the gastrointestinal tract, we are gaining an understanding of the extent to which environmental habitats can serve as sources and sinks of this group. Although the environmental stressors discussed above negatively impact the survival of enterococci, many studies have clearly demonstrated the persistent nature of some *Enterococcus* spp. and strains in extraenteric habitats. Figure 1 illustrates our current understanding of the major sources and sinks of enterococci in environmental habitats. The concepts presented in this graphical illustration are discussed in Use of Enterococci as Fecal Indicator Bacteria. Table 3 provides an overview of studies and findings on the occurrence, persistence, growth, and population genetics of enterococci in extraenteric habitats.

Aquatic and terrestrial vegetation. *Cladophora*, a macrophytic green alga, is found in both fresh and marine waters. Until recently, the impact of *Cladophora* on beach water quality was unexamined, but in a seminal work, Whitman et al. (358) showed that the algal mats collected along shorelines of southern and northern Lake Michigan in the Great Lakes were a significant source of FIB (*E. coli* and enterococci), with densities often exceeding 100,000 CFU/g (dry weight). These findings have been confirmed by a number of studies of Lake Michigan and elsewhere (342). Aside from FIB, enteric pathogens, such as Shiga toxin-producing *E. coli* (STEC), *Shigella*, *Salmonella*, and *Campylobacter*, have also been isolated from these mats, indicating that *Cladophora* may serve as an environmental source of these pathogens in recreational water (41). Enterococci survived in sun-dried algal mats stored at 4°C for over 6 months and displayed the ability to grow to high concentrations ($\sim 10^8$ CFU/g) upon rehydration (358).

The high densities of FIB, including enterococci, in fresh *Cladophora* mats have been attributed to *in situ* growth (42). For instance, enterococci grew over 100-fold in undiluted algal leachate at 35°C in 24 h, suggesting that *Cladophora* provides enough nutrients to sustain these bacteria, which are known to have fastidious growth requirements, as evident by the commercial media used for these bacteria (6). *Cladophora* is perennial in nature, and in temperate waters (e.g., Great Lakes), it overwinters, leaving behind scattered basal stumps; however, there have been no reports of residual enterococci or other FIB surviving in these stumps under wintery conditions.

Other aquatic macrophytes that have been identified as sources of enterococci include decaying seaweed (11, 131, 167). In New Zealand, Anderson et al. (11) observed that the densities of enterococci in drifting seaweed exceeded those in seawater by 2 to 4 orders of magnitude; Grant et al. (131) found densities of enterococci as high as a 450,000 most probable number (MPN)/100 g in a marsh in southern California. In addition to seaweed, recent

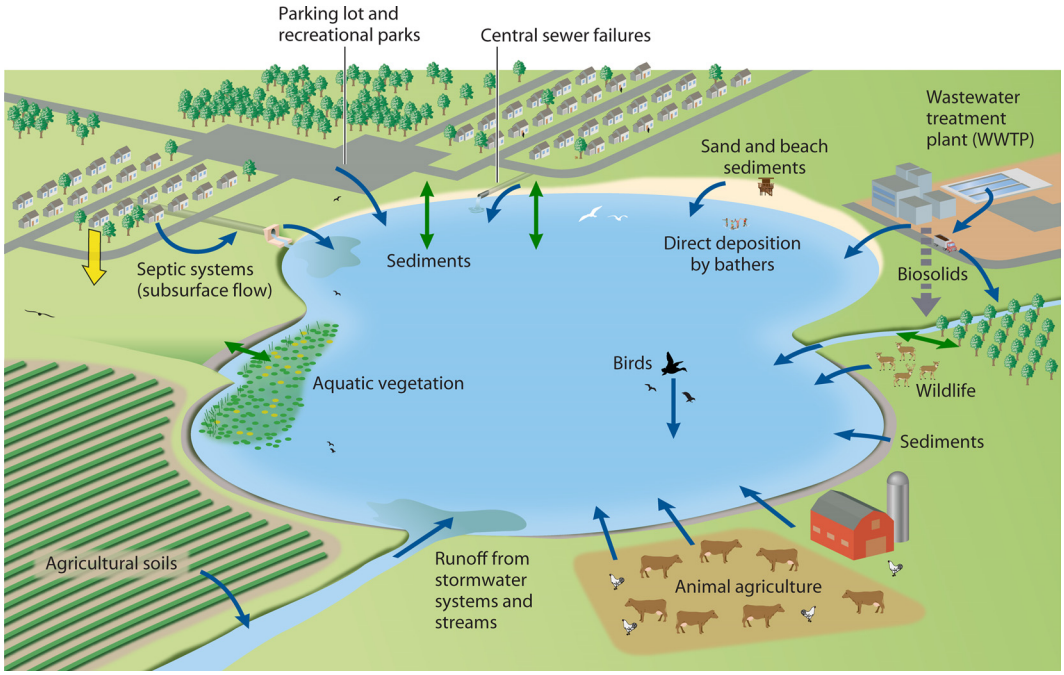

FIG 1 Sources of enterococci in water bodies (blue arrows) as well as sinks where enterococci are immobilized (yellow arrow) and areas of flux, in which enterococci can transition from a reservoir to the water column and vice versa (green arrows). Fluxes act as secondary sources or sinks depending upon the conditions.

studies have expanded the occurrence of enterococci to submerged aquatic vegetation (SAV) (mostly *Hydrilla verticillata*) (15, 16). In laboratory studies, Badgley et al. (15) found that enterococci survived longer and at much higher densities in mesocosms containing SAV than in those without SAV. Furthermore, the recovery of a dominant *E. casseliflavus* strain indicated that this genotype was likely adapted to or naturalized on this vegetation. Aside from macrophytic plants, enterococci have also been associated with planktonic communities and macroinvertebrates (221, 299). Data reported by Signoretto et al. (299) suggested that attachment and the shift to a VBNC state contribute to the prolonged survival of enterococci in marine waters. Furthermore, the time of survival of enterococci is longer in sediments than in water (see "Sediments" below) and in the presence of aquatic vegetation (14, 358).

Some of the earliest findings for the association of enterococci with terrestrial vegetation were demonstrated by Mundt (236), who recovered these bacteria from flowers and buds of different plant species. Recent studies have expanded these findings to forage and crop species (235, 261). Mundt (237) initially suggested that the occurrence of enterococci in plants was seasonal, with maximum recovery in late summer (September), and that these bacteria were transient populations most likely introduced by insects and wind (237). Shortly thereafter, Mundt et al. demonstrated the ability of *E. faecalis* to grow on plants (238), and many other studies have argued for the existence of epiphytic enterococci (204, 235, 261). Furthermore, the finding that certain strains of *Enterococcus* spp. may have environmental adaptations, for instance, *E. casseliflavus* in submerged aquatic vegetation (15) and *E. casseliflavus*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. mundtii*, *E. sulfureus*, and many other strains resembling *E. faecalis* from forage crops (50, 235, 261), strongly supports the existence of plant-associated enterococci.

Beach sand. The sanitary quality of beach sand, i.e., the extent of contamination by FIB, has been a subject of public health concern in recent years (reviewed in reference 142). Numerous investigations across marine and freshwater beaches have repeatedly shown that enterococci and other FIB and pathogens (e.g., *E. coli*, *Salmonella*, and *Campylobacter*) are common microbial contaminants in beach sand, with potential implications for shoreline water quality (120, 278, 290, 355, 363). Whether enterococci are part of the natural resident or transient microflora of beach sand remains unknown; different *Enterococcus* spp. have been recovered in sand from freshwater and marine beaches, e.g., *E. faecium*, *E. casseliflavus*, *E. durans*, and many unidentified species from Lake Michigan (44), and *E. faecalis*, *E. faecium*, *E. hirae*, *E. casseliflavus*, and *E. mundtii* from marine sediments (16, 102). However, the recent demonstration of biofilm-associated enterococci in beach sands (265) argues that some of these populations are resident.

In recent years, efforts have been directed toward an understanding of the sources and contributions of FIB and their potential interactions in the "beachshed" ecosystem, which comprises the various sources of FIB, and their influences, within the beach and its related watershed, as described by Whitman et al. (356) (Fig. 1). Shoreline birds, particularly geese and gulls, have received significant attention because of their abundance and potential influence on water quality (105, 136, 240). Other potential contributors may include beach visitors themselves as carriers of sand-borne bacteria during recreational activities (99, 262); an individual bather might contribute as many as 6.0×10^5 CFU of enterococci through sand particles adhered to the skin (99). Hydrological processes, such as overflows, runoff, and wave surges (27, 136, 271), are among the contributors to diffuse nonpoint sources of enterococci that influence water quality at recreational beaches. A growing body of data suggests that *in situ* bacterial

TABLE 3 Occurrence, persistence, growth, and population genetics of enterococci in extraenteric habitats

Habitat for enterococci	Occurrence (reference[s])	Persistence/survival (reference[s])	Growth (reference[s])	Population genetics (reference[s])
Soil	Recovered in tropical and temperate soils (36, 40, 85, 110, 145, 237)	Persist longer than *E. coli*; survive longer than other FIB (37, 305)	May grow in soil under certain conditions (40, 85)	Different *Enterococcus* spp. have been recovered in tropical soils (40); metabolically diverse strains of *Enterococcus* spp. are found in soil (40, 111)
Sediment	Enterococci are found in both freshwater and marine water sediments (102, 116, 207, 252, 258)	Survive longer in sediments than in water (10, 282); found mostly in surficial layers, with no seasonal trends in distribution (258); hydrometeorological events influence the bacterial flux between sediments and water (116, 134, 282); differential survival observed for freshwater and marine water sediments (75)	May grow in sediments under certain conditions (85, 102, 116)	
Beach sand	Found in both freshwater and marine beaches (44, 120, 142, 264, 278, 363, 366); bacterial distribution is patchy over space and time (44, 142)	Persists longer in moist beach sand and in nearshore and backshore areas (44)	*In situ* growth has been suggested for persistent populations of enterococci in sand (148, 205, 367)	Numerous *Enterococcus* spp., including *E. faecalis*, *E. faecium*, *E. casseliflavus*, *E. mundtii*, and *E. hirae*, have been recovered from beach sand/intertidal sediments (44, 102)
Vegetation	Found in both aquatic and terrestrial vegetation, including algae (358), beach wrack (11, 131, 167), submerged vegetation (14, 15), flowering plants (237), and forage crops (235, 261)	Few days to months for submerged vegetation (14, 15), dried algae (358)	High bacterial densities in vegetation have been attributed to growth (14, 42, 358)	*Enterococcus* spp. have been recovered from the rhizosphere and the phyllosphere (235, 261); evidence for clonal populations (14)
Freshwater	Found in both tropical and temperate freshwaters (110, 114, 136, 145, 181, 193)	Differential survival of enterococci compared to other FIB has been observed (10, 308)	Sporadic growth in nutrient-rich waters has been recorded in the presence of algae (42)	Strains of environmental origin found in catchments (3, 15); diverse *Enterococcus* spp. and strains observed (14, 244)
Marine water	Found in marine waters (27, 297)	Survive longer than *E. coli* (208)	Prolonged survival and potential growth are most likely in moist sand/sediments and associated vegetation (102, 131, 167, 178, 179, 367)	Diverse *Enterococcus* strains have been observed (14)

growth may be an alternative explanation for high levels of enterococci in beach sand (148, 367). Studies conducted in sand-filled columns found that transient growth of enterococci occurred after intermittent wetting of sand (367). At the same time, the replication of enterococci under natural conditions is likely to be limited because of desiccation and other environmental stresses. Despite these limitations, the widespread occurrence of enterococci and other FIB in beach sand (44, 120, 355, 363) can be attributed to repeated seeding from birds and other sources (105, 240) as well as residual surviving populations, especially in moist, subsurface sand in both foreshore and backshore areas (44).

Previous studies have shown that the densities of enterococci in sand vary within and between locations (44, 264, 366). Densities of enterococci as high as 7,200 CFU/100 g were reported in a study conducted in coastal California (366). Lower densities of enterococci in beach sand have been reported in other studies; e.g., densities in samples of wet sand at Avalon Beach, CA, averaged a 310 MPN/100 g (ranging from nondetected to a 4,200 MPN/100 g) (143). Likewise, in a 13-month study at two Great Lakes beaches along southern Lake Michigan, Dunbar, and West Beach, the densities of enterococci (log MPN ± standard error [SE]) in moist subsurface sand near the water table averaged 1.1 (±0.15) and 1.1 (±0.08) MPN/100 g (44). Such variations in densities of enterococci probably reflect contaminant sources, the locations of sampling (e.g., foreshore and backshore areas) (44), and, importantly, methods of sample collection and analysis (28). Currently, there is no established or approved method(s) for FIB analysis in beach sand/sediment substrates, and a patchy distribution of these bacteria in sand environments is highly probable, as observed for other substrates (e.g., soil) (36, 40).

Despite concerted efforts, the management of beach sanitary quality has proven difficult. Various management strategies, such as sand replacement (355), beach grooming (190, 191), and bird harassment using trained dogs, have been attempted, with various degrees of success. The total eradication of FIB and other microbial contaminants might be difficult or simply impractical. For instance, enterococci can survive and persist in moist subsurface sand (44), countering the effects of surface treatments. Other beach management practices, such as beach grooming—a tool routinely applied for esthetic purposes—might help reduce the problem but can be counterproductive when the particulate-associated FIB get dispersed and are deposited deeper, further protecting them and prolonging their survival in sand environments (191). In these situations, the sand can serve as a continuous source or reservoir of FIB and associated pathogens to nearshore waters.

Sediments. Numerous studies have shown that both freshwater and marine sediments are significant sources or reservoirs of enterococci (116, 119, 258, 296), with (sediment) bacterial densities being typically several orders of magnitude higher than those in the overlying water on a per-mass basis (10, 72, 102, 129, 252). High bacterial densities in sediments have been attributed to better resistance to environmental stressors, in particular predation, solar inactivation, starvation, possible regrowth, vegetation, and related factors (10, 141, 167, 179) (see also "Responses to Environmental Stressors"). The prolonged survival of enterococci has similarly been observed in freshwater (10, 15, 141) and estuarine (10, 179) sediments.

Whether enterococci can grow under most environmental conditions remains speculative; however, Mundt et al. (238) demonstrated the growth of *E. faecalis* on germinating seeds and plants. High bacterial densities in sediments (85, 102, 205), in aquatic vegetation (15, 42, 358), and in detritus and planktonic communities (234) suggest that enterococci grow in these nonenteric habitats under certain conditions. Growth of enterococci has been observed in several mesocosm studies: in beach sand (367), algal washings (42), rehydrated algal (*Cladophora*) mats (358), and aquatic vegetation (15). These findings collectively support their growth capabilities in the environment; however, more studies are needed to better understand this ecological process.

Because of their close interaction with surface water, sediments play a major role in influencing shoreline water quality through the resuspension of the particle-bound bacteria in the water column. While the quantification of bacterial loads from sediments by conventional methods might be difficult, alternative techniques such as hydrodynamic or empirical modeling have increasingly been used in recent years to better understand this process (118, 130). In large bodies of water, such as the upper Chesapeake Bay, more than 80% of indicator organisms, including fecal streptococci and fecal coliforms, were found to be associated with suspended sediments (282). Such processes are often mediated by mechanical disturbances during recreational activities (7), hydrometeorological events (including high-flow, wind, and erosional conditions [116, 175, 274, 312] and river outfalls [98, 249]), as well as dredging operations (134, 135). Collectively, such events can increase FIB densities in the water even in the absence of any significant human inputs.

Soil. Some of the earliest research on the survival of enterococci in soils was conducted with experimental plots by van Donsel et al. (340), who observed that the rates of survival of *Streptococcus faecalis* were higher than those of fecal coliforms during spring and winter, and while there was no difference in the survival patterns in the autumn, fecal coliforms survived longer than *S. faecalis* during summer months. Interestingly, many of the early investigations of the survival and persistence of enterococci/fecal streptococci in soil environments focused on watersheds impacted by anthropogenic activities, particularly cattle grazing and field lot operations (92, 165, 176). Recent studies confirmed that populations of FIB (*E. coli* and enterococci) are equally abundant in relatively less-impacted soils (46, 85, 110, 200). For instance, a survey of soils on the island of Oahu, HI, showed that enterococci were nearly ubiquitous compared to *E. coli* (98% and 54% frequencies in surveyed soils, respectively), with enterococcal counts often exceeding a 1,000 MPN/g soil (40).

High densities of enterococci in soils may be attributed, in part, to the greater survival abilities of Gram-positive bacteria (e.g., enterococci and staphylococci) than of Gram-negative bacteria (e.g., *E. coli*, *Pseudomonas* spp., and *Rhizobium* spp.) in the face of environmental stresses, particularly cellular injury and desiccation (17, 245). In one mesocosm study, densities of seeded *E. faecalis* remained nearly constant (~6.0 log CFU/g dry soil) for 8 days when the moist soil (35% moisture, corresponding to a 60% water-holding capacity) was allowed to desiccate (12% moisture) under laboratory conditions (25°C). *E. coli* densities, on the other hand, declined drastically from 6.0 log CFU/g to <1 CFU/g in 4 days but returned to the original levels upon rehydration (37). Similarly, enterococci survive longer than other enteric bacteria under certain field conditions: in cow feces, a 90% inactivation of enterococci occurred after 56 days, followed by *E. coli* (48 days),

Salmonella enterica (38 days), nonenterococcal fecal streptococci (35 days), and *Campylobacter jejuni* (6.2 days) (305).

It has been argued that soil environments provide the necessary niche for populations of FIB to survive, adapt, and grow in these heterothermic habitats (115, 171, 361). While the growth requirements of *E. coli* are relatively simple because of its ability to synthesize cellular macromolecules from glucose and minerals (12), enterococci require complex nutrients (e.g., growth factors), even when grown under laboratory conditions on commercial media such as m-Enterococcus agar and mEI (6). Although enterococci are relatively common in some tropical soils (40, 110, 145), studies of growth characteristics in these environments are rather limited. In one study, enterococci grew only marginally in the presence of full competition from the native microbiota; however, in the presence of nutrients (peptone) and reduced competition (achieved by the addition of sodium azide), enterococci grew more than 100,000-fold over 13 days (38).

The paucity of available nutrients may thus limit the growth of FIB in soil environments, yet a likely habitat that provides conditions for spurts of growth is the plant rhizosphere region, where microbial activity is known to be severalfold higher than in the adjacent bulk soil (310). The various compounds released by plant roots as exudates into the surrounding soil are highly diverse and complex, including amino acids, growth-promoting and growth-inhibiting substances, low-molecular-weight sugars, organic acids, polysaccharides, and proteins (139, 310, 349). Additional studies are needed to better understand pathogen and FIB ecology in the rhizosphere.

Populations of enterococci represent only a small part of the soil microflora. For instance, in six soil samples collected on the campus of the University of Hawaii, culturable heterotrophic bacteria were about 10,000- to 10,000,000-fold more numerous than enterococci (37). Furthermore, the widespread range of these bacteria in soils throughout the island of Oahu (40), comprised of at least six different species of *Enterococcus* (40, 111), strongly supports the hypothesis of environmentally adapted or autochthonous populations in soil environments.

While the original source of populations of enterococci in soil is debatable in some cases, potential sources include human and animal (including wildlife) waste (Table 1 and Fig. 1), and over time, a subset of the original population may have adapted to the soil environment. In summary, aquatic and terrestrial vegetation, beach sand, freshwater and marine water sediments, and soil have been identified as some of the major environmental sources of enterococci and other FIB. FIB derived from these sources can potentially impact the water quality of associated beaches and watersheds, and thus, there is a need for a better understanding of their fate in these ecosystems.

USE OF ENTEROCOCCI AS FECAL INDICATOR BACTERIA

For over a century, FIB have been used to assess water quality and protect humans from the myriad of enteric pathogens that are transmitted by the waterborne route by acting as fecal indicators (reviewed in references 277 and 362). FIB are generally commensal inhabitants of the gastrointestinal tracts of many warm-blooded animals and are shed in feces at high densities; thus, they are easily detected in contaminated waters. Ostrolenk et al. (260) were among the first to suggest that the enterococci might be more appropriate FIB than *E. coli* (260), and studies conducted in the 1970s confirmed this suggestion for marine waters (49, 96). More recently, multiple studies have shown a correlation between elevated concentrations of enterococci and the risks of humans contracting gastroenteritis during recreational water use, particularly when point source contamination is present (186, 267, 333, 335).

The use of enterococci as FIB has been criticized almost since their adoption as a regulatory tool (64), because the epidemiology studies on which the standards were based were focused solely on waters contaminated by point source (particularly human sewage) pollution (47, 333). Little was known about the relationship of enterococci and other FIB to human health in recreational waters contaminated by nonpoint sources when the regulations were promulgated (115). Recently, some studies found an association between densities of enterococci and illness rates at beaches impacted by nonpoint sources of contamination (104, 304). While there was an increased incidence of gastrointestinal illness, respiratory illness, and skin illness in bathers in one study (104), the only health effect with a dose-response relationship to concentrations of indicator bacteria in both studies was skin illnesses (104, 304). Furthermore, in a comparison of analytical methods, the dose-response relationship for skin illness was seen only with samples analyzed by membrane filtration (304). Enterococci are currently the only FIB recommended by the U.S. Environmental Protection Agency (EPA) for brackish and marine waters, since they correlate better with human health outcomes than other FIB, such as fecal coliforms or *Escherichia coli* (346–348). Several epidemiological studies have also shown a correlation between concentrations of enterococci in beach sands and gastrointestinal illness in bathers (31, 157).

Characteristics associated with "ideal" FIB include a lack of virulence; the existence of a simple, rapid methodology for enumeration; survival characteristics that are similar to those of pathogens in extraenteric environments; and a strong association with the presence of pathogens (49). In contrast to this ideal, studies have shown that populations of enterococci may be endogenous in sediments and soils and not exclusively of fecal origin, which may confound accurate water quality assessments (37, 85). Furthermore, many domestic and wild animals can contribute enterococci to water bodies (Table 1), which complicates the FIB-pathogen relationship since the suite of pathogens associated with various animal gastrointestinal tracts and the risk associated with fecal contamination are highly variable (309, 364). Figure 1 depicts some of the many possible sources (blue arrows) of enterococci in environmental waters, which include human sources, such as sewage and its many derived products, e.g., biosolids, and fecal shedding from recreational water users. Other important sources are agricultural contributions, which may come directly from animals, e.g., cattle or swine defecating in and near water bodies, or indirectly from activities such as the spreading of manure or poultry litter on fields (334). Wildlife (e.g., birds, deer, feral hogs, and raccoons) (Table 1) can be sources of enterococci in urban and rural environments, either via direct deposition (represented by the gull depicted mid-lake in Fig. 1) or in runoff. The particulate matter in storm water contributes to the transport of enterococci in receiving waters and eventual deposition into sediments (90). Enterococci may also attach to aquatic vegetation and detritus (14–16, 234). When sediment is disturbed by high flow, waves, or the activity of humans or animals, enterococci can recontaminate the water column in what can be considered a flux (Fig. 1, green arrows). Here, we define a flux as a transport pathway for enterococci that begins with the primary source (e.g., fe-

ces), followed by deposition to a sink, in which enterococci are temporarily sequestered (e.g., sediments). In the case of a flux, the sink is temporary and eventually becomes a secondary source when organisms reenter the water column following a disturbance (183, 207). Likewise, fluxes of enterococci from aquatic vegetation to the water column or runoff from a field to a stream can constitute a secondary source. Permanent sinks, in which enterococci are deposited into an area from which they have very little probability of being transported to water, are less common (Fig. 1, yellow arrow); appropriate examples would be properly functioning on-site wastewater disposal systems such as septic systems and pit toilets.

Several studies have reported difficulty in finding media that can effectively enumerate the broad range of *Enterococcus* spp. without sacrificing specificity to the genus (204, 288), and the identification of isolates of enterococci from environmental matrices (e.g., plants, soil, sediments, sand, and water) remains challenging (14, 43, 89, 151, 235). Upon the initial introduction into an extraenteric environment, enterococci may become rapidly inactivated (see also "Responses to Environmental Stressors"), which could potentially result in false-negative results when enterococci are used as pathogen surrogates (226, 281). Conversely, the underlying sediments and aquatic vegetation can act as reservoirs for enterococci (see also "Environmental Reservoirs and Extraenteric Habitats") (14–16, 85, 205), which may lead to overestimates of health risks when pathogens are not similarly persistent.

Several methods for the detection and enumeration of enterococci have been successfully used and are prescribed by regulatory agencies to predict health risks. The epidemiological studies conducted in the 1970s that were used to set recreational water quality criteria (48, 49) concentrated enterococci by membrane filtration and cultured them on mEI medium; consequently, membrane filtration methods are the current "gold standard" for water quality assessments (336). In addition to standard methods using membrane filtration, numerous monitoring laboratories have also relied on alternative culturing techniques. A comparison of membrane filtration with multiple-tube fermentation and chromogenic substrate methods, i.e., Enterolert (34), showed that results did not vary significantly by method (132) and were being used interchangeably to manage beaches across a large portion of Southern California. With the increasing frequency and number of beaches being monitored for enterococci since the passage of the BEACH Act (22), many locations are using either chromogenic substrate or membrane filtration analytical techniques, with results being used interchangeably across jurisdictions for beach management (246).

The benefits of both culture-based methodologies discussed above are that the techniques are easily learned and the methods are not costly (254). Furthermore, concentrations of enterococci obtained by using culture-dependent methods have shown significant correlations with human health risks in estuarine and marine waters (47, 333). Despite the demonstrated advantages, the drawback of these culture-based methods is that they have a lengthy time lag (18 to 24 h) before results are obtained (201). This lag results in the postponement of decisions on risk management for recreational water use, potentially exposing humans to health threats between the sample collection time and the reporting of results, as FIB concentrations can vary widely across small spatial and temporal scales (32, 201, 250, 254, 354). In other words, by the time the testing results are reported, the contamination that caused the elevated FIB concentrations may have dissipated, leaving the water body safe for use by the time a warning is eventually posted.

EMERGING TECHNOLOGIES FOR DETERMINING CONTAMINATION SOURCES, ASSESSING WATER QUALITY, AND DETERMINING HUMAN HEALTH RISKS

Microbial Source Tracking

While the presence of enterococci in the feces of a wide range of animals is a useful characteristic for a general indicator of fecal contamination, no information on the contamination source is provided by the quantification of the group as a whole. Knowledge of fecal contamination sources is useful or required in many scenarios, e.g., for total maximum daily load (TMDL) assessment, risk assessment for water use, and remediation of polluted water bodies (137, 153, 337). Microbial source tracking (MST) methods, which target host-specific microorganisms as identifiers of fecal or sewage sources in water bodies, have repeatedly addressed the lack of specificity of conventional FIB (recently reviewed in references 137, 153, and 275).

The enterococci have been the focus of the development of several MST methodologies (reviewed in reference 315). Library-dependent methods require a large database of FIB from the feces of host species; FIB are isolated from feces and genotyped or phenotyped (149) to identify specific characteristics or traits for discrimination among strains. Once the accuracy of the library categorization of isolates by host source is ascertained, isolates from water or other matrices are then compared to library isolates for assignment to source categories. Although field studies that used enterococci as source identifiers for library-dependent MST methods initially indicated promise for use in a regulatory context (138, 154, 359), the expense, difficulty in the interpretation of results, and uncertain accuracy of such methods (315) have discouraged their general use. The potential for the extended persistence and possible growth of enterococci in extraenteric habitats (Fig. 1) further complicates the interpretation of results from library-dependent MST methods. Instead, the focus of MST has turned to library-independent methods, which generally rely on PCR to identify gene fragments (markers) specific for microorganisms that are host associated (153).

The *esp* gene of *E. faecium* (esp_{fm}) is strongly human associated (5, 287, 357), although a low frequency of cross-reactivity with nonhuman feces has been noted, and it is not readily detected in some sewage sources, such as on-site (septic) systems (4, 357). The occurrence of esp_{fm} was correlated with human polyomaviruses in polluted surface waters in Florida (227) and with fecal coliforms in another study (197). It has also been used in field studies in Florida, the Great Lakes, and Australia (5, 39, 196, 209). A quantitative PCR (qPCR) method for esp_{fm} has been developed and used in field studies in Australia (4). Interestingly, the presence of the *esp* gene was found to affect the transport of *E. faecium* in saturated quartz sands by lowering bacterial mobility through increased attachment to sand particles (184).

A novel approach for identifying MST markers of enterococci associated with various hosts was proposed by Soule et al. (311), who used DNA microarrays to identify candidate host-specific *Enterococcus* species and associated genes. The use of bacteriophages specific to certain strains of *Enterococcus* spp. was also recently explored (32, 268) (see also "Responses to Environmental

Stressors"). Many other MST methods rely on microbial groups other than enterococci (recently reviewed in references 275 and 365), including anaerobes such as the *Bacteroidales* (e.g., see references 26, 291, and 292) and *Methanobrevibacter smithii* (331) and viruses (106, 227, 228). The correlation of MST marker detection or concentrations with concentrations of enterococci has varied across studies: Harwood et al. (150) found no correlation between concentrations of enterococci and levels of human sewage markers in untreated sewage; however, enterococci and the human Lachno2 marker were strongly correlated in a freshwater harbor that received combined sewer overflows (251). As is the case for currently recognized FIB such as enterococci, the usefulness of MST for water quality assessment is ultimately predicated on the correlation of human health risk and pathogen presence with host-specific markers; however, there are many data gaps remaining in this growing area of research.

Quantitative Microbial Risk Assessment

The term quantitative microbial risk assessment (QMRA) refers to a risk analysis framework and process for defining the type(s) of microbial hazard that is likely to be encountered in a given situation and the magnitude of the probable harm (risk), usually to some human population (177). Over the past decade, QMRA has increasingly been applied to hazard estimations for recreational water quality, and enterococci are frequently employed in these models (13, 329). Schoen et al. (285) found that measurements of levels of enterococci by culture methods are likely to underestimate the risk of gastroenteritis caused by enteric viruses in recreational waters where contamination is from mixed sources; in contrast, qPCR estimates of densities of enterococci were more reliable predictors of norovirus and human health risk. QMRA has been used to estimate the relative risk from contamination by human sewage versus animal sources in models that use the U.S. EPA's recreational water quality criterion for enterococci (35 CFU/100 ml) as one reference point (284, 309). Among the sources examined, gull fecal contamination carried the least human health risk, and cattle contamination carried the greatest (309). Another study estimated that rain events and storm water runoff increase health risks to surfers (329). QMRA has been recommended as an important component of a "holistic" approach to recreational water quality assessment (13), which includes extensive knowledge of the watershed(s), including potential pathogen sources and transport pathways. An important caveat in all risk assessment models that use FIB as surrogates is that the ratio of FIB to pathogens is highly variable in contaminating fecal material and in water samples (13); therefore, users must be cognizant of the limitations of such models.

Rapid Testing Methods

While epidemiological studies conducted at sewage-impacted beaches continue to support the association between concentrations of enterococci and rates of swimming-related illnesses (345, 346), the time lapse between sample collection and the availability of results severely compromises their usefulness in making appropriate decisions regarding the opening or closing of beaches. Efforts to overcome these shortcomings have included the use of rapid enumeration methods such as qPCR (155), alternative indicators that are more specific to the contaminant sources (e.g., human-associated *Bacteroides*, *Catellicoccus* gull fecal markers, and *Brevibacterium* poultry fecal markers) (26, 213, 351, 352), direct monitoring for potential pathogens and QMRA (see above) (13), and predictive modeling (164, 249), but cost, ease of use, and sustainability as a monitoring program must all be considered in optimizing the application of any newer method or monitoring technology. More information on the sensitivities and specificities of individual tests are provided in other sections.

Alternate methods for the enumeration of enterococci in surface waters that do not rely on bacterial growth and therefore are more rapid and have the potential to become "real-time" tools for water quality assessment have been developed in recent years. Among these methods, qPCR (e.g., see reference 155) has been the most widely tested method and is currently under consideration for application in beach programs. While membrane filtration relies on the detection of living and culturable enterococci, qPCR quantifies DNA from both living and dead cells, a difference with potential implications for regulatory and management decisions. Some studies identified a correlation between the two endpoints in side-by-side comparisons with culture-dependent methods (45, 201, 298). Direct comparisons of the results of the two tests have been discouraged due to differences in variation along a concentration gradient and fluctuations in outcomes due to the original source of the enterococci (201, 353). Inhibition during qPCR analysis has been a significant issue, and efforts to refine the technique have led to numerous modifications of the original protocol, including purification kits, additional filtration steps, and smaller sample volumes (256). The lack of universal standards, calibrators, and methods complicates the use of this test as a monitoring standard. Recent method validation studies have begun to address these concerns (93, 201, 350).

Another emerging technology that has been widely tested is immunomagnetic separation-ATP (IMS-ATP) (206). In this analysis, target enterococci or other FIB are concentrated and separated through the use of specific, antibody-coated immunomagnetic beads; the cells are then quantified by measuring the bioluminescence response from the bacterium's ATP (206). Unlike qPCR, IMS-ATP targets only metabolically active cells; however, the use of ATP as the target can result in the detection of organisms that may not be culturable by using standard culture methods (35). Because IMS-ATP depends on active cellular metabolism, it may underestimate target concentrations compared to methods that measure total cells, such as qPCR. In a comparison study of numerous test methods, IMS-ATP analysis and culturable enterococci showed a strong correlation between the two results, with the exception of one location (35). Further comparisons indicated that IMS-ATP suffered from a large number of false-positive results (133). The cost of the equipment and analytical reagents and the need for technical expertise/personnel may limit its application for routine monitoring.

Further analytical approaches have sought to target multiple potential indicators and pathogens simultaneously by using molecular techniques. For example, the Luminex (Luminex Corporation, Austin, TX) detection system has been developed to test multiple targets through the detection of DNA, RNA, or proteins; this technique has been used to analyze FIB (e.g., *E. coli* and *Enterococcus* spp.) and pathogenic bacteria such as *Shigella* spp. in a multiplex format (21). In brief, the extracted DNA is marked with probes, and a detection system determines the overall concentration of the target microbes. Experiments using natural waters found that the methodology worked best on river water samples. The targets were not as concentrated in beach water and sand;

therefore, group-specific primers were developed to optimize the technique for these natural waters (21). Initial results indicated that the system could detect *Enterococcus* spp., but quantities were often quite different from those obtained by culture methods (21). Further method validation indicated that the Luminex system had the highest specificity and sensitivity for *Enterococcus* over those of IMS-ATP or any of the currently used DNA-based methods, such as PCR and qPCR, with no false-positive results for the negative controls (133). That study cautioned that the system has yet to be fully developed for use with natural water samples; as with many molecular methods, natural water samples introduce inhibition and other obstacles to accurate detection (313).

Aside from comparisons among analytical methods, the issue of primary importance is the usefulness of a given method for predicting health risk. Thus, any new application with a weak or no clear relationship between the measured parameter and human health would be less useful for the management of recreational water quality. Overall, qPCR results for enterococci have generally correlated well with illness rates at sites impacted by point sources (345, 346, 348). However, if the contaminants are from nonpoint sources (e.g., storm water), evidence for effects on health is thus far conflicting; i.e., several studies did not find a correlation between qPCR for enterococci and health effects (2, 64, 104, 304), but one did (63).

Predictive Modeling of Levels of FIB

In addition to advancing molecular technologies to develop rapid tests for enterococci, efforts have been made to improve reporting accuracy by predicting concentrations of enterococci *in situ* by using statistical models. Predictive models have also been encouraged by the U.S. EPA (332), and as such, they have been used in numerous locations in the United States. Unlike many of the rapid analytical tests developed, predictive models are not hindered by interference from other materials suspended in natural beach water. Typically, beach monitoring data for FIB are collected in concert with data for hydrometeorological variables, such as wave height, solar insolation, and wind direction, and the combination of parameters that best predicts the concentration of enterococci is determined through statistical modeling (246). Statistical models have included regression (246, 298), Bayesian analysis (69), and neural networks (219); the complexity of the type of model that is tested is determined by need and application: more simple models can be used for daily predictions, while complex models integrate numerous parameters and may be used for determining contamination sources and pathways in order to develop mitigation plans. Simple models, such as rainfall threshold, allow for immediate management decisions (314), while more complex models, incorporating multiple predictive variables, require additional technology and expertise (246). Predictive models have met with uneven success; the source of contamination and characteristics of the beach structure generally influence the predictability of concentrations of FIB (247, 298). Common predictors can be linked directly to the physical persistence of enterococci in water: solar insolation affects bacterial die-off, wave height influences the resuspension of settled particle-attached bacteria, and wind direction influences the advection of bacterium-containing plumes from point sources such as rivers and streams (246). Predictive models can be developed which provide results in a fraction of the time currently required for FIB culturing techniques and even rapid molecular methods. Public health improvements with the use of a predictive model have not been adequately assessed, but one study indicated an improvement in overall health protection with the use of a model over standard, culture-dependent techniques (248).

CONCLUSIONS

Major advances in the understanding of the phylogeny of the enterococci and the ecology of the group in secondary habitats have been made over the past several decades. While these advances aid in the protection of human, animal, and environmental health, many gaps remain. From our perspective, among the most important areas for further research is the gaining of a better understanding of the relationship between environmental enterococci and a range of human pathogens (e.g., *Campylobacter*, *Salmonella*, enterotoxigenic *E. coli*, *Vibrio* spp., and *Listeria monocytogenes*) commonly transmitted through contaminated water and food networks. Additional epidemiological studies of waters impacted by nonpoint source pollution that include the identification of enterococci to the species level and the quantification of pathogens are also needed to better understand the human health risks associated with elevated levels of FIB. Measurements of nutrient levels (e.g., total organic carbon, nitrogen, phosphorous, and micronutrients) in conjunction with survival studies that are realistic simulations of aquatic environments will provide a further understanding of the drivers of the fate of enterococci in extraintestinal habitats. The possible ecological roles of enterococci in extraintestinal habitats, e.g., the decomposition of organic matter, competition with other members of the microbial community, and the protection of plants from pathogens, warrant further investigation. When used in conjunction with the quantification and species-level identification of enterococci, next-generation sequencing technologies may well revolutionize our understanding of the ecology of these organisms and their continued usefulness as FIB for recreational waters worldwide. A coordinated, international effort that focuses on the issues outlined above and that involves academic and regulatory research scientists could produce a comprehensive data set that allows us to define the impact of ecological factors on the survival of enterococci and the relationships among enterococci, pathogens, and human health in environmental settings.

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