Comparison of *Enterococcus* species selectivity using Enterolert and EPA Method 1600

Donna M. Ferguson, John F. Griffith, Charles D. McGee[1] and Stephen B. Weisberg

Abstract

Enterococcus species selectivity between Environmental Protection Agency (EPA) Method 1600 and Enterolert® was compared using 17 environmental samples (influent and effluent of four wastewater treatment plants, ambient marine water from seven different beaches, and urban runoff). *Enterococcus* colonies from EPA 1600 agar plates and Enterolert wells were identified and species composition was compared using Vitek® and biochemical testing. *Enterococcus faecium* and *Enterococcus faecalis* were the dominant species among the 1345 isolates, followed by *Enterococcus gallinarum* and *Enterococcus casseliflavus*. EPA Method 1600 and Enterolert produced comparable ratios of enterococcal species for most beach water and urban runoff samples; however, Enterolert was selective for *E. faecalis* in wastewater samples. This selectivity was experimentally confirmed in the laboratory through analysis of samples with known concentrations of *E. faecium* and *E. faecalis*. The species distribution of *Enterococcus* isolated from most environmental samples differed only slightly between the two methods; however, when distributions did differ, EPA Method 1600 characterized *Enterococcus* populations more accurately. EPA Method 1600 and Enterolert are used interchangeably in beach-water quality monitoring programs that measure *Enterococcus*, with comparable reliability for enumeration purposes in most cases. However, the species selectivity observed in Enterolert may explain why numerical results occasionally differ when both methods are used to measure concentrations of *Enterococcus* in the same sample. Due to this selectivity, the EPA Method 1600 is preferred over Enterolert for characterizing the species distribution of culturable *Enterococcus* in the environment.

Introduction

EPA Method 1600 and Enterolert are two frequently used methods to measure *Enterococcus*, a fecal indicator bacteria group used to assess recreational bathing water quality (Weisberg *et al*. 2007). EPA Method 1600 is a membrane filtration approach, in which water is passed through a membrane that is subsequently placed atop *Enterococcus* Indoxyl-β-D-glucoside (mEI) agar and following incubation, examined for colonies with blue halos. Enterolert is a defined substrate methodology that measures a fluorescent endpoint based on enterococci metabolizing 4-methylumbelliferone-β-D-glucoside in liquid media.

Both of these methods are EPA approved and often used interchangeably in beach water quality monitoring programs. A number of studies have found that these methods are generally comparable for enumerating enterococci, but the methods sometimes produce markedly different results on a small percentage of samples (Budnick *et al*. 1996, Eckner 1998, Noble *et al*. 2004, Griffith *et al*. 2006). One possible explanation for these differences is species selectivity, particularly as related to differences in the growth media used for each method. Here we compare the distribution of *Enterococcus* species cultured by the two methods.

Methods

Two tests were conducted to compare *Enterococcus* species identification between EPA Method 1600 and Enteroler (IDEXX, Westbrook,

[1] *Orange County Sanitation District, Fountain Valley, CA*

ME, USA). The first involved 17 environmental samples, while the second involved a laboratory-created sample with known species composition.

The environmental samples were collected from seven marine beach sites, two freshwater urban runoff sites, four wastewater treatment plants (WWTP) primary influent, and four WWTP secondary effluents (Table 1). The beach water was collected using 100-ml plastic bottles at ankle depth upon an incoming wave. Freshwater was collected as 1-L samples just below the water surface. Wastewater was collected as 1-L samples from influent or effluent pipes.

Samples were analyzed within six hours of collection following EPA standards (USEPA 2002) and the Enterolert manufacturer's instructions. For EPA Method 1600, isolates were obtained by selecting colonies with blue halos from mEI agar (Northeast Laboratory, Waterville, ME) and subculturing them onto tryptic soy agar (TSA) agar with 5% sheep blood (Northeast Laboratory). After 24 hours of incubation at 35°C, the blood agar plates (BAPs) were assessed for purity. Up to five colonies from the BAPs were subcultured onto a TSA slant (Northeast Laboratory) and incubated as before. The TSA slants were stored at 4°C until speciation was performed.

Isolates from Enterolert Quanti-Trays® were obtained following the method described by Kinzelman *et al*. (2003). The back of the Quanti-Tray was disinfected with 70% alcohol, and media from up to 5 fluorescing (positive) wells was withdrawn using sterile syringes and needles. The media was then inoculated into brain heart infusion (BHI) broth containing 6.5% NaCl (BD, Franklin Lakes, NJ) at a 1:20 dilution. Inoculated broth was then incubated at 41°C for 48 hours; cultures with growth were subcultured onto BAPs. Colonies from the BAPs were subcultured onto TSA slants, incubated and stored at 4°C.

Isolates from the TSA slants were subcultured onto BAPs 24 hours prior to species identification using the Vitek microbial identification system (bioMérieux, St. Louis, MO). Isolates identified as *Enterococcus* species but at low discrimination (<80% confidence level) were categorized as "unidentified". Isolates identified as species other than *Enterococcus* were categorized as "non-*Enterococcus*". Motility and pigment testing was performed as per Ferguson (*et al*. 2005) on *E. casseliflavus*/*E. gallinarum* isolates that could not be discriminated using Vitek. Since *Enterococcus mundtii* is not identified by Vitek (Moore *et al*. 2006), 10% of all isolates were screened for this organism based on published biochemical tests, including: motility; pigment production; and fermentation of arabinose, sucrose, and mannitol (Facklam and Collins 1989).

The laboratory created sample consisted of clean seawater inoculated with approximately 1,000 colony forming units per 100 ml each of *E. faecium* and *E. faecalis* . The seawater was collected 18 km offshore, at 10-meter depth with no measurable enterococci present. The enterococci cultures were prepared using three strains isolated from environmental samples that were enumerated using EPA Method 1600 and Enterolert and identified to species using Vitek.

Table 1. Sample source location/agency by type.

Beach Water
Imperial Beach, San Diego
San Mateo Beach, San Clemente
Doheny State Beach, Dana Point
Cabrillo Beach, Los Angeles
Surfrider Beach, Malibu
Paradise Cove, Malibu
Big Sycamore, Malibu
Urban Runoff
Dominguez Channel, Los Angeles
Tijuana River, San Diego
Wastewater Influent and Effluent
Joint Water Pollution Control Plant of the Los Angeles County Sanitation District
Orange County Sanitation District, Huntington Beach
South Orange County Wastewater Authority, Dana Point
Encina Wastewater Authority, Carlsbad

Results

In this study, 1,345 presumptive enterococci isolates were examined (Table 2), which were found to include 9 species of *Enterococcus* (Figure 1). *Enterococcus faecium* and *E. faecalis* were the most frequent species obtained using Enterolert, with each species comprising about one-third of the isolates (Figure 1). Among the isolates obtained using EPA Method 1600, *E. faecium* was more common (44.3%) than *E. faecalis* (12.8%); *E. gallinarum* and *E. casseliflavus* were the next most frequently identified species by both methods. Non-*Enterococcus*

Table 2. Number of samples and isolates analyzed by EPA Method 1600 and Enterolert.

Source (Number of Samples)	Number of Isolates		
	EPA Method 1600	Enterolert	Total
Beaches (7)	275	303	578
Urban Runoff (2)	91	99	190
Wastewater Influent (4)	126	130	256
Wastewater Effluent (4)	129	126	255
Culture (1)	20	46	66
Total	**641**	**704**	**1345**

species comprised 9.5% of the isolates derived from Enterolert and 5.4% from EPA Method 1600. The most common non-enterococcal bacteria isolated were *Proteus mirabilis* from Enterolert wells and *Aerococcus viridans* from EPA Method 1600. Other species identified by both methods included *Streptococcus bovis*, *Streptococcus uberis*, *Streptococcus mutans* and *Streptococcus pneumonia*. Approximately 4% of the isolates were identified with a low level of certainty and classified as "unidentified".

Overall, the distribution of the most common species found (i.e., *E. faecalis* , *E.* faecium, *E. gallinarum* and *E. casseliflavus* and non-*Enterococcus*) using Enterolert and those found using EPA Method 1600 were not significantly different (X^2, (1, N = 1,104) = 47.4, p <0.001)). However, differences attributable to the relative dominance of *E. faecium* and *E. faecalis* were prominent for the wastewater samples, with *E. faecalis* observed to be the most frequent species identified using Enterolert and *E. faecium* found to be dominant based on EPA Method 1600 (Figure 2). Although the *Enterococcus* species distribution among urban runoff and beach water samples were generally comparable, Enterolert selectivity for *E. faecium* was observed for one of seven beach samples (Paradise Cove Beach), in which

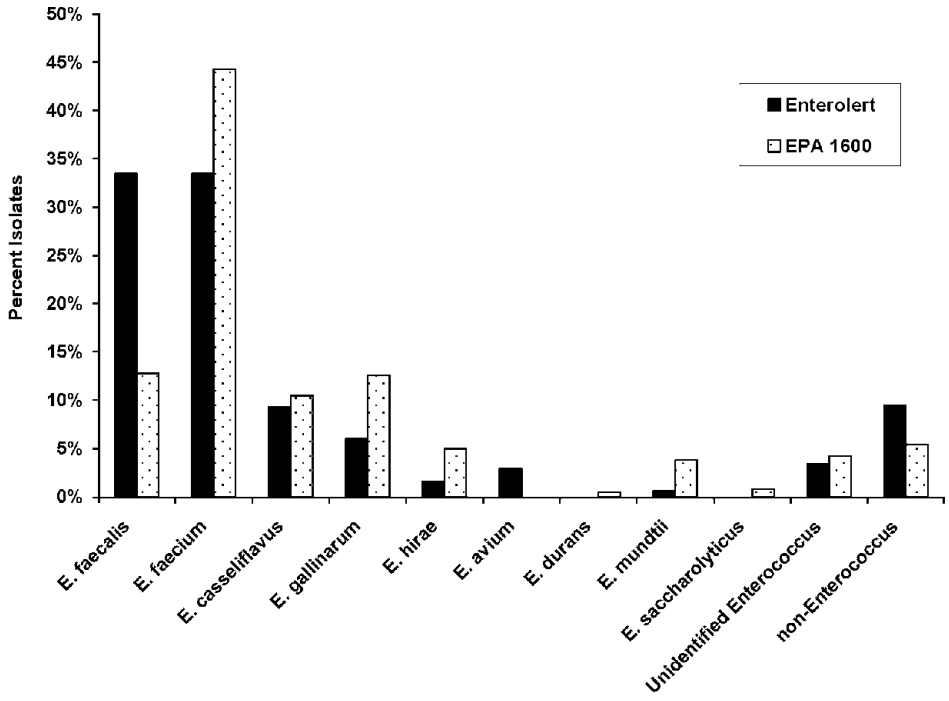

Figure 1. *Enterococcus* species found overall using Enterolert vs EPA Method 1600.

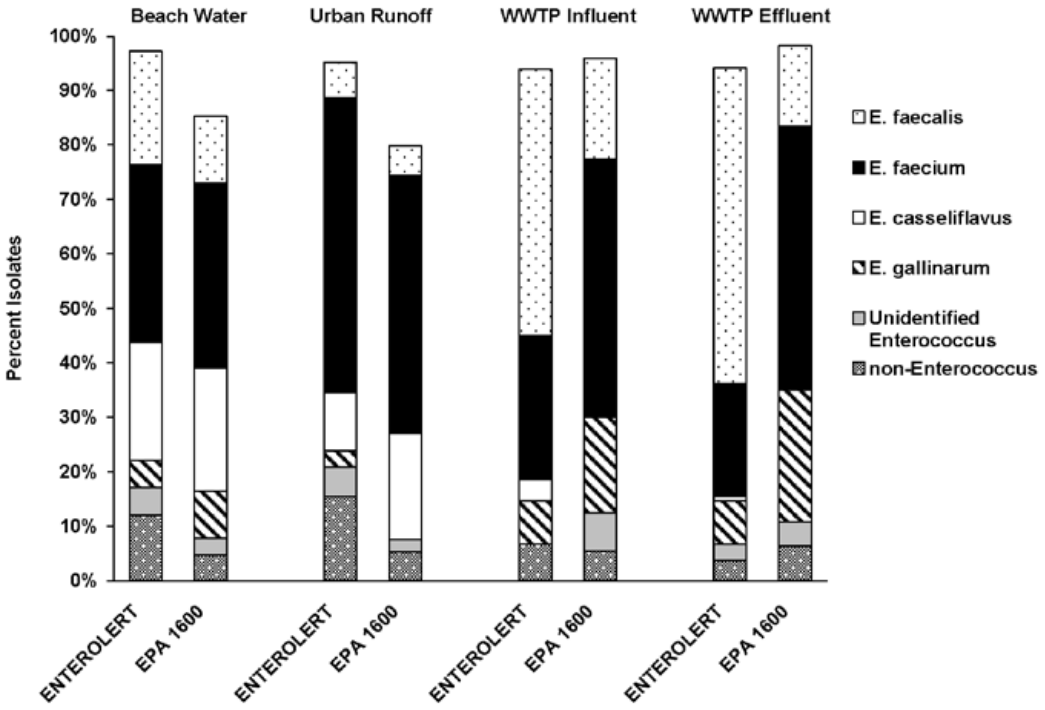

Figure 2. Distribution of predominant species found among environmental sources.

Enterolert, identified 96% of its isolates as *E. faecium*, while EPA Method 1600 identified only 47% as *E. faecium* (data not shown).

The same selectivity observed in the wastewater samples was also observed in the laboratory sample spiked with similar concentrations of *E. faecalis* and *E. faecium*. EPA Method 1600 resulted in 65% of the isolates identified as *E. faecalis*, 30.0% as *E. faecium*, and 5.0% as "unidentified". In contrast, 98% of the Enterolert isolates were identified as *E. faecalis* (Figure 3).

Eighteen (16.8%) of the isolates that underwent additional biochemical testing had discrepant identifications, including six that had been identified as *E. gallinarum* or *E. casseliflavus* using Vitek or *E. mundtii* and conventional biochemical testing. In this case, the identification by biochemical testing was assumed to be correct, as testing for pigment, which can discriminate between certain strains of *E. gallinarum* and *E. casseliflavus*, is not done by Vitek, and because *E. mundtii* is not included in the Vitek database.

Discussion

While EPA Method 1600 and Enterolert generally yielded the same species, some differences, mostly related to a preferential culturing of *E. faecalis* by Enterolert, were observed. There are a number of reasons why EPA Method 1600 and Enterolert might differ in species selectivity. The most obvious is that Enterolert uses liquid media and EPA Method 1600 uses solid media. Mixed bacterial cultures in liquid media allow faster growing or more resilient species to quickly reach densities that increase their likelihood of being selected (Schoenborn *et al*. 2004). In contrast, bacterial cells growing on a membrane placed on solid media are spatially separated and develop into colonies independently, providing less opportunity for competition.

Another difference is media formulation. The mEI media used in EPA Method 1600 contains additives, such as triphenyltetrazolium chloride to differentiate *Enterococcus* from other gram-positive cocci, sodium azide and nalidixic acid to inhibit the growth of gram-negative bacteria, and cycloheximide to suppress the growth of fungi; Enterolert media is proprietary, and it is unknown whether similar additives to increase specificity are present. The media also differ in the reporter molecule they use to detect enterococci. While mEI media relies on the ability of *Enterococcus* to metabolize indoxyl-β-D-glucoside to produce a blue compound, Enterolert media relies on 4-methylumbelliferone-β-D-glucoside to produce a fluorescent metabolite.

Another potential mechanism for the differences observed in species isolated from the two methods is

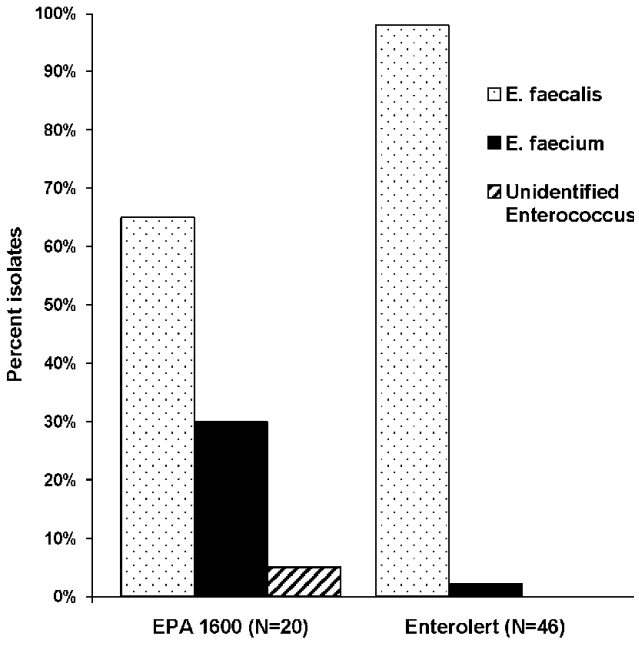

Figure 3. Percent *E. faecalis* and *E. faecium* isolates found using Enterolert vs EPA Method 1600 from culture sample containing 1:1 ratio of both species.

oxygen availability. Enterolert media are heat sealed within a Quanti-Tray, creating a more anaerobic environment compared to mEI agar in a petri dish. Enterococci are facultative anaerobes (Facklam and Collins 1989), but it is unknown whether there are varied growth rates among species under differing oxygen levels.

One concern with our study is that we used BHI broth with 6.5% NaCl to facilitate isolation of enterococci from Enterolert, which could have introduced species selection bias. To address this, we compared the growth rates of *E. faecalis* (ATCC 29212), *E. faecium* (ATCC 35667) and *E. casseliflavus* (ATCC 700527) in BHI with 6.5% NaCl versus Enterolert media and determined the doubling time of each species using optical density (600 nm; UV160U Spectrophotometer, Shimadzu Scientific Instruments, Columbia, MD) and plate counts on mEI. *Enterococcus faecalis* had the highest growth rate in Enterolert and in BHI broth with 6.5% NaCl. These results would seem to indicate that species selectivity also occurred with BHI with 6.5% NaCl. However, since the selection bias of BHI with 6.5% NaCl appeared to be in the same direction as that of Enterolert, it did not appear to be the reason for the observed differences in species selectivity between Enterolert and EPA Method 1600.

Another concern is that automated identification systems, such as Vitek, have limited databases that could lead to misidentifications (Moore *et al*. 2006). For example, *E. mundtii*, a species that is present in environmental waters (Ferguson *et al*. 2005) is not included in the database. In a previous study, Moore *et al*. (2006) found that 14% of the isolates categorized by Vitek were misidentified. Our results were similar in that approximately 16.8% of the isolates identified by Vitek had discrepant results based on biochemical testing. Though this would normally be cause for concern, this error rate is relatively small compared to the differences we observed in species selectivity between the methods and did not appreciably change our results or conclusions.

While there were some differences in species selectivity between Enterolert and EPA Method 1600, these differences were much smaller than the species composition differences that both methods found among sample types. For example, both methods found much higher percentages of *E. casseliflavus* in beach samples than in wastewater samples. Both methods also found similar enterococcal species distribution among sewage influent from the four wastewater treatment plants, despite differences in size, treatment processes, and the nature of service areas. The dominance of *E. faecium* and *E. faecalis* in the wastewater systems is consistent with previous studies that have examined species distributions of *Enterococcus* in wastewater, including those conducted in other countries (Sinton and Donnison 1994, Blanch *et al*. 2003, Moore *et al*. 2008). Higher percentages of *E. faecalis* and *E. faecium* in wastewater streams is also consistent with clinical studies that have established that these species comprise a significant fraction of the enterococci found in human and animal feces (Murray 1990, Sinton and Donnison 1994, Tannock and Cook 2002). Similarly, the prevalence of *E. casseliflavus* in samples from watersheds with minimal human influence and its lower occurrence in wastewater samples is also consistent with clinical human fecal samples (Ruoff *et al*. 1990, Stern *et al*. 1994) and its known association with plants (Ott *et al*. 2001, Aarestrup *et al*. 2002).

The observed differential species selection by EPA Method 1600 and Enterolert does not seem large or frequent enough to be problematic for continued interchangeable use of these methods for enumerating enterococci. However, our results indicate that due to the selection bias exhibited by Enterolert,

subculturing colonies from EPA Method1600 plates is the preferred technique to isolate *Enterococcus* to determine the species composition of environmental samples.

Literature Cited

Aarestrupp, F.M., P. Butaye and W. Wolfgang. 2002. Nonhuman reservoirs of enterococci. p. 60 *in*: M.S. Gilmore, D.B. Clewell, P. Courvalin, G.M. Dunny, B.E. Murray and L.B. Rice (eds.), The Enterococci: Pathogenesis, Molecular Biology and Antibiotic Resistance. American Society for Microbiology, Press. Washington, DC.

Budnick, G.E., R.T. Howard and D.R. Mayo. 1996 Evaluation of Enterolert® for enumeration of enterococci in recreational waters. *Applied and Environmental Microbiology* 62:3881-3884.

Blanch, A.R., J.L. Caplin, A. Iversen, I. Kühn, A. Manero, H.D. Taylor and X. Vilanova. 2003. Comparison of enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions. *Journal of Applied Microbiology* 94:994-1002.

Eckner, K.F. 1998. Comparison of membrane filtration and multiple-tube fermentation by Colilert® and Enterolert® methods for detection of waterborne coliform bacteria, *Escherichia coli* , and enterococci used in drinking and bathing water quality monitoring in southern Sweden. *Applied and Environmental Microbiology* 64:3079-3083.

Facklam, R.R. and M.D. Collins. 1989. Identification of *Enterococcus* species from human infections by a conventional test scheme. J*ournal of Clinical Microbiology* 27:731-734.

Ferguson, D.M., D.F. Moore, M.A. Getrich and M.H. Zhowandai. 2005. Enumeration and speciation of enterococci found in marine and intertidal sediments and coastal water in southern California. *Journal of Clinical Microbiology* 99:598-608.

Griffith, J.F., L.A. Aumand, I.M. Lee, IC.D. McGee, L.L. Othman, K.J. Ritter, K.O. Walker and S.B. Weisberg. 2006. Comparison and verification of bacterial water quality indicator measurement methods using ambient coastal water samples. *Environmental Monitoring and Assesment* 116:335-344.

Kinzelman, J., C. Ng, F. Jackson, S. Gradus and R. Bagley. 2003. Enterococci as indicators of Lake Michigan recreational water quality: Comparison of two methodologies and their impacts on public health regulatory events. *Applied and Environmental Microbiology* 69:92-96.

Moore, D.F., J.A. Guzman and C. McGee. 2008. Species distribution and antimicrobial resistance of enterococci isolated from surface and ocean water. *Journal of Applied Microbiology* 105:1017-1025.

Moore, D.F., M.H. Zhowandai, D.M. Ferguson, C. McGee, J.B. Mott and J.C. Stewart. 2006. Comparison of 16S rRNA sequencing with conventional and commercial phenotypic techniques for identification of enterococci from the marine environment. *Journal of Applied Microbiology* 100:1272-1281.

Murray, B.E. 1990. The life and times of the *Enterococcus*. *Clinical Microbiology Reviews* 3:46-65.

Noble, R.T., M. Leecaster, C.D. McGee, S.B. Weisberg and K. Ritter. 2004. Comparison of bacterial indicator analysis methods in stormwater-affected coastal waters. *Water Research* 38:1183-1188.

Ott, E.M., T. Müller, M. Müller, C.M.A.P. Franz, A. Ulrich, M. Gabel and W. Seyfarth. 2001. Population dynamics and antagonistic potential of enterococci colonizing the phyllosphere of grasses. *Journal of Applied Microbiology* 91:54-66.

Ruoff, K.L., L.D.L. Maza, M.J. Murtagh, J.D. Spargo and M.J. Ferraro. 1990. Species identities of enterococci isolated from clinical specimens. *Journal of Clinical Microbiology* 28:435-437.

Schoenborn, L., P.S. Yates, B.E. Grinton, P. Hugenholtz and P.H. Janssen. 2004. Liquid serial dilution is inferiror to solid media for isolation of cultures representative of the phylum-level diversity of soil bacteria. *Applied and Environmental Microbiology* 70:4363-4366.

Sinton, L.W. and A.M. Donnison. 1994. Characterization of faecal streptococci from some New Zealand effluents and receiving waters. *New Zealand Journal of Marine and Freshwater Research* 28:145-158.

Stern, C.S., M.G.S. Carvalho and L.M. Teixeira. 1994. Characterization of enterococci isolated from human and nonhuman sources in Brazil. *Diagnostic Microbiology and Infectious Disease* 20:61-67.

Tannock, G.W. and G. Cook. 2002. Enterococci as members of the intestinal microflora of humans. p. 104 *in*: M.S. Gilmore, D.B. Clewell, P. Courvalin, G.M. Dunny, B.E. Murray and L.B. Rice (eds.), The Enterococci: Pathogenesis, Molecular Biology and Antibiotic Resistance. American Society for Microbiology, Press. Washington, DC.

United States Environmental Protection Agency (USEPA). 2002. Enterococci in Water by Membrane Filtration Using membrane-*Enterococcus* Indoxyl-β-D-Glucoside Agar (mEI). EPA 821-r-02-022. USEPA. Washington, DC.

Weisberg, S.B., R.T. Noble and J.F. Griffith. 2007. Microbial indicators of marine recreational water quality. pp. 280-289 *in*: C.J. Hurst, R.L. Crawford, J.L. Garland, D.A. Lipson, A.L. Miles and L.D. Stetzenbach (eds.), Manual of Environmental Microbiology. 3rd edition. American Society for Microbiology, Press. Washington, DC.

Acknowledgements

The authors wish to thank Matthew Hamilton, Melissa Madison, Linda Tran, and Andriana Gallegos-Amparo, Los Angeles County Sanitation District, Orange County Sanitation District, South Orange County Wastewater Authority, Encina Wastewater Authority, and Orange County Public Health Laboratory for their assistance.