INVESTIGATION OF PROPOSED WATER QUALITY INDICATOR ORGANISMS FOR MARINE MAMMAL ENCLOSURES

By

Christopher Owen

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ABSTRACT

INVESTIGATION OF PROPOSED WATER QUALITY INDICATOR ORGANISMS FOR MARINE MAMMAL ENCLOSURES

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A USDA APHIS proposal would require institutions housing marine mammals to limit concentrations of total coliforms to 500 CFU/100 mL and enterococci, staphylococci, and/or pseudomonads to 35, 10, and/or 10 CFU/100 mL, respectively. Little is known about the amount of water treatment necessary to meet these levels, or significance of said levels, if any, in relation to the total microbial community of marine mammal housing systems and the health of the mammals housed.

Using membrane filtration and growth on selective media, concentrations of these indicators were monitored in a system housing Pacific white-sided dolphins over a period of 5 days and compared between exhibit water and water from exhibit plumbing after sand filtration but before ozone treatment. Concentrations were also examined after ozone treatment. Isolates from each media were taxonomically identified using 16S rRNA gene sequencing, and colony counts were analyzed as predictors of 16S rRNA gene community data.

All indicator counts were significantly reduced either after sand filtration or after ozone treatment, but none were significantly reduced at both points. Genetic sequencing of isolates from selective and differential media revealed that 10% of presumptive pseudomonads, 19% of presumptive staphylococci, 100% of presumptive enterococci, and 91% of lactose-fermenting total coliforms were members of the expected taxa. Several correlations between indicator counts and individual OTUs from the community as well as overall dissimilarity between community samples were detected.

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KEY TO ABBREVIATIONS

ANOVA Analysis of Variance

APHIS Animal and Plant Health Inspection Service

BLAST Basic Local Alignment Search Tool

bp Base Pairs

C.F.R. Code of Federal Regulations

CFU Colony Forming Unit(s)

DNA Deoxyribonucleic Acid

EDTA Ethylenediaminetetraacetic Acid

EPA Environmental Protection Agency

FDR False Discovery Rate

MPN Most Probable Number

NCBI National Center for Biotechnology Information

OTU Operational Taxonomic Unit

PBW Phosphate Buffered Water

PCoA Principal Coordinates Analysis

PCR Polymerase Chain Reaction

QIIME Quantitative Insights into Microbial Ecology

qPCR Quantitative Polymerase Chain Reaction

Rep-PCR Repetitive Extragenic Palindromic Polymerase Chain Reaction

rRNA Ribosomal Ribonucleic Acid

TBE Tris/Borate/EDTA

TTC 2,3,5-Triphenyltetrazolium Chloride

UIUC University of Illinois at Urbana-Champaign

USDA United States Department of Agriculture

UV Ultraviolet

VBNC Viable but not Culturable

WHO World Health Organization

1. LITERATURE REVIEW

1.1 Overview of Current and Proposed Regulations for Marine Mammal Bacteriological Standards

In the United States, institutions housing marine mammals are required to abide by regulations promulgated by the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS). These regulations give minimum standards for housing, care, and transportation of marine mammals. The current bacteriological standards for waters containing captive marine mammals are given in 9 C.F.R., Chapter I, Subchapter A, Part 3, Subpart E, § 3.106 (Published online at http://www.ecfr.gov/cgi-bin/text-idx?SID=9cc0a635dd221f42fa434e1829a63b73&mc=true&node=se9.1.3_1106&rgn=div8). This overview focuses on the portion of the regulations that set bacteriological standards intended to indicate water quality.

Standards for water quality in marine mammal habitats require that a count of cultivable total coliform bacteria be performed at least weekly. Total coliforms in the primary enclosure must not exceed 1,000 most probable number (MPN) units per 100 mL of enclosure water. In the event of a coliform count above 1,000 MPN per 100 mL, the institution holding the animal(s) may immediately take corrective action (e.g. increasing chemical disinfection) or take two subsequent samples 48 h and 96 h after the initial sample[1]. If the follow-up samples give coliform counts low enough that the average MPN between all three drops below 1,000 per 100 mL, no further action is required. However, if the average MPN between the initial sample and

[1] The language of the regulation states: "Should a coliform bacterial count exceed 1,000 MPN, two subsequent samples *may* [emphasis added] be taken at 48-hour intervals and averaged with the first sample." The use of the word "may", interpreted literally, implies that follow-up samples are not legally required.

the two subsequent samples remains above 1,000 per 100 mL, the condition "must be corrected immediately" (9 C.F.R. § 3.106). Records of these tests must be maintained for one year and be available for inspection upon request. Although the regulation does not give recommendations specifically for correcting unsatisfactory conditions, it does state that water quality is to be maintained by "filtration, chemical treatment, or other means". In addition, water samples must be tested daily for any chemical additives that are used to maintain the required bacteriological standards (though no action limits are given for such chemicals). No examples of chemicals that may be used are given in the regulation text, but standard chemicals used for disinfection of marine mammal water include ozone and chlorine (Coakly, Crawford, & Technical Coordinators, 1998).

In 2016, a proposal was put forth to update the above bacteriological standards (USDA APHIS, 2016). The update is based on the Environmental Protection Agency's 2012 Recreational Water Quality Criteria (EPA, 2012) for humans. The proposed changes would allow colony counting methodologies with solid agar to be used as a substitute for MPN. The limit on coliform counts would be lowered to 500 colony-forming units (CFU) per 100 mL, with institutions given the option to instead test fecal coliforms with a proposed limit of 400 CFU per 100 mL. In addition, institutions housing marine mammals would be required to test for at least one of *Enterococcus*, *Pseudomonas*, or *Staphylococcus*. The proposed limits are 35 CFU per 100 mL for Enterococci and 10 CFU per 100 mL for *Pseudomonas* or *Staphylococcus* (Table 1 summarizes the current and proposed regulations for bacterial counts). In addition, testing for suspect pathogens would be required when there is evidence of health problems or a potential health hazard is suspected. The testing schedule and averaging procedure given in the current regulation would be retained.

Indicator	Current action limit (CFU/100 mL)	Proposed action limit (CFU/100 mL)
Total coliforms	1000	500
Enterococcus	No limit	35
Staphylococcus	No limit	10
Pseudomonas	No limit	10

Table 1: Current and proposed (USDA APHIS 2016) microbial regulations for water containing captive marine mammals.

The proposed update states a few reasons for testing the additional organisms. Enterococci are included because, like coliforms, they come primarily from the intestinal tract, but may be more related to pathogens in marine waters because of their survivability in high-salinity environments (Fisher and Phillips, 2009). *Pseudomonas* spp. are very common in water but can also produce opportunistic lung infections in marine mammals; thus, they are intended to indicate pathogen contamination (although it is worth noting that many pathogens are opportunistic and their presence alone does not indicate risk to an otherwise healthy animal). *Staphylococcus* spp. are generally associated with skin and mucus membranes, can be pathogenic or non-pathogenic, and are indicators of "contamination and/or possible danger" arising from both fecal and non-fecal sources on the animals themselves (APHIS, 2016).

While there is justification for the use of these indicators, there are no data to demonstrate that they are significantly associated with marine mammal health. Thus, when they are present at the limits suggested, there is no indication of a clear, scientifically validated risk. There is therefore a need for inquiry into the use of these standards to indicate compromised water quality impacting marine mammal health.

1.2 History of Indicators and the Indicator Concept for Water

Historically, indicator organisms used for water are culturable bacterial or viral species whose presence shows that an environment has fecal contamination, though more recently, indicator organisms have been suggested for the detection of other types of pollution as well.

Ashbolt et al. (2001), for instance, identified three categories of indicators that are grouped by the type of pollution they indicate. The first group, process indicators, demonstrates the efficacy of a disinfection process (total coliform bacteria fall into this category). The second group are fecal indicators; these are organisms found in excreta of warm blooded animals (including humans) and are thus used to infer fecal contamination (this includes fecal coliform bacteria, *E. coli* and enterococci). Third, there are index and model organisms, which are intended to indicate the presence and behavior of pathogens. Specifically, these species behave in similar ways and survive in similar environments as pathogens, so their presence is used to infer pathogen presence; *E. coli* and enterococci may in some cases may be associated with enteric bacterial pathogens. Some indicators can serve in more than one of these three capacities.

Griffin et al. (2001) have summarized the characteristics of the ideal indicator (from Bonde, 1966) as follows: They should occur where pathogens do, should not be able grow in the environment, should be more resistant to disinfection than pathogens, should be easy to isolate and count, should be able to be isolated from all types of water (e.g., freshwater and seawater), should not be subject to antibiosis (antagonistic interaction with other microbes), should only be found in sewage (or in the source of contamination), should be found in higher numbers than pathogens, and should show a relationship between their density and the degree of contamination, as well as a relationship between their density and a health hazard or type of pollution. There is no known indicator that meets all of these criteria.

Several issues affect the ability of indicator organisms to predict pathogen presence. Notably, there is no correlation between indicators and enteric pathogens in many environments (Grabow, 1996). In addition, indicator organisms are subject to varying environmental conditions, and their ability to survive these conditions affects their validity (Ashbolt et al.,

2001). If they can reproduce in environmental conditions, then they may not indicate legitimate or recent contamination. Thus, there is a need for a set of indicators or a "tool box" approach.

1.2.1 Total coliforms as indicators

Total coliform bacteria are defined as the subset of the family *Enterobacteriaceae* that can ferment lactose, producing acid and gas (Edberg, Rice, Karlin, and Allen, 2000). The use of the total group to indicate water quality originated from tests for *Escherichia coli*. *E. coli* was suggested as an indicator of fecal presence in water during the 1890s (documented in Prescott and Winslow, 1915, who refer to the now-obsolete name *Bacillus coli*), but culture methods available at the time only allowed for the total coliform group. This includes all aerobic or facultatively anaerobic prokaryotes that grow at 35 ^{o}C on lactose-based media and ferment the sugar to acid and gas. The Eijkman test (described in Singleton and Sainsbury, 2006) was developed in 1904 by Christiaan Eijkman, to differentiate members of the total coliform group that grew at higher temperatures of 44.5 °C, implying that they originated in the feces of warm blooded animals. Such organisms are now known as "fecal coliforms" or "thermotolerant coliforms". Although Eijkman originally used a glucose-based medium, this test has since been adapted to use lactose-based media (Batty-Smith, 1942). Total coliforms include fecal coliforms, but are not necessarily thermotolerant (Edberg et al., 2000). Thus, total coliforms include but are not limited to fecal coliforms, which in turn include but are not limited to *E. coli*.

A disadvantage of using total coliforms as indicators of fecal pollution is that some species and strains are found in soil, and can persist and even replicate in non-enteric environments. For example, coliform bacteria, including *E. coli*, have been recovered from natural biofilms that formed on riverbed pebbles and sediments in higher concentrations than those found in overlying water (Hirotani & Yoshino, 2010; Balzer et al., 2010), suggesting that

they proliferate in the riverbed and contribute to the total coliform population of the water. Soils can also contribute to aquatic *E. coli*, and thus to total coliforms. Populations that originate from soil may be genetically distinct from those in animal feces as determined using repetitive extragenic palindromic polymerase chain reaction (Rep-PCR[2]; Byappanahalli et al., 2006). The ability of *E. coli* to proliferate in soils depends on soil moisture content, suggesting that in some cases, coliform counts may be elevated due to the moisture of the soil surrounding the tested system rather than the volume of fecal material or soil entering the water (Solo-Gabriele et al., 2000).

In some water treatment systems using chlorine, ozone, or ultraviolet (UV) light, concentrations of coliform bacteria are lowered during disinfection, but reach higher levels in storage reservoirs and distribution systems, suggesting that regrowth occurs (Jjemba et al., 2010). Coliform bacteria have also been shown to significantly increase in storage containers where high levels of assimilable organic carbon (AOC) were available in a community that had no distribution plumbing (Mellor et al., 2013). It is not clear whether total coliforms reliably indicate fecal contamination in marine waters. Davies et al. (1995) found that a net die-off of fecal coliforms occurred over a period of 85 days in nonsterile marine waters and sediment. Because the die-off did not follow an exponential decay curve, it was assumed that the decline in numbers was due to predation rather than starvation or salt toxicity. In a highly controlled marine environment, there is a possibility that predation will not take place at sufficient levels to

[2] Rep-PCR involves the use of primers that bind to several locations in the bacterial genome, producing multiple amplicons of different lengths that can then be visualized using gel electrophoresis. The specific set of amplicons produced, and thus the band pattern produced by electrophoresis, is highly variable and can be used to differentiate individual strains of bacteria.

produce a decline in coliform numbers, and coliform bacteria cannot be assumed to indicate recent contamination. Thus, the use of coliform counts in such an environment should be subject to validation.

1.2.2 Staphylococci as Indicators

Staphylococci (members of the genus *Staphylococcus*), particularly *Staphylococcus aureus,* have been suggested for use in human recreational waters as indicators of bacterial shedding from human skin, mucous membranes, and, to some extent, excreta. Early on, Favero et al. (1964) designated staphylococci as ideal candidates for the detection of swimming pool pollution from human swimmers. Unlike total coliforms, which are generally enteric bacteria, staphylococci are mostly derived from the mouth, nose, throat, and skin. The authors found that staphylococci were more resistant to chlorination in swimming pools than were total coliforms. This finding is potentially significant for marine mammal health, as chlorination is also a common method of disinfection for marine mammal enclosures (Spotte, 1991). In addition, the authors found that staphylococci were present in higher numbers in swimming pools that were utilized by many people than in those that saw little use. It is important to note that these data were collected from swimming pools used exclusively by humans, and they may or may not translate to marine mammal enclosures.

Yoshpe-Purer and Golderman (1987) examined the effect of human bather load on the presence of *S. aureus* in ocean water. *S. aureus* was monitored in 628 samples taken from coastal seawater along with total coliforms, fecal coliforms, and *Pseudomonas aeruginosa*. *S. aureus* was found to be associated with heavier beach use by humans. In addition, *S. aureus* was found in several samples that did not contain total or fecal coliforms. This result suggests that the use of *S. aureus* as an indicator organism could provide evidence of contamination from human sources

other than the intestinal tract, and would be a good supplement to the use of enteric indicator organisms. Plano et al. (2011) also examined the contributions of adult human bathers to the *Staphylococcus aureus* present in marine waters. To isolate *S. aureus* from the bathers, cultures were taken from nasal swabs. They then placed subjects into inflatable pools filled with marine water collected from an area where no humans were present. After the subjects bathed in the pools for two 15-minute cycles, levels of *S. aureus* increased by two orders of magnitude. *S. aureus* were typed by sequencing the *spa* gene, which encodes protein A and possesses a highly variable repeat region that has been used to differentiate strains of *S. aureus* (*spa* typing; Shopsin et al., 1999). Twelve of fifteen methicillin-resistant *S. aureus* isolated were identical to those from two participants, and 13 of 17 methicillin-sensitive *S. aureus* isolates were identical to those from three other subjects. These results suggest that *S. aureus* is a good indicator of human contact with water, although not all humans contributed equally to the measured *S. aureus* loads.

It is not clear whether *S. aureus* or other staphylococci are also shed by marine mammals in the waters they inhabit. Some staphylococci have been found in marine mammals, and these seem to have little overlap with isolates taken from humans. For example, Streitfeld and Chapman (1976) compared staphylococci isolated from humans and captive bottlenose dolphins (*Tursiops truncatus*); of 31 healthy dolphins and one with a respiratory tract infection, coagulase-positive staphylococci were isolated from 7 healthy dolphins and the ill dolphin. In addition, of 32 healthy personnel, coagulase-positive staphylococci were isolated from 14. There were considerable differences in antibiotic resistance patterns between the dolphin and human isolates. This difference suggests that staphylococci strains were not shared between the humans and dolphins. Buck et al. (1988) isolated unidentified *Staphylococcus* spp. from the blowhole and anus of two out of three stranded Atlantic white-sided dolphins (*Lagenorhynchus acutus*) that

were brought into captivity. One of these dolphins died and *Staphylococcus* spp. were also recovered from its heart and lungs. A separate strain identified as *S. aureus* was also isolated from the blowhole of one of these dolphins and the anus of the other. *Staphylococcus* spp. (including *S. aureus* and *S. epidermidis*) have also been cultured from the exhalations of wild killer whales (*Orcinus orca*; Raverty et al., 2017), while several species of *Staphylococcus* were cultivated from blowhole and anal swabs of two wild-caught, captive beluga whales (*Delphinapterus leucas*) during a longitudinal study (Buck et al., 1989). Species isolated from the blowhole included *S. aureus, S. cohnii, S. epidermidis, S. haemolyticus, S. hominis, S. hyicus, S. saprophyticus, S. sciuri, S. warneri,* and *S. xylousus.* A subset of these were isolated from the anus, including *S. aureus, S. epidermidis, S. hominis, S. saprophyticus,* and *S. warneri.* Although several isolates were collected, none of these species were consistently isolated from either body site over the duration of the 945-day study, suggesting transient colonization or variation depending on their exposures or general health status.

S. aureus can pose some risks to dolphin health. In a survey of 2,586 bacterial isolates cultured from bottlenose dolphins (*Tursiops truncatus*), *S. aureus* comprised 44 out of the 64 isolates considered to be primary pathogens resulting in mortality (Venn-Watson et al., 2008). It must be noted that all samples included in this survey were isolated from internal tissues and fluids, not feces, skin, or blowholes. Thus, it remains a possibility that *S. aureus* can innocuously occupy such sites. In fact, staphylococci have been cultured on Baird-Parker agar from healthy bottlenose dolphin feces and gastric juices, but these were not identified at the species or strain level (Fiorucci et al., 2016).

In phocids and pinnipeds, *Staphylococcus* spp. have been isolated from sick or stranded individuals, in particular from ocular lesions and abscesses (Thornton et al., 1998; Johnson et al.

1998), as well as the ears, nose, umbilicus, and some infected wounds (Lockwood et al., 2006). Although Lockwood et al. tested fecal samples, they did not find any staphylococci in them.

It is worth noting that in cetaceans, phocids, and pinnipeds, there is no body site where staphylococci are consistently found. It is possible that they are present but the culture techniques used do not always capture them, but it is also possible that they are not present in marine mammals to the same degree that they are found in humans, and therefore would not be ideal indicators of pollution from marine mammal sources. Further investigation will be necessary to determine whether either of these hypothetical scenarios are correct.

1.2.3 Enterococci as indicators

Enterococci are commonly used as fecal indicator bacteria because they are found in high concentrations in the feces of humans and certain animals and in water that contains feces (Slanetz & Bartley, 1957). Domestic animals whose feces contain enterococci include cattle, sheep, horses and dogs (Slanetz & Bently, 1957) as well as poultry and swine (Hamerum, 2012). Enterococci are also found in the feces of many, but not all, wild animals, including mammals, birds, and reptiles (Mundt, 1963), though their presence and abundance in feces varies both by individual and species.

There is little information regarding the presence of enterococci in marine mammals. Enterococci have been isolated from wild bottlenose dolphins (*Tursiops truncatus*), though they were more prevalent in blowhole swabs than in fecal samples (Buck et al., 2006). The authors mentioned the possibility, however, that the enterococci could have originated from human fecal contamination of the water surrounding the animals sampled. Enterococci have also been isolated from fecal samples collected from dead cetaceans including a dwarf minke whale (*Balaenoptera acutorostrata*), a humpback whale (*Megaptera novaeangliae*) and a Risso's

dolphin (*Grampus griseus*). The carcasses were in an intact state suggesting that significant decomposition had not taken place; thus, the enterococci were presumed to have been present while the animals were alive (Prichula et al., 2016).

Regarding phocids and pinnipeds, *Enterococcus* spp. DNA has been identified in the feces of South American fur seals (*Arctocephalus australis*) and Subantarctic fur seals (*A. tropicalis*) using qPCR (Medeiros et al., 2017). *Enterococcus* spp. have also been isolated from abscesses in stranded California sea lions (*Zalophus californianus*), harbor seals (*Phoca vitulina*) and northern elephant seals (*Mirounga angustirostris*) using nonselective media. However, fecal samples taken concurrently from the same animals did not yield any *Enterococcus* spp. (Johnson et al.,1998). Based on these data, there are a few possibilities to consider: a) *Enterococcus* spp. are present in the feces of *A. australis* and *A. tropicalis*, but not in *P. vitulina*, *Z. californianus*, or *M. andustirostris;* b) *Enterococcus* spp. are present in a viable but not culturable (VBNC) state in pinnipeds in general; c) *Enterococcus* DNA is present in pinniped feces, but it originates from dead cells; d) the difference in *Enterococcus* detection between Medieros et al. (2017) vs. Johnson et al. (1998) is due to individual variation between the microbiota of the animals studied; or e) the use of qPCR in Medieros et al. (2017) was more sensitive for detection of *Enterococcus* than the culture-based isolation method used in Johnson et al. (1998). If *Enterococcus* spp. are not reliably present in a culturable state in the feces of all marine mammals, it does not make sense to utilize them as indicators of marine mammal fecal presence. That said, if there are certain marine mammal species whose feces do reliably contain *Enterococcus* spp., then they would have potential as fecal indicators in enclosures that house only those species.

1.2.4 Pseudomonads as indicators

Pseudomonas spp. can be used as process indicators for the effectiveness of sanitation or the impact of water storage on bacterial regrowth. Like coliform organisms, their presence in drinking water is correlated with human gastrointestinal illness (de Victoria and Galván, 2001). However, *P. aeruginosa* is not strictly correlated with fecal coliforms, and it has therefore been suggested as a supplemental indicator for human recreational water quality monitoring (Mates, 1992). Members of the family *Pseudomonadaceae* are also not correlated with total coliforms in drinking water systems (Ribas et al., 2000), suggesting that their presence can provide information that is not given by coliform tests.

Pseudomonas spp. can survive and proliferate in oligotrophic waters due to their powerful affinity for biodegradable organic carbon (Ribas et al. 2000; Van der Kooij et al., 1982). This characteristic makes them highly sensitive indicators of bacterial growth in waters that are expected to be very clean or have undergone some form of sanitization (as is typical in marine mammal enclosures). In particular, the presence of *Pseudomonas* in treated waters can indicate that treatment has not successfully removed all bacteria from the water, and that bacteria are growing post-treatment (e.g. in distribution systems or animal enclosures).

1.3 Isolation and Enumeration Methods for Indicator Organisms

1.3.1 Membrane filter technique for enumerating total coliforms

One of the methods for coliform bacteria is growth as colonies and formation of a metallic sheen (due to the acid production) on LES Endo agar (sold by Difco™ as m-Endo LES). This medium contains lactose (which coliform bacteria ferment to produce acids and gas and other aldehydes), as well as sodium desoxycholate and sodium lauryl sulfate to inhibit the

growth of non-coliform organisms. The medium also contains fuchsin and sodium sulfite, which turn red and develop a golden or green metallic sheen in the presence of the acids produced during lactose fermentation by coliform bacteria, serving to differentiate them from other colonies that may grow on the medium. Plates are incubated for 22 ± 2 h at 35 ± 0.5 °C, and all colonies that develop a metallic sheen are counted as total coliforms (Rice et al., 2012; Difco and BBL Manual).

1.3.2 Membrane filter technique for enumerating staphylococci

Baird-Parker agar with egg yolk tellurite enrichment is a selective and differential medium for colonies of staphylococci. Baird-Parker agar contains sodium pyruvate as an enrichment, and glycine and lithium chloride to inhibit non-staphylococci. The egg yolk is an enrichment that can help differentiate *Staphylococcus aureus* by producing a transparent area around colonies (though this isn't visible under the membrane filter, it can be useful for non-filtration based methods). The tellurite inhibits non-staphylococci and produces a black coloration when reduced by staphylococci. Plates are incubated for 48 ± 4 h at 35 ± 0.5 °C, and black or gray colonies with smooth surfaces and entire margins are counted as staphylococci (Rice et al., 2012; Difco and BBL Manual).

1.3.3 Membrane filter technique for enumerating enterococci

The simplest membrane filter method for colony enumeration of enterococci is the use of mEI agar with nalidixic acid and 2,3,5-triphenyltetrazolium chloride (TTC). Directions for the preparation of mEI agar are published in Rice et al. (2012) but commercial preparations are also available. The nalidixic acid is an antibiotic that selects for enterococci and TTC turns red when metabolized by enterococci and certain other organisms. In addition, the medium contains indoxyl-β-D-glucoside, which is also metabolized by enterococci, leaving behind indoxyl, which

then reacts with atmospheric oxygen to produce a blue dye (Difco and BBL Manual). This produces a visible blue halo around enterococci colonies which can be used to differentiate them from other organisms that may be able to grow on the medium. Plates are incubated for 24 ± 2 h at 41 ± 0.5 °C, and all colonies that develop a blue halo are counted as enterococci.

1.3.4 Membrane filter technique for enumerating Pseudomonas

Modified M-PA agar (sold by Hardy Diagnostics and Difco™ as m PA-C and M-PA-C, respectively) is a selective agar for the cultivation of *Pseudomonas* colonies. It was developed for *P. aeruginosa* tests, and is recommended for this purpose in Rice et al. (2012). This medium contains kanamycin to inhibit gram-positive organisms and nalidixic acid to inhibit most gram-negative bacteria. It also contains phenol red, which becomes yellow in acidic conditions produced by fermentation (though not all *Pseudomonas* will produce this coloration). Plates are incubated for 72 ± 4 h at 41.5 ± 0.5 °C. *P. aeruginosa* produce flat colonies with light outer rims and brownish to greenish-black centers, but other *Pseudomonas* spp. may produce different morphologies.

1.4 Significance of Regulations for Marine Mammal Health

1.4.1 How will aquaria meet the count requirements?

It is common for aquaria to utilize general disinfection[3] techniques to maintain waters that hold marine mammals within microbial limits. Such treatments include the use of UV radiation, chlorine-based oxidants, and ozone. Chlorine-based treatments are a form of bulk disinfection, meaning that the chemicals used are dispersed throughout the water system,

[3] Note that in a document entitled *Sterilization of Marine Mammal Pool Water,* published by USDA APHIS, Spotte (1991) refers to UV radiation, chlorine, and ozone treatments as "sterilization" methods even though they are not intended to eliminate all microbial life because they are not intended to target specific microbial taxa.

including the part where animals reside. Ozone and UV radiation are point-contact disinfection methods, meaning that water leaves the exhibit to be pumped through a contact chamber which either injects ozone or shines UV light into the water. In general, bulk disinfection levels involve low levels of chemical disinfectant in all system water, while point-contact disinfection methods have very high levels of disinfectant in a small part of the system and no disinfectant in the rest of the system (Spotte, 1991).

1.4.2 Potential Consequences of treatment

Both chlorine- and ozone-based treatments and can produce chemical byproducts which, at high enough concentrations, can be toxic to mammals (Spotte, 1991). While this is not known to be an issue with UV radiation treatment, it would take an enormous amount of energy to significantly impact the microbial load in the amount of water required to comfortably house a marine mammal (Spotte and Buck, 1981). Therefore, a chemical treatment method must almost always be used, making chemical byproducts of disinfection a potential cause of concern in marine mammal enclosures.

It is difficult to assess the impact of microbial load on marine mammal health because there is not a standard baseline for a "healthy animal". While the presence of an infection is an obvious sign of poor health, there is not published data comparing rates of marine mammal infection with the microbial load of their waters. There are documented differences between the immune systems of captive and wild marine mammals. For example, a study comparing two populations of wild Atlantic bottlenose dolphins (*Tursiops truncatus*) with one captive population found that the captive population had a significantly lower total white blood cell count, but a higher monocyte count, when compared to the wild populations (Nouri-Shirazi et al., 2017). Another study comparing two captive populations and two wild populations (not the same

populations as in the previous citation) of *T. truncatus* showed that the wild dolphins had more upregulated immune activity across many parameters (Fair et al., 2017). There are some important caveats when interpreting these studies. While these results do show differences between captive and wild dolphin immune function, it is difficult to assess whether this means one population is healthier than the other. In addition, it must be noted that disinfection is only one difference among many between captive and wild environments, and while it may contribute to the observed differences between captive and wild dolphin immune function, there is not sufficient published data to conclude that it is the primary variable responsible for these differences.

Consequences to the animal and water microbiota from using ozone, chlorine-based treatments, and UV radiation are not well understood in marine mammal systems, but several studies have been performed in other types of systems. It is important to understand that, while these treatments are considered nonselective in that they are not designed to target specific microbial taxa, this does not mean that certain taxa are not more likely to survive these treatments. In a study designed to simulate the effects of ozone and UV treatment on ballast water, exposure to ozone or UV radiation initially reduced cell counts in treated water below the limit of detection. Following exposure by 2-6 days, cell counts returned to or exceeded initial levels, but the diversity of these cells was greatly reduced (Hess-Erga, 2010). A study testing the bacterial community composition along various points in a drinking water treatment system also showed a reduction in gram-negative bacteria and an increase in gram-positive bacteria after both ozone and chlorine treatment when compared with untreated water, but the community composition of tap water that had passed through the entire system resembled that of untreated water (Vaz-Moreira et al., 2012). Because of these effects on microbial diversity, it is important

to study multiple taxa or entire microbial communities when evaluating the effects of water disinfection in order to understand how disinfection impacts these communities.

2. RESEARCH OBJECTIVES

The research described herein was conducted in the Abbott Oceanarium exhibit at the Shedd Aquarium. This exhibit houses Pacific white-sided dolphins (*Lagenorhynchus obliquidens*), beluga whales (*Delphinapterus leucas*), and California sea lions (*Zalophus californianus*). It is divided into several habitats, each of which has its own dedicated life support system consisting of mechanical sand filtration and ozone treatment. It is not well understood how the exhibit's life support systems influence the microbial communities of both the marine mammals and the water. The overall goal of this research was to determine the abundance and genetic characteristics of the bacterial indicators proposed by USDA APHIS in the Oceanarium exhibit while using two different water treatments (sand filtration and ozone). This goal was addressed by comparing concentrations of CFUs that grow on m Endo LES, Baird-Parker, mEI, and m PA-C media between water samples collected at three sites: a) the Oceanarium exhibit surface; b) the life-support system downstream of sand filtration and upstream of the ozone treatment point; and c) the life-support system immediately downstream of ozone treatment and upstream of returning to the exhibit. It was hypothesized that indicator concentrations would differ significantly between these three sites. Since it is not known what typical concentrations of staphylococci, pseudomonads, or enterococci are in a recirculating system housing marine mammals, nor how often these organisms would exceed the proposed maximum concentrations, samples taken on five consecutive days were tested to determine the stability of bacterial concentrations over time.

Next, the identities of microbes grown on selective and differential media were determined using genetic tools. Baird-Parker, mEI, m PA-C and m Endo LES are designed to be selective for *Staphylococcus*, *Enterococcus*, *Pseudomonas*, and total coliforms, respectively.

While these media are standard for human recreational and drinking water assays (Rice et al., 2012), the Abbott Oceanarium has many novel characteristics (including being a manmade closed system containing recirculating, artificial seawater and utilizing ozone disinfection). Hence, it is not well understood how the use of these assays will translate to this system and it is therefore important to evaluate whether these standard media can be used to test concentrations of the proposed indicators in such a system. A subsample of colonies was isolated from each media type and classified at the genus level using 16S rRNA gene sequencing. These identities were used to determine the selectivity of m Endo LES for total coliforms, Baird-Parker for *Staphylococcus*, mEI for *Enterococcus*, and m PA-C for *Pseudomonas* in the Oceanarium system. It was hypothesized that the selective media would detect a greater diversity of bacterial species and not just the organisms that these media are designed to capture.

Genetic sequence data was also used to compare the taxonomic identities of organisms isolated from each site, as well as indicator organisms isolated from the feces and chuff (material including mucous and water expelled from the blowhole by rapid exhalation), of Pacific white-sided dolphins (*Lagenorhynchus obliquidens*). If certain taxa are found primarily in the life support system, those taxa may originate from regrowth within the system rather than from animal sources. Phylogenetic relationships of isolates recovered were visualized in tree format so that their physical distribution could be compared to their taxonomic distribution.

Enterococcus, *Pseudomonas*, *Staphylococcus*, and total coliforms are intended to indicate microbial risk (i.e., the presence of fecal or other animal-derived material that may contain potentially disease-causing microbes). Therefore, it is of interest whether the abundance of these indicator organisms predict aspects of the total microbial community in the water from which they are isolated. To address this, I also examined whether there are associations between

specific indicator counts and 16S rRNA gene community sequence data, both in terms of community dissimilarity between samples (as measured using adonis permutational regression), and in terms of relationships to specific taxa detected by 16S rRNA gene sequencing.

3. MATERIALS AND METHODS

3.1 Sampling Locations

The research for this thesis was conducted in the Oceanarium system at the Shedd Aquarium. This is a contiguous system of approximately 3 million gallons total volume. It is made up of interconnected habitats that housed Pacific white-sided dolphins, beluga whales, and California sea lions during the present study. The habitats are referred to as the Large Habitat, Small Habitat, Sea Lion Exhibit, Sea Lion Reserve, and IsoMed. Under normal operation, water flows freely, but is not deliberately pumped, between the habitats. Each habitat has its own dedicated life-support system consisting of mechanical sand filtration and an ozone contact chamber. The present study was conducted in the Large Habitat and its associated life support system, which contained Pacific white-sided dolphins (*Lagenorhynchus obliquidens*) for the duration of the sampling period. Specific sampling locations within this system are listed in Table 2.

Sample Location	Abbreviations used	Description
Life support system between sand filtration and ozone contact	Pre; PreOz	Sample port connected to ozone tower influent in Large Habitat life support system
Ozone contact chamber effluent	Post; PostOz	Sample port connected to ozone tower effluent in Large Habitat life support system
Large Habitat Surface	LH; Surface; Exhibit	Surface water in Large Habitat exhibit

Table 2: Sampling locations in the Oceanarium system from which water was collected.

3.2 Sample Collection and Processing

Water sampling took place each day from May 8-12, 2017. A total of 5 water samples were obtained per site divided among four media types with three replicates each, or 180 plates in total. Sterile sampling containers were rinsed three times with water from the sample source

prior to sample collection. Exhibit water was collected directly from the Large Habitat pool surface. Water from the life-support system was taken from spigots installed in the system plumbing. Water was flushed through the spigots for at least one minute before samples were taken. Samples were vacuumed through individually sterilized Supor® PES Membrane Disk Filters with a pore size of 0.2 µm and diameter of 47 mm (Pall Corporation, catalog no. 66234). Samples from each source were filtered using a separate funnel apparatus. Sample volumes (listed in Table 3) ranged from 0.1-1000 mL depending on the source and the indicator organism being tested. These volumes were chosen to obtain countable bacterial colonies and were based on preliminary data collected from the same sites. For sample volumes smaller than 10 mL, 10-50 mL of autoclave-sterilized phosphate buffered water (PBW; [40.55 mg $MgCl_2$ + 53.75 mg KH_2PO_4] × L^{-1}) was added directly to the funnel to increase the uniformity of bacterial CFU distribution across the membrane. Samples were filtered in order from smallest to largest volume to minimize cross-contamination. After filtration, membranes were placed onto selective and differential media and incubated at the appropriate temperature and time for each organism being tested. Media types, incubation temperatures, and incubation times are listed in Table 4. All samples were processed in triplicate. Samples for total microbial community analysis (n = 5 per site, with each sample being partitioned into three subsamples and processed in triplicate, for a total of 45 data points) were collected and processed as described in the previous paragraph up to and including the filtration step. 1 L of water was filtered per replicate and filters were placed into Mo Bio MagAttract PowerWater DNA/RNA Kit bead tubes (Mo Bio, catalog number 27800-4-EP) rather than petri dishes and stored at -80 °C until DNA extraction was performed.

Organism	Sample source	Volumes filtered (mL)
Pseudomonads	Pre-ozone	0.1, 1.0, 10
Enterococci	Pre-ozone	1000
Staphylococci	Pre-ozone	10, 100, 1000
Total coliforms	Pre-ozone	100, 1000
Pseudomonads	Post-ozone	1000
Enterococci	Post-ozone	1000
Staphylococci	Post-ozone	100, 1000
Total coliforms	Post-ozone	100, 1000
Pseudomonads	Large Habitat surface	100, 1000
Enterococci	Large Habitat surface	1000
Staphylococci	Large Habitat surface	10, 100, 1000
Total coliforms	Large Habitat surface	10, 100, 1000

Table 3: Volumes of water filtered for indicator samples.

Organism	Media type	Product reference	Incubation temperature (°C)	Incubation time (hours)
Pseudomonads	m PA-C	Criterion™ by Hardy Diagnostics, cat. no. C7961	41	72 ± 4
Enterococci	mEI	BD Difco™ cat. no. 214881	41	24 ± 2
Staphylococci	Baird-Parker	BD Difco™ cat. no. 276840	35	48 ± 4
Total coliforms	m Endo LES	Criterion™ by Hardy Diagnostics, cat. no. C7411	35	22 ± 2

Table 4: Culturing procedures.

All media were prepared according to manufacturer's instruction with additives specified. 50 mL of PBW was filtered at the start and end of each filtering session and placed on one plate of each media type to control for contamination. The following positive controls were used for each freshly-prepared batch of media: *Pseudomonas aeruginosa* ATCC 27853™ for m PA-C, *Enterococcus faecalis* ATCC 19433™ for mEI, *Escherichia coli* ATCC 25922™ for m Endo LES, and *Staphylococcus aureus* ATCC 25923™ for Baird-Parker.

Fecal samples from 5 dolphins were collected using a sterile 2.7 mm rubber catheter tube which was inserted into the rectum. The dolphins were trained to allow this procedure on command for routine health surveillance. Chuff samples (material expelled from the blowhole during rapid exhalation) from the same 5 dolphins were collected by holding a sterile petri dish above the blowhole and having the dolphins exhale chuff upon command. The rubber tubes and petri dishes were swabbed using sterile cotton swabs, which were then placed into brain-heart infusion broth and incubated at 35 °C for 24 hours for enrichment. Based on separately collected data, the weight of fecal material per swab (average ± standard deviation) is approximately 2.0 mg ± 3.8 mg, and the weight of chuff per swab is 0.1 mg ± 0.5 mg (some samples displayed negative weights, presumably either due to measuring error or because fibers from the swabs used to collect samples were shed during swabbing, resulting in a net loss of material). 0.01 mL and 0.1 mL volumes of each enrichment culture were then spread onto plates of m PA-C, mEI, m Endo LES and Baird-Parker media and incubated at the same temperatures and durations used for water samples.

After incubation, colonies from water samples were counted, and dolphin fecal and chuff samples were checked for the presence or absence of colonies. Greenish-brown to yellow colonies with dark centers (after Rice et al., 2012) and light pink colonies (based on the manufacturer's image of *P. aeruginosa* colonies on m PA-C; https://catalog.hardydiagnostics.com/cp_prod/product/images/catalog/G150_mPA-C%20Agar_web.jpg) colonies on m PA-C were counted as presumptive *Pseudomonas*; all colonies surrounded by a blue halo on mEI were counted as presumptive *Enterococcus*; black and grey colonies on Baird-Parker were counted as presumptive *Staphylococcus*; and dark red colonies with a metallic sheen on m Endo LES were counted as presumptive total coliforms.

Because the positive control for the batch of m Endo LES used during May 8-10 developed a metallic sheen at 48h, colonies were counted at this time. Averages and standard deviations were calculated between replicates and converted to CFU/100 mL. Counts of all indicators were log transformed and compared between sampling sites using the Analysis of Variance (ANOVA) test and means were separated using Tukey post-hoc tests.

3.3 Molecular Analysis

A subsample of colonies from each media type was subcultured by streaking onto blood agar (Hardy Diagnostics catalog no. A10) or brain-heart infusion (BHI) agar (Criterion™ by Hardy Diagnostics catalog no. C5141 with 15g agar × L^{-1}). Morphological characteristics (color, shape, margin and elevation) were recorded for all isolates when grown on the selective and differential media. A subset of atypical colonies that did not produce a metallic sheen on m Endo LES agar ("atypical coliforms") were isolated in addition to sheen-producing colonies, based on the recommendation from Rice et al. (2012) that a subset of atypical colonies be verified. Isolates were stored at 4 °C for up to 75 days prior to characterization. Colony PCR was performed on each isolate to amplify the nearly-complete 16S rRNA gene for sequencing following the protocol described by Muyzer et al, 1993. Individual colonies were lysed using PrepMan™ Ultra sample preparation reagent (Applied Biosystems™ catalog no. 4318930) following the manufacturer's protocol, and lysates were subjected to PCR using universal primers GM3F (5´-AGAGTTTGATCMTGGC-3´) and GM4R (5´-TACCTTGTTACGACTT-3´) (Muyzer et al., 1993). PCR conditions included the addition of 12.5 µL 2X Kapa High Fidelity Hot Start Readymix (Kapa Biosystems catalog no. KK2601) or Phusion® Hot Start Flex Master Mix (New England Biolabs® catalog no. M0536S) to 10 µL water, 0.75 µL each of 10 µM GM3F and GM4R primers, and 1 µL lysate as template (25 µL total reaction volume).

The thermal cycling protocol was 3 min at 95 °C for initial denaturation followed by 25 cycles of 20 s denaturation at 98 °C, 15 s annealing at 45 °C, and 30 s extension at 72 °C; and final extension at 72 °C for 90 s. PCR products were confirmed by gel electrophoresis (1% agarose gel with TBE buffer) at 150 V for 35 minutes, and the DNA was visualized using GelRed™ Nucleic Acid Gel Stain (Biotium catalog no. 41002). For isolates that failed to amplify the product by PCR, lysates were diluted in 9 parts water per 1 part lysate to reduce the concentration of PCR inhibitors and the PCR was repeated. If no band was observed after dilution and repeated PCR, then the isolate was not subjected to further analysis. For the remainder of the samples, PCR products were purified using a ZR-96 DNA Clean-up Kit™ (Zymo Research catalog no. D4017) and eluted in water. Purified products were quantified fluorometrically using a Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen™ by Thermo Fisher Scientific catalog no. P11496) and amplicons were sequenced at the University of Illinois Urbana-Champaign Core Sequencing Facility's Sanger sequencing service (UIUC Core Sequencing Facility, 334 Edward R. Madigan Laboratory, 1201 W. Gregory Drive, Urbana, IL 61801). Several colonies could not be successfully isolated, amplified, or sequenced, bringing the final number of sequences available for analysis to 33 presumptive enterococci, 16 presumptive staphylococci, 28 presumptive pseudomonads, 23 presumptive total coliforms, and 18 atypical coliforms.

Genomic DNA for 16S rRNA gene-based community analysis was extracted from samples using a Mo Bio MagAttract PowerWater DNA/RNA Kit (Mo Bio, catalog number 27800-4-EP) following the manufacturer's directions for DNA isolation. Extracted DNA was quantified using a Qubit 3.0 fluorometer and the V4 region of the 16S rRNA gene was amplified by PCR using as described previously (Caporaso et al., 2011, 2012): 1 µL of sample extract was

used as template in 13 µL of PCR-grade water, 10 µL of 2X Kapa HiFi Mastermix, 0.5 µL of 10 µM 515F primer (5´-GTGYCAGCMGCCGCGGTAA-3´), and 0.5 µL of 10 µM 806RB primer (5´-GGACTACNVGGGTWTCTAAT-3´) with Golay barcodes for a total reaction volume of 25 µL. The thermal cycling protocol was 3 min at 94 °C followed by 35 cycles of 45 s at 94 °C, 60 s at 50 °C, and 90 s at 72 °C; then 5 min at 72 °C and a final holding temperature of 4 °C. Amplicons were visualized using gel electrophoresis and quantified using PicoGreen. They were then pooled to a single sequencing library and sequenced using an Illumina MiSeq.

3.4 Bioinformatics Approach

During Sanger sequencing, fluorescently tagged base pairs are added to the template sequence, and the color of the fluorescence is used to determine the identity of each base pair. The raw output of a Sanger sequencing run is a roughly sinusoidal graph of the fluorescence of the entire DNA molecule. This graph is called a chromatogram. Each peak on the graph corresponds to one base pair. Because there were tens of thousands of base pairs, the base identities were determined from Sanger chromatograms automatically using Geneious software version 10.2.3 (http://www.geneious.com, Kearse et al., 2012). Some (n=37) sequences contained mostly ambiguous bases and these were omitted from the downstream analysis. Sequences were submitted to GenBank (Benson et al. 2005) under accession numbers MH208823-MH208837 (presumptive staphylococci), MH208857-MH208883 (presumptive enterococci), MH208922-MH208946 (presumptive pseudomonads), and MH208884-208921 (presumptive total coliforms and atypical coliforms). Forward-read sequences were identified by aligning to the NCBI Nucleotide Collection database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the built-in BLAST search feature in Geneious 10.2.3. Specific parameters used were as follows: the BLAST algorithm used was Megablast, word size = 28 bp, linear gap cost, and

match/mismatch scoring = (1, -2). Reverse-read sequences were not analyzed as they contained mostly ambiguous bases.

Region	Start position	End position
Complete	1	1,542
V1	69	99
V2	137	242
V3	433	507
V4	576	682
V5	822	879
V6	986	1,043
V7	1,117	1,173
V8	1,243	1,294
V9	1,435	1,465

Table 5: Positions of hypervariable regions of the E. coli 16S rRNA gene (Yang et al., 2016).

Sequences were mapped to the *Escherichia coli* K12 16S rRNA gene and hypervariable regions were selected according to Yang et al. (2016). The positions of these regions on the *E. coli* K12 genome are shown in Table 5. Phylogenetic trees were constructed using Geneious Tree Builder (Kearse et al., 2012) configured with the following settings: Tamura-Nei genetic distance model (Tamura and Nei, 1993), neighbor-joining tree build method, no outgroup, and bootstrap resampling with 500 replicates to create a consensus tree (as described in Felsenstein 1985). A support threshold of 80% was used, which means that nodes were only included in the consensus tree if they appeared in at least 80% of bootstrap replicates. For each hypervariable region, separate trees were generated to include isolates from each medium used. Sequences from type strains of the genera selected for by each media were included in trees for reference. These strains are listed in Table 6.

Media	Positive control species	GenBank accession number
mEI	*Enterococcus faecalis*	AB012212
Baird-Parker	*Staphylococcus aureus*	L37597
m PA-C	*Pseudomonas aeruginosa*	HE978271
m Endo LES	*Escherichia coli*	AB681728

Table 6: Positive controls for phylogenetic tree analysis.

The most specific taxonomic level to which all sequences could be identified was the genus level. The closest-matching genus identities from BLAST search results were plotted according to their relative abundance among the pre-ozone, post-ozone, and exhibit surface isolates, and isolates cultured from dolphin fecal and chuff samples.

FASTQ-formatted Illumina reads from total community samples were processed using Quantitative Insights into Microbial Ecology (QIIME) version 1.9.1 (Caporaso et al., 2010). Sequences were demultiplexed according to their barcodes. Sequence data then underwent merging of paired sequence reads, denoising, and removal of eukaryote plastid and chimera sequences. Operational taxonomic unit (OTU) clustering was performed de novo based on a 97% sequence similarity threshold, and taxonomy assignment of resulting OTUs was performed using the SILVA 1.2.8 database. The number of sequences was rarified to 30,000 per sample replicate. Data were then partitioned by sample site. The partitions were visualized using principal coordinates analysis (PCoA) and ordination along the first three principal coordinates. One data point each was removed from the pre-ozone and post-ozone partitions because including those points caused all remaining points within the same partition to form a single cluster that was relatively distant from that point, indicating that the point was an outlier. Thus, the number of samples in the pre-ozone and post-ozone partitions was reduced to 14 each. After outliers were removed, adonis permutational analysis of variance with 999 permutations, implemented in

QIIME using the R Vegan package (Oksanen et al., 2017) was used to determine how much the Bray-Curtis distance between sample communities could be explained by associated indicator counts. To further examine which specific aspects of the communities could be explained by indicator counts, Pearson correlation of all OTUs against indicator counts was performed. Because of the large number of statistical analyses involved with this step, p-values were corrected using the false discovery rate (FDR) method (Benjamini and Hochberg, 1995) to reduce the proportion of experiment-wide type I error to 0.05.

4. RESULTS

4.1 Comparison of Counts between Sampling Sites

Colony counts on mEI, Baird-Parker, m PA-C and m Endo LES were compared between water samples collected upstream of ozone contact, downstream of ozone contact, and from the exhibit surface using an analysis of variance (ANOVA) test on log-transformed counts (Table 7; all counts were increased by 1 prior to log transformation to account for non-detects). There were significant between-site differences on all media, but the source of these differences, as determined using Tukey post-hoc tests, varied between the media. For presumptive enterococci, geometric mean count in the exhibit was 0.14 CFU/100 mL, which was significantly greater than the counts in plumbing pre- or post-ozone (0.00 and 0.0061, respectively, $p = 6.67 \times 10^{-6}$; note that this and following p-values in this paragraph represent the probability of the null hypothesis that all three sites have identical counts). For presumptive staphylococci, the geometric means in the exhibit and pre-ozone (1.3 and 1.7 CFU/100 mL, respectively) were significantly higher than post-ozone (0.0047 CFU/100 mL, $p = 0.025$). For presumptive pseudomonads, the geometric mean pre-ozone was 19,000 CFU/100 mL, which was significantly greater than post-ozone or in the exhibit (0.16 and 0.57 CFU/100 mL, respectively, $p < 2 \times 10^{-16}$). Finally, for presumptive total coliforms, the geometric mean count in the exhibit (0.24 CFU/100 mL) was higher than post-ozone (0.0047 CFU/100 mL) but neither were significantly different from pre-ozone (0.12 CFU/100 mL; $p = 0.025$).

	CFU per 100 mL at each site[4]			
Indicator	**Pre-ozone**	**Post-ozone**	**Exhibit**	**P-value**
Pseudomonads	19,000[1]	0.16[2]	0.57[2]	<2e-16
Enterococci	0.0061[1]	0.00[1]	0.14[2]	6.67e-06
Total coliforms	0.12[1,2]	0.0047[1]	0.24[2]	0.025
Staphylococci	1.7[1]	0.09[2]	1.3[1]	0.002

Table 7: Comparison of geometric mean colony counts in water samples upstream of ozone contact, downstream of ozone contact, and from the Oceanarium exhibit surface water.

Dolphin fecal and chuff swabs were tested for the presence or absence of growth on each of the four media tested. Among the fecal samples from 5 dolphins, 5 (100%) produced presumptive total coliforms, while 3 (60%) produced presumptive enterococci, staphylococci, and pseudomonads. Among chuff swabs from the same 5 dolphins, 4 (80%) produced presumptive enterococci, staphylococci, and pseudomonads; and 3 (60%) produced colonies presumptive total coliforms. The percent of positive samples for all sample sources, as well as detection limits, maximum observed counts, and geometric means in water samples, are shown in Table 8.

[4]Each geometric mean is computed from 15 samples. P-values are from ANOVAs performed on log-transformed colony counts and represent the probability that there is no difference between the three sample sites. Superscripts represent results of Tukey post-hoc tests; values in the same row are not significantly different if they have the same superscript ($p > .05$ according the Tukey test).

Sample Site	Organism tested	Medium	Number of samples	% Positive samples	Detection limit (CFU/100 mL)	Maximum observed count (CFU/100 mL)	Geometric mean (CFU/100 mL)
Pre-ozone	*Enterococcus*	mEI	15	7%	0.1	0.1	0.0061
Pre-ozone	*Pseudomonas*	m PA-C	15	100%	1000	164000	19000
Pre-ozone	*Staphylococcus*	Baird-Parker	15	73%	1	4	1.71
Pre-ozone	Total coliforms	m Endo LES	15	47%	0.1	1.7	0.12
Post-ozone	*Enterococcus*	mEI	15	0%	0.1	0	0
Post-ozone	*Pseudomonas*	m PA-C	15	87%	0.1	0.6	0.16
Post-ozone	*Staphylococcus*	Baird-Parker	15	33%	0.1	0.4	0.093
Post-ozone	Total coliforms	m Endo LES	15	7%	0.1	0.1	0.0047
Exhibit	*Enterococcus*	mEI	15	93%	0.1	0.9	0.14
Exhibit	*Pseudomonas*	m PA-C	15	47%	1	3	0.57
Exhibit	*Staphylococcus*	Baird-Parker	15	93%	0.1	7.2	1.3
Exhibit	Total coliforms	m Endo LES	15	60%	0.1	3	0.24
Dolphin Chuff	*Enterococcus*	mEI	5	80%	NA	NA	NA
Dolphin Chuff	*Pseudomonas*	m PA-C	5	80%	NA	NA	NA
Dolphin Chuff	*Staphylococcus*	Baird-Parker	5	80%	NA	NA	NA
Dolphin Chuff	Total coliforms	m Endo LES	5	60%	NA	NA	NA
Dolphin feces	*Enterococcus*	mEI	5	60%	NA	NA	NA
Dolphin feces	*Pseudomonas*	m PA-C	5	60%	NA	NA	NA
Dolphin feces	*Staphylococcus*	Baird-Parker	5	60%	NA	NA	NA
Dolphin feces	Total coliforms	m Endo LES	5	100%	NA	NA	NA

Table 8: Summary of bacteriological data collected from 5/8/2017-5/12/2017 in the Large Habitat and associated life support system.

4.2 Characterization of counts over time

Colony counts were taken from water samples over 5 consecutive days to characterize the dynamics of the USDA APHIS-proposed water quality indicators over time. These results are visualized in Figures 1 and 2. At no point did CFU concentrations in the Oceanarium exhibit water rise above the proposed limits of 500 CFU/100 mL presumptive total coliforms, 35 CFU/100 mL presumptive enterococci, 10 CFU/100 mL presumptive staphylococci, or 10 CFU/100 mL presumptive pseudomonads. The only exceedances of any of these values occurred with presumptive pseudomonad counts in samples collected downstream of mechanical filtration and upstream of ozone contact, where these counts ranged from 5,000 to 164,000 CFU/100 mL, with a geometric mean of 19,000 CFU/100 mL. All types of indicators varied within each sampling site by less than two orders of magnitude over the sampling period.

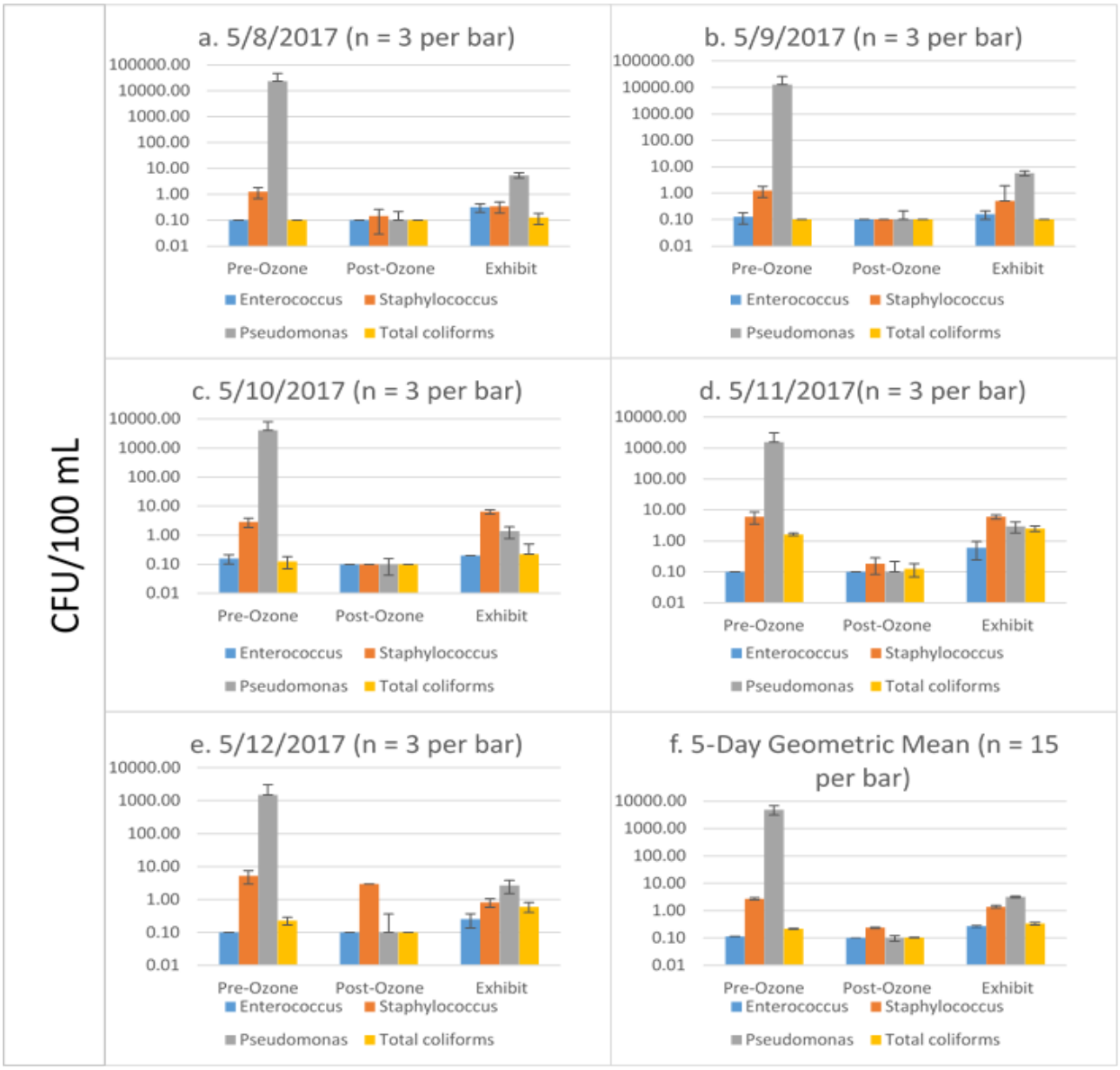


Figure 1: Colony counts of presumptive Enterococcus, Staphylococcus, Pseudomonas, *and total coliforms in water sampled from the Oceanarium exhibit and life support system.* Each bar in a.-g. represents the geometric mean of three samples. Each bar in f. represents the geometric mean of 15 samples (three per day for five days). Error bars represent one standard deviation. Data without error bars were below the detection limit for all replicates, making error estimation impossible. For samples with CFU concentrations below the detection limits of the assays used, a value of 0.1 CFU/100 mL was assigned (the detection limit for a 1000 mL sample).

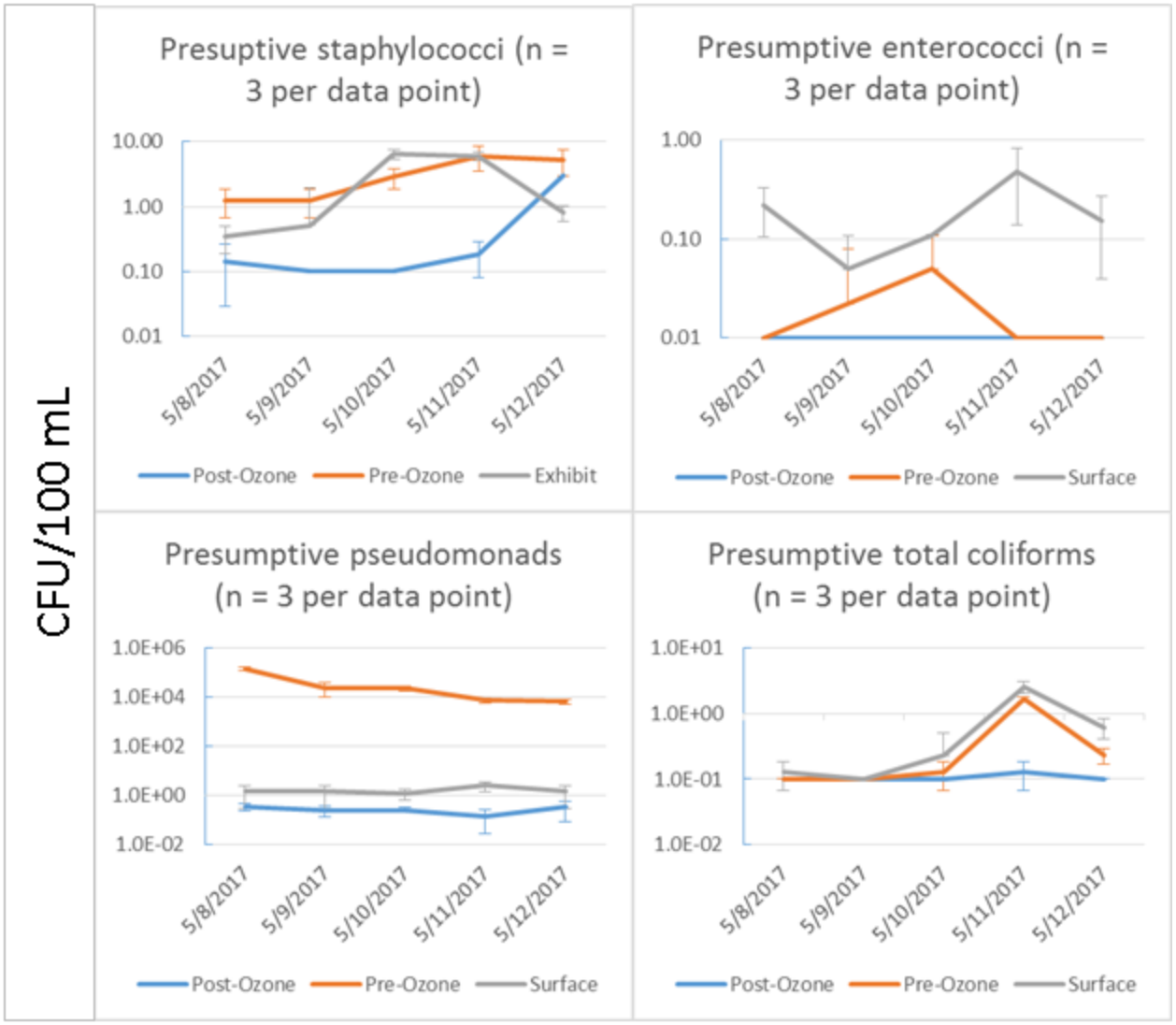


Figure 2: Each indicator organism plotted by day for each sampling site in the Large Habitat system. Each point represents three replicate samples. Error bars represent standard deviations. The positive and negative error bars are equal in magnitude, but appear asymmetrical due to log transformation of the data. Points without error bars were below the detection limit for all three replicates, rendering error estimation impossible. A value of 0.1 CFU/100 mL (the detection limit for a 1000 mL sample) was assigned when CFU concentrations were below the detection limit.

4.3 Genetic characterization of colonies

In order to evaluate the accuracy of the standard methods used to enumerate proposed indicator taxa in the unusual system of recirculating artificial sea water containing cetaceans and pinnipeds, a random subsample of colonies was isolated and identified using 16S rRNA gene sequencing. The results of these identifications are shown in Figs. 3-8. 27 of 27 (100%) of colonies from mEI (presumptive enterococci) were identified as *Enterococcus* spp., 3 of 16 (19%) of colonies from Baird-Parker (presumptive staphylococci) were identified as *Staphylococcus* spp., and 3 of 29 (10%) of colonies from m PA-C (presumptive pseudomonas) were identified as *Pseudomonas* spp. Among lactose-fermenting colonies on m Endo LES (presumptive total coliforms), 21 of 23 (91%) were *Enterobacteriaceae*, while 4 of 14 (29%) of non-lactose-fermenting colonies ("atypical coliforms") were *Enterobacteriaceae*.

Phylogenetic trees of the isolates were produced to visualize the relatedness of colonies from each sample site. V5-V9 regions of the 16S rRNA gene sequences were too low in quality to use for analysis. In addition, other variable regions were unusable on several sequences; thus, the trees produced for each of the V1-V5 regions each represent a different subset of isolates (with some overlap). Trees generated from V4 regions are shown in Figures 9-12; trees generated from the other hypervariable regions are shown in Appendix 1.

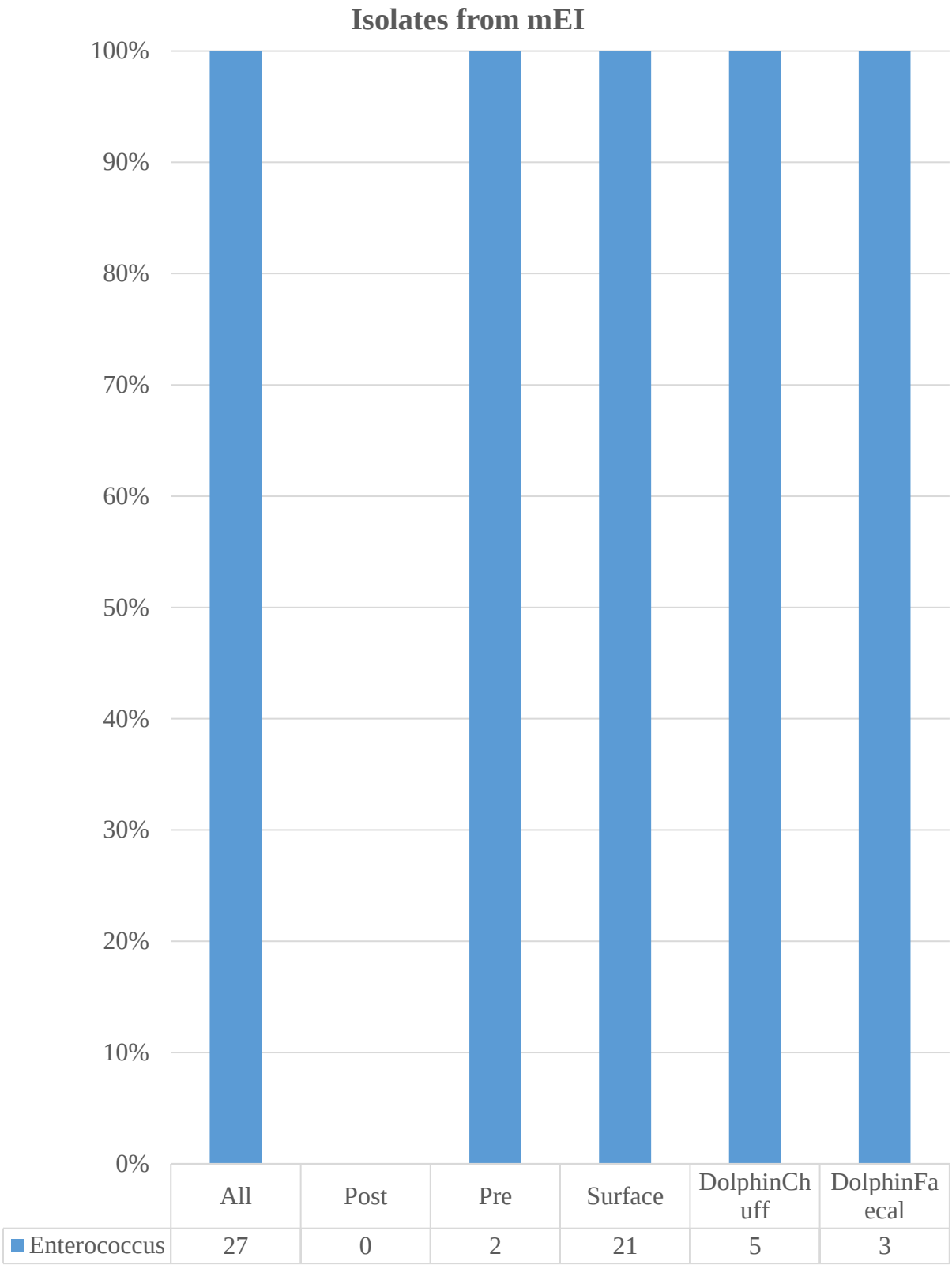
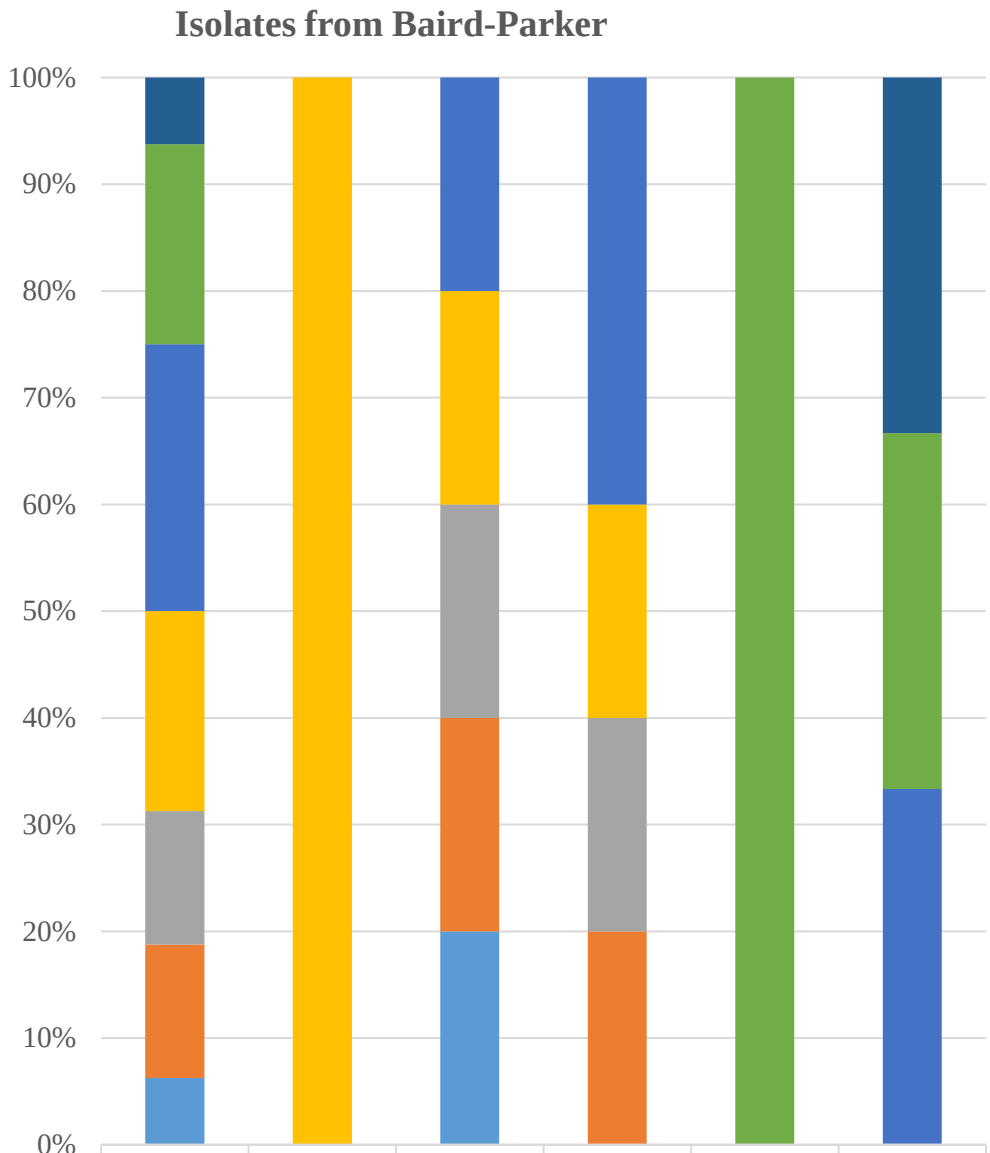
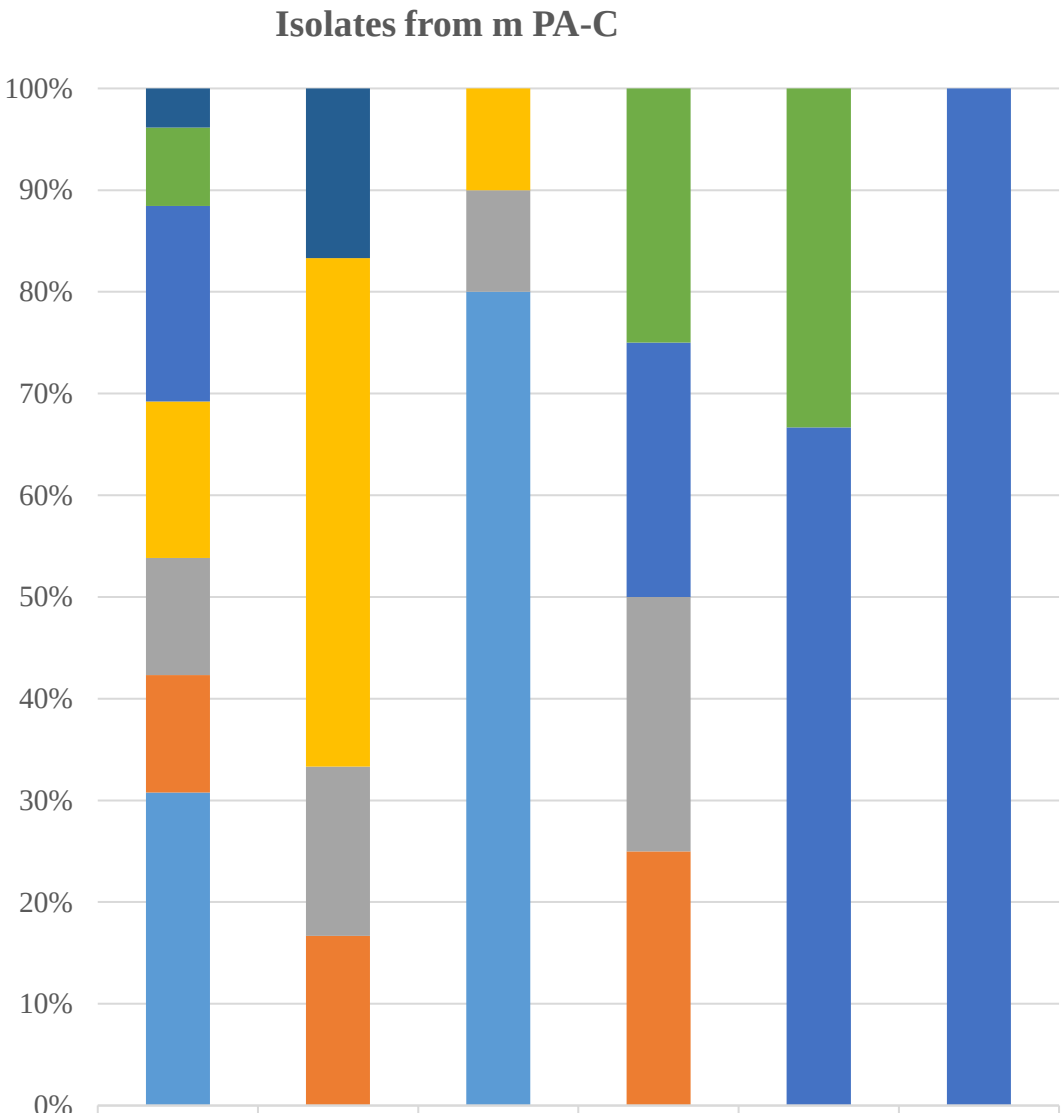


Figure 3: Genus identities of colonies isolated from mEI media (expected genus is Enterococcus*).* Identities were determined using Sanger-based 16S rRNA gene sequencing.



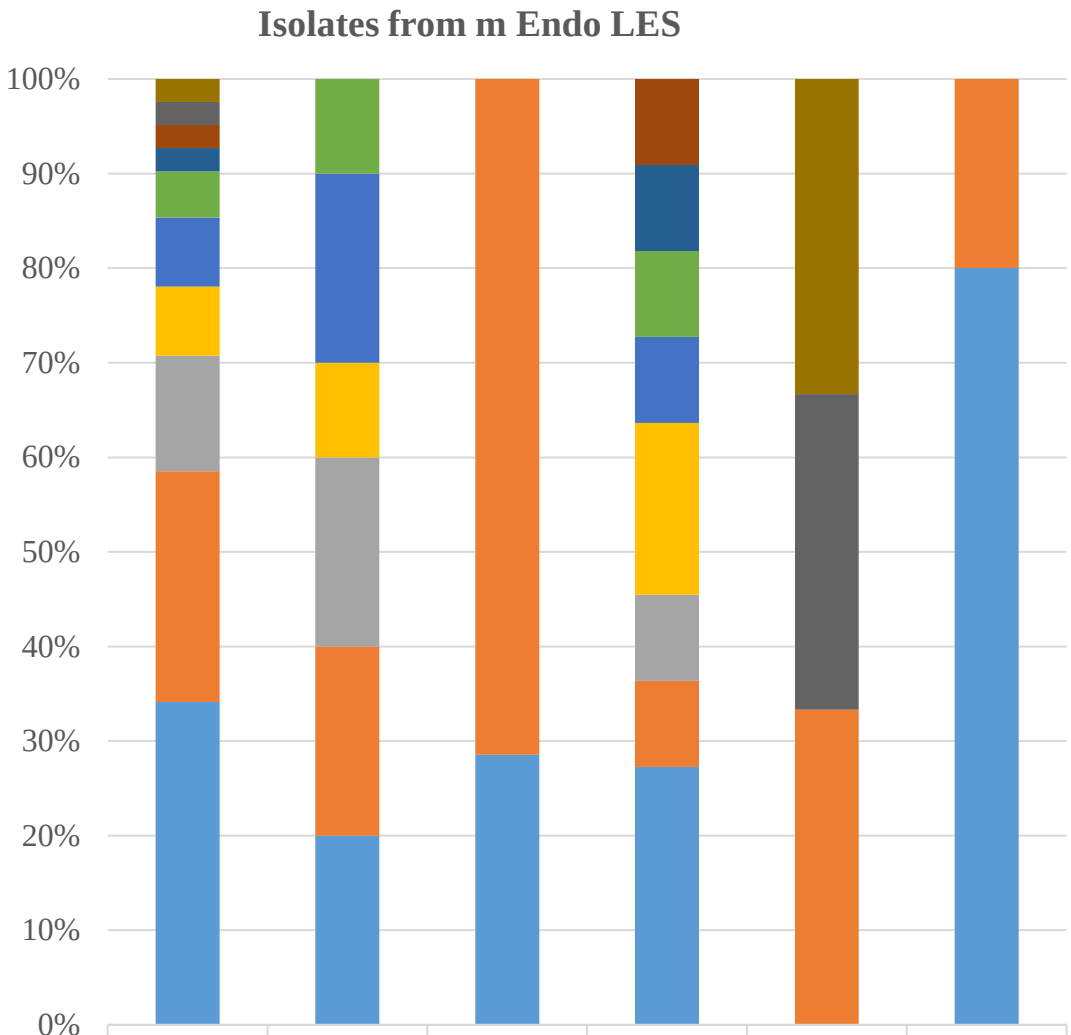
	All	Post	Pre	Exhibit	DolphinChuff	DolphinFaecal
Escherichia	1	0	0	0	0	1
Proteus	3	0	0	0	2	1
Pseudoalteromonas	4	0	1	2	0	1
Staphylococcus	3	1	1	1	0	0
Kocuria	2	0	1	1	0	0
Shewanella	2	0	1	1	0	0
Microbacterium	1	0	1	0	0	0

Figure 4: Genus identities of colonies isolated from Baird-Parker media (expected genus is Staphylococcus*).* Identities were determined using Sanger-based 16S rRNA gene sequencing.



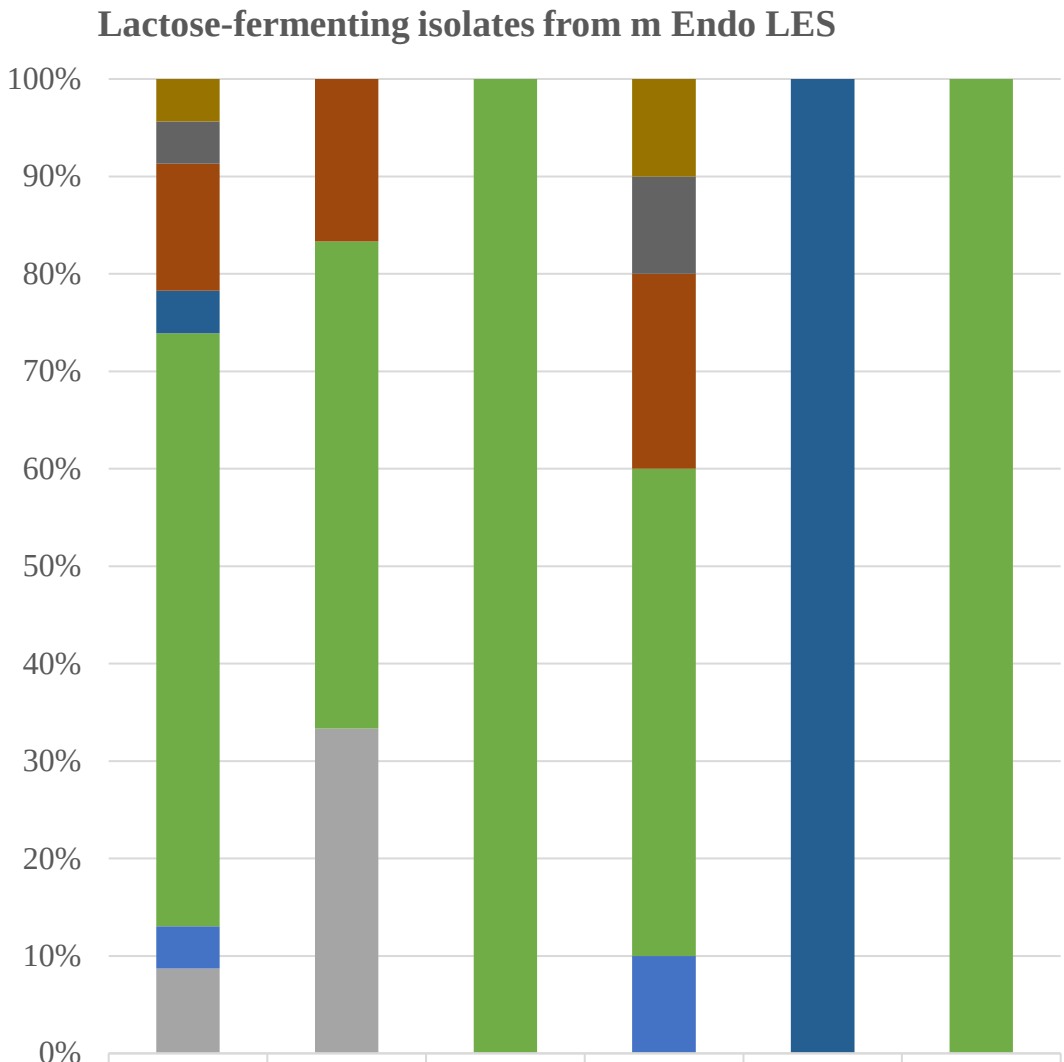
	All	Post	Pre	Exhibit	Dolphin Chuff	Dolphin Faecal
Paenibacillus	1	1	0	0	0	0
Pseudomonas	2	0	0	1	1	0
Enterococcus	5	0	0	1	2	2
Staphylococcus	4	3	1	0	0	0
Brevibacterium	3	1	1	1	0	0
Micrococcus	3	1	0	1	0	0
Alcanivorax	8	0	8	0	0	0

Figure 5: Genus identities of isolates from m PA-C media (expected genus is Pseudomonas*).* Identities were determined using Sanger-based 16S rRNA gene sequencing.



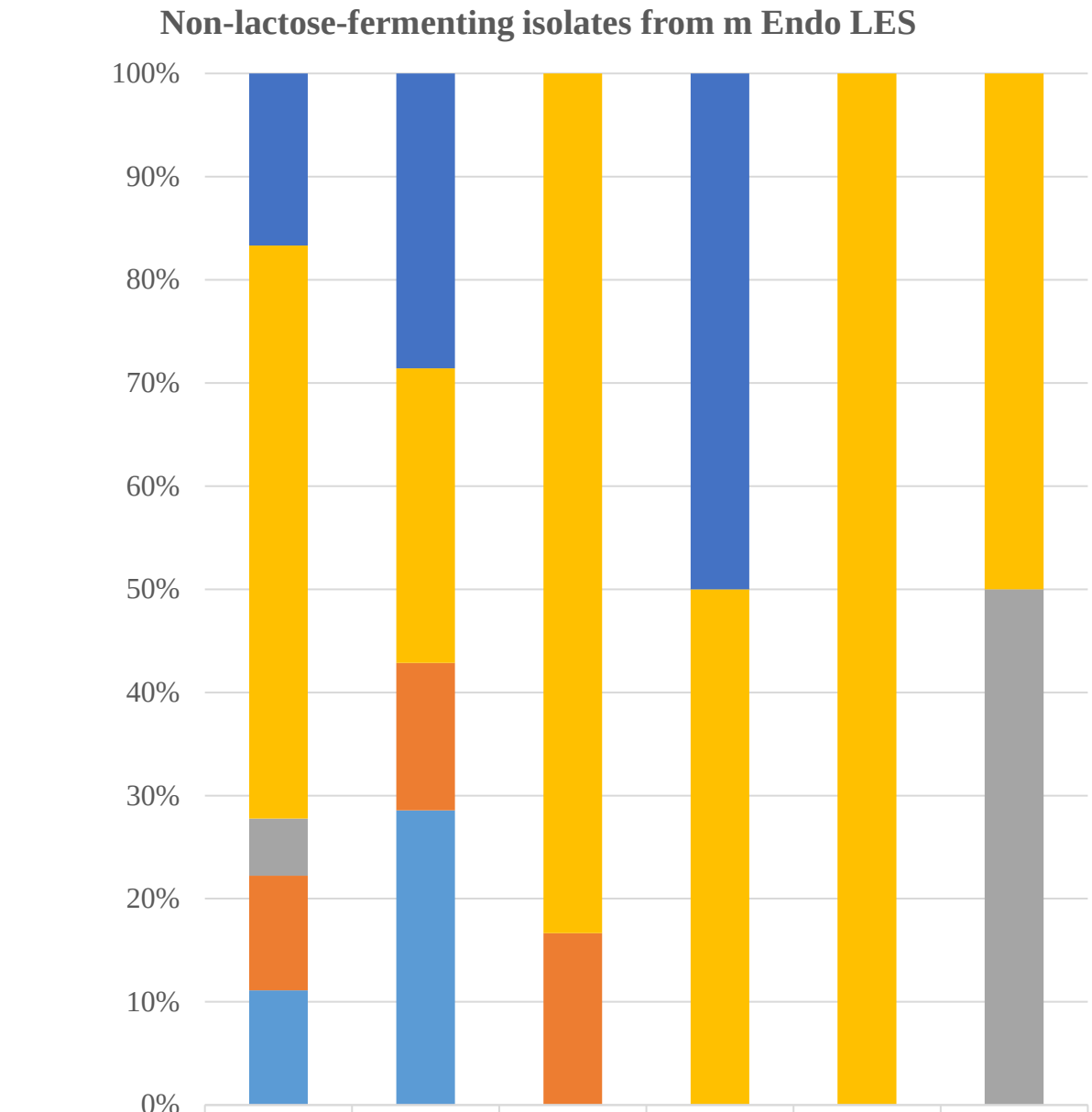
	All Sites	Pre ozone	Post ozone	Exhibit surface	Dolphin chuff	Dolphin fecal
Morganella	1	0	0	0	1	0
Proteus	1	0	0	0	1	0
Cronobacter	1	0	0	1	0	0
Rhodococcus	1	0	0	1	0	0
Enterococcus	2	1	0	1	0	0
Acinetobacter	3	2	0	1	0	0
Shigella	3	1	0	2	0	0
Serratia	5	2	0	1	0	0
Pseudomonas	10	2	5	1	1	1
Escherichia	14	2	2	3	0	4

Figure 6: Genus identities of isolates (with or without a sheen) from m Endo LES media, Members of the family Enterobacteriaceae *are expected.* Identities were determined using Sanger-based 16S rRNA gene sequencing.



	All sites	Pre ozone	Post ozone	Exhibit surface	Dolphin chuff	Dolphin fecal
Cronobacter	1	0	0	1	0	0
Rhodococcus	1	0	0	1	0	0
Shigella	3	1	0	2	0	0
Proteus	1	0	0	0	1	0
Escherichia	14	3	2	5	0	4
Acinetobacter	1	0	0	1	0	0
Serratia	2	2	0	0	0	0

Figure 7: Genus identities of isolates that produced a metallic sheen indicating lactose fermentation on m Endo LES media (called typical coliforms). Members of the family Enterobacteriaciae *are expected.* Identities were determined using Sanger-based 16S rRNA gene sequencing.



	Total	Pre-Ozone	Post-Ozone	Exhibit surface	Dolphin feces	Dolphin Chuff
Serratia	3	2	0	1	0	0
Pseudomonas	10	2	5	1	1	1
Morganella	1	0	0	0	0	1
Enterococcus	2	1	1	0	0	0
Acinetobacter	2	2	0	0	0	0

Figure 8: Genus identities of isolates from m Endo LES media that did not produce a metallic sheen, indicating lack of lactose fermentation (or weak fermentationThese are typically not expected to be members of the family Enterobacteriaceae. Identities were determined using Sanger-based 16S rRNA gene sequencing.

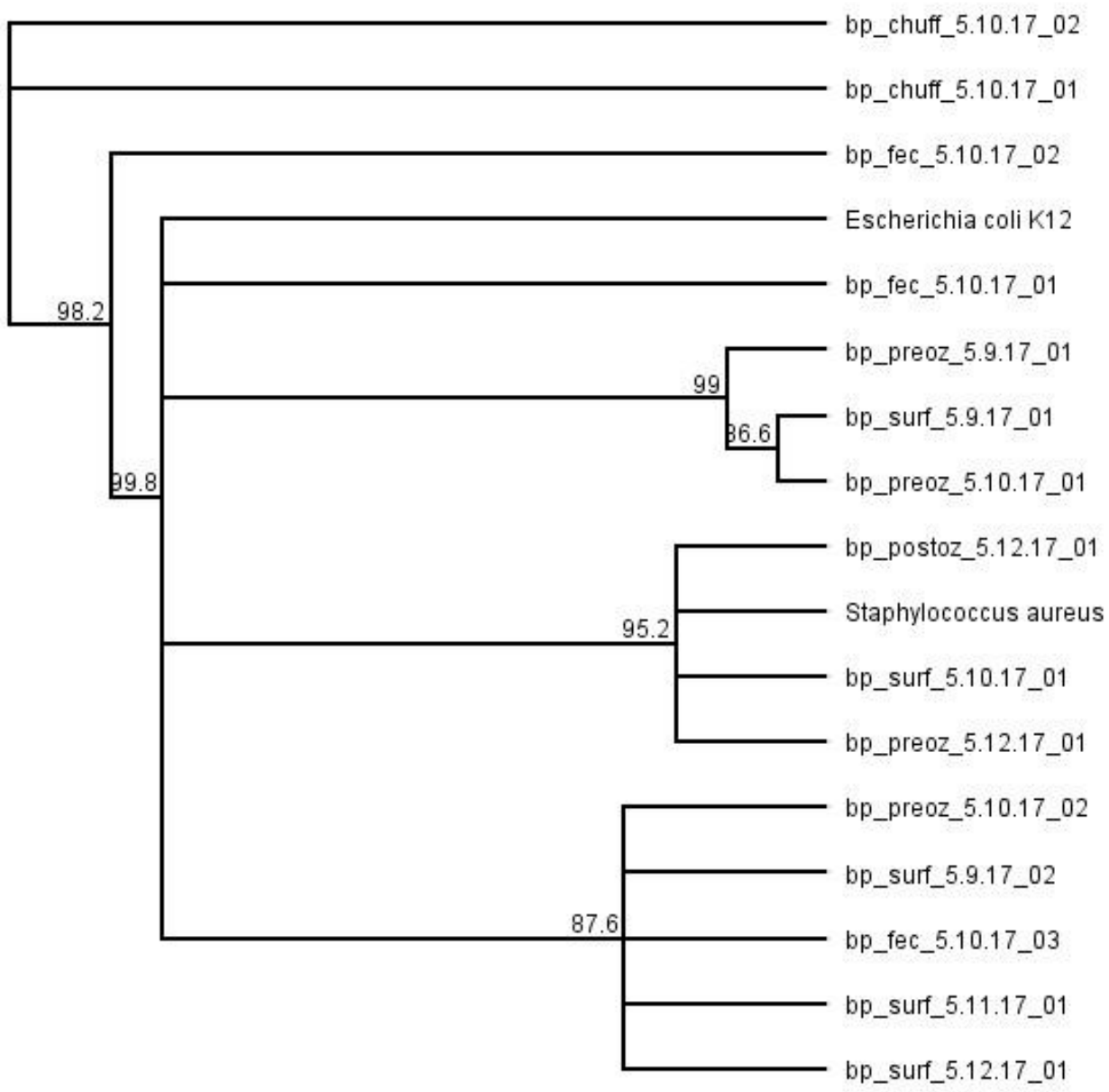


Figure 9: Phylogenetic tree of V4 hypervariable regions of 16S rRNA genes from presumptive staphylococci isolates. Numbers next to nodes represent consensus support values based on 500 bootstrap replications. Sample names consist of a media identifier (bp = Baird-Parker), sample source identifier (surf = exhibit, preoz = life support system between sand filtration and ozone contact, postoz = life support system after ozone contact, fec = dolphin feces, and chuff = dolphin chuff), collection date of the sample from which the isolate was taken, and an identification number to distinguish samples for which the preceding label elements are the same. *Escherichia coli* K12 and *Staphylococcus aureus* are included for reference.

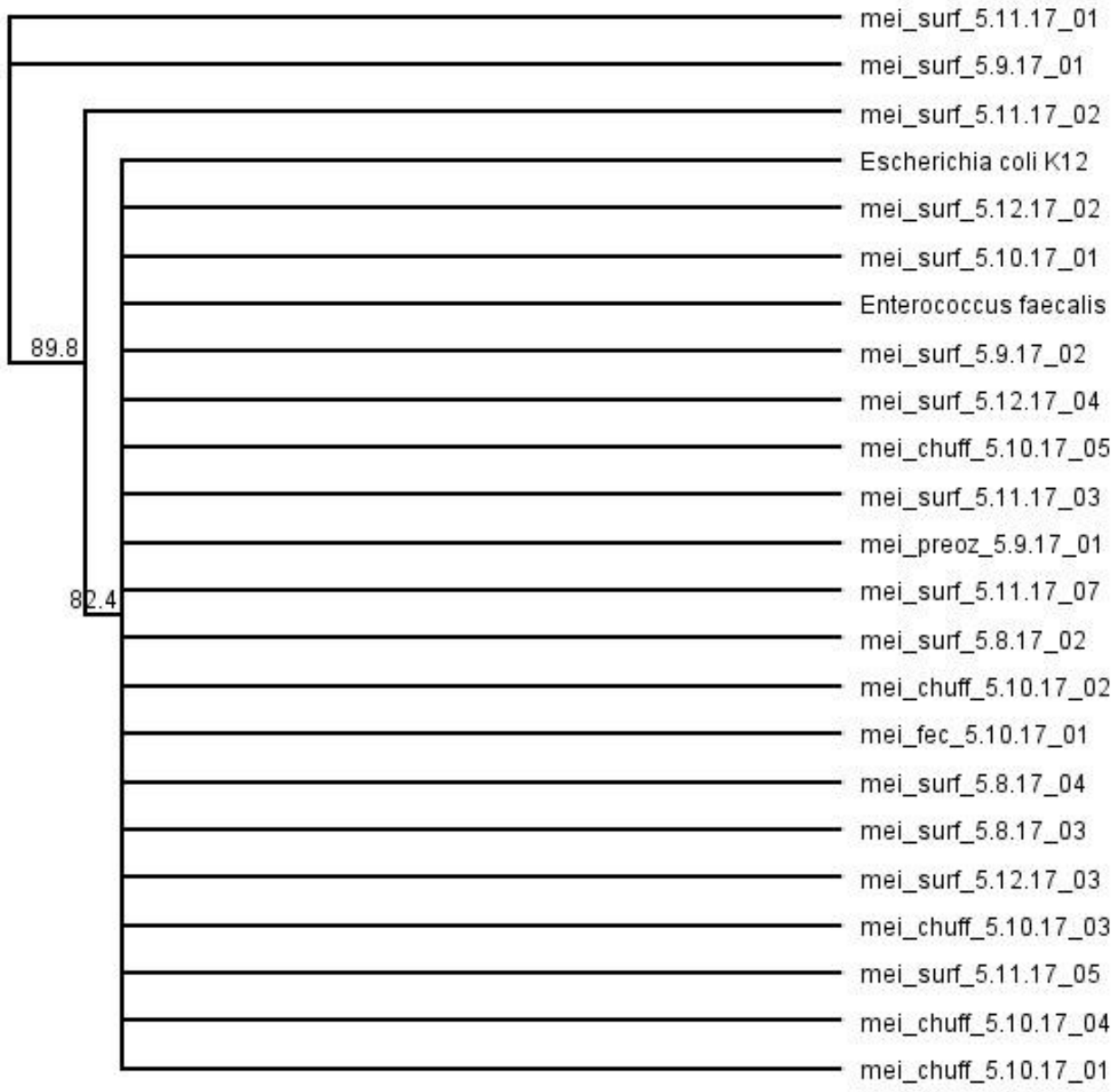


Figure 10: Phylogenetic tree of V4 hypervariable regions of 16S rRNA genes from presumptive enterococci isolates. Numbers next to nodes represent consensus support values based on 500 bootstrap replications. Sample names consist of a media identifier (mei = mEI), sample source identifier (surf = exhibit, preoz = life support system between sand filtration and ozone contact, postoz = life support system after ozone contact, fec = dolphin feces, and chuff = dolphin chuff), collection date of the sample from which the isolate was taken, and an identification number to distinguish samples for which the preceding label elements are the same. *Escherichia coli* K12 and *Enterococcus faecalis* are included for reference.

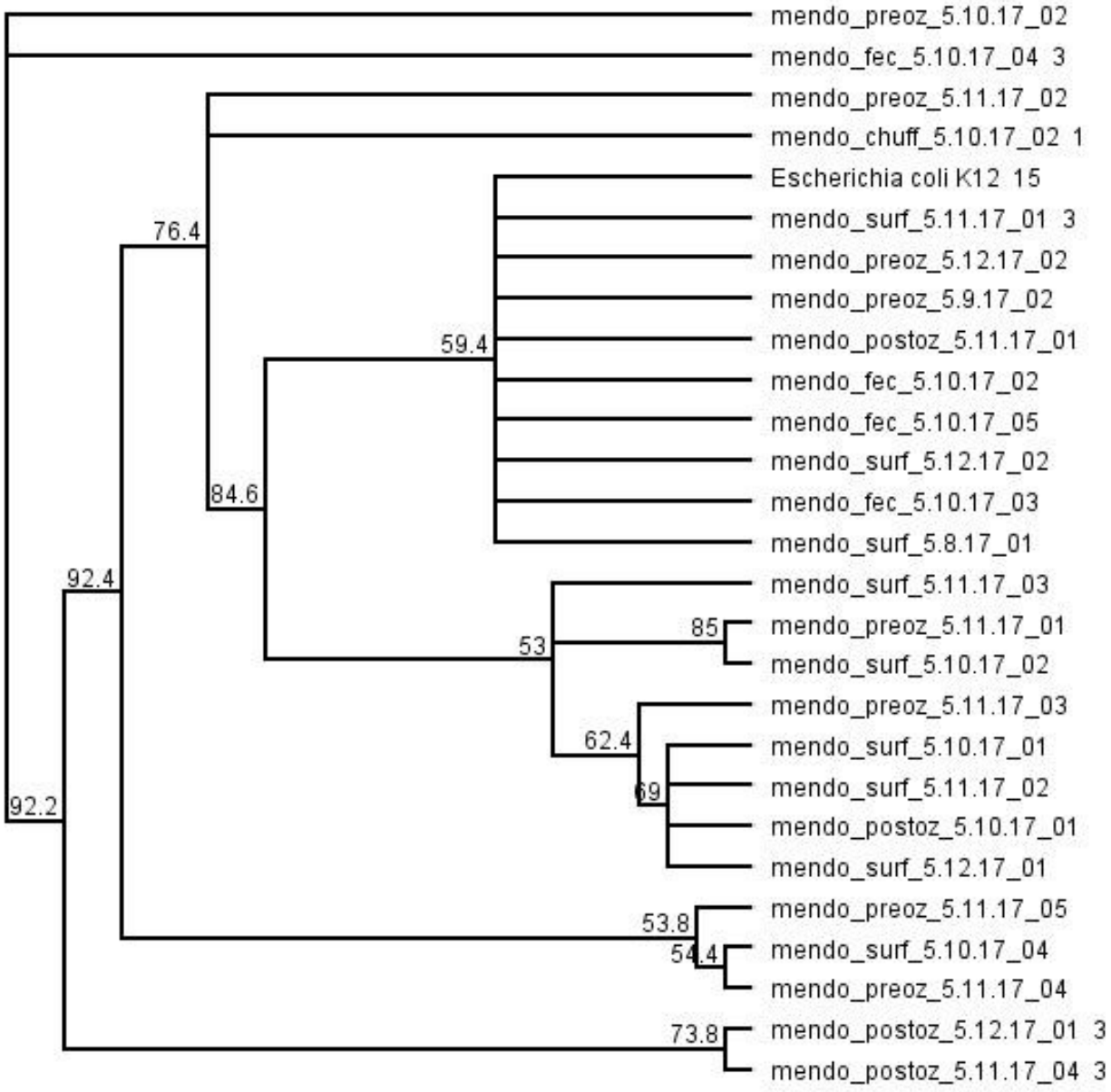


Figure 11: Phylogenetic tree of V4 hypervariable regions of 16S rRNA genes from presumptive total coliform isolates. Numbers next to nodes represent consensus support values based on 500 bootstrap replications. Sample names consist of a media identifier (mendo = m Endo LES), sample source identifier (surf = exhibit, preoz = life support system between sand filtration and ozone contact, postoz = life support system after ozone contact, fec = dolphin feces, and chuff = dolphin chuff), collection date of the sample from which the isolate was taken, and an identification number to distinguish samples for which the preceding label elements are the same. *Escherichia coli* K12 is included for reference.

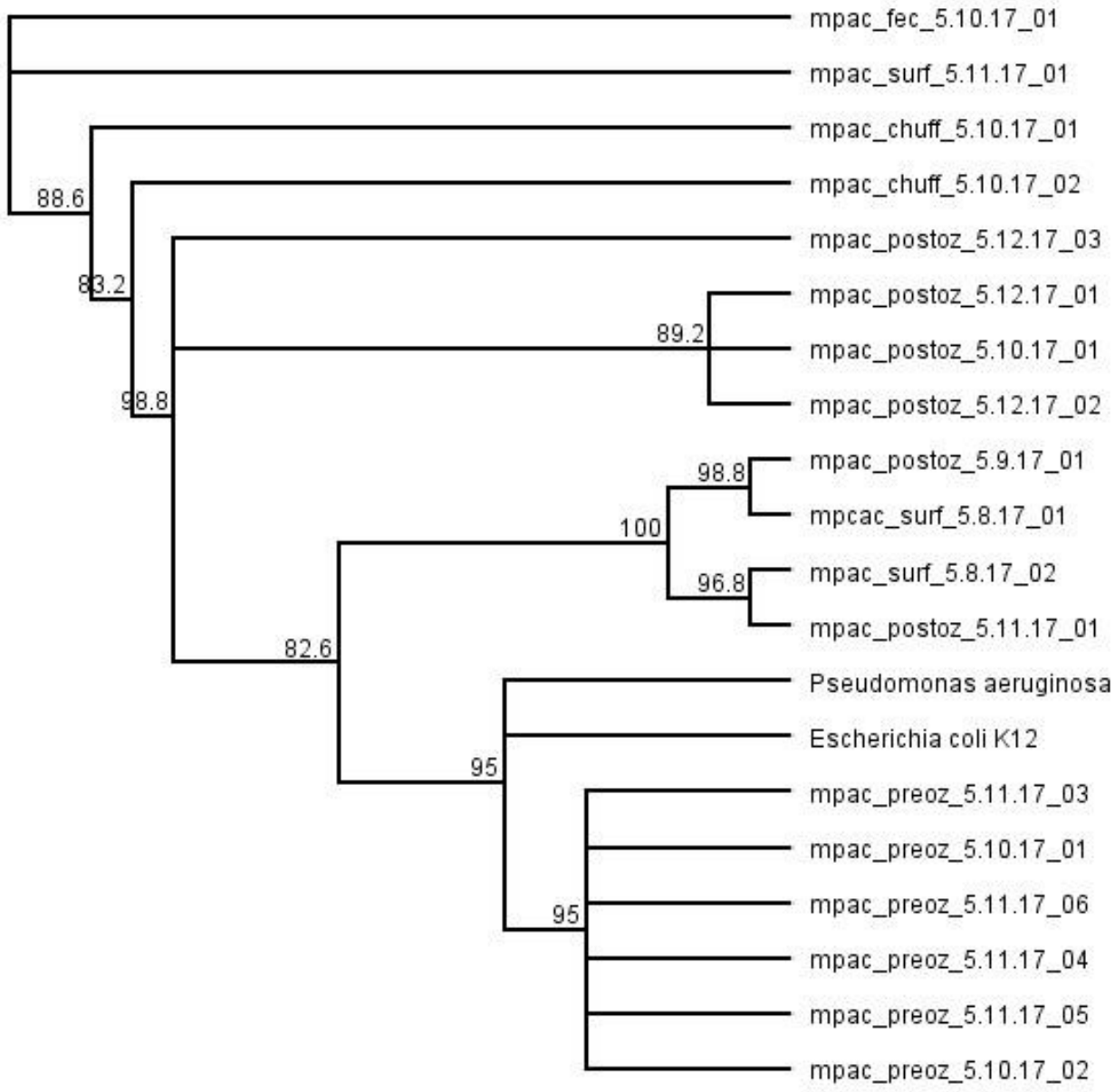


Figure 12: Phylogenetic tree of V4 hypervariable regions of 16S rRNA genes from presumptive pseudomonad isolates. Numbers next to nodes represent consensus support values based on 500 bootstrap replications. Sample names consist of a media identifier (mpac = m PA-C), sample source identifier (surf = exhibit, preoz = life support system between sand filtration and ozone contact, postoz = life support system after ozone contact, fec = dolphin feces, and chuff = dolphin chuff), collection date of the sample from which the isolate was taken, and an identification number to distinguish samples for which the preceding label elements are the same. *Escherichia coli* K12 and *Pseudomonas aeruginosa* are included for reference.

4.4 Relationship between indicators and total microbial community

Adonis permutational analysis of variance was used to determine how much of the Bray-Curtis distance between total microbial community samples could be explained by indicator counts. These results are shown in Table 9.

Organism tested	Sample site	DF (counts, residuals)	SS	MSE	F statistic	R-squared	P-value
Presumptive staphylococci	Pre-Ozone	1, 12	0.012, 0.13	0.012, 0.011	1.07	0.08	0.311
	Post-Ozone	1, 12	0.032, 0.15	0.032, 0.013	2.56	0.18	0.001
	Exhibit	1, 13	0.034, 0.18	0.034, 0.014	2.44	0.16	0.011
Presumptive enterococci	Pre-Ozone	1, 12	0.020, 0.013	0.020, 0.011	1.9	0.14	0.046
	Post-Ozone	-	-	-	-	-	-
	Exhibit	1, 13	0.016, 0.20	0.016, 0.015	1.04	0.07	0.394
Presumptive total coliforms	Pre-Ozone	1, 12	0.035, 0.11	0.035, 0.01	3.79	0.24	0.001
	Post-Ozone	1, 12	0.016, 0.17	0.016, 0.014	1.13	0.09	0.264
	Exhibit	1, 13	0.026, 0.19	0.026, 0.014	1.79	0.12	0.034
Presumptive pseudomonads	Pre-Ozone	1, 12	0.056, 0.30	0.056, 0.025	2.25	0.16	0.001
	Post-Ozone	1, 12	0.014, 0.17	0.014, 0.014	1.02	0.08	0.444
	Exhibit	1, 13	0.028, 0.19	0.028, 0.015	1.9	0.13	0.034

Table 9: Adonis results. The R-squared value is the proportion of Bray-Curtis distance between total community samples collected from the specified site that can be explained by raw colony counts on the media named in the leftmost column. All post-ozone samples were below the detection limit for enterococci, so no regression analysis was possible involving post-ozone enterococci counts. DF = degrees of freedom, SS = sums of squares; MSE = mean square error.

To determine relationships between indicator organisms and individual taxa detected through 16S rRNA community sequencing, colony counts were log-transformed before performing Pearson regressions against each OTU. In the exhibit, presumptive pseudomonads and staphylococci were not correlated with any OTUs detected in the total community; presumptive total coliform counts were correlated with the proportional abundance of an *Oceanivirga* sp. OTU ($R^2 = 0.89$, FDR-corrected $p = 0.012$); and presumptive enterococci counts were correlated with the same OTU ($R^2 = 0.89$, FDR-corrected $p = 0.007$). In samples collected downstream of sand filtration and upstream of ozone contact, presumptive staphylococci counts were not correlated with any OTUs; presumptive total coliform counts were correlated with a Gammaproteobacteria that was not identifiable below the class level ($R^2 = 0.88$, FDR-corrected $p = 0.015$); presumptive enterococci counts were correlated with an unidentified bacterium from the phylum TM6 ($R^2 = 0.91$, FDR-corrected $p = 0.002$); and presumptive pseudomonad counts were correlated with 20 OTUs (shown in Table 10). Downstream of ozone contact, no presumptive enterococci were detected, so no correlation was possible, and the other indicators were not correlated with any OTU.

R^2	FDR-corrected p value	Most Specific Taxonomic Level	Identity
0.92	0.0009	Genus	*Marixanthomonas*
0.92	0.002	Family	*Simkaniaceae*
0.88	0.004	Order	Alphaproteobacteria
0.88	0.004	Genus	*Polaribacter*
0.88	0.004	Class	Marine Group 1
0.88	0.004	Phylum	TM6
0.87	0.005	Family	*Rickettsiaceae*
0.86	0.008	Order	Sphingobacteriales
0.86	0.010	Order	Chlamydiales
0.85	0.010	Order	HTA4
0.85	0.010	Family	*Cryomorphaceae*
0.85	0.010	Family	*Simkaniaceae*
0.85	0.011	Family	*NS11-12 marine group*
0.85	0.011	Genus	*Aliivibrio*
0.85	0.012	Family	*Parachlamydiaceae*
0.84	0.016	Order	*Chlamydiales*
0.82	0.037	Order	Alphaproteobacteria
0.82	0.037	Family	*NS7 marine group*
0.82	0.037	Family	*Simkaniaceae*
0.82	0.037	Family	*Parachlamydiaceae*

Table 10: OTUs correlated with log-transformed colony counts on m PA-C media and the identity of the most specific taxonomic level to which they could be identified. Note that each row in this table represents one OTU, so the first row represents a single OTU that is a member of the genus *Marixanthomonas*, not all *Marixanthomonas* spp.

5. DISCUSSION

5.1 Differences between sampling sites: effects of sand filtration and ozone disinfection

To determine the effect of sand filtration and ozone treatment on indicator abundance, colony counts on selective and differential media were compared between water from the Oceanarium exhibit, water from the plumbing between sand filtration and ozone treatment, and water from the plumbing after ozone treatment.

The geometric mean total coliform count was higher in the exhibit compared to post-ozone treatment. There were not significant differences between exhibit and post-sand-filtration/pre-ozone counts, or between pre-ozone and post-ozone counts. Both sand filtration and ozone treatment were therefore necessary to produce a significant reduction in total coliform counts within the context of the present study. Because the sample size was small (n = 5), a more robust study consisting of more samples would be useful in determining whether there is an effect of sand filtration or ozone treatment individually.

Presumptive enterococci counts were higher in exhibit water compared to both post-sand-filtration/pre-ozone and post-ozone samples. Although all samples collected post-ozone were below the limit of detection (BLD) for enterococci (0.03 CFU/100 mL), there was not a significant difference in counts before and after ozone treatment. This may have been because the ozone influent had such low enterococci concentrations (BLD-0.07 CFU/100 mL) that any further reduction by ozone treatment would not be statistically noticeable. The significant reduction in enterococci between exhibit and post-sand-filtration/pre-ozone samples suggests a die-off in enterococci by the time water travels through the sand filter. Since other indicator counts were not significantly reduced after sand filtration, it is unlikely that the reduction in

enterococci is due to their being filtered out of the water. Further investigation should be done to assess the survivorship of enterococci in this system.

Presumptive staphylococci counts were significantly lower post-ozone than either of the other two sample sites, suggesting that they were reduced by ozone treatment but not by sand filtration.

Finally, presumptive pseudomonads were significantly higher (by 4-5 orders of magnitude) post-sand-filtration/pre-ozone than either of the other two sites, but the exhibit and post-ozone samples did not differ significantly from each other. The increase after sand filtration may be due to a biofilm formed within the water treatment plumbing that contributed cells to the samples. If this is the case, the ozone contact tower would be receiving water with pseudomonad counts in the tens of thousands. Ozone treatment or a substitute disinfection method would therefore be essential in preventing these bacteria from returning to the exhibit where animals are held and potentially violating the USDA APHIS-proposed limit of 10 CFU/100 mL.

5.2 Levels of indicators in exhibit water

None of the indicator organisms tested had CFU concentrations in the exhibit above the limits proposed by USDA APHIS. Because this study took place over 5 consecutive days, it provides only a brief glimpse into what abundances to expect for these organisms. In order to determine the long-term stability of these abundances, it would be ideal to collect many more samples over a longer time period (i.e. a year or more, in order to ensure that any seasonal variation could be detected). It would also be useful to assess the effects of potential disturbances to the system, such as the removal or addition of new animals, cleaning, or changes in animal feeding routines.

5.3 Identities of indicator isolates and specificity of media

Examination of phylogenetic trees reveals several distinct lineages among isolates from each medium. Most pre-ozone/post-sand-filtration presumptive pseudomonad isolates (from m PA-C agar) form a single polytomy, suggesting that the large number of CFUs observed the associated samples are members of a clonal population originating near that site. There is no discernable location pattern among the other isolates, suggesting that these taxa travel throughout the system. Some polytomies contain isolates from fecal or chuff samples along with water samples, consistent with the possibility that animals are the source of these taxa in the system (although the same pattern could occur if organisms originating in the water were able to survive in the animals up until the point of sampling, or if animal samples were contaminated with water containing those organisms).

Comparison of 16S rRNA gene sequences obtained from isolates to taxonomic databases showed that many organisms that grew on the selective and differential indicator media were not members of the taxa those media were designed to capture. mEI was an exception; all sequences of mEI isolates were identified as *Enterococcus* spp., as expected for that medium. M Endo LES was expected to produce total coliforms, here defined as lactose-fermenting members of the family *Enterobacteriaceae*. While all presumptive coliform isolates from dolphin feces and chuff were *Enterobacteriaceae*, several from the water samples were not. A more thorough survey that includes animal excreta collected over a broader time period, and samples from animals residing in adjacent exhibits with shared water, would be necessary to confirm whether these non-*Enterobacteriaceae* originate from animal sources or the environment itself. Most presumptive staphylococci and pseudomonad isolates were not members of *Staphylococcus* or *Pseudomonas* (respectively). There is limited data on the previous evaluation

of Baird-Parker in water available to compare to this result. One report (Klapes, 1983) evaluated this method for swimming pool water and found that 58 of 73 isolates (79%) from unchlorinated freshwater and 122 of 142 (86%) isolates from chlorinated freshwater were *Staphylococcus* spp. (where positive identity as *Staphylococcus* was defined by susceptibility to the bacteriocin lysostaphin), compared to 3 of 16 isolates (19%) in the present study. This difference may be due to several factors, including differences in environment (freshwater vs. saltwater), animals in the water (humans in the Klapes study vs. humans and marine mammals in the present study), treatments (chlorine/none vs. ozone) or methodology (lysostaphin susceptibility vs. sequence identity).

m PA-C agar has been previously evaluated for its specificity at the species level in differentiating *Pseudomonas aeruginosa* (Brodsky and Ciebin, 1978), but does not seem to have been evaluated for specificity at the genus level. This evaluation used the membrane filter technique to capture colonies from raw sewage influent and freshwater canal and lake samples, and found that 99.4% of typical colonies (which the authors defined as "Circular, raised colonies ≥1 mm in diameter, with entire or undulate margins, tan to dark-brown in color with dark-brown centers or pink with nucleated centers or surrounded by an amber halo") were verified as *P. aeruginosa* by morphology and growth on other selective media. In the present study, 2 of 26 colonies (8%) were confirmed through sequence identity as *Pseudomonas* spp. Presumptive pseudomonad counts also included unnucleated pink colonies due to their presence in an image of *P. aeruginosa* on m PA-C provided by the media manufacturer (https://catalog.hardydiagnostics.com/cp_prod/product/images/catalog/G150_mPA-C%20Agar_web.jpg); however, most non-*Pseudomonas* spp. were consistent with Brodsky and

Ciebin's morphological parameters. I was not able to find any published specificity test of m PA-C in marine waters.

Aside from fecal contamination, pseudomonads are also sometimes used as indicators of regrowth within water treatment systems (e.g. Ribas et al., 2000). Since presumptive pseudomonad counts increased drastically between exhibit samples and sand filter effluent, it appears that they were successful in this regard, even though most of the isolates from sand filter effluent were identified as *Alcanovorax* spp. rather than *Pseudomonas* spp.

5.4 Relationship between indicators and total microbial community

The use of indicator bacteria in marine mammal enclosures is intended to inform enclosure managers about microbiological water quality as it relates to marine mammal health. Therefore, it is important to understand whether the indicators predict aspects of the community of microbes living within the water, and if so, how those community aspects inform managers about the animals' health and safety.

Indicator counts were weak predictors of overall community dissimilarity (based on low R^2 values), even when their predictive values were significant (see Table 9). This means that community structure is mostly dependent on other factors, and future research should investigate what these factors are. There were also several correlations observed between presumptive total coliform, enterococci, and pseudomonad counts and individual OTUs within the total microbial community as detected through 16S rRNA gene sequencing. It is difficult to infer what meaning, if any, these correlations have for the health of animals in the system. None of the OTUs correlated with increased indicator counts were on Venn-Watson et al.'s (2008) list of pathogens of concern for marine mammals. The observed correlations involved different OTUs in each of

the three sample sites studied, suggesting that the ecological meaning of increased indicator counts is varied depending on the individual community of which the indicators are a part.

Of particular interest are the correlations of both total coliform and presumptive enterococci colony counts in exhibit water with the relative abundance of an *Oceanivirga* sp. OTU. The genus *Oceanivirga* consists of a single species, *O. salmonicida,* an intracellular pathogen of Atlantic salmon (Eisenberg et al., 2016). However, the 16S gene of *O. salmonicida* shares 99% sequence homology with some uncultured clones from the digestive tracts of bottlenose dolphins (data from Bik et al., 2016) with no associated pathology recorded. It is therefore reasonable that the OTU identified as *Oceanivirga* sp. in the present study is in fact a normal member of the marine mammal gut microbiota that is shed along with coliforms and enterococci. This would support use of coliforms and enterococci as indicators of marine mammal fecal presence, though it remains unknown what level of fecal presence and associated indicator counts should be considered "safe" for marine mammals.

Ozone disinfection, and other common practices such as chlorination, are broad methods of microbial inactivation that are not intended to specifically target disease-causing or otherwise harmful organisms. These methods may be employed to maintain indicator counts within the proposed limits, and the impact of doing so on potentially beneficial microbes is not well-characterized. Therefore, an important topic for future research will be to determine what minimum level of ozone disinfection is necessary to maintain acceptable indicator counts, and whether such a level has other undesirable effects. Other future research should test further for relationships between indicators and the rest of the microbial community, using longer-term data collection to determine whether the correlations observed in the present study were merely sporadic and limited to the 5-day sampling period, or whether the predictive value of indicators

persists over the long term. It will also be necessary to develop metrics that can help evaluate the health of marine mammals in order to determine whether and how they are impacted by changes in the microbial community associated with indicator counts and with water disinfection methods used to maintain indicators within the proposed limits.

APPENDIX

PHYLOGENETIC TREES GENERATED FROM V1-V3 HYPERVARIABLE REGIONS OF 16S RRNA GENES OF COLONY ISOLATES (REFER TO CHAPTERS 3.3-3.4 FOR METHODS USED TO GENERATE THESE TREES)

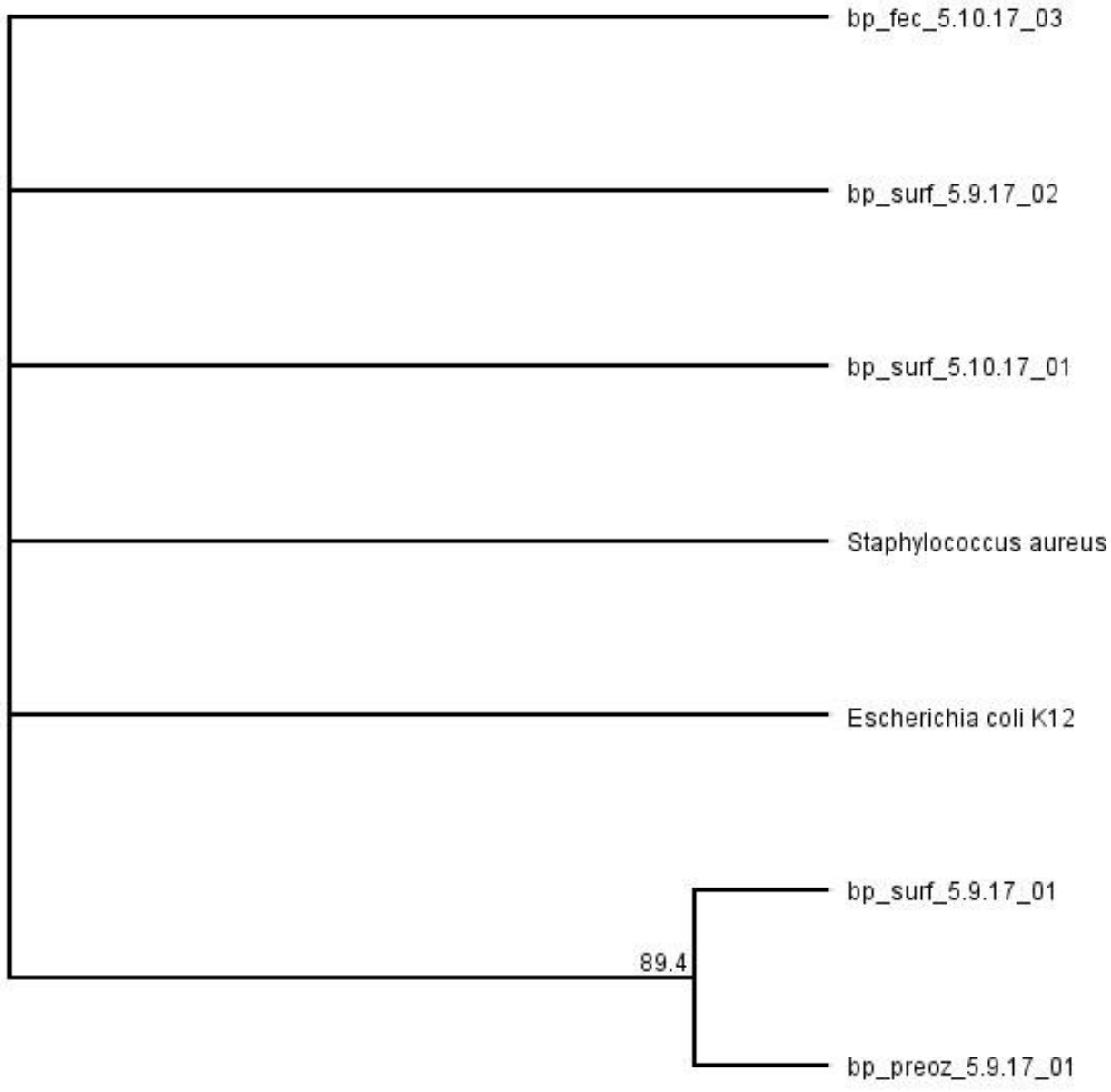


Figure 13: Tree of sequences from the V1 hypervariable region of 16S rRNA genes of presumptive staphylococci isolates.

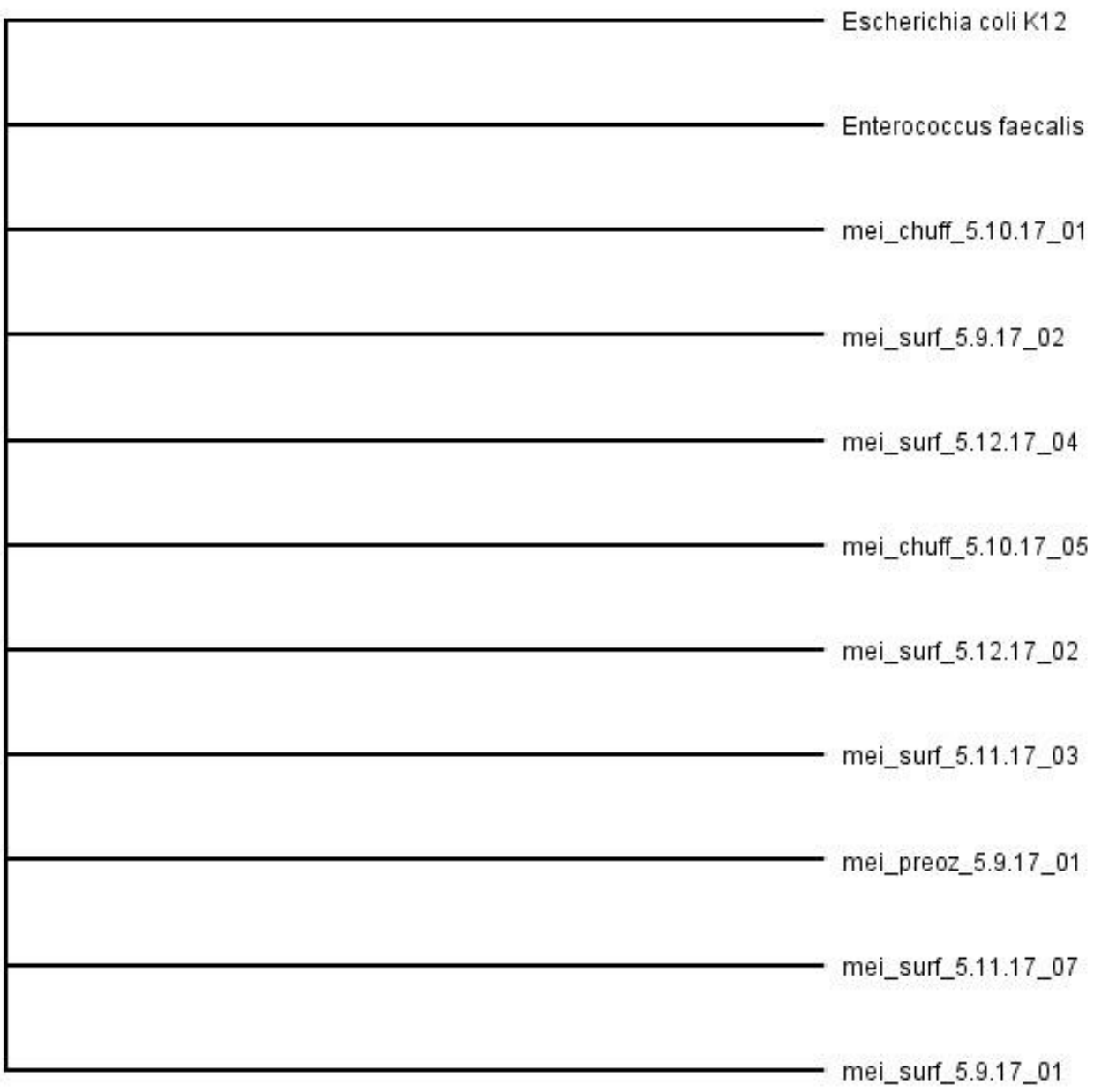


Figure 14: Tree of sequences from the V1 hypervariable region of 16S rRNA genes of presumptive enterococci isolates.

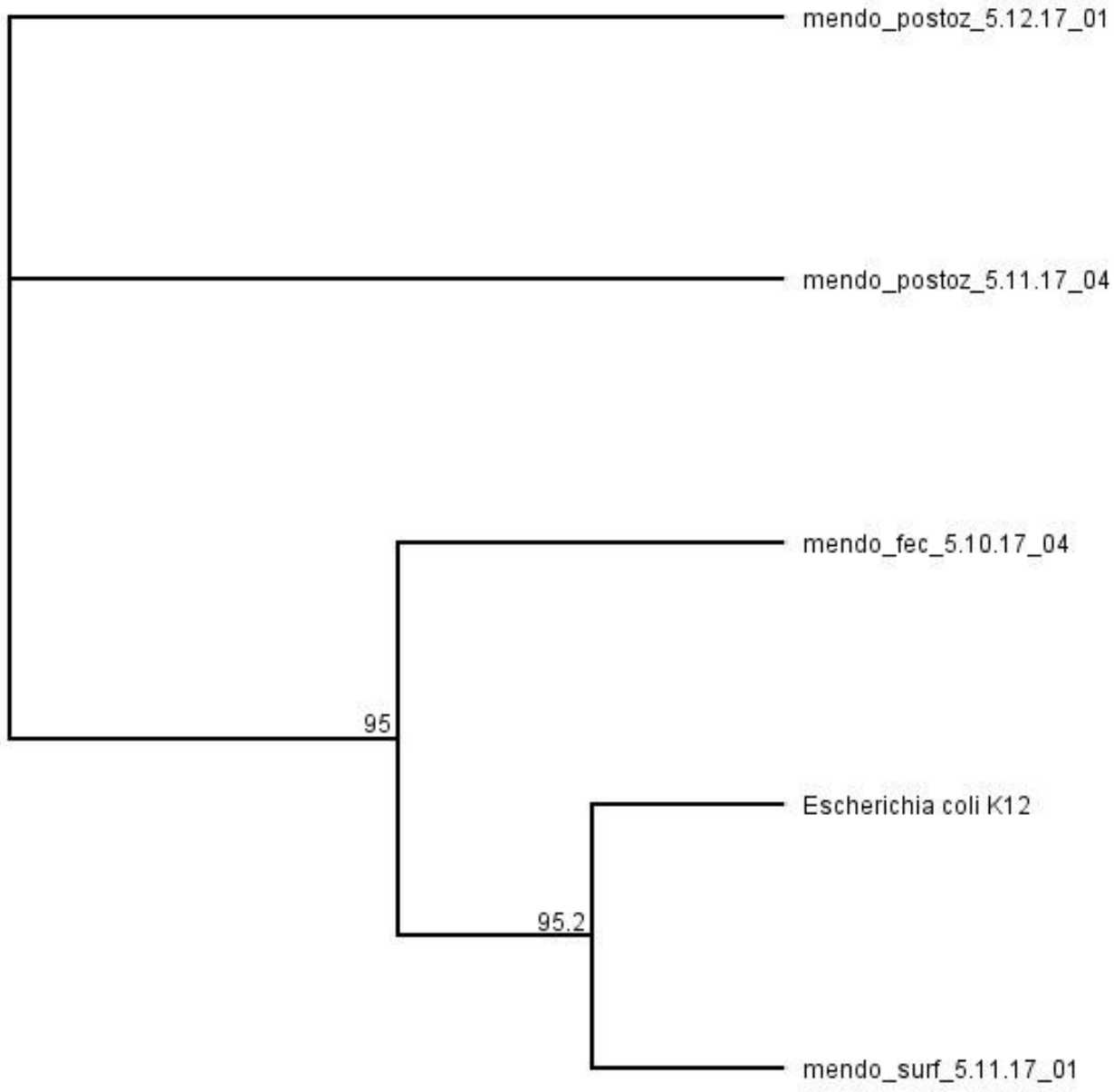


Figure 15: Tree of sequences from the V1 hypervariable region of 16S rRNA genes of presumptive total coliform isolates.

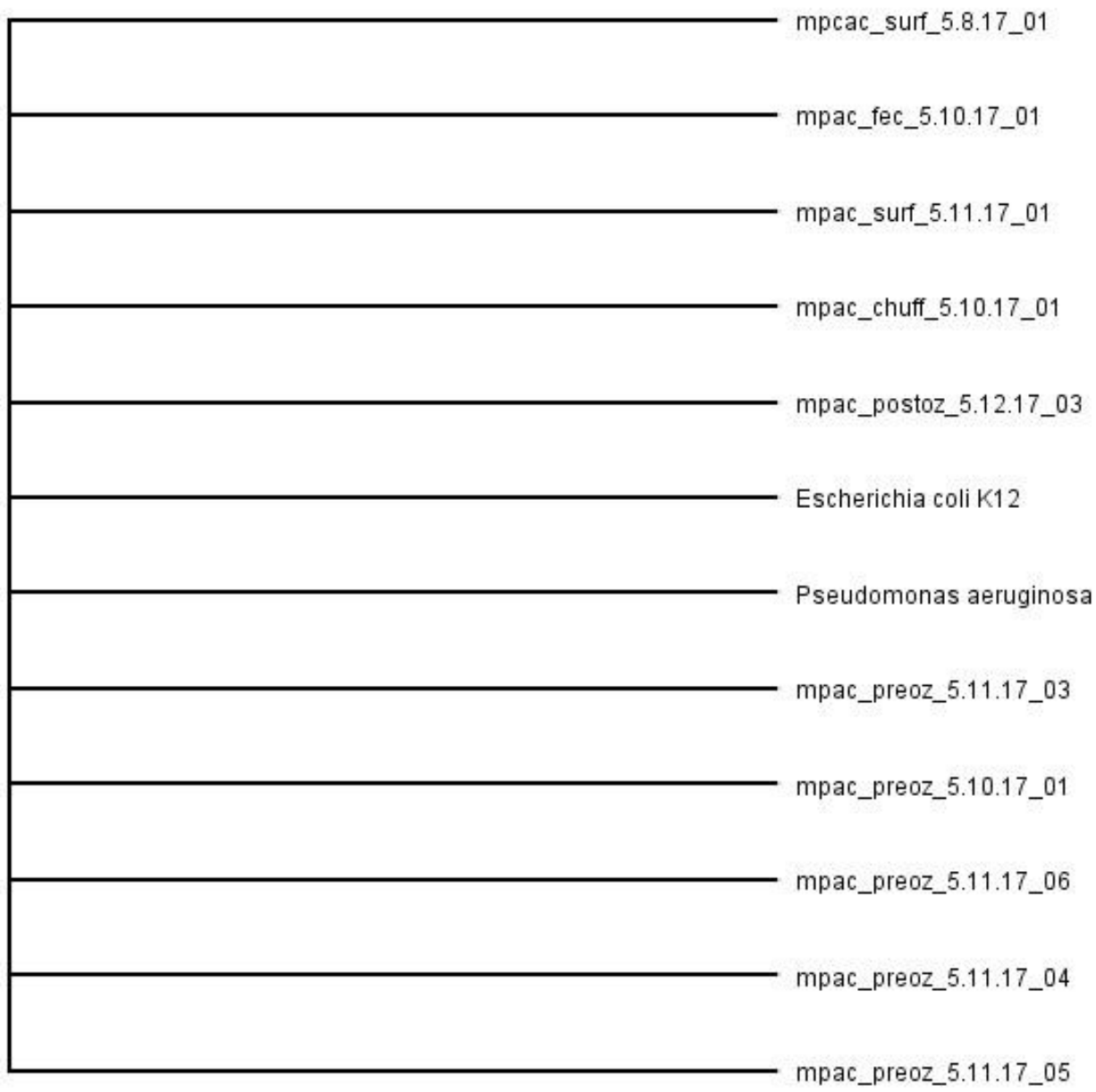


Figure 16: Tree of sequences from the V1 hypervariable region of 16S rRNA genes of presumptive pseudomonad isolates.

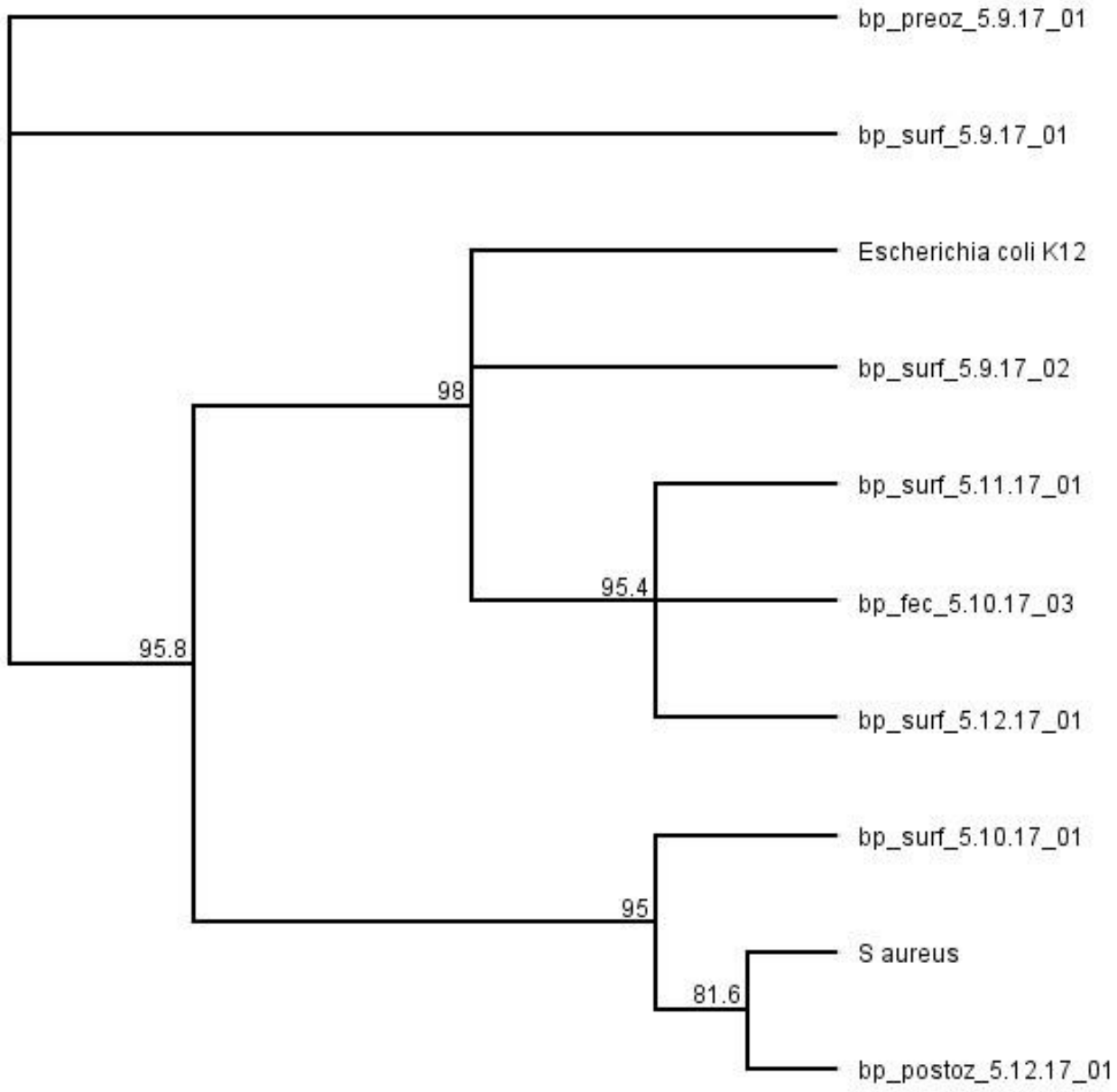


Figure 17: Tree of sequences from the V2 hypervariable region of 16S rRNA genes of presumptive staphylococci isolates.

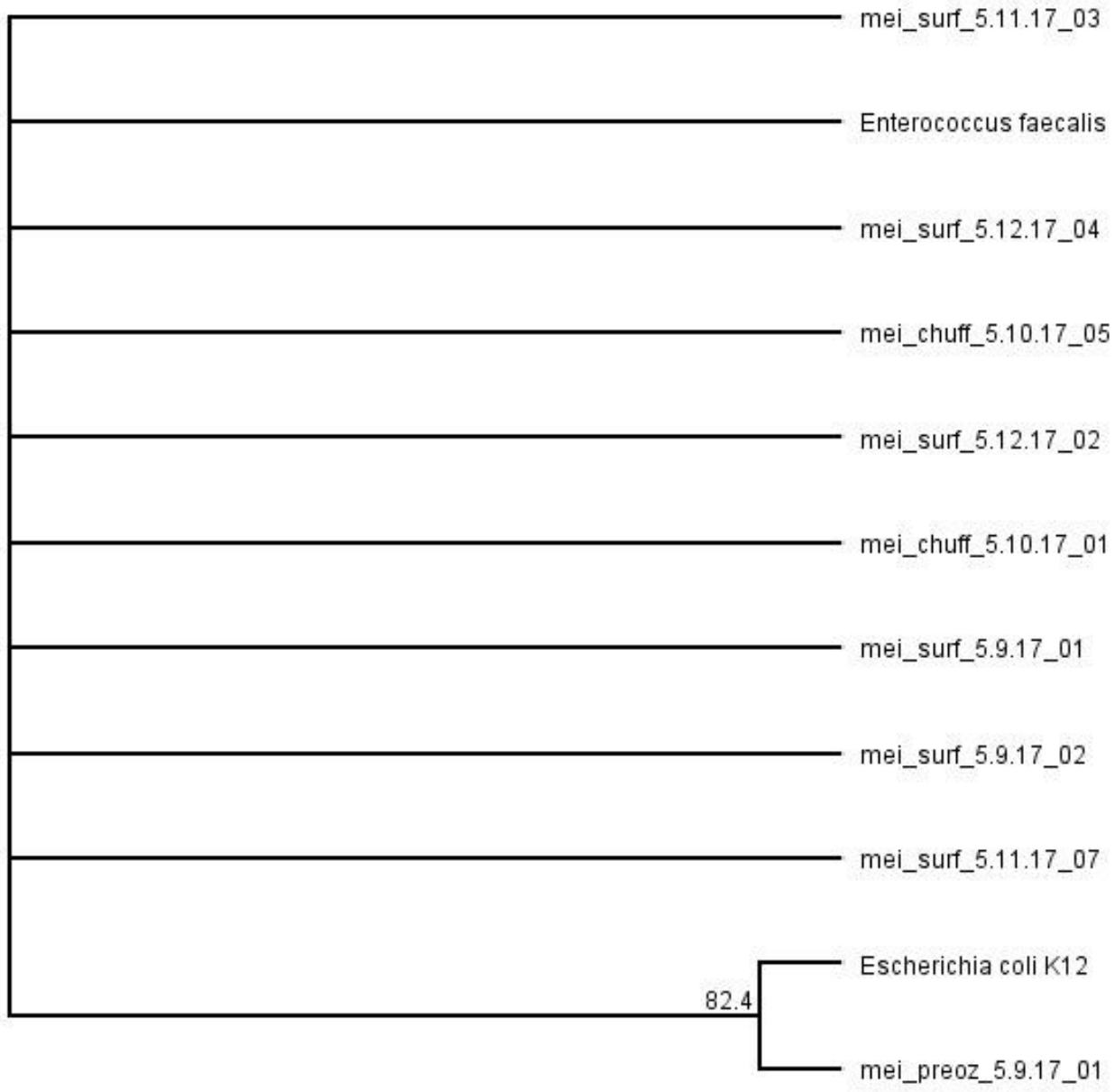


Figure 18: Tree of sequences from the V2 hypervariable region of 16S rRNA genes of presumptive enterococci isolates.

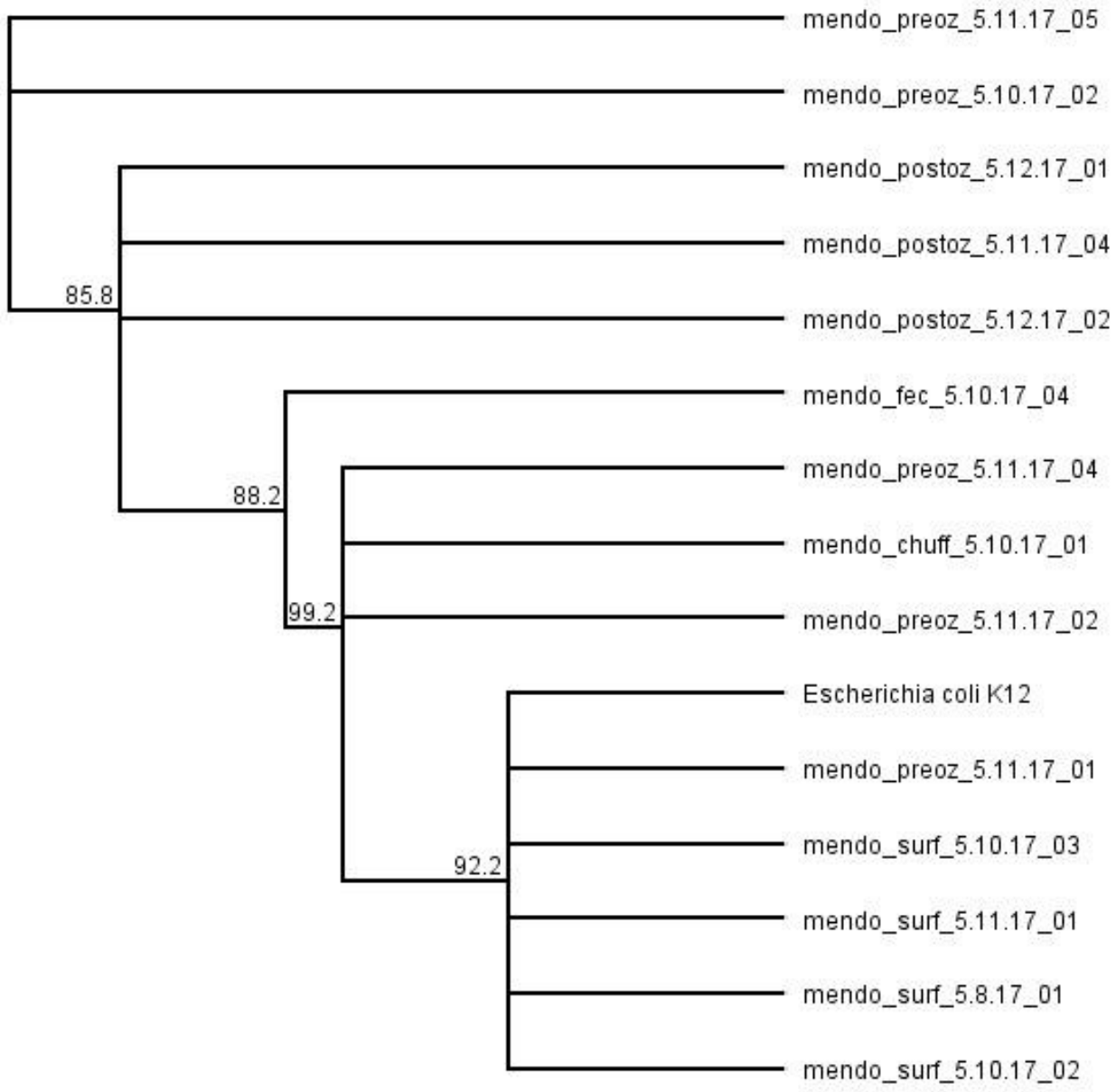


Figure 19: Tree of sequences from the V2 hypervariable region of 16S rRNA genes of presumptive total coliform isolates.

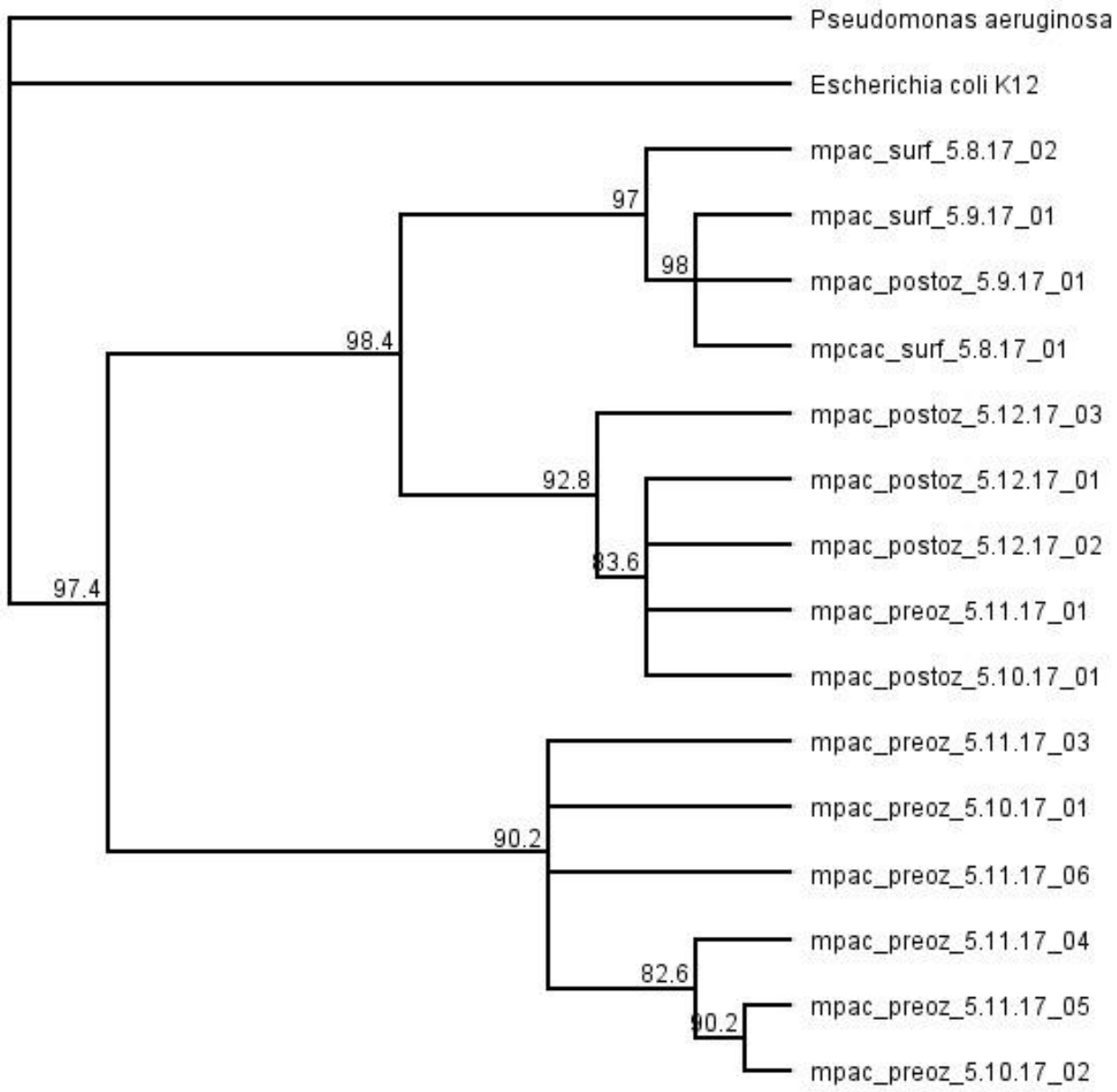


Figure 20: Tree of sequences from the V2 hypervariable region of 16S rRNA genes of presumptive pseudomonad isolates.

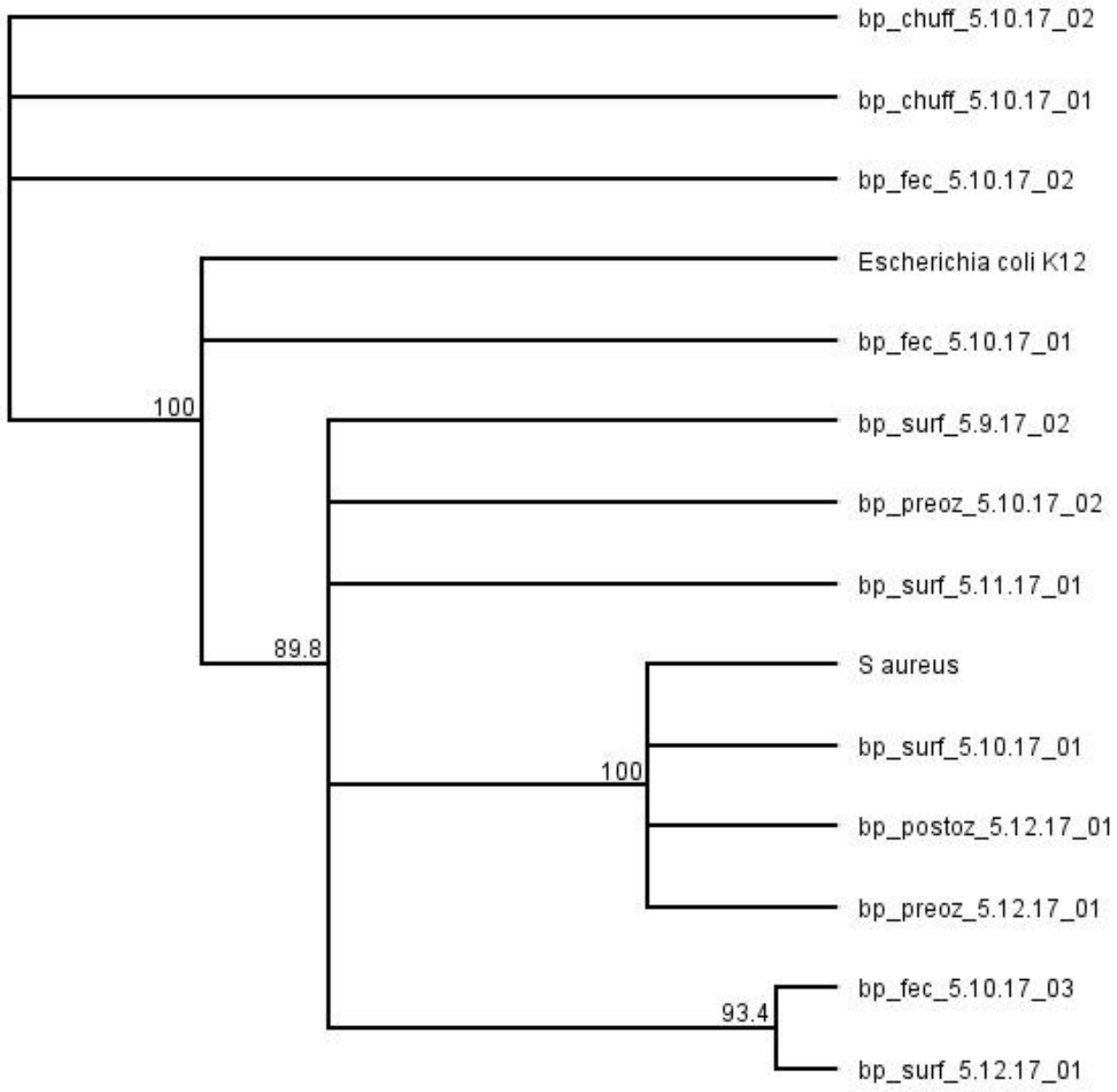


Figure 21: Tree of sequences from the V3 hypervariable region of 16S rRNA genes of presumptive staphylococci isolates.

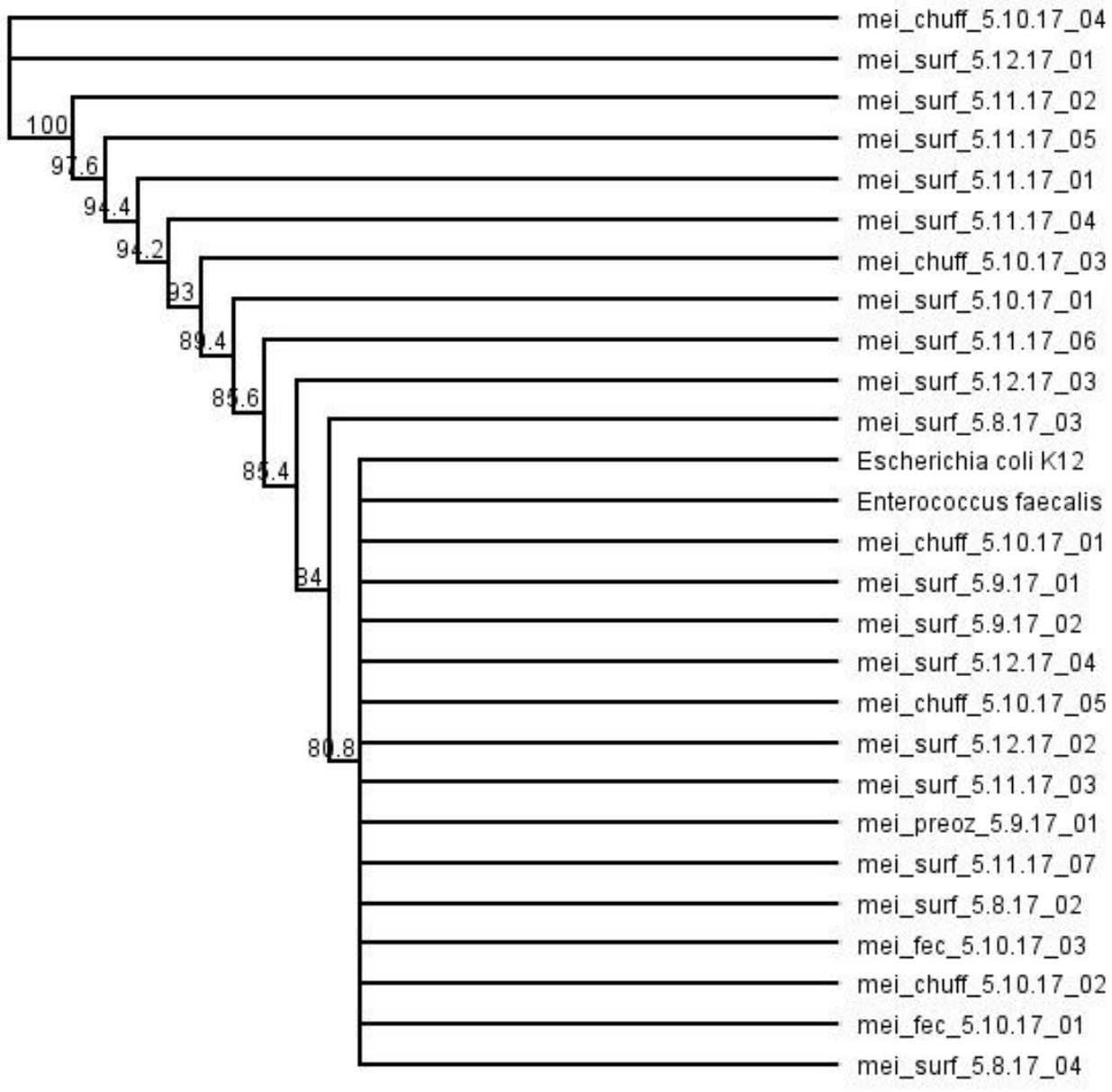


Figure 22: Tree of sequences from the V3 hypervariable region of 16S rRNA genes of presumptive enterococci isolates.

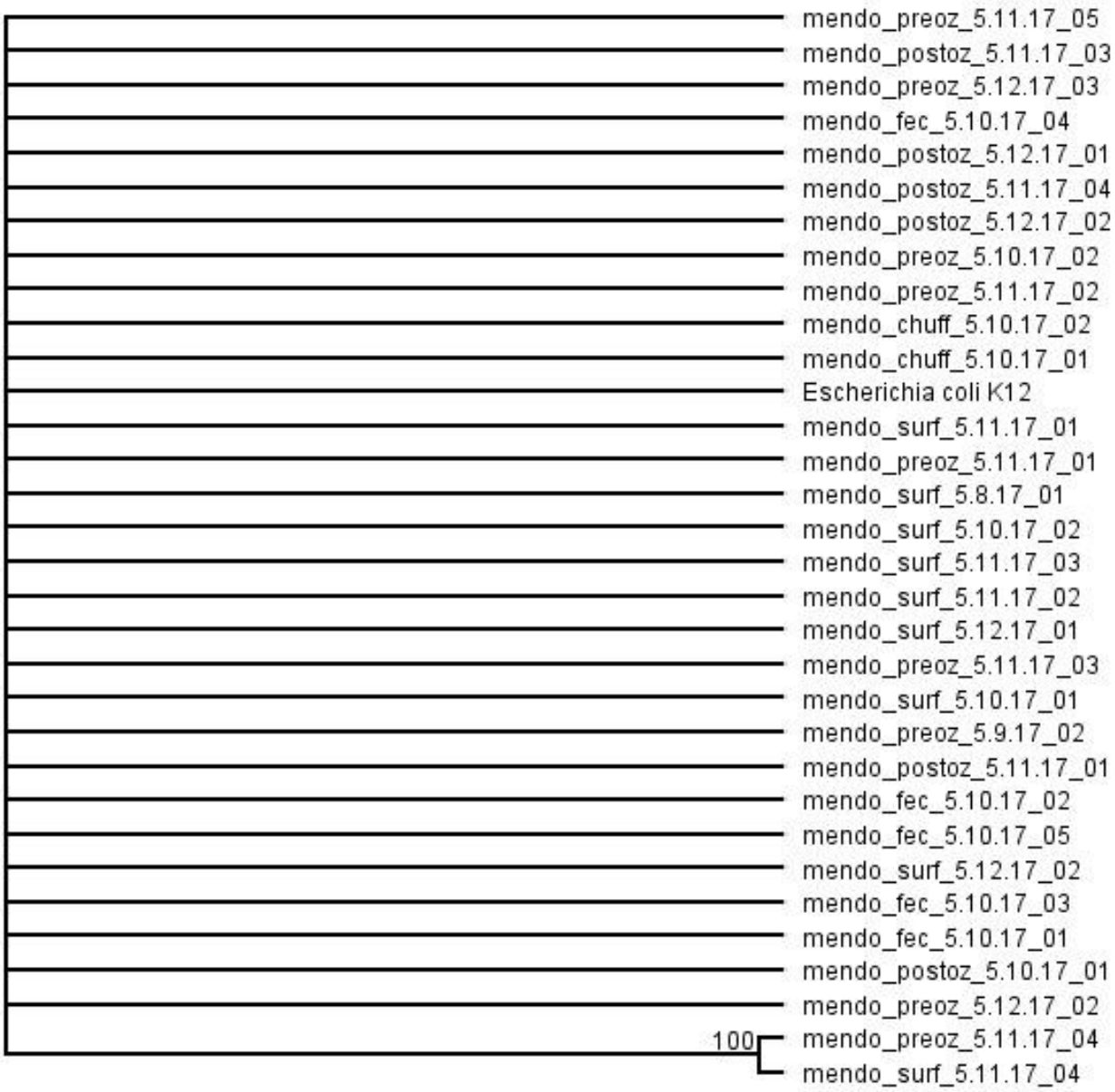


Figure 23: Tree of sequences from the V3 hypervariable region of 16S rRNA genes of presumptive total coliform isolates.

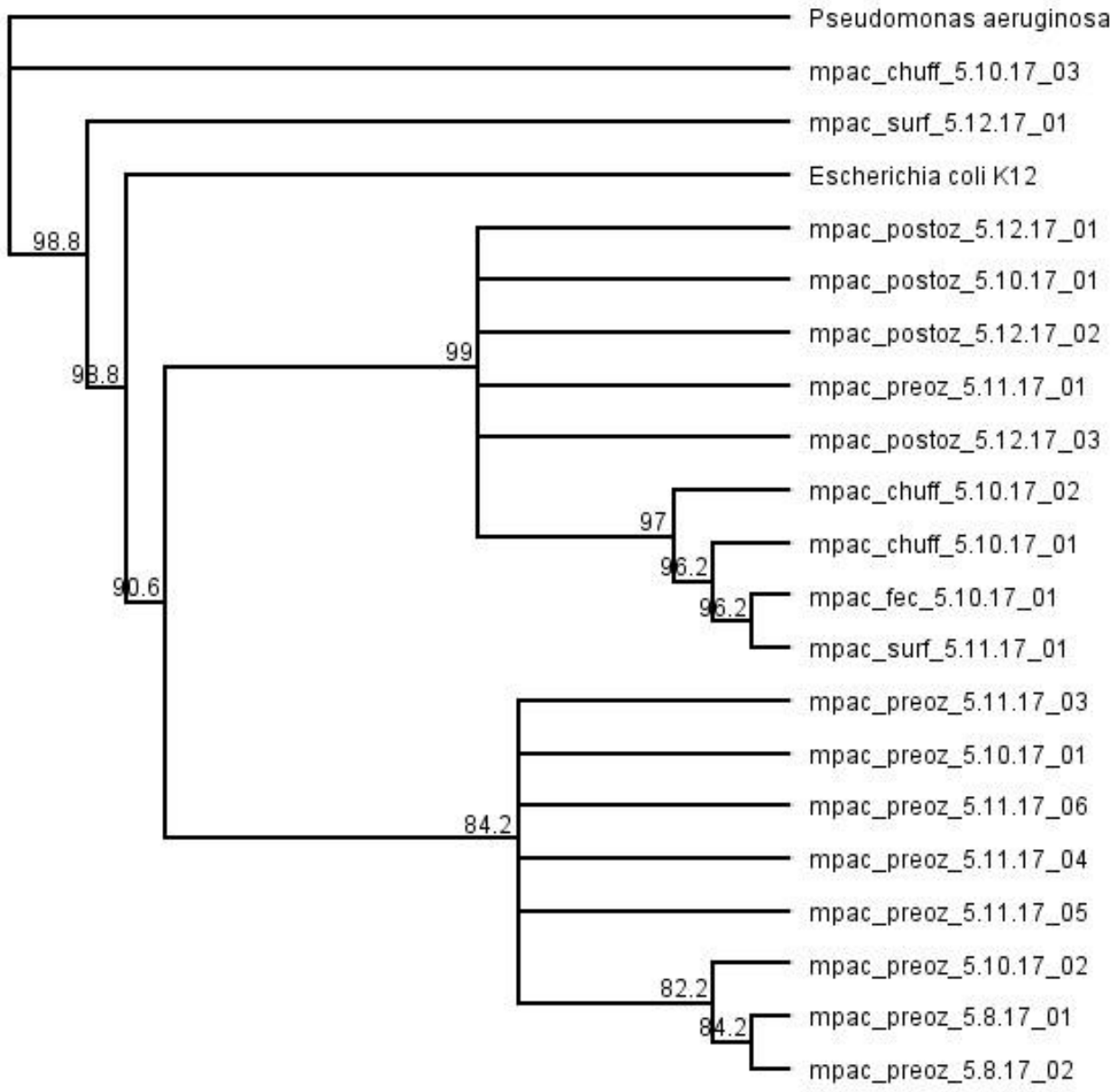


Figure 24: Tree of sequences from the V3 hypervariable region of 16S rRNA genes of presumptive pseudomonad isolates.

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