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Enterococcus Growth on Eelgrass (*Zostera marina*); Implications for Water Quality

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Abstract Enterococci are fecal indicator bacteria used to monitor fecal pollution of recreational waters. When enterococci levels exceed health standards, fecal pollution is assumed as the cause. Enterococci growing on plants limit their usefulness as fecal indicator bacteria. Here we examined enterococcal growth on eelgrass in Mission Bay, CA where enterococci levels have exceeded water quality thresholds. 69 eelgrass samples were collected from six sites, shaken to remove enterococci attached to plant surfaces and the eluant filtered onto culture media. Isolates were then identified to species using biochemical methods, and DNA typing by pulsed-field gel electrophoresis was done to assess clonality of strains. Enterococci concentrations among eelgrass ranged from 8 – 14,000 CFU/g dry weight. The most predominant enterococcal species found were *E. casseliflavus* and *E. hirae* followed by *E. faecalis*. Cluster analysis indicated a high level of clonality among isolates across all species, with clonal isolates consistently associated with individual eelgrass samples. Finding high densities of *E. casseliflavus*, *E. hirae* and *E. faecalis* on eelgrass that included clonal strains indicates the capability of enterococcal growth on eelgrass. Amplification of enterococci on eelgrass presents challenges for regulatory agencies that interpret elevated levels of these bacteria as an indication of fecal pollution.

Keywords Fecal Indicator bacteria, Enterococci, Environment, Growth

Introduction

Regulatory agencies use enterococci as fecal indicator bacteria to determine the microbiological suitability of marine waters used for recreational purposes. Enterococci are normal inhabitants of the gastrointestinal tracts of humans, birds and animals; thus, water bodies with enterococci densities that exceed regulatory standards are assumed to be contaminated with fecal waste and the public is warned of increased risk for acquiring gastrointestinal illnesses (USEPA 1986). However, the measurements of enterococci may be confounded because recent studies show that enterococci are capable of growing on algae (*Cladophora* spp.) (Whitman et al., 2003), sea wrack (*Macrocystis*) (Imamura et al., 2011), beach sand (Yamahara et al., 2009) and soil (Byappanahalli et al., 2012a). Amplification of enterococci in the beach environment presents challenges in interpreting enterococci water quality standards that were developed without current knowledge regarding enterococci growth in extra-intestinal habitats.

Eelgrass, a type of submerged aquatic vegetation (SAV) that grows in shallow waters such as estuaries, bays and lagoons has not been studied as supporting enterococcal growth. The goal of this study was to examine whether enterococci grow on eelgrass washed up on beaches in Mission Bay, CA. Enterococci levels in the Bay continue to exceed enterococci water quality standards despite numerous mitigation measures to reduce fecal bacterial pollution, including replacing sewer mains and trunk sewers, upgrading pump stations and installing a state-of-the-art low-flow drain diversion system that encircles the Bay (Henry et al., 2005). Approximately 54% of the bottom surface of Mission Bay is covered with eelgrass (Merkel 2008). Mounds of exposed eelgrass are frequently observed on beach sand near regulatory sampling sites historically exceeding enterococci standards raising the question whether enterococci growing on eelgrass could be contributing cells to the water column. We set out to

examine whether enterococci were growing on eelgrass deposited onto beach sand near water sampling sites.

Materials and Methods

To assess enterococcal growth on eelgrass stranded on beach sand, we isolated enterococci from multiple eelgrass samples, determined enterococci concentrations per plant, identified isolates to species level and assessed clonality of isolates using DNA typing by pulsed-field gel electrophoresis (PFGE). We hypothesized that enterococcal growth would be indicated by high densities of enterococcal cells including clonal strains, i.e., isolates with similar DNA fingerprints. If enterococci growth was occurring on eelgrass plants, we expected to find clonal strains among multiple plants collected on different days.

Sampling design. Eelgrass plants (N=69) were collected from 6 sampling sites at Visitor Center beach where mounds of eelgrass are typically observed on beach sand (Fig. 1). Sampling sites were located about ~50m to 130m apart, spanning a total distance of ~360m. Eelgrass plants were collected once a day on 12 different days from September 17, 2012 – October 11, 2012. Only the top portions of eelgrass washed up on beach sand were collected, avoiding contact with sand.

Enterococcus isolation and enumeration. Eelgrass samples were collected ~8 a.m. on 12 different days from September 17, 2012 – October 11, 2012. The samples were obtained on beach sand in the intertidal range, placed into zip lock bags, transported to the lab on ice and processed within 4 hours of collection. Approximately 25g of sample was transferred to another

zip lock bag and weighed. Sterile phosphate-buffered saline (PBS) (250 ml) was added to the bag and the bag was gently shaken by hand for around 20 seconds to remove enterococci attached to plant surfaces. Dilutions of the eluant were filtered onto mEI (indoxyl-ß-D-glucoside) agar (EPA Method 1600) to obtain 20 – 60 colonies per filter. After 24h incubation at 42°C, presumptive enterococci isolates were enumerated. Portions of the undiluted eelgrass sample were placed into a drying oven overnight to normalize enterococci concentrations per gram of dry weight of eelgrass.

Enterococcus species and strain typing. After enumerating enterococci on mEI, the isolates were subcultured onto tryptic soy agar with 5% sheep blood agar and incubated for 24h at 37°C. Presumptive enterococci isolates from 10 different eelgrass samples yielding at least 10 – 30 enterococcal isolates per plant were selected for species identification using Vitek II (bioMériuex, St. Louis, MO, USA) plus additional biochemical testing including pigment, motility, mannitol, sucrose, arabinose and methyl-alpha-D-glucopyranoside, as described previously (Ferguson et al. 2013). ATCC strains of *E. faecalis*, *E. faecium*, *E. hirae*, *E. casseliflavus*, *E. gallinarum* and *E. mundtii* were used as controls for supplemental biochemical tests. Isolates identified by Vitek II as *Enterococcus* species with discrimination at <80% confidence were categorized as "*Enterococcus,* species indeterminant". Eight isolates identified as *E. faecalis*, *E. hirae* and *E. mundtii* were confirmed by 16S rRNA gene sequencing (GenoSeq, University of California Los Angeles). To increase the probability of identifying clonal strains by PFGE, we selected isolates with similar phenotypic profiles (i.e., bionumber) based on Vitek II species identification results.

PFGE. DNA typing was done using the Centers for Disease Control (CDC) PulseNet 1-day PFGE protocol for gram positive bacteria (CDC 2012). A standardized suspension of each isolate was made in Tris-EDTA (TE) and pre-lysed with lysozyme (20mg/mL) for 30 min. The DNA was embedded in agar plugs made with 1% Seakem Gold Agarose (Lonza, Rockland, ME). The DNA embedded agar plug was lysed with cell lysis buffer and proteinase K (20mg/mL). The plugs were washed twice with type I, mili-Q water and 4 times with 1X Tris-EDTA. A 1 mm slice was restricted with SmaI at RT for 2h. 1mm slices of agarose containing the chromosomal DNA fragments were placed on their respective lanes, and the restriction fragments resolved into a pattern of discrete bands using a CHEF DR III system (Bio-Rad Laboratories, Irvine, CA). After 19h, DNA restriction patterns of the isolates were then compared with one another to determine clonal relatedness. PFGE types were evaluated by visual analysis followed by computer analysis using FPQuest (Bio-Rad). Cluster analysis of electrophoretic band patterns was performed using similarity levels calculated by the Dice coefficient and clustering was achieved by the unweighted-pair group method (UPGMA) using arithmetic average algorithms. Isolates that were indistinguishable by Sma1 were verified using the same protocol but with Not1 or Spe as the restriction endonuclease.

Isolates with PFGE patterns that were 100% similar were considered "indistinguishable" (clones) if their restriction patterns had the same number of bands and the corresponding bands were of the same apparent size. Isolates with patterns that were 85% to 99% were considered "closely related".

PFGE was repeated on 20 isolates at the Orange County Public Health Laboratory (OCPHL) using the same PFGE protocol to assess method reproducibility.

Results

Densities of enterococci in eelgrass. Enterococci isolated from 69 eelgrass samples had concentrations ranging from 8 – 14,000 CFU/g dry weight (average = 1148; median =140). Of these, 28 (41%) samples had enterococcal concentrations that were <100 CFU/g dry weight; 27 (39%) and 14 (20%) of samples had counts ranging from 101 – 999 CFU/g dry weight and 1000 – 14,000 CFU/g dry weight, respectively.

Enterococcus species and biotype diversity based on phenotypic analysis. A total of 140 presumptive enterococci isolates obtained from 10 individual eelgrass plants (10 isolates each from 8 plants and 30 isolates each from 2 plants) were identified to species level. Of these, *E. hirae*, *E. casseliflavus* and *E. faecalis* comprised 39%, 29% and 21% of isolates (respectively); the remaining isolates were identified as *E. gallinarum* (5%), *E. faecium* (2%), *E. mundtii* (1%) and *E. durans* (<1%). Two isolates were unidentified to species level by Vitek plus additional biochemical and categorized as "*Enterococcus,* species indeterminant".

Only one or two *Enterococcus* species were predominant among isolates obtained from all individual samples, indicating low species diversity within samples (Fig. 2). Among the majority of samples, *E. casseliflavus* was the most predominant species found, closely followed *E. hirae*.

All 10 isolates obtained from 5 of 8 samples (63%) were identified as the same species. Analyzing more than 10 isolates per eelgrass plant did not increase *Enterococcus* species diversity, even when additional isolates (30 isolates per sample) were analyzed (data not shown).

The majority of biotypes (64.7%, 72.2% and 46.2%) among isolates of *E. casseliflavus*, *E. hirae* and *E. faecalis*, respectively were unique, indicating a high diversity of strains (Table 1). There were a higher percentage of non-unique biotypes (35.3%, 27.7% and 38.5%) among isolates of *E. casseliflavus*, *E. hirae* and *E. faecalis*, respectively from the same eelgrass plant as compared to biotypes occurring in more than one plant.

Clonality of Enterococcus strains based on PFGE. A total of 48 enterococci isolates were selected from 8 samples for DNA typing using PFGE to assess clonality of *E. hirae, E. casseliflavus and E. faecalis* strains. All but 5 enterococci strains generated evaluable PFGE banding patterns using Sma1. Examples of PFGE patterns representative of clonal strain types obtained using Sma1 and Not1 are shown in Fig 3. and Fig 4.

E. hirae. PFGE typing of 18 *E. hirae* isolates resulted in 11 different PFGE pattern types (Fig. 5). Eleven (61.1%) isolates were of indistinguishable pattern types (i.e., 100% similar). Two isolates were 95% similar and considered closely related and 8 isolates resulted in unique (unrelated) pattern types.

E. casseliflavus. PFGE typing of 18 *E. casseliflavus* isolates resulted in 13 PFGE pattern types (Fig. 6). Seven (38.9%) isolates had indistinguishable pattern types that comprised 3 different clusters. Two isolates were closely related (85% similar) and 11 isolates resulted in unique pattern types.

E. faecalis. PFGE typing of 7 *E. faecalis* isolates resulted in 4 PFGE pattern types (Fig. 7). Three (42.9%) isolates had indistinguishable pattern types that grouped into a single cluster. Four isolates resulted in unique pattern types.

All clonal isolates within each cluster, regardless of species group, were obtained from the same eelgrass sample; i.e., no clonal strains appeared to be shared across eelgrass samples, though most of the samples were collected from different sites on different days.

***PFGE Reproducibility*.** PFGE results of twenty *Enterococcus* isolates were verified by OCPHL using the same protocol with Sma1. Isolates that OCPHL found as indistinguishable using Sma1 were also indistinguishable after restriction with Not1. Six *E. hirae* isolates that were found to be clonal using Sma1 failed to digest using Not1 and Spe even after repeated efforts, likely due to a lack of enzyme restriction sites among these strains.

Discussion

Important criteria for ideal fecal indicator bacteria are that they must be incapable of growing in water and their densities should have some direct relationship to the degree of fecal pollution (USEPA 2006). Eelgrass has been shown to support bacterial growth at rates similar to that of pure cultures and 100% of the produced biomass of bacteria (unspecified species) was lost to surrounding waters on a daily basis (Törnblom and Søndergaard 1999). In this study, we found high concentrations of enterococci on eelgrass including clonal strains indicative of replication that could lead to overestimation of fecal pollution.

The average concentration of enterococci found on eelgrass was similar to that reported previously for sea wrack (*Macrocystis)* obtained from 5 other beaches in Los Angeles County and Cowell Beach in Santa Cruz County, California (Imamura et al., 2011) and higher than typical levels reported for beach water and sand in California (Yamahara et al., 2007).

Enterococci concentrations on eelgrass were also comparable to densities reported (26 to 61,600 CFU/g dry weight) for enterococci colonizing forage grass (*Phalaris arundiacea*, *Phalaris arundinacea*, *Cala palustris* and *Poa pratensis*) in Germany (Ott et al., 2001).

Beach water samples tested for regulatory monitoring purposes are collected at ankle depth in the surfzone. Beached eelgrass samples were collected on near water line under the assumption that wave action removes enterococci attached to the plants, thus releasing them into surfzone water. The concentrations of enterococci on eelgrass underwater were not determined to avoid including enterococci that may have been present in water.

Eelgrass consisted of both wet and dry samples exposed to air and UV. The effect of these environmental stressors on enterococci present on eelgrass is uncertain. Previous studies found higher densities of enterococci on dry and decaying seawrack (Imamura et al., 2011; Byappanahalli et al., 2012b). It is possible that decomposing plants release nutrients that allow enterococcal growth.

The most predominant enterococcal species found among most eelgrass samples were *E. casseliflavus* and *E. hirae*, followed by *E. faecalis*. These three species have also been frequently isolated from ocean water at various beaches in southern California (Ferguson et al., 2005; Moore et al., 2008; Maraccini et al., 2012); thus, sand and beach water may contribute enterococci to eelgrass. To minimize enterococci contributions from these sources, we collected eelgrass plants away from sand and thoroughly rinsed the plants to remove enterococci attached to plant surfaces; however, we acknowledge that the isolates may include clonal enterococcal cells present in sand and Bay water.

PFGE typing showed multiple enterococcal strain types and no predominant strain that was shared across all eelgrass samples. It is uncertain whether the enterococci isolated from

eelgrass found in this study are representative of the plant microflora, though *E. casseliflavus*, which was abundant among eelgrass, has been associated with plants and SAV studied elsewhere (Ulrich and Müller, 1998; Ott et al., 2001; Badgley et al., 2010). Unlike most species of *Enterococcus*, *E. casseliflavus* produces carotenoid, a yellow, cell-bound pigment that may confer a selective advantage to epiphytic bacteria by protecting them from UV irradiation and oxidative stress (Taylor et al., 1971; Hirano and Upper, 2000).

Enterococcus species typically associated with humans, such as *E. hirae* and *E. faecalis*, were also found on eelgrass. *E. faecalis* strains have also been isolated from terrestrial plants (Mundt 1961; Ott, et. al, 2001; Jha et al., 2005); however, there are no similar reports regarding *E. hirae*. Interestingly, the *E. hirae* isolates characterized in this study were slightly yellow pigmented, which is not typical of human clinical strains. The species identity of pigmented *E. hirae* was verified using 16S rDNA gene sequencing. Also, Kirschner et al., 2001 suggested that slight pigment production by certain *E. hirae* strains may be due to low levels of carotenoid.

We found a high diversity of *Enterococcus* species from eelgrass, which is in accordance with previous studies that characterized enterococci from marine water, soil and aquatic vegetation (Whitman et al., 2005; Grant et al., 2009; Nayak et al., 2011; Ran et al., 2013). Thus, conducting DNA typing on isolates representative of the most abundant enterococcal species and biotypes may have improved our ability to find a high percentage of clonal isolates (61.1%, 42.9% and 38.9%) among *E. hirae*, *E. faecalis* and *E. casseliflavus*, respectively, though it is worth noting that 42% of all isolates of similar biotypes were not clonal (data not shown).

PFGE typing is still considered the gold standard for typing clinically relevant bacteria, including *Enterococcus* (Turabelidze et al., 2000; Werner et al., 2015). It is widely used during outbreaks to match bacterial strains isolated from patients with isolates from suspect sources.

PFGE involves separating large pieces of DNA comprising greater than 90% of the genome. Thus, insertions or deletions of mobile genetic elements and large recombination events within genomic DNA results in varied PFGE patterns (Sabat et al., 2013). PFGE typing of enterococci requires 3 – 4 days to complete. This is the first report on using a rapid one-day, unified PFGE protocol for assessing the clonal relatedness of environmental enterococcal strains. The rapid protocol was developed and validated by CDC to standardize molecular strain typing of clinically-important *Enterococcus* spp (CDC 2012). The validation process includes assessing inter and intra-laboratory method reproducibility. Analysis of PFGE fingerprints can be somewhat subjective; however, in this study the banding patterns of clonal isolates were easily discernable.

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Table 1. Distribution of *Enterococcus* biotypes* among isolates from eelgrass plants

Species	No. plants	No. isolates	No. biotypes	No. (%) unique biotypes	No. (%) biotypes shared within plants	No. (%) biotypes found >1 plant
E. casseliflavus	6	40	17	11 (64.7%)	6 (33.3%)	2 (11.8%)
E. hirae	5	5	18	13 (72.2%)	5 (27.7%)	3 (16.5%)
E. faecalis	4	30	13	6 (56.2%)	5 (38.5%)	3 (23.1%)

*biotype is based on bionumber assigned by Vitek II

Figure 1 Mission Bay, San Diego

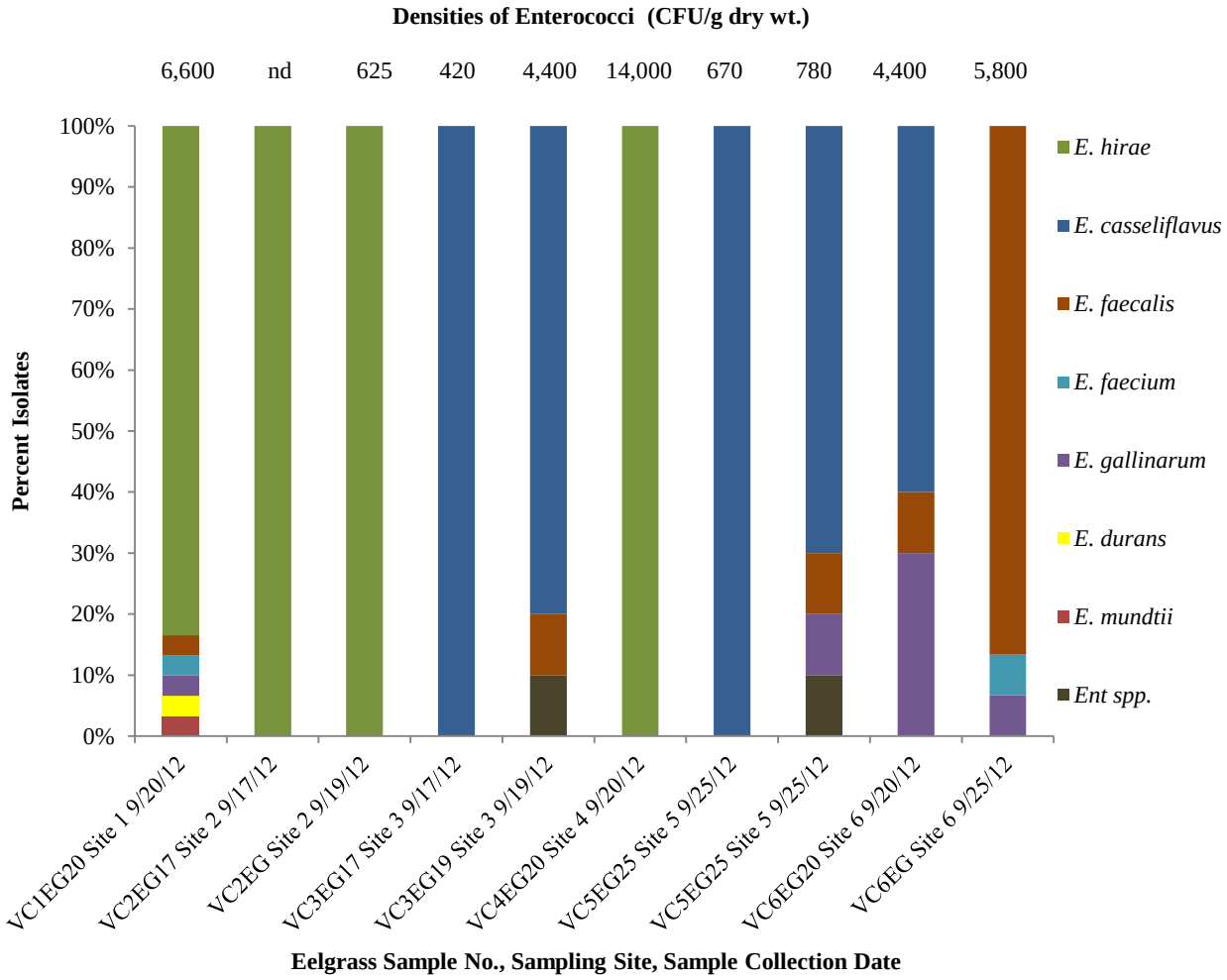

Figure 2 Distribution of *Enterococcus* species found among individual eelgrass plants

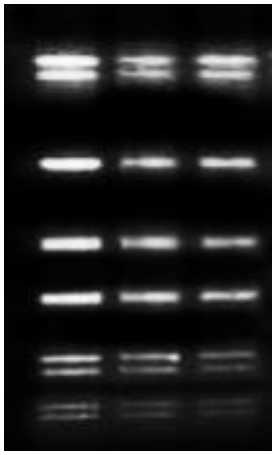

Figure 3 Chromosomal digestion patterns of *E. hirae* isolates from eelgrass sample VC4EG

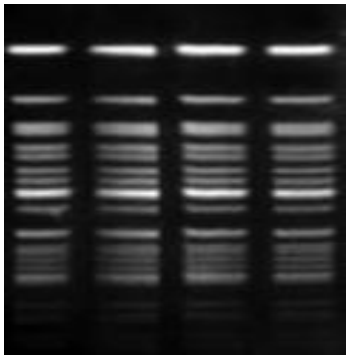

Figure 4 Chromosomal digestion patterns of *E. faecalis* isolates from eelgrass sample VC6EG

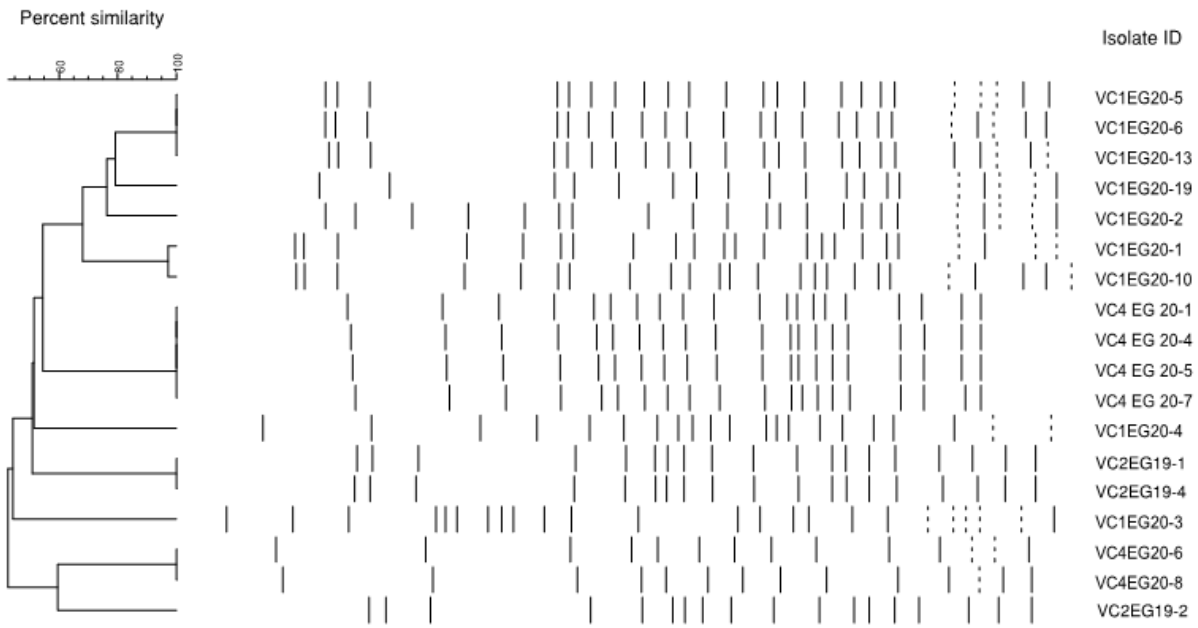

Figure 5 PFGE dendrogram of *E. hirae* isolates from eelgrass plants VC1EG20, VC2EG and VC4EG20. Isolate number corresponds to sampling sites (VC1EG-VC6EG), followed by sampling date (19-Sept-2012, 20-Sept-2012) and isolate number (1-19)

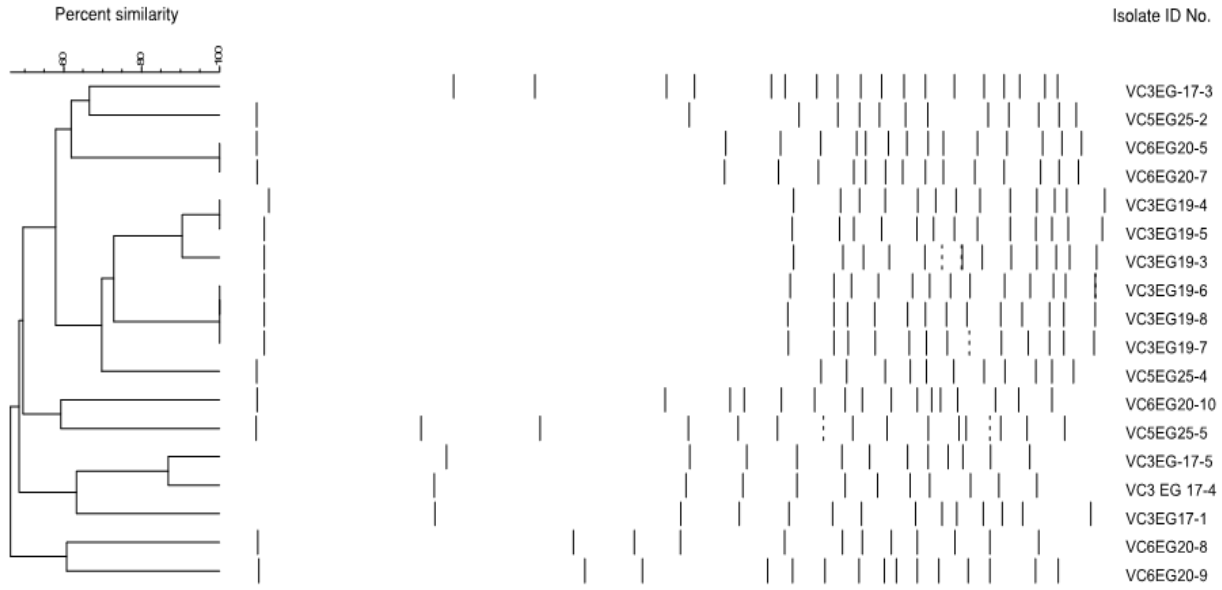

Figure 6 PFGE dendrogram of *E.casseliflavus* from eelgrass samples VC3EG17, VC3EG19, VC5EG25, VC6EG20. Isolate number corresponds to sampling sites (VC1EG-VC6EG), followed by sampling date (17-Sept-2012, 19-Sept-2012, 20-Sept-2012, 25-Sept-2012) and isolate number (1-10)

Figure 7 PFGE dendrogram of *E. faecalis* isolates from eelgrass plant VC6EG25. Isolate number corresponds to sampling sites (VC6EG), followed by sampling date (25-Sept-2012) and isolate number (1-21)