Fall 2018

Characterizing potential bacterial pathogens of Pisaster ochraceus sea stars with wasting disease

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Characterizing potential bacterial pathogens of *Pisaster ochraceus* sea stars with wasting disease

By

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Accepted in Partial Completion
of the Requirements for the Degree
Master of Biology

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Chelsea Hutchinson

September 13, 2018

Characterizing potential bacterial pathogens of *Pisaster ochraceus* sea stars with wasting disease

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
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Abstract

In 2013 sea star wasting disease (SSWD) caused an epizootic in over 20 species of asteroids along the west coast of North America. To see if SSWD was still affecting wild populations, we surveyed populations of the sea star, *Pisaster ochraceus*, in Birch Bay, WA. Our surveys indicated that advanced symptoms (lesion formation) increased 73% from July to September in 2017. To understand the role of bacteria in SSWD, we isolated bacteria with tissue-degrading potential from epidermal tissues of *P. ochraceus* animals in Birch Bay, WA. Next, we identified these isolates via 16S rRNA gene sequencing. Our results indicated that the community structure differed between SSWD-affected and healthy stars. In addition, we found that many of the *Vibrio* species isolated primarily from diseased sea stars lyse red blood cells, avidly degrade gelatin, and are related to species that cause disease in other marine animals. Other researchers have suggested that SSWD is caused by a microbial agent smaller than most bacterial cells (0.2 microns). Although none of the bacteria we isolated were smaller than 0.2 microns, we theorized that their signaling molecules could transmit disease. Quorum sensing signals (QSSs) are bacterially-produced, autoinducing molecules that regulate gene expression in a population-dependent manner within bacterial populations. We hypothesized that quorum sensing signals from diseased animals could facilitate development of SSWD on healthy individuals by triggering expression of virulence genes in opportunistic bacterial pathogens creating a state of dysbiosis. We tested bacterial isolates and tissue extracts from diseased and healthy sea stars for the presence of a subset of QSSs, acyl homoserine lactones (AHLs). We found that organic extracts from healthy and diseased stars differed in how strongly they activated a QSS bioreporter, and that multiple of the bacterial isolates associating with diseased stars produced AHLs. Thus, quorum sensing might contribute to transmission of SSWD.

Acknowledgements

Many undergraduate research assistants donated their time to facilitate this thesis work and we wholeheartedly thank them for their contributions. Fran Carpenter provided valuable assistance in the sea star surveys as well as caring for animals housed in aquaria. Trevor Grimm assisted to PCR amplify the 16S rRNA gene from bacteria isolates. Kayla Russell assisted with aligning sequences and optimizing techniques to sterilize the surfaces of sea star epidermis tissues for use in bioassays. Emma Kooyman contributed her time monitoring sea stars and maintaining aquaria for experiments. Serena Riley, Alastair Laing, Nathan Hannah, and Logan Wallace assisted in multiple experiments involving testing the abilities of isolated bacteria to degrade gelatin and other media. Cassandra McHugh assisted with many aspects of this study including testing the isolates for production of hemolysins and SSWD surveys and sample collection. Beth Loper played a key role in determining morphology of our bacterial isolates and provided valuable assistance in many experiments. A special thanks goes to stockroom employees Peter Thut, Sam Danforth, Erin Macri, and Joe Somera for their assistance with getting materials and equipment to complete this thesis work. In addition, I would like to thank Western Washington University and specifically the Biology Department of WWU for the use of facilities as well as generously providing me with funding for Sanger sequencing of the 16S rRNA gene and quorum sensing bioassay reagents. We would also like to thank the North Cascades Audubon Society for funding the 16S rRNA gene sequencing. Zoë Zilz contributed to this thesis work by teaching molecular biologists how to survey sea stars and she generously helped us collect animals for use in this study. Finally, we would like to thank the entire Pete Greenberg lab at the University of Washington and especially Amy Schaefer for her invaluable insights, teaching us the techniques for testing our samples for quorum sensing activity, and providing us with the quorum sensing bioreporter *A. tumefaciens* strain KYC55 (pJZ410)(pJZ384)(pJZ372) used in this study.

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Introduction

Sea star wasting disease (SSWD). Sea star wasting disease describes a particular set of signs that researchers have observed in asteroid populations since the 1970's (Dungan et al. 1982). In sea stars (asteroids), the signs of SSWD typically develop as follows: limbs twist, bodies appear to deflate, lesions form, limbs autonomize, tissues degrade, and the animal dies (Bates et al. 2009; Hewson et al. 2014; Fuess et al. 2015; Gudenkauf and Hewson 2015; Eisenlord et al. 2016; Kohl et al. 2016; Menge et al. 2016). The marine mass-mortality event that began in 2013 affected at least 20 echinoderm species (Eisenlord et al. 2016; Menge et al. 2016; Montecino-Latorre et al. 2016; UC Santa Cruz 2018). In addition to the wide taxonomic range affected by SSWD, this event is currently unparalleled due to the speed with which SSWD spread among populations and the vast geological range that SSWD was observed, ranging from Alaska to Mexico (Miner et al. 2018; Moritsch and Raimondi 2018).

SSWD and *Pisaster ochraceus.* *P. ochraceus* is a keystone species in the intertidal zone (Paine 1966) that suffered unprecedented declines from 2014 to 2015 along nearly its entire geographic distribution (Miner et al. 2018). *P. ochraceus* preys extensively on mussels; therefore, when sea star populations are low, mussels are left unchecked and usurp available rocky substrata (Paine and Trimble 2004). As would be predicted, SSWD-related mortality caused a significant reduction in mussel predation in Oregon following the 2014 outbreak of SSWD (Menge et al. 2016) and recently researchers have shown a significant decrease in mussel bed coverage in many sites of Washington, Oregon, and California (Moritsch and Raimondi 2018), which could lead to lasting community structure changes if populations of *P. ochraceus* do not recover (Paine and Trimble 2004; Moritsch 2018). Importantly, while sea star counts are generally increasing along the west coast, recruitment is varied among sites, and post-SSWD survivorship and

biomass remain lower than during pre-SSWD periods (Miner et al. 2018; Moritsch 2018). Moreover, mortality from SSWD is still occurring in populations along the coast (B. Miner, personal communication). Therefore, recovery and the ecological implications from this epizootic is unclear.

Mortality events due to epizootics are a primary factor in recent and dramatic population declines of many marine species (Harvell et al. 1999b, 2002; Ward and Lafferty 2004). Crashing populations of echinoderms exemplify the trend towards a higher frequency of marine epizootics. These events need to be thoroughly investigated to better understand how disease spreads in the wild so we can more effectively prevent and manage devastating epizootics.

SSWD is caused by a small infectious agent. Early in the SSWD epizootic, there were multiple observations that suggested that SSWD was likely caused by an infectious agent transmitted via seawater or food sources. Some evidence included the manner that SSWD progressed and devastated populations of sea stars from 2013-2014 and the observations by professional aquarists that sea stars in their aquaria would develop signs of SSWD when exposed to non-UV treated incoming seawater (Hewson et al. 2014). Additionally, other researchers maintaining sea stars in aquaria noted that stars would develop symptoms when fed wild-harvested mussels while SSWD was ongoing, while animals that were fed frozen mussels that had been collected prior to a disease outbreak remained healthy (A. Nguyen, personal communication). In an effort to determine the identity of a biological pathogen causing SSWD, researchers harvested tissues from sea stars affected with SSWD, homogenized them, and filtered them to exclude anything smaller than 0.22 µm (Hewson et al. 2014). Subsequent injection of the filtered tissue homogenate into unaffected sea stars appeared to transmit SSWD, while heat-killed samples did not (Hewson et al. 2014). Satisfying Koch's postulates, the researchers sacrificed sea star tissues

from the stars that were previously injected and showing signs of SSWD, and using the same method, successfully infected another previously heathy star (Hewson et al. 2014). This study corroborates the hypothesis that a biological agent causes SSWD symptoms. This same study also claimed, despite weak evidence, that there was a correlation between viral load of a densovirus, termed Sea Star associated Densovirus (SSaDV), and disease state in some species of sea stars (Hewson et al. 2014). Late research, however, indicated that there was only a correlation between SSaDV load and one species, *Pycnopodia helianthoides*, which no longer supports the original conclusion that SSaDV is associated with SSWD in multiple species (Hewson et al. 2018). Our studies were initiated between the publication dates of these two reports, and the first paper greatly influenced our hypotheses. We proposed that: bacteria might act as co-agents of viral disease, and/or bacteria might produce non-viral, SSWD-transmitting agents smaller than 0.22 µm. One such bacterially produced component that could have been present in the filtrate from the Hewson et al. (2014) study and cause SSWD is quorum sensing signals (QSSs).

An alternative etiological agent: quorum sensing signals. QSSs are small bacterially-produced molecules that communicate among individual cells information about local population density. Many types of marine bacteria produce a subset of QSS, termed N-acyl-homoserine lactones (AHLs) (Swift andWilliams 1999). AHLs are produced at low levels constitutively, and readily diffuse through the cell membrane, into the aqueous environment and across the membrane of neighboring cells (Miller and Bassler 2001). Therefore, as populations of bacteria become denser, concentrations of these signals increase both inside and outside the cells in that population. When a threshold density is reached, these signals activate target genes in the bacterial population that perceives the signal (Miller and Bassler 2001). The mechanism for QS

was first described in *Vibrio fischeri* where it requires the proteins *LuxI* and *LuxR* (Swift and Williams 1999). The *LuxI* protein is responsible for making AHLs, while *LuxR* binds the AHL, dimerizes, and binds to promoters of target genes (Fuqua et al. 1994). The *luxI* promoter is autoinduced by LuxR bound to AHL (Fuqua et al. 1994). This positive feedback loop is responsible for the exponential expression of target genes that rapidly coordinate population-level phenotypes, such as the genes required by bacteria to form biofilms or for bacterial pathogens to successfully infect a host (Davies et al. 1998; de Kievit et al. 2000).

The role of quorum sensing in disease transmission. For QSS to be an agent of SSWD disease transmission, opportunistic pathogens must be present in association with healthy animals and have virulence genes that are regulated by QSS. Hypothetically, after these opportunistic bacteria reached a sufficient population density to cause disease in one host, QSS could diffuse to bacteria on neighboring hosts, initiate expression of virulence-associated genes, and cause autoinduction of QSS (Williams et al. 2000). Supporting this idea, the addition of a synthetic AHL, N-hexanoyl-DL-homoserine lactone, to healthy corals (*Acropora cervicornis)* caused dramatic tissue loss that mimicked signs of corals suffering from white band disease (Certner and Vollmer 2015), and the addition of quorum sensing inhibitors to corals prevented disease progression (Certner and Vollmer 2018). Although the genes of the coral microbiome that were induced by AHL addition were not characterized, AHLs are known to govern virulence genes in bacteria associated with marine animals. For example, AHLs regulate expression of serine proteases which enable infection of salmonid species by the opportunistic pathogen *Aeromonas salmonicida* (Swift et al. 1997).

Factors involved in disease development. Disease is often characterized by a state of dysbiosis occurring when there is an imbalance between commensal and opportunistically pathogenic

bacteria. This imbalance can be precipitated by changes with the host, environment, pathogen, or an interaction between these factors (Ritchie 2006; Thurber et al. 2009; Alverdy and Luo 2017; Longo and Zamudio 2017; Peixoto et al. 2017). Due to the intricacies of these relationship, the etiologies of many marine diseases remain unsolved (Harvell et al. 1999b), but new studies using metagenomic, metatranscriptomic and other molecular techniques have linked environmental stressors and changes in bacterial communities with disease (Sutherland et al. 2004; Mydlarz et al. 2006; Bally and Garrabou 2007).

Environmental stressors can facilitate diseases in a variety of ways (Alverdy and Luo 2017). Environmental stressors can cue virulence in pathogens; for example, in mammals under stress, host stress signals such as adrenaline induce the pathogenic bacterium *Escherichia coli* O157:H7 to form host-damaging lesions and manufacture toxins (reviewed in Hughes and Sperandio 2008). It is also possible that environmental changes can directly cue an increase in the pathogenicity of certain members of an animal's microbiome. Metagenomic analyses on coral holobionts showed that increased temperatures triggered the expression of genes related to virulence (e.g., those for iron sequestration and invasion) in the associated microbiome (Thurber et al. 2009). Environmental stressors can also weaken mutualistic members of a microbiome, thereby allowing pathogens to infect the host. A notable example of protection by the native microbiome comes from researchers studying a disease that causes mass mortalities in amphibians worldwide. These scientists showed that during the cool-dry season in the El Yunque National Forest of Puerto Rico, continual recruitment and replacement of beneficial bacteria on frog epidermis is reduced, making frogs more susceptible to infection by the chytrid pathogen *Batrachochytrium dendrobatidis* (Longo and Zamudio 2017).

Scientists have explored the possibility that there are some potential environmental factors that have driven outbreaks of SSWD. Specific to marine diseases, seawater temperature and population density are often significant factors influencing or causing an increase in disease (reviewed in Lafferty et al. 2004; Burge et al. 2014); however, there is conflicting evidence on the role of temperature in the patterns of SSWD incidence or differences in severity of SSWD among affected sites (Eisenlord et al. 2016; Menge et al. 2016; Miner et al. 2018). The impact of temperature on SSWD is a difficult variable to test because it affects the host, the host's microbiome, and the pathogen simultaneously. Although, there is also some evidence that exposure due to low tides increases a population's risk for SSWD (Moritsch and Raimondi 2018). Regarding population density, there is no clear evidence supporting the hypothesis that density influences the susceptibility or severity of SSWD, though underlying genetic variability to SSWD within sea star populations could obscure the effect of density (Wares and Schiebelhut 2016; Miner et al. 2018; Schiebelhut et al. 2018). Much remains to be discovered about the complex interactions between the host, resident bacteria and other microbes, and environmental conditions in SSWD. Identifying the causal agent(s) of SSWD will significantly improve our ability to study the patterns and mechanisms of disease emergence and provide tools for conservation efforts for sea stars and other animals impacted by aquatic diseases.

We had three central objectives. First, we sought to understand prevalence and temporal changes in SSWD in a wild population of *P. ochraceus.* To this end, we conducted field surveys in Birch Bay, WA. Second, we wanted to understand the role that bacteria associating with sea stars could play in SSWD. To accomplish this, we collected bacteria from the epidermis of healthy and SSWD-affected *P. ochraceu*s individuals, identified them, and characterized their tissue-degrading abilities. Because of our focus on epidermal bacterial symbionts, we also tested

whether *P. ochraceus*, like other echinoderms, harbored subepidermal bacterial symbionts (Kelly et al. 1995; Burnett and Mckenzie 1997; Lawrence et al. 2010), so we optimized a method for isolating them. Third, we wanted to explore whether QSSs are involved in SSWD. While we did not directly test the ability of QSS to incite disease, we reasoned that if it were possible for QSS to transmit SSWD, we should observe differences in overall QSS between the tissues of healthy and diseased individuals. To test this, we used a bioreporter assay to compare the amount of total AHL activity induced by organic extracts of healthy and diseased sea stars tissue homogenates.

Methods

Sea star wasting disease surveys. To assess how disease prevalence changed over time, we completed six surveys over a six-week period during the summer of 2017. Surveys were performed during low tides in the rocky intertidal zone of Point Whitehorn in Birch Bay, Washington (Figure 1). This site is within the Salish Sea and has been surveyed periodically since the major epizootic in 2013-2015 (UC Santa Cruz 2018). We haphazardly chose three fifteen by fifteen-meter plots from areas that represented ideal substrata for *P. ochraceus*, sites containing large rocks and boulders that remain partially submerged during low tide. From each plot we removed, photographed, and determined disease state of ten sea stars. To quantify disease state, we used a scale modified from the "Intertidal Sea Star Protocol" by University of California Santa Cruz (UC Santa Cruz 2018). In our survey, 0 = healthy, 1 = one to two superficial lesions smaller than one centimeter in diameter (typically isolated to a single limb or body), 2 = two to five lesions, some of which are greater than one centimeter in diameter, and 3 = more than five lesions; an entire limb might be degrading, 4 = loss of limbs or death (Figure 2). We also photographed all surveyed individuals and measured the population density.

Seawater. For all experiments, seawater was obtained off the coast of Anacortes, WA and kept at outdoor temperature in a holding tank. From this reservoir, we obtained seawater as needed. For aquaria, unfiltered seawater was pre-chilled to the temperature of the cold room (10-12^{o}C). For growth media, seawater was filtered prior to use using Whatman #1 cellulose filters (11 µm retention cutoff) and sterilization was completed via autoclaving.

Culture conditions. Bacterial isolates were maintained routinely on filtered seawater containing 2.87 % (w/v) Knox gelatin, 0.1% (w/v) yeast extract, and 1.6% (w/v) agar. When testing for gelatin degrading ability, 2.87 % (w/v) Knox gelatin was dissolved in seawater and isolates were grown in broth cultures. Agar degradation was tested on filtered seawater containing 1.6 % (w/v) agar. Premixed thiosulfate citrate bile salts (TCBS; BD Difco, Franklin Lakes, NJ) medium and sheeps blood agar were purchased from Hardy Diagnostics (Santa Maria, CA).

Collection and isolation of epidermal bacteria. To compare the types of bacteria associating with healthy and diseased sea stars, *P. ochraceus* stars were haphazardly chosen during low tides at Birch Bay, WA on June 8, 2016 (hereafter called "BB1") and June 22, 2016 (hereafter referred to as "BB2") (Figure 1). Three distinct epidermal zones were sampled by gently rubbing a sterile swab moistened with sterile seawater across region near the madreporite on the aboral surface for 15 seconds. Tissue from apparently healthy stars were swabbed near the madreporite. Diseased stars were swabbed at two different zones on the epidermal surface: tissue that was apparently intact and unaffected (no lesion) and zones that contained lesions. Swabs were transported on ice to the lab, where they were stored at 4 ^{o}C and processed within 24 h. To dislodge bacteria from the swabs, the swabs were sonicated for ten minutes and vortexed for one minute in 1.5 mL of sterile seawater. The seawater used to collect the bacteria was subsequently dilution plated onto 2.87 % (w/v) Knox gelatin solidified with 1.6% agar, in seawater. Gelatin, which is partially

hydrolyzed collagen, was one of several substrates we expected SSWD pathogens to use because the predominant polymer in sea star epidermis is collagen (Lee 2009). From each plate representing bacteria from a single swab (one tissue type and one animal), when fewer than ten isolates were present on the plate, they were all chosen for further analyses. When more than ten colonies grew on a plate, the population was sub-sampled by choosing ten colonies that included at least one representative of each colony morphology.

Collection and isolation of sub-epidermal bacteria. An individual *P. ochraceus* star was collected from Birch Bay, then kept in an aquarium for multiple weeks at 10-12 ℃, and it became sick during captivity. An arm was excised by cutting along the natural fracture plane where the arm meets the main body (Zilz 2018). Two pieces of arm tissue (excluding digestive and reproductive organs) were cut into 0.5 g pieces. Each tissue sample was then individually surface sterilized as follows: samples were placed into glass culture tubes with sterile seawater, sonicated for ten minutes, transferred to a 0.6% NaOCl solution, vortexed for one minute, allowed to sit for five minutes, vortexed another minute, and finally rinsed by vortexing sequentially in ten tubes containing sterile seawater. To check for successful surface sterilization, 100 µL of each rinse solution was plated in duplicate on seawater gelatin plates. Plates were incubated at room temperature and checked daily for growth over the course of seven days. The surface sterilized sea star tissues were then incubated in three mL of sterile seawater at room temperature with shaking. Optical density of the seawater with the tissue was read at 595 nm every 24 hours over the course of a week with a Victor 3V multiwall plate reader (Perkin Elmer). After 168 hours of incubation (one week), tissue-containing seawater from one sample was dilution-plated onto seawater gelatin agar to determine if the change in optical density

corresponded with bacterial growth. Fifteen individual colonies grew on the agar. All were identified via Sanger sequencing of the 16S rRNA gene.

Amplification of the 16S rRNA gene. PCR amplification of the 16S rRNA gene was performed on all bacterial isolates for subsequent Sanger sequencing. To obtain DNA template for PCR, isolates were grown in seawater gelatin broth until logarithmic phase. Cultures were then centrifuged and excess medium was removed to obtain cell pellets. Next, 15 µL of sterile water was added to cell pellets, and cells were lysed by heating at 94℃ for ten minutes immediately before PCR. When this technique was unsuccessful, a phenol chloroform extraction was used to extract genomic DNA from bacterial cell pellets, as described previously (Wilson 1994) except that DNA was resuspended in 10 mM Tris HCl instead of Tris EDTA to avoid the inhibitory effects of EDTA on PCR (Rossen et al. 1992). Before PCR of the 16S rRNA gene, DNA concentration was estimated with a NanoDrop UV-Vis spectrophotometer (Thermofisher Scientific). When necessary, samples were diluted in HPLC-grade water prior to PCR to achieve a concentration below 100 ng/µL; this helped to minimize deleterious effects of excessive DNA template or contaminants on PCR.

PCR reactions consisted of 1X New England BioLabs Reaction Buffer, 0.2 mM (each) deoxynucleotide triphosphates, 0.2 µM universal bacterial primers 27f-CM or 27f-YM and 1492R (Table 1), and sterile HPLC-grade water. For each 50 µL reaction, 0.25 U Taq Polymerase (New England BioLabs M0495S) was used. To each reaction, 1 µL of either extracted genomic DNA or cell lysate was added. The following thermal cycler method was used: 94℃ for 4 min, followed by 30-35 cycles of 94℃ for 1 minute, 42℃ for 30 seconds, and 72℃ for 1 minute 30 seconds, with a final extension at 72℃ for 4 minutes. To check the quality of PCR amplicons, samples were tested with gel electrophoresis. Reactions yielding an amplicon

of approximately 1,500 bp in length were sent out for Sanger sequencing. Approximate amplicon length and concentration were determined based on comparison to the 1 Kb Plus Ladder (ThermoFisher # 10787018) using Image Studio Lite from LI-COR®.

Sequencing, identification, and phylogenetic analyses. To identify the epidermal and sub-epidermal isolates, the partial 16S rRNA gene of each isolate was Sanger sequenced at Molecular Cloning Laboratories (San Francisco, CA) with the same two primers used for PCR, resulting in two overlapping sequences. Once preliminary sequences were aligned, previously described primers (Frank et al. 2008; Klindworth et al. 2013) and custom designed internal primers (named according to conventions outlined in Alm et al. 1996), were used that would anneal to central regions of the gene and generate four-fold coverage (Table 1 and Figure 3). All four sequences were trimmed in Bioedit (Hall 1999) and aligned using the algorithm BLASTN from NCBI to make a full sequence of at least 1,100 bp of two-fold minimum coverage. The consensus sequences were then compared to the NCBI nucleotide database with Megablast (Hall 2013), excluding uncultured, environmental sample sequences from the search query. Alignments were made using the Multiple Sequence Comparison by the Log-Expectation (MUSCLE) method in Molecular Evolutionary Genetics Analysis (MEGA 7) software was used to generate a phylogentic tree, which though not specifically shown, was used to phylogenetically organize species for Figure 6 and Table 3.

Cellular morphology. In addition to tentatively identifying the isolates with 16S rRNA gene sequencing, isolates were also observed for size and cellular morphology. Each tested bacterium was streaked to isolation on seawater gelatin agar. Once growth was observed, each bacterium was Gram stained. Images were taken via light microscopy and cell length, width, and morphology were recorded.

Production of hemolysins. One representative isolate from each 16S rRNA consensus group was tested for its ability to produce hemolysins because bacteria that produce hemolysins can lyse animal cells. Each isolate was streaked and stabbed onto three separate sheep's blood agar plates. All plates were incubated at 20℃. Every 24 hours over the course of four days, hemolysis was assessed. A significant clearing zone as caused by lysing of red blood cells was interpreted as β-hemolysis, while clearing only in the stabbed portion of agar was interpreted as anaerobic β-hemolysis.

Agar degradation. Because agar is originally derived from red algae and these isolates were cultured from a marine habitat, it was important to test the isolates for their ability to degrade agar. All isolates were streaked onto solid medium made from 1.6% (w/v) agar autoclaved in filtered seawater as described above. Plates were incubated at approximately 20±2℃ for 72 hours. Growth was categorized as absent (-), or present (+) but collected data were not quantitative.

Gelatin degradation. To obtain relative comparisons of the rates at which the unique isolates degrade gelatin, isolates were first inoculated into three mL of seawater plus 2.87% (w/v) gelatin as a sole nutrient source and incubated at room temperature at 150 rpm for three to five days depending on growth rates. Samples were then diluted to an optical density of approximately 0.01 in seawater gelatin medium with two technical replicates each, in 96-well plates incubated with shaking at 150 rpm at either 10 ±0.5 ℃ or 20 ± 2℃. Optical density (OD) was measured at 450 nm. Each assay was performed three separate times. Each plate was read every 24 hours, technical replicate values were averaged for each assay, and blank corrections were made by subtracting the absorbance of seawater gelatin medium. The growth rates (r) were determined by using the GrowthCurver package in R, which combined all OD measurements for a single isolate

and fit a logistic regression. GrowthCurver then calculated the growth rate (as well as other growth parameters) based on the logistic regression (Figure 6). To obtain the best fit, data were removed past the point that the population had reached stationary phase (Sprouffske and Wagne 2016).

Testing individual isolates, tissue homogenates, and seawater for AHLs. Bacteria isolated from the epidermal swabs were tested for production of AHLs. Water samples and animal tissue samples tested for AHLs were collected directly from Birch Bay, WA or from tanks and animals therein that had been previously collected and held at 10-12 ℃. Sea stars were collected with a permit from the Washington Department of Fish and Wildlife.

Preparation of bacterial cultures for extraction. All cultures were grown by inoculating 100 mL of culture to an OD of 0.01 in seawater gelatin broth and shaking at 150 rpm at room temperature (25 ^{o}C $\pm$ 2 ^{o}C). OD was measured every 24 hours (as previously described). Once cultures reached late exponential or early stationary phase, as indicated by growth curves generated previously in seawater gelatin broth as above, they were centrifuged at 4,000 rpm for 5 minutes. The supernatant was collected for extraction.

Collection of tissues from the field. Animals were haphazardly selected from separate tidal pools on October 2, 2017. Because nearly every individual showed signs of SSWD at this time of the year in Birch Bay, only apparently SSWD-affected individuals were sampled. One to two arms without lesions (unaffected) and one to two arms with lesions (affected) were carefully excised from the same individual along the natural fracture plane. Unaffected and affected arms from each animal were then placed into separate sterile Whirl-pak bags (Nasco, Fort Atkinson, WI). Tidal pool and offshore seawater were sampled by using a Niskin bottle or by submerging a 200

mL bottle when water was prohibitively shallow. In total, ten sea stars were sampled (representing affected and unaffected tissues from five individual stars), five tidal pool seawater samples, and five offshore seawater samples were collected. Samples were stored in a cooler containing ice during transport and were processed within 24 h.

Collection of tissues from aquaria. We excised arms from five asymptomatic and symptomatic *P. ochraceus* stars that had been either collected from Point Whitehorn in Birch Bay at various times six months prior to use, or Post Point in Bellingham, WA on June 12, 2017. Animals were held in individual aquaria until tissues were harvested November 2, 2017-November 11, 2017. Seawater was collected from the corresponding tank within 12 h of sea star removal.

Tissue homogenization. Tissues were homogenized rather than swabbed to capture AHLs produced by the whole microbiome, including potential endosymbionts. Tissue samples were weighed and added to a blender. Filtered (Whatman #1) and autoclaved seawater was added to the blender in a volume equal to that of the tissue to facilitate blending. Tissues were blended for 30 seconds. Homogenate was poured into sterile centrifuge bottles, leaving behind remains of the endoskeleton. To separate the insoluble tissues such as spicules and endoskeleton fragments from the homogenate, tissues samples were centrifuged at 10,000 rpm for 15 minutes at 4°C. The volume of supernatant was measured with a sterile pipette. To further remove small debris, the supernatant was filtered into a sterile vacuum flask using a Buchner funnel lined with Whatman #1 cellulose filters. Seawater samples (100 mL) were filtered through Whatman #1 filters into a sterile vacuum flask.

Extraction of AHLs from bacterial cultures, sea star tissue homogenate, and seawater. Bacterial supernatant, seawater, and tissue filtrate were extracted twice with an equal volume of 0.01%

(v/v) acetic acid in ethyl acetate. The first extraction was performed over 24 hours at 4 ℃. Tissue extracts formed an emulsion that prevented separation of the aqueous and organic layers of tissue extracts, so mineral oil and small amounts of NaCl were added to facilitate separation. After the aqueous layer was extracted for the second time, extracts were stored at -20 ℃.

Rotary evaporation. To concentrate the samples, all samples were rotary evaporated until approximately one mL remained, transferred to 20 mL scintillation vials, dried via evaporation at approximately 20 ºC in a chemical fume hood, and resuspended in 0.2X of the original sample volume in acidified ethyl acetate. For example, a one g sample was assumed to equal approximately one mL, and therefore was concentrated into 200 µL of solvent extract to achieve a five-fold concentration.

Agrobacterium tumefaciens bioreporter assay for the detection of AHLs. A. tumefaciens strain KYC55 (pJZ410)(pJZ384)(pJZ372) was chosen as an ideal bioreporter because it responds to a wide range of AHLs at nanomolar concentrations. It is most sensitive to its native signal, 3-oxooctanoyl-homoserine lactone, to other 3-oxo AHLs, and to those with similar chain lengths (Zhu et al. 2003). The plasmid pJZ372 contains a *traI*::*lacZ* fusion that drives production of β-galactosidase (but not TraI) when AHLs recognize and bind the portion of the *traI* region. The plasmid pJZ372 contains a gene encoding tetracycline resistance as a selectable marker. The plasmid pJZ384 includes the T7 promoter fused to *traR* (the gene encoding the native AHL receptor of *A. tumefaciens*) for strong sensitivity to AHLs and bears a gene for spectinomycin resistance as a selectable marker. The plasmid pJZ410 encodes the T7 RNA polymerase and bears a gene for gentamicin resistance as a selectable marker. This reporter strain lacks *traI*, and cannot make its cognate signal, 3-oxooctanoyl-HSL. Therefore, production of β-galactosidase only occurs in the presence of exogenous AHLs. β-galactosidase activity was detected via

cleavage of an artificial substrate (Tropix™; Thermofisher Scientific), resulting in luminescence (Zhu et al. 2003).

Preparing the culture, samples and standards for the AHL bioreporter assay. A. tumefaciens was grown in tryptone yeast (TY) medium supplemented with spectinomycin (50 µg/mL), tetracycline (4.5 µg/mL), and gentamycin (50 µg/mL) for two days at 200 rpm at 30 ℃. It is not possible to determine OD because *A. tumefaciens* aggregates in culture; therefore, cultures were diluted 1:100 in TY medium. Each AHL sample extract or synthetic AHL standard were added to 1.5 mL Eppendorf tubes, and ethyl acetate was evaporated in the fume hood as described above. Next, 0.5 mL of diluted *A. tumefaciens* culture was added to each tube. Tubes were shaken at 200 rpm at an angle, at 30 ℃ rpm for 16-18 hours. Standards consisted of dilutions of pure synthetic N-hexanoyl homoserine lactone (Cayman Chemical, Ann Arbor, MI) at concentrations of 0 (blank), 50, 100, 150, 200 and 250 nM.

Completing the bioreporter assay. After incubation for 16-18 hours, cells were lysed to release β-galactosidase from *A. tumefaciens* KYC55 cells by adding 10% (v/v) of chloroform, and then briefly vortexed. Sample from each lysed cell supernatant was added to a 96-well plate. Tropix® reagent (Thermofisher Scientific) was added. Plates were then covered and incubated at 25℃ for one hour in the dark. Just prior to reading the luminescence with a plate reader, the accelerator reagent (Thermofisher Scientific) was added to each well. A standard curve relating concentration of synthetic AHLs to luminescence was used to interpolate concentration of test samples of one g of extracted tissue or one mL of extracted seawater.

Results

Sea star wasting disease surveys. Over the six weeks of monitoring populations of *P. ochraceus* in Point Whitehorn in Birch Bay, WA, incidence of disease increased 33.3%. Sea stars with advanced symptoms (score of 3) spiked from 0% to 73.3% (Figure 4). No sea stars with a score of 4 were documented from the surveys, indicating that the most severe disease symptoms were not prevalent during this timeframe at the sites we surveyed in Birch Bay, WA.

Identification of epidermal isolates based on the 16S rRNA gene. There was a total of 118 two-fold coverage sequenced isolates (Table 2) and an additional 17 sequences that were either short (500-700 bp) or single coverage (Supplementary Table 1). While there appear to be at least 34 unique isolates based on comparison with the most current GenBank database (excluding uncultured/environmental sequences), species and strain-level designations cannot be made confidently based on 16S rRNA gene sequencing alone (Janda and Abbott 2007). Genus-level identification; however, was predicted (Table 2). All but three epidermal isolates belonged to the phylum *Proteobacteria*. Lesion swabs yielded a single *Staphylococcus* (phylum *Firmicutes*) and two *Polaribacter* (phylum *Bacteroidetes*) isolates (Table 2). Overall, *Psychrobacter, Vibrio, and Pseudoalteromonas* were the most prevalent genera that we collected.

Trends in the types of gelatin-degrading isolates associated with epidermal tissues. Gelatin-degrading bacteria were isolated from only one healthy animal in the BB1 collection, but were isolated from three healthy animals from the BB2 collection. In contrast, gelatin degraders were collected from the lesions of four out of five sick BB1 animals, and from lesions of all five BB2 animals sampled. In addition, four symptomatic BB1 animals and three symptomatic animals from BB2 yielded gelatin-degraders from the unaffected tissue swabs. Overall, gelatin-degrading

bacteria were cultivated at lower frequencies from the epidermis of healthy animals than from sick specimens (Table 2).

The percentage of each genus within the total number of isolates from each tissue zone of five healthy and five SSWD-affected individuals is represented by Figure 5. Each genus was only counted once per swab. There was a temporal shift in the structure of gelatin-degrading bacterial populations from the time of the BB1 collection to the time of the BB2 collection. Most notably, members of the genus *Psychrobacter, Paracoccus, and Thioclava* were largely absent from BB2, while *Vibrio* and *Pseudoalteromonas* dominated BB2 populations. The types of bacteria isolated from epidermal swabs of *P. ochraceus* also shifted from being primarily poor gelatin degraders to avid gelatin degraders (Figure 5, Table 3).

Healthy tissues. Gelatin-degrading isolates from healthy animals at BB1 were equally divided among *Psychrobacter, Thioclava, and Paracoccus*. From BB2 healthy animals, the gelatin-degrading bacterial isolates changed to 78% *Pseudoalteromonas* and 22% *Vibrio* (Figure 5). Members of the genera *Paracoccus* and *Thioclava* were only isolated from intact (healthy and unaffected) tissues and were not encountered in the BB2 collection (Figure 5). There were fewer types of gelatin-degrading bacteria isolated from healthy stars than from symptomatic animals (Figure 6).

Unaffected tissues of symptomatic animals. Isolates derived from unaffected tissues of sick animals were composed of the same genera as healthy tissues (except *Thioclava* from the BB1 collection) but in addition included isolates of the genus *Neptunomonas. Neptunomonas* was the only genus specific to unaffected tissues (found neither on healthy animals nor on lesions) and was present in both sampling trips (6% of BB1, 17% of BB2). From BB1, *Pseudoalteromonas*

(17%) and *Vibrio* (17%) are only present in association with the unaffected tissues, although in the BB2 collection these genera were isolated from all tissue zones of both healthy and sick animals. *Cobetia* represented 11% of isolates from unaffected tissues from BB1 but was not isolated from unaffected tissues from BB2. Notably, *Cobetia* and *Neptunomonas* were only isolated from sea stars exhibiting SSWD symptoms (Figure 5).

Lesions of symptomatic animals. Psychrobacter was a dominant genus of lesion-associated tissues of BB1 (33%) but was absent from BB2 lesion swabs, which consisted of mostly *Pseudoalteromonas* and *Vibrio* (39% each). Genera specific to actively degrading tissues were *Marinomonas* (8% and 4% for BB1 and BB2, respectively), *Polaribacter* (0%, 9%), *Staphylococcus* (8%, 0%), and *Glaciecola* (0%, 4%) (Figure 5). These isolates might represent saprophytic organisms that take advantage of the actively degrading tissues, rather than primary pathogens. While species and strain-level designations are only tentative, based on 16S rRNA gene sequencing there were nine unique *Vibrio* isolates, seven of which were only found on diseased animals, and five that were only recovered from actively degrading tissues (Figure 6a and b).

Isolation of endosymbiotic bacteria. After surface sterilization of excised sea star tissue, none of the sterile seawater rinses yielded colonies after plating on seawater gelatin agar, indicating that the sterilization of surface tissues was successful. Sterile seawater inoculated with tissue pieces exhibited an increase in OD over the course of a week, from 0.04 to 0.21 at 450 nm, and it also yielded fifteen colonies after being spread atop seawater gelatin medium. The 16S rRNA gene from all fifteen colonies matched the genus *Bacillus*, with a 99% identity to *Bacillus aquimaris* strain OX1213 (GenBank ID MG575973.1).

Production of hemolysins. All the isolates that showed β-hemolysis (full lysing of red blood cells) as indicated by clearing on sheep's blood agar plates belonged to the genus *Vibrio* (Table 3). In addition, these β-hemolytic *Vibrio* isolates were only isolated from actively degrading tissues on diseased sea stars.

Utilization of agar as a nutrient source by bacterial isolates. Some isolates were able to grow with agar as the sole nutrient source. Many agar degraders belonged to the genera *Pseudoalteromonas* and *Polaribacter.* A single species of *Vibrio* also grew on agar as a sole nutrient source (Table 3).

Gelatin degradation by bacterial isolates. Isolates belonging to the genera *Staphylococcus, Polaribacter, Pseudoalteromonas, and Vibrio* degraded gelatin at both 10 ℃ and 20 ℃, often with higher growth rates observed at 20 ℃ (Figure 6c). *Vibrio* and *Pseudoalteromonas* isolates had the greatest differences in growth rates between the two temperatures, but there was high variability of the growth rate within a genus. For example, *Pseudoalteromonas tentraodonis* strain GFC exhibited growth rates seven times higher than other isolates belonging to the genus *Pseudoalteromonas*. While all isolates were originally cultivated in seawater gelatin medium, *Paracoccus, Thioclava, Neptunomonas, Marinomonas, Psychrobacter, and Cobetia* had no detectable growth at these two temperatures from cultures revived from storage at -80 ºC. The single isolate of the genus *Glaciecola* grew in seawater gelatin medium as shown by visible dense aggregates of cells; however, its propensity to aggregate skewed optical density measurements.

AHL activity for bacterial isolates. While many of the isolate extracts showed some weak activation of the reporter strain, six isolates activated the bioreporter strain well above detection

levels (Table 3). These isolates included five species of *Vibrio* that also avidly degraded gelatin and produce hemolysins, and a single isolate of the genus *Glaciecola* found in association with the lesion of a SSWD-affected sea star (Figure 6, Table 3).

AHL activity of tissue and seawater extracts. The AHL bioreporter *A. tumefaciens* strain KYC55 (pJZ410)(pJZ384)(pJZ372) was used to detect AHLs in crude tissue and seawater extracts because it sensitively detects multiple AHL derivatives at biologically relevant concentrations. AHL extracts derived from tissues of aquarium-housed healthy animals activated the bioreporter 54% more than tissue extracts from SSWD-affected animals (Fig. 7a). Unaffected and affected tissues from diseased animals; however, did not appear to differ from one another in activating the bioreporter (Fig. 7b). All seawater samples from tanks of healthy and diseased animals, tidal pools, and shore water activated the bioreporter strain less than the tissue samples. Offshore water and tidal pool water also did not appear to differ from one another in induced AHL activity (Figure 7c and 7d). Seawater from aquaria housing sick and healthy animals resulted in similar bioreporter activation, except for one diseased tank, which was 20-fold greater in activity than the other four tanks (data not shown).

Discussion

Quorum sensing and SSWD. Our study demonstrates a difference in the overall complement of AHLs in the microbiome of healthy and diseased *P. ochraceus*. Because the bioreporter responds to multiple AHL species, activation differences might represent differences in AHL species, concentration, or both. Bioreporter activation differences could also reflect signal inhibitors produced by the microbiome. Additionally, AHLs regulate many processes aside from virulence-associated gene expression, making it impossible to infer functional significance of our results.

Isolates from diseased animals could represent pathogens or saprophytes; genes for both virulence and tissue degradation are commonly regulated by AHLs (de Kievit et al. 2000).

Although extracts from healthy wild sea star tissues activated the bioreporter more successfully than extracts from wild sick sea stars, the individual isolates (*Vibrio* spp. and *Glaciecola*) that activated the bioreporter were exclusively isolated from diseased stars. It is important to note that while bacterial isolates were collected in 2016, animals for AHL extraction were collected in 2017. Thus, it is unknown whether the microbiomes of the animals tested for AHL activity resemble those from the BB1 collection, the BB2 collection, or neither. In addition, pure culture conditions are very different from that of their natural environment, and might not lead to the same type of AHL production. Further occluding any conclusive inferences, AHLs from sea star tissues could have been produced by non-gelatin-degrading bacteria that were missed by our census. We hypothesized that the differences that we observed in AHL activity in sea star tissues also could be caused by quorum sensing inhibitors in the tissue extracts; however, preliminary experiments where we spiked extracts with pure AHLs did not indicate that healthy and diseased tissue extracts were differentially inhibiting the bioreporter strain from detecting the pure AHLs (data not shown). Because we did not directly address the role of AHLs in SSWD, we suggest that future work should try to identify the signals present in microbiome extracts as well as those produced by bacterial isolates. It would be informative to query if AHLs from sea star isolates induce expression of genes responsible for potential virulence traits, such as collagen degradation and hemolysis.

Quorum sensing and the environment. Quorum sensing represents a unique and relatively unexplored mechanism for disease transmission in marine diseases. Much like other etiological agents, QSS are susceptible to environmental changes. In the marine environment, chemical

degradation and dilution reduce AHLs (Hmelo 2017). The ocean is typically alkaline with an average pH ranging from 7.5 to 8.2. In this pH range, AHLs are sensitive to base-catalyzed hydrolysis of the lactone ring, and high pH decreases the longevity of these signals in microbial mat communities (Decho et al. 2009; Hmelo 2017). As oceanic pH decreases due to ocean acidification, AHLs will be less prone to degrading (Decho et al. 2009). Consequently, at a lower pH there would be a decrease in the overall threshold of AHL production needed to elicit bacterial population responses, and increased maximum distance over which quorum sensing maintains effectiveness (Decho et al. 2009; Hmelo and Van Mooy 2009; Hmelo 2017). As oceans acidify, therefore, diseases that involve quorum sensing by bacterial populations could worsen.

QSSs are protected from dilution in the marine environment by geomorphological features that prevent the majority of water flow such as rock crevices (Kim et al. 2016). Research suggests that *P. ochraceus* living in tidal pools or sheltered had significantly higher rates of SSWD (Bates et al. 2009; Menge et al. 2016). While many factors could explain this finding, tidal pools certainly represent environments that could prevent dilution of QSS, and maintain the presence of disease-transmitting agents (Kim et al. 2016). Nevertheless, our results indicate that AHL activity in the seawater directly offshore, where it is subject to tidal flow, is similar to the AHL activity in seawater from sheltered tidal pools. That both the tidal pools and offshore seawater samples contained AHLs detectable by the bioreporter suggests that these signals persist at biologically relevant concentrations in the environment. It is worth acknowledging that AHLs might be derived from multiple sources of bacterial communities, only one of which is sea stars.

QSSs can be maintained in the environment by bacterial biofilms (Kim et al. 2016). Researchers have found that bacteria that successfully colonized surfaces in temperate marine habitats are initially Rhodobacterales but are replaced by biofilm-forming communities that comprise some of the same genera we isolated from sea star epidermal tissues, including *Pseudoalteromonas, Glaciecola, Vibrio, Psychrobacter* (Roger Anderson 2016) This suggests that the microbiome colonizing the epidermis of a sea star might be derived from other surfaces in the marine environment, and the most successful colonizers of sea star epidermal tissues are likely species that form biofilms. Biofilms found on sea stars and other surfaces slow diffusion of substances into the surrounding environment and could concentrate AHLs (Kim et al. 2016).

Impacts of QSS on the host. The effect of quorum sensing is not limited to communicating solely with bacterial populations. In mammals, administration of AHLs in low concentrations impaired immune response, while high concentrations triggered reactions by the immune system, such as an increase in production of proteins that help attack infecting organisms by macrophages (Hughes and Sperandio 2008). *Pycnopodia helianothoides* sea stars injected with 0.22 micron filtered homogenate from a symptomatic star developed SSWD symptoms and had a significant increase in immune response gene expression (Fuess et al. 2015). While there are different components in the filtrate that could induce this immune response, QSS have been shown to modulate host immunity. For instance, at high concentrations, the AHLs produced by *Pseudomonas aeruginosa* actively facilitate disease in humans by inhibiting the ability of immune cells to produce antibodies (Telford et al. 1998). Long-chained AHLs can also trigger apoptosis in host cells of mammals, specifically attacking cells such as macrophages, thus allowing the infecting bacteria to evade the innate immune response (Hughes and Sperandio

2008). While our study did not address the host response to these bacterial signaling molecules, it is possible that quorum sensing could enable development of SSWD in multiple ways.

Sea star microbiome. Some research has been done to explore the microbiome of sea stars; researchers that sought to characterize the whole microbiome via a metagenomics approach of different sea stars found that epidermal tissues of many sea star species were dominated by α, β, and γ-Proteobacteria, while phyla *Bacteroides* and *Firmicutes,* and *Actinobacteria* were found less frequently (Jackson et al. 2018). However, the microbiome of *P. ochraceus* specifically has not been characterized. To our knowledge, a metagenomics comparison of SSWD-affected versus healthy animals is also lacking.

Multiple studies have focused on endosymbiotic subcuticular bacteria of echinoderms (Kelly et al. 1995; Burnett and Mckenzie 1997; Lawrence et al. 2010), but not in relation to disease development. We focused on sea star-associated bacterial species that have pathogenic characteristics; specifically, the ability to degrade collagen/gelatin and to lyse cells. Our results suggest that the types of bacteria associating with diseased tissues have more of these pathogenicity and/or saprotroph-associated traits, i.e. they degrade gelatin avidly and produce hemolysins (Table 3; Figure 6). Overall, these experiments represented a straightforward approach for finding potential pathogens within a larger microbiome, so that future analyses could focus on likely pathogens. The caveat is that this biased approach could have missed pathogens that do not degrade gelatin. Importantly, this study was not designed to elucidate protective microorganisms whose absence could facilitate disease; for instance, our culturing techniques might have missed mutualistic bacteria that might provide disease resistance and other benefits to the host star. It would be informative to examine the sea star microbiome of healthy and SSWD-affected sea stars on different tissues, including epidermal, subcuticular, and

coelomic fluid samples, using an unbiased metagenomics approach to compare community structures between healthy and diseased sea stars and predict potential roles in SSWD development.

Trends in bacterial community composition. A study found that corals suffering from white band disease have distinct microbiomes at the lesion edge as compared to unaffected tissues (Pollock et al. 2017). This same study; however, did not find a significant difference between the microbiomes of unaffected tissues of diseased corals and healthy tissues (Pollock et al. 2017). In contrast, our experiments showed some distinct differences between the types of bacteria recovered from healthy animals vs. unaffected tissues from sick animals when compared within a sampling trip. Notably, when comparing unaffected tissues to lesion and healthy tissues from the first sampling trip, unaffected tissues included *Pseudoalteromonas* and *Vibrio* isolates, while the other two tissue samples did not. During the second sampling trip when SSWD prevalence was higher, unaffected tissues still included *Vibrio* and *Pseudoalteromonas* isolates but differed from the other two tissue types because they included *Psychrobacter*. Moreover, *Neptunomonas* was only isolated from unaffected tissues. Similar community composition between healthy and unaffected sea star tissues might indicate that bacterial community changes as the disease front advances. We observed, however, that unaffected tissues had distinct community compositions, suggesting that the entire diseased animal differs in microbiome composition, regardless of whether the tissue is actively degrading or not.

Factors driving differences in bacteria between healthy and diseases stars. Echinoderms have a more rudimentary immune system than vertebrates but some immune mechanisms present in vertebrates and mammals are found in echinoderms (Fuess et al. 2015). One such immune mechanism found in both sea stars and mammals are antimicrobial peptides (Blair and Hedges

2005; Li et al. 2010; Fuess et al. 2015). In mammals under stress, these antimicrobial agents are primarily used to defend against invading microbes but in extreme situations they could also harm beneficial symbiotic bacteria (Alverdy and Luo 2017). If a sea star was producing antimicrobial peptides harmful to symbiotic bacteria, sea stars would be left more susceptible to infection (Strahl et al. 2002; Zaborin et al. 2014), which could then allow for a consortium of opportunistic pathogens to thrive in their stead. For instance, members of the genera *Polaribacter*, *Glaciecola*, and *Staphylococcus* were associated only with lesions of diseased *P. ochraceus* stars, and thus could represent saprophytes exploiting the rich nutrient source of degraded tissue of the sick animals. To understand the interactions among bacteria that likely facilitate, if not directly lead to disease, future studies should monitor how bacterial populations change with respect to individual sea stars while disease symptoms progress. Sampling the same individual during disease progression would also increase the statistical power of the study by reducing variation caused by comparing different individuals.

***Vibrio* and disease.** Members of the genus *Vibrio* are implicated as pathogens in diseases affecting animals ranging from corals, oysters, sea cucumbers, salmon, and humans (Swift et al. 1997; Kushmarol et al. 1998; Ben-Haim and Rosenberg 2002; Bally and Garrabou 2007; Eiston et al. 2008). Many species of *Vibrio* have a suite of virulence factors that enable them to infect a host animal. For example, *V. tasmaniensis* produces outer membrane vesicles that deliver degradative enzymes, enabling infection of oyster immune cells (Vanhove et al. 2015). Furthermore, we showed that many of the species of *Vibrio* that we isolated were able to lyse red blood cells and degrade the primary protein in sea star epidermal tissues, gelatin (partially degraded collagen).

Multiple species of *Vibrio* have also been characterized by their ability to enter a state in which the cell-membrane is intact, gene expression continues, but cellular division is mostly suspended, a state referred to as viable-but-nonculturable (VBNC) (Oliver 1993, 2010). In general, the ability to enter the VBNC state enables some pathogenic species of *Vibrio* to persist in seawater and even within a host to resist a variety of stressors, such as unfavorable temperatures or low nutrient availability (Oliver et al. 1995; Oliver 2010).

Quorum sensing signals have been shown to resuscitate some VBNC *Vibrio* species (Bari et al. 2013; Ayrapetyan et al. 2014). Herein, we found that many species of bacteria associating primarily with diseased stars (all *Vibrio* apart from a single isolate) that were also isolated when SSWD was widespread, produced AHLs. This finding could indicate that quorum sensing could be awakening these species and causing their emergence as SSWD progresses through a population of sea stars. However, until a connection is made between quorum sensing and upregulation of virulence genes in these bacterial isolates, this is only preliminary evidence that quorum sensing could facilitate SSWD by enabling opportunistic pathogens.

In addition to quorum sensing signals, improved environmental conditions trigger these bacteria to resuscitate from the VBNC state (Oliver 2010). Researchers have shown that two species of *Vibrio* isolated from diseased corals were able to be resuscitated from a VBNC state by increasing nutrient availability (Vattakaven et al. 2006). There are multiple manners in which nutrient enrichment can occur in marine environments and thus trigger resuscitation of bacterial populations. Upwelling, which occurs seasonally, brings fresh nutrients from the sea floor (Lester et al. 2010). At our study site of Point Whitehorn in Birch Bay, WA, storms or snowmelt could also have increased river flow or have brought runoff into the bay, increasing nutrients and influencing the differences that we saw in bacterial populations. Nutrient enrichment, whether as

a consequence of seasonal upwelling or anthropogenic activities, is often cited as a driver of marine disease (as reviewed by Harvell et al. 1999a).

Temperature is known to significantly influence changes in *Vibrio* populations (Thompson et al. 2004), likely due in part to the resuscitation of vibrios from the VBNC state (Oliver 2010). Warmer temperatures might therefore explain the increased prevalence of *Vibrio* isolates from our second collection of epidermal bacteria. Additionally, the species of vibrios that we isolated in association with diseased stars exhibited increased growth rates in seawater gelatin broth from 10℃ to 20℃, which reflects a temperature that would occur during the warm summer months in the intertidal zone in the Salish Sea.

Intriguingly, during a spike in disease incidence in the wild at Birch Bay, animals from the same site but distantly housed in individual tanks in a temperature and light-controlled environment developed SSWD symptoms and died [data not shown]. It is possible that the tank water (taken from offshore near Shannon Point in Anacortes, WA) or the food sources (mostly the mussel *Mytilus edulis*) harvested from various local sites could carry infectious agents. Often, we observed that moving individuals from one tank to another (despite transferring the water with the individual to minimize shock) resulted in multiple animals deteriorating and dying. These observations might suggest that the pathogenic agent was able to remain dormant and revive under aquarium conditions.

The epidemiology of SSWD remains a mystery; however, a pathogen that can persist dormant in the environment and be revived by environmental cues that would differ regionally could explain the currently unpredictable pattern of SSWD emergence (Miner et al. 2018; Moritsch 2018). Much like SSWD, diseases affecting other marine animals such as corals have

unexplained patterns of severity, and researchers have posited that difference in nutrient enrichment at different sites could explain the patchiness of disease severity (Bruno et al. 2003). It logically follows that these potential pathogens persist in the environment and a culmination of environmental cues trigger their reemergence and virulence. Because our study could not elucidate whether the bacteria that we isolated from sea stars reflect changes in the host immunity and health of the animals, or are predominantly influenced by environmental changes such as temperature, we collected and preserved epidermal swabs from *P. ochraceus* stars progressing from health to death in aquaria and wish to perform metagenomic analyses on these samples in the future.

Multiple pathogens *V. tasmaniensis* has been shown to induce symptoms of skin ulceration disease in the sea cucumber, *Apostichopus japonicus*, which include the development of white lesions that expose underlying muscle, loss of spicules, inability to adhere to surfaces, appetite loss, evisceration (Deng et al. 2009). Another study exploring the causative agent of skin ulceration disease in *A. japonicus,* showed that both inoculation with bacteria (*Pseudoalteromonas tetraodonis* and *Pseudoalteromonas* sp.) isolated from lesions and the injection of crude viral extracts could manifest itself symptoms of this disease and lead to death of the sea cucumber (Liu et al. 2010). The ability of multiple types of bacteria (*Vibrio* sp. and *Pseudoalteromonas* sp.), in addition to viruses, being able to induce nearly identical disease symptoms in the same species of sea cucumber illustrates the possibility that the same set of symptoms can have multiple causes. While *V. tasmaniensis* or another bacterium might not the sole causative agent of SSWD, these bacteria still likely play a role in disease progression. Going forward, studies that research marine epizootics including SSWD cannot exclude the possibility of multiple pathogens, especially as these marine animals face environmental stressors associated

with climate change and nutrient influxes that might weaken their ability to maintain a healthy balance with their microbiome.

Conclusions. We found that a subset of the *P. ochraceus* microbiome that can degrade collagen/gelatin was different between SSWD-affected and apparently healthy animals. No single genus or strain could be unequivocally correlated with disease, suggesting that function, more than phylogenetics, might be key to maintaining a balanced microbiome. The isolation of multiple unique isolates within the genus *Vibrio*, all with the ability to lyse cells and produce quorum sensing signals, supports the notion that any of them (or many of them together) might incite necrosis. Epizootic diseases like coral bleaching and amphibian decline illustrate the importance of understanding the interactions of the host with its entire microbiome (and metabolome) and with a complex environment. A similar mindset will be required to understand SSWD.

Implications and future areas of study. Sea stars were continuously developing SSWD symptoms in the summer of 2017 in Birch Bay, WA; however, dying or melting stars were rarely observed in the field. This observation might reflect that we discontinued surveys too early, or alternatively, that SSWD has decreased since the 2013-2014 outbreak. Other researchers have indicated that SSWD might be attenuating even if populations have not fully recovered (Miner et al. 2018; Moritsch and Raimondi 2018). We propose that future studies should track disease as it progresses in individual animals to elucidate whether individuals that sicken then recover, or whether they die. The latter scenario would suggest that SSWD selected for naturally resistant individuals and that these could dominate the population, as suggested by recent genetics-based surveys of sea star populations (Wares and Schiebelhut 2016). A more nuanced understanding of the current disease status would provide insight about whether SSWD is still an immediate

ecological concern, or if populations of *P. ochraceus* are likely to recover and continue to shape their resident ecosystems.

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Tables

Table 1. Primers used for PCR and Sanger sequencing of the 16S rRNA gene.
Standard nomenclature used: "M" is either adenine or cytosine, "Y" is cytosine or thymine, "B" is any except for adenine, and "D" is any nucleotide except cytosine.

Sequence 5'-3'	Name	Source
AGAGTTTGATCMTGGCTCAG	27f-CM	(Frank et al. 2008)
AGAGTTTGATYMTGGCTCAG	27f-YM	(Frank et al. 2008)
CGGTTACCTTGT TACGACTT	1492R	(Frank et al. 2008)
CGTCAATTCMTTTGAGTT	S-D-Bact-0908-a-A-18	(Klindworth et al. 2013)
AGGATTAGATACCCTGGTA	S-D-Bact-0784-a-S-19	(Klindworth et al. 2013)
GGMTTAGATACCCBDGTA	S-D-Bact-0785-a-S-18	(Klindworth et al. 2013)
ATTAGATACCCTGGTAGTC	S-D-Bact-0787-a-A-19	(Klindworth et al. 2013)
AGCATGTGGTTTAATTCGA	S-Sc-Bact-0943-a-A-19	This Study
GCGGTCTACTTAACGCGTT	S-Sc-Bact-0861-a-A-19	This Study

Table 2. Results of Sanger sequencing of the 16S rRNA genes of bacterial isolates. Bacteria were isolated from the epidermis of *P. ochraceus* stars on seawater gelatin agar, then cultured in seawater containing gelatin as a sole nutrient source. The Genbank ID is from the best match to the partial 16S rRNA gene, using the megaBLAST algorithm and comparing sequences of two-fold minimal coverage against the NCBI database. Swab IDs reflect the animal number (1-5) and tissue type from which swabs were taken: H = healthy (no shading), U = unaffected tissues (light gray shading), E/C = edge and center of lesions respectively (dark gray shading). All tentative identifications have an E-value of 0 and are a minimum of 1,100 bp in length.

BB1 (6/8/2016)

Swab ID	Class	Order	Family	NCBI Match	Similarity to GenBank ID (%)	Gen Bank ID
5H	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Paracoccus* sp. A30V	100	LC094992.1
5H				*Thioclava indica* strain DT23-4	100	KX618918.1
5H	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
5H				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
5H				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
5H				*Psychrobacter nivimaris* strain OUCMDZ4219	99	KU579272.1
5H				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
5H				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
5H				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
4U	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Paracoccus* sp. A30V	99	LC094992.1
2U				*Paracoccus* sp. HIJAc-3c	99	JX515659.1
2U	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Psychrobacter glacincola* strain OUCMDZ4202	100	KU579265.1
4U				*Psychrobacter glacincola* strain OUCMDZ4202	100	KU579265.1
4U				*Psychrobacter glacincola* strain OUCMDZ4202	100	KU579265.1
4U				*Psychrobacter glacincola* strain OUCMDZ4202	100	KU579265.1
2U				*Psychrobacter nivimaris* strain OUCMDZ4219	99	KU579272.1
2U				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
3U				*Psychrobacter nivimaris* strain OUCMDZ4219	99	KU579272.1
3U				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
3U				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
4U				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
4U				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
4U				*Psychrobacter* sp. 4-Z18	100	HM584480.1
4U				*Psychrobacter* sp. 4-Z18	100	HM584480.1
4U				*Psychrobacter* sp. 4-Z18	99	HM584480.1
4U				*Psychrobacter* sp. 4-Z18	100	HM584480.1
4U				*Psychrobacter* sp. 4-Z18	100	HM584480.1
2U		*Oceanospirillales*	*Halomonadaceae*	*Cobetia amphilecti* strain DHQ 19	100	KP126616.1
2U				*Cobetia marina* strain NBRC 102605	100	NR_114162.1
2U				*Cobetia marina* strain NBRC 102605	100	NR_114162.1
2U				*Cobetia marina* strain NBRC 102605	100	NR_114162.1
2U				*Cobetia marina* strain NBRC 102605	100	NR_114162.1
5U			*Oceanospirillaceae*	*Neptunomonas* sp. BZm-1	99	LC006855.1
5U		*Vibrionales*	*Vibrionaceae*	*Vibrio cyclitrophicus* strain LMG21359	100	NR_115806.1
5U				*Vibrio cyclitrophicus* strain LMG21359	100	NR_115806.1
5U				*Vibrio* sp. V020	100	DQ146971.1
5U		*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas marina* strain S411	100	FJ457131.1
5U				*Pseudoalteromonas marina* strain S411	100	FJ457131.1
5U				*Pseudoalteromonas marina* strain ECSMB14103	100	CP023558.1
5U				*Pseudoalteromonas paragorgicola* strain KMM 3548	100	NR_025654.1
5C	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Staphylococcus sciuri* strain 2-6	99	MH491952.1
2C	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Psychrobacter glacincola* strain OUCMDZ4202	100	KU579265.1
2C				*Psychrobacter glacincola* strain OUCMDZ4202	100	KU579265.1
4E				*Psychrobacter glacincola* strain OUCMDZ4202	100	KU579265.1
1E				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
2C				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
2C				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
2C				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
2C				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
2C				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
2C				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
2E				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
2E				*Psychrobacter nivimaris* strain OUCMDZ4219	99	KU579272.1
2E				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
4E				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
4E				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
4E				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
4E				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
4E				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1

5C				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
5C				*Psychrobacter nivimaris* strain OUCMDZ4219	99	KU579272.1
5C				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
5C				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
5E				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
5E				*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
4E				*Psychrobacter nivimaris* strain 20KNS10L2	99	MH478312.1
4E				*Psychrobacter* sp. strain CJKOP-52	100	MF537097.1
5E				*Psychrobacter* sp. strain CJKOP-52	99	MF537097.1
5E		*Oceanospirillales*	*Halomonadaceae*	*Cobetia amphilecti* strain 58115	100	KX418494.1
5E				*Cobetia marina* strain NBRC 102605	100	NR_114162.1
5C				*Cobetia marina* strain NBRC 102605	99	NR_114162.1
5C				*Cobetia marina* strain NBRC 102605	100	NR_114162.1
5C			*Oceanospirillaceae*	*Marinomonas arenicola* strain KMM 3893	99	NR_112826.1
BB2 (6/22/2016)						
H1	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Vibrio cyclitrophicus* strain S2-7	99	MF327708.1
H3				*Vibrio cyclitrophicus* strain S2-7	100	MF327708.1
H1		*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas marina* strain ECSMB14103	100	CP023558.1
H1				*Pseudoalteromonas paragorgicola* strain KMM 3548	100	NR_025654.1
H1				*Pseudoalteromonas paragorgicola* strain KMM 3548	100	NR_025654.1
H4				*Pseudoalteromonas paragorgicola* strain KMM 3548	100	NR_025654.1
H1				*Pseudoalteromonas* sp. BSi20396	99	EU330365.1
H1				*Pseudoalteromonas* sp. BSi20396	99	EU330365.1
H1				*Pseudoalteromonas* sp. UST981101-004	99	EU982340.1
H1				*Pseudoalteromonas tetraodonis* strain GFC	100	CP011041.1
U2		*Pseudomonadales*	*Moraxellaceae*	*Psychrobacter nivimaris* strain OUCMDZ4219	100	KU579272.1
U4		*Oceanospirillales*	*Oceanospirillaceae*	*Neptunomonas* sp. BZm-1	99	LC006855.1
U4		*Vibrionales*	*Vibrionaceae*	*Vibrio cyclitrophicus* strain S2-7	100	MF327708.1
U4				*Vibrio cyclitrophicus* strain S2-7	100	MF327708.1
U4				*Vibrio tasmaniensis* strain Mj28	99	GQ455006.1
U5		*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas paragorgicola* strain KMM 3548	100	NR_025654.1
E3	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Polaribacter* sp. S-6	99	DQ978987.1
E3				*Polaribacter* sp. S-6	99	DQ978987.1
C4	*Gammaproteobacteria*	*Oceanospirillales*	*Halomonadaceae*	*Cobetia marina* strain NBRC 102605	99	NR_114162.1
E4			*Oceanospirillaceae*	*Marinomonas arenicola* strain KMM 3893	99	NR_112826.1
E2		*Vibrionales*	*Vibrionaceae*	*Vibrio cyclitrophicus* strain S2-7	100	MF327708.1
E2				*Vibrio cyclitrophicus* strain S2-7	100	MF327708.1
E5				*Vibrio rumoiensis* strain DN10	99	KY474375.1
C1				*Vibrio* sp. H1309/5I	99	LN871553.1
C1				*Vibrio* sp. H1309/5I	99	LN871553.1
C1				*Vibrio* sp. H1309/5I	99	LN871553.1
C1				*Vibrio* sp. H1309/5I	99	LN871553.1
E2				*Vibrio* sp. H1309/5I	100	LN871553.1
E5				*Vibrio* sp. H1309/5I	100	LN871553.1
C1				*Vibrio* sp. S278	100	FJ457312.1
E5				*Vibrio* sp. V004	100	DQ146970.1
C1				*Vibrio tasmaniensis* strain Mj28	99	GQ455006.1
C1				*Vibrio tasmaniensis* strain Mj28	99	GQ455006.1
C1				*Vibrio tasmaniensis* strain 04102	99	AM422801.1
C1				*Vibrio tasmaniensis* strain 04102	99	AM422801.1
E5		*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas aliena* strain EH1	100	CP019628.1
E2				*Pseudoalteromonas marina* strain ECSMB14103	99	CP023558.1
E2				*Pseudoalteromonas marina* strain ECSMB14103	100	CP023558.1
E2				*Pseudoalteromonas marina* isolate NSP562	100	FR750954.1
E2				*Pseudoalteromonas marina* isolate NSP562	99	FR750954.1
E2				*Pseudoalteromonas nigrifaciens* strain HG J4.3	99	MG681171.1
C4				*Pseudoalteromonas paragorgicola* strain KMM 3548	100	NR_025654.1
E2				*Pseudoalteromonas paragorgicola* strain KMM 3548	100	NR_025654.1
E2				*Pseudoalteromonas tetraodonis* strain GFC	99	NR_041787.1
E3			*Alteromonadaceae*	*Glaciecola mesophila* strain KMM 241	99	NR_025546.1

Table 3. Phenotypes of representative bacterial isolates. 32 representative isolates characterized by their abilities to grow on different medium types, morphology, and the production of AHLs. Gelatin degradation refers to growth on gelatin agar; (+/-) indicates weak growth and (+) indicates robust growth with a halo of degradation surrounding colonies. Agar degradation was determined as either growth (+) or no growth (-) on water agar. β-hemolysis refers to clearing on blood agar plates. Conversely, γ-hemolysis refers to a lack of visible clearing. "ND" refers to "not determined".

Tentative ID	AHL Activity (per ml)	Hemolysin	Agar	Gelatin	TCBS	Cell Width (μm)	Cell Length (μm)	Cell Morphology
Staphylococcus sciuri strain DSM 20345	-	γ	+	+	-	0.5	0.5	coccus
Polaribacter sp. S-6	-	γ	+	+	-	1.5	2	rod
Paracoccus sp. HIJAc-3c	ND	γ	-	+/-	-	ND	ND	ND
Paracoccus sp. A30V	ND	γ	+	+/-	-	1	1.5-2	rod
Thioclava indica strain DT23-4	ND	γ	-	+/-	-	1	1.5-2	oblong/rod
Neptunomonas sp. BZm-1	ND	γ	-	+/-	-	0.75	1.5-2	rod
Marinomonas arenicola strain KMM 3893	ND	γ	-	+/-	-	1	2	rod
Psychrobacter glacincola strain OUCMDZ4202	ND	γ	-	+/-	-	1.5	2	fat rod
Psychrobacter nivimaris strain OUCMDZ4219	ND	γ	+	+/-	-	1	2	small rod
Psychrobacter nivimaris strain 20KNS10L2	ND	ND	ND	ND	ND	ND	ND	ND
Psychrobacter sp. 4-Z18	ND	γ	-	+/-	-	1.5	1.5	coccus
Psychrobacter sp. CJKOP-52	ND	ND	ND	ND	ND	ND	ND	ND
Cobetia marina strain NBRC 102605	ND	γ	-	+/-	-	0.5	0.5	coccus
Cobetia amphilecti strain 58115	ND	γ	+	+/-	-	1	1	coccus
Cobetia amphilecti strain DHQ 19	ND	γ	-	+/-	-	0.25	0.25	coccus
Vibrio tasmaniensis strain 04102	35.5	β	-	+	+ (Green)	0.5	1.5	curved rod
Vibrio sp. S278	98.6	β	-	+	+ (Yellow)	1	2-3	curved rod
Vibrio tasmaniensis strain Mj28	33.7	β	-	+	+ (Green)	0.75	1-2	curved rod
Vibrio sp. V004	66.2	β	-	+	+ (Yellow)	0.5	0.75	oblong/curved rod
Vibrio sp. H1309/5I	450.0	β	+	+	+ (Green)	0.3	0.75	curved rod
Vibrio sp. V020	-	β, anaerobic	-	+	+ (Yellow)	0.5	1-2	curved rod
Vibrio cyclitrophicus strain S2-7	-	γ	+	+	+ (Yellow)	0.5	1-2	curved rod
Vibrio cyclitrophicus strain LMG21359	-	β, anaerobic	+	+	+ (Yellow)	1	2	fat curved rod
Vibrio rumoiensis strain DN10	-	γ	-	+/-	+ (Yellow)	0.5	1-1.5	curved rod
Glaciecola mesophila strain KMM 241	6,722.5	γ	+	+	-	1	2	rod
Pseudoalteromonas tetraodonis strain GFC	-	γ	+	+	-	0.25	1	rod
Pseudoalteromonas paragorgicola strain KMM 3548	-	γ	-	+	-	0.5	1	rod/oblong
Pseudoalteromonas sp. BSi20396	-	γ	+	+	-	1	2-3	rod
Pseudoalteromonas aliena strain EH1	-	γ	-	+	-	0.25	1-1.5	
Pseudoalteromonas nigrifaciens strain HG J4.3	-	γ	+	+	-	1	3	rod
Pseudoalteromonas sp. UST981101-004	-	γ	+	+	-	1	2	rod
Pseudoalteromonas marina strain S411	-	γ	+	+	-	0.5	0.75	coccus/oblong
Pseudoalteromonas marina strain ECSMB14103	-	γ	+	+	-	0.75	1-1.5	rod
Pseudoalteromonas marina isolate NSP562	-	γ	+	+	-	0.5	2	rod

Figures

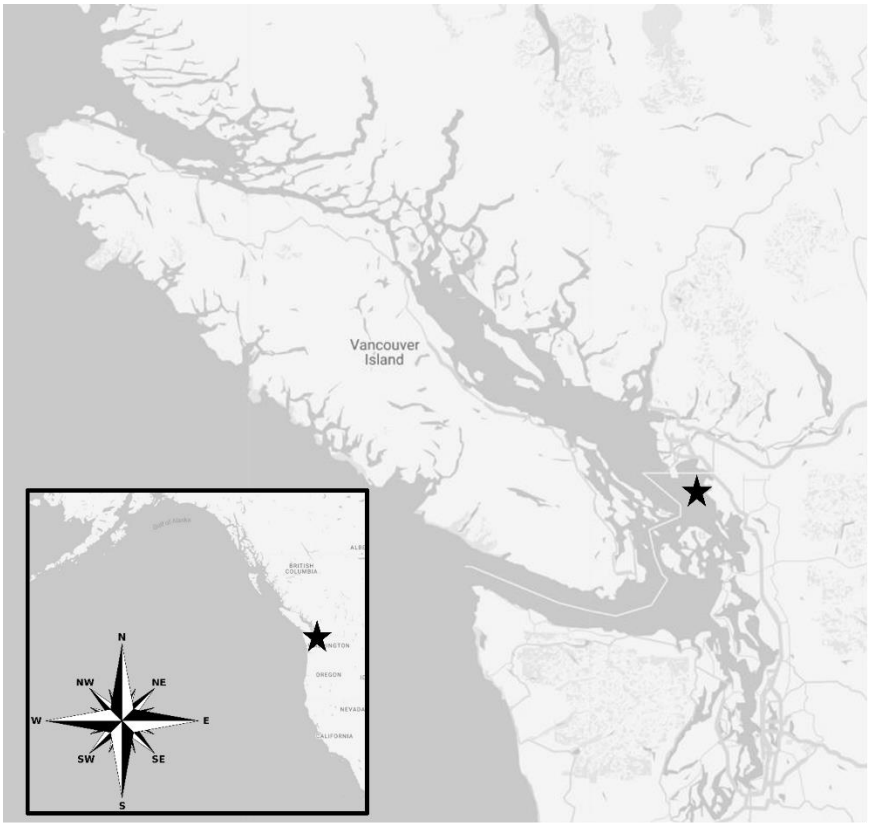

Figure 1. Geographic region sampled in surveys. Disease prevalence was assessed from *P. ochraceus* individuals at Point Whitehorn in Birch Bay, WA, which is in the Salish Sea. This region represents a central area for SSWD observations that span from Alaska to California.

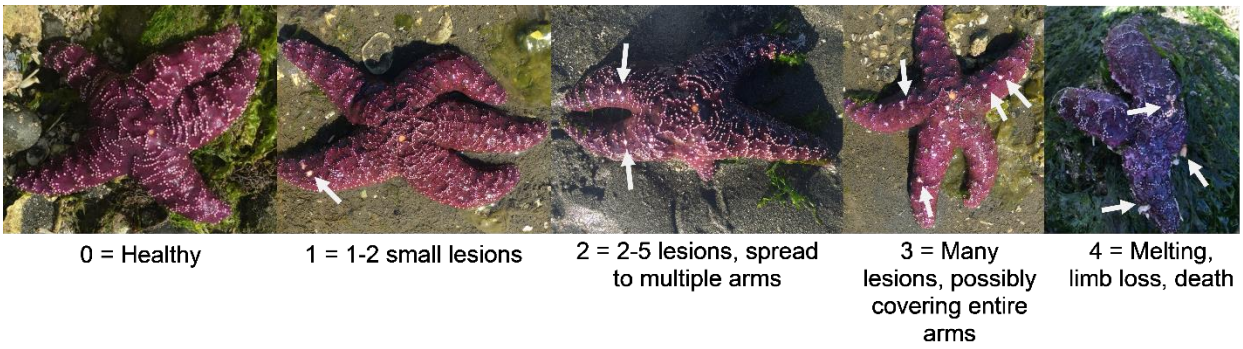

Figure 2. SSWD scoring system for *P. ochraceus* stars. Arrows indicate lesions.

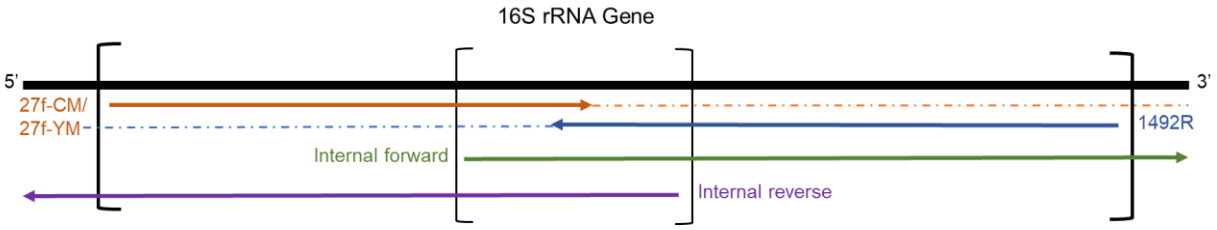

Figure 3. Sequence alignment strategy of the 16S rRNA gene. After PCR amplification with 27f-CM or 27f-YM and 1492R primers, these primers were used to Sanger sequence the 16S rRNA gene starting near the ends of the sequence. Once 27F and 1492R sequences were aligned, internal primers were custom-made to match each bacterial sequence, to achieve 2X coverage across a $\geq$ 1,100 bp region.

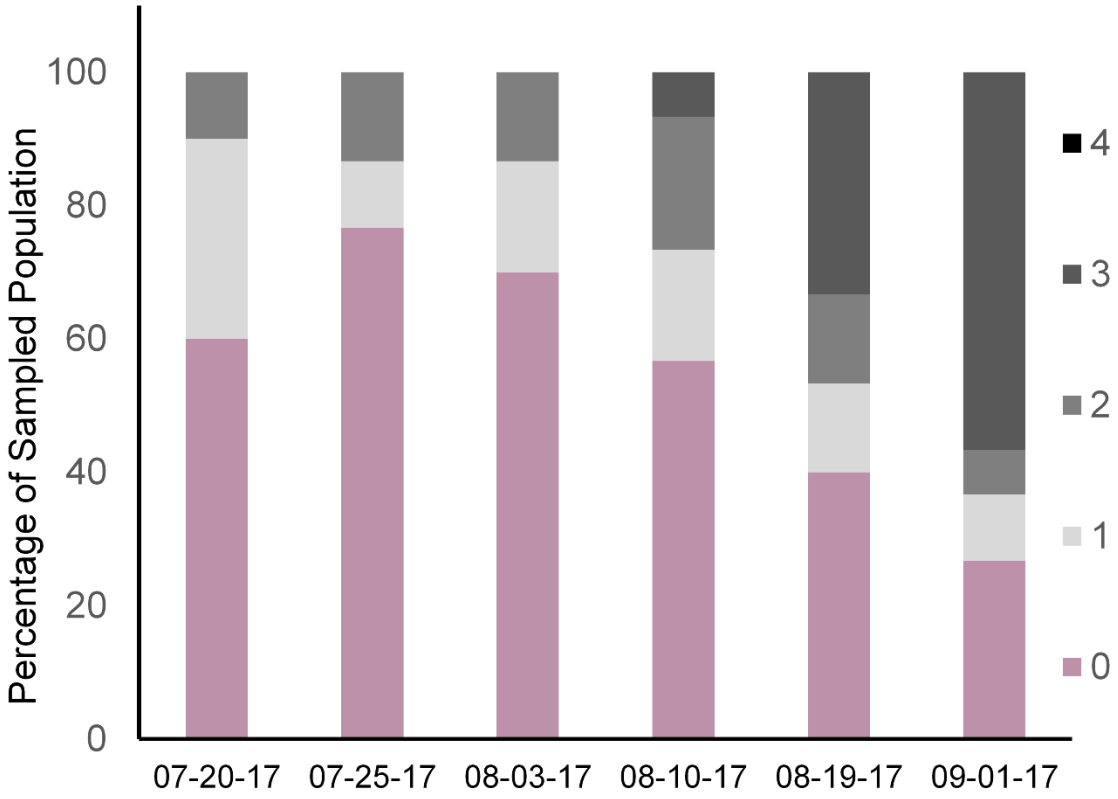

Figure 4. SSWD progression at Point Whitehorn, WA. Each date represents thirty haphazardly chosen *P. ochraceus* stars in three plots at Birch Bay, WA (n = 10 per plot). Severity in SSWD signs are represented on a scale from 0-4 where 0 = healthy, 1 = one to two superficial lesions (typically isolated to a single limb), 2 = two to five lesions, spanning multiple limbs, and 3 = five lesions, and/or degradation of an entire limb, and 4 = loss of limbs or death. Percentage represents number of individuals at each disease stage out of all thirty animals.

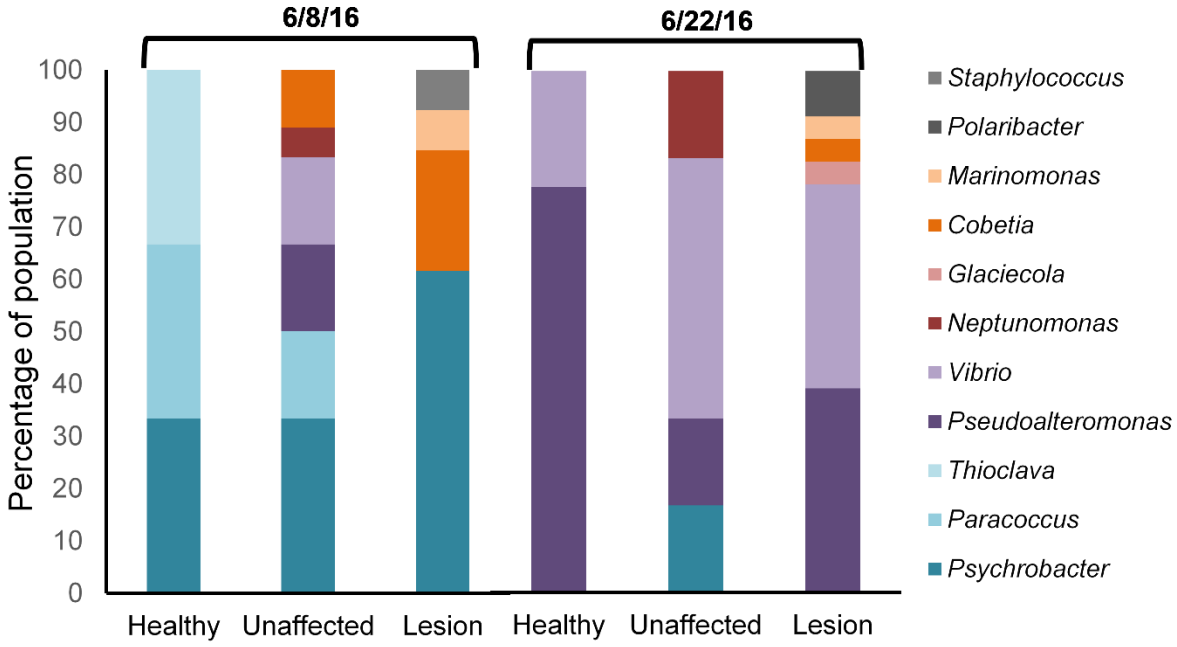

Figure 5. Genera of gelatin-degrading bacteria by tissue zone. Bacterial isolates from swabs taken from *P. ochraceus* individuals in Birch Bay, WA were cultured in seawater containing gelatin as a sole nutrient source. "Healthy" samples were from visually asymptomatic animals. "Unaffected" samples were from sea stars with SSWD, but on tissues free of lesions. "Lesion" samples were taken from lesions on SSWD-affected sea stars. 16S rRNA sequences were used in identifications. Five healthy and five diseased sea stars were swabbed, but not all samples had an equal number of cultivatable isolates. Percentages represent the number of isolates belonging to a genus per the total number of cultivatable isolates from all five swabs in a single tissue zone. Isolates that appeared to be identical via 16S rRNA gene sequencing that were represented multiple times from a single swab were only counted once for that swab.

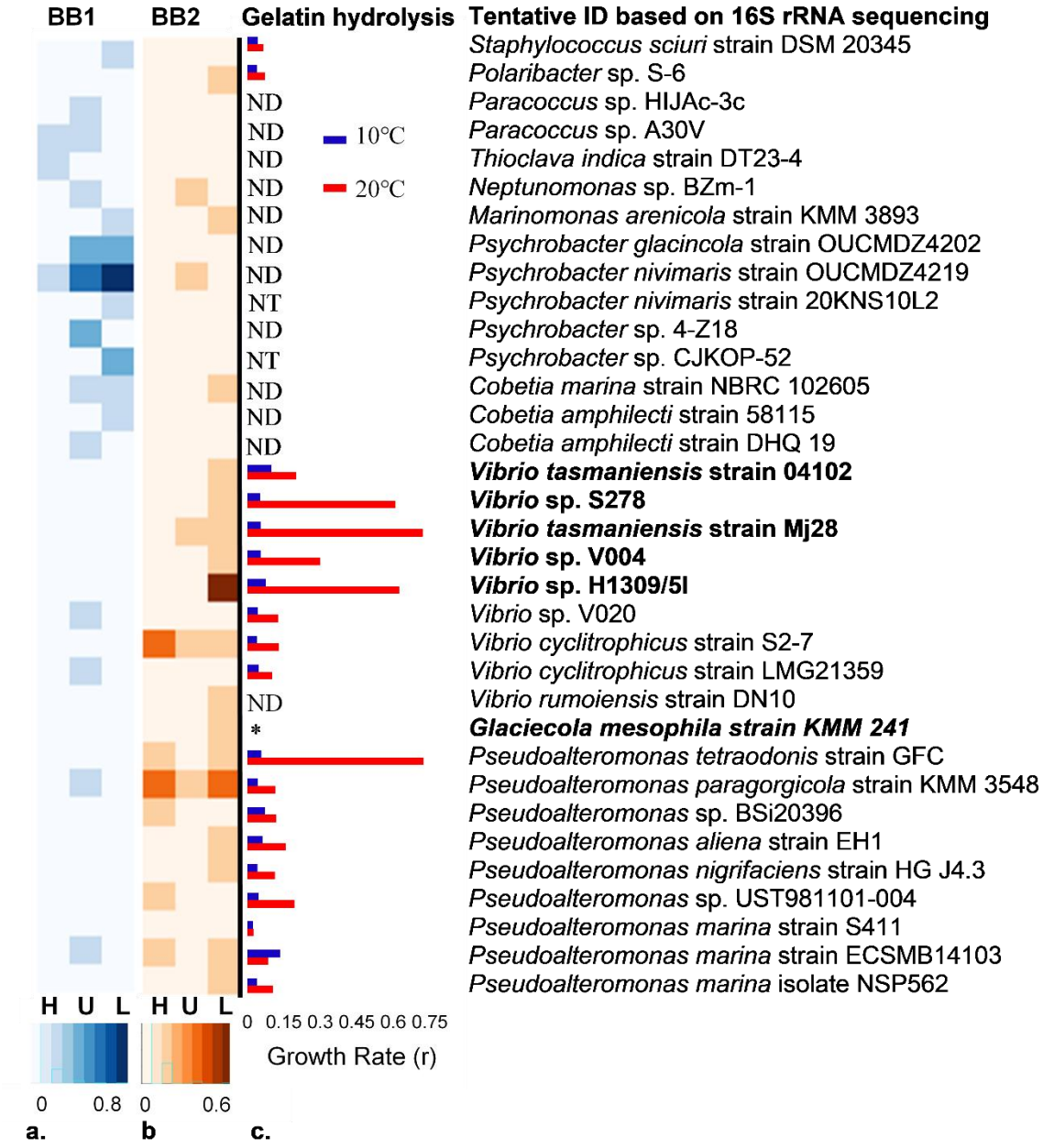

Fig. 6 Prevalence, gelatin utilization, and AHL production of isolates from the epidermal tissues of *P. ochraceus.* The proportion of sea stars from which each isolate was found for each sampling trip, (**a**) BB1 (6/8/16) and (**b**) BB2 (6/22/16), n = 5. H = healthy tissue, U = unaffected tissues of a symptomatic sea star, and L = lesion of a symptomatic sea star. (**c**) Growth rate in seawater with gelatin as a sole nutrient source, as determined by fitting a logistic regression using the GrowthCurver package in R (n = 3).
* = aggregated in culture, NT = not tested, ND = no detectable growth, b**oldface type** for tentative IDs indicates isolates that produced AHLs in culture.

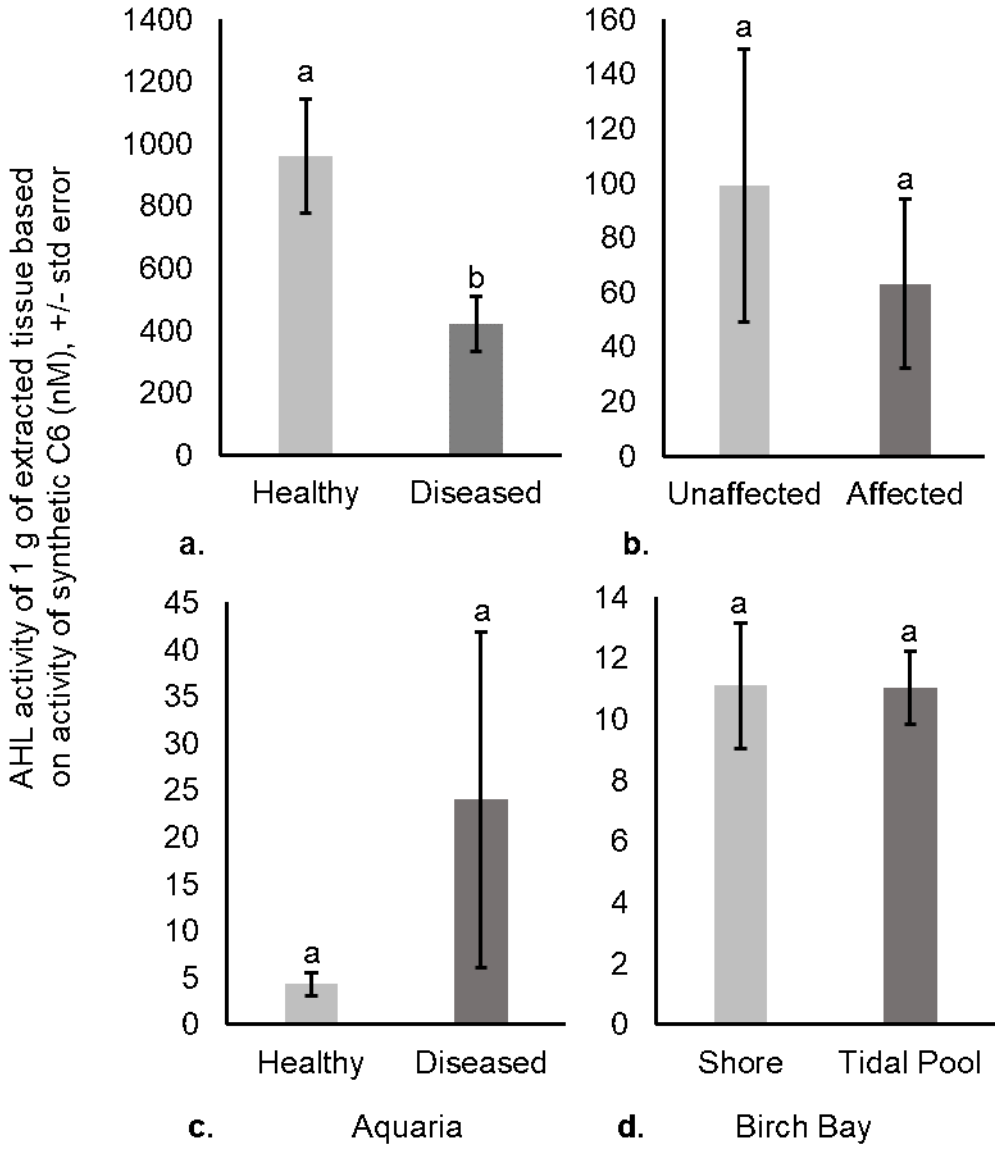

Figure 7. Activation of the bioreporter *Agrobacterium tumefaciens* KYC55 (pJZ410)(pJZ384)(pJZ372) by organic tissue extracts taken from *P. ochraceus* arms or seawater. The bioreporter strain was incubated with extracts for 16-18 hours. Luminescence was the result of cleavage of Tropix™ substrate by β-galactosidase that is produced in response to exogenous AHLs. Values represent activation interpolated from a standard curve generated activation of the bioreporter with synthetic C6 (nM), but do not represent actual AHL concentrations or identities. Extracts were tested from (**a**) the arms of healthy or diseased stars kept in aquaria (n = 5) (**b**) the unaffected (lesion-free) or affected arms of diseased stars collected in the field at Birch Bay, WA (n = 5) (**c**) seawater from the tanks of healthy or diseased animals (n = 5) (**d**) seawater taken directly offshore or from tidal pools that were inhabited by sea stars in the field at Birch Bay, WA (n = 5).

Appendix A

Supplementary Table 1. Results of Sanger sequencing of the 16S rRNA genes of bacterial isolates, sequences that were short or lacked two-fold coverage. Isolates that were identified via 16S rRNA gene sequencing; however, the sequences were either short (500-600 bp) or do not have two-fold coverage due to failed Sanger sequencing reactions. Swab IDs reflect the animal number (1-5) and tissue type from which swabs were taken: H = healthy (no shading), U = unaffected tissues (light gray shading), E/C = edge and center of lesions respectively (dark gray shading).

BB1 (6/8/2016)					
Swab ID	**Class**	**Order**	**Family**	**NCBI Match**	**Similarity to GenBank ID (%)**
5H	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Paracoccus* sp. HIJAc-3c	100
3U	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Paracoccus* sp. HIJAc-3c	99
5U	*Gammaproteobacteria*	*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas marina* strain S411	99
5U				*Pseudoalteromonas* sp. BSw20244	99
5U				*Pseudoalteromonas undina* strain XH124	100
BB2 (6/22/2016)					
H1				*Pseudoalteromonas paragorgicola* strain KMM 3548	100
H1				*Pseudoalteromonas* sp. strain 70007	99
H4				*Pseudoalteromonas tetraodonis* strain GFC	100
H5				*Pseudoalteromonas tetraodonis* strain GFC	100
U4		*Vibrionales*	*Vibrionaceae*	*Vibrio tasmaniensis* strain HS10	99
E5		*Alteromonadales*	*Pseudoalteromonadaceae*	*Vibrio cyclitrophicus* strain LMG21359	100
C1				*Pseudoalteromonas paragorgicola* strain KMM 3548	100
E3				*Pseudoalteromonas paragorgicola* strain KMM 3548	100
E3				*Pseudoalteromonas paragorgicola* strain KMM 3548	100
E3				*Pseudoalteromonas* sp. 2099	99
E3				*Pseudoalteromonas tetraodonis* strain IAM 14160	100
E5				*Pseudoalteromonas undina* strain XH124	99