Variations in Sea Star Bacteria Related to Sea Star Wasting Disease from Kachemak Bay, AK

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Introduction

- Sea stars are keystone species that maintain diversity within intertidal communities.[1] In Kachemak Bay, sea stars were found to have a greater impact on clam populations than sea otters[2]
- Many species present in Kachemak Bay. Those studied include mottled star (*Evasterias troschelii*), sunflower star (*Pycnopodia helianthoides*), ochre star (*Pisaster Ochraceus*), little six-rayed star (*Leptasterias hexactis*), leather star (*Dermasterias imbricata*), Stimpson's sun star (*Solaster stimpsoni*), red-banded star (*Orthasterias koehleri*)[2, 3]
- Since 1970's multiple mass mortality events occurred along Pacific coast of United States. By 2013, over 20 star species affected by sea star wasting disease (SSWD)[4]
- SSWD: infectious disease found in sea stars
- Symptoms: abnormally twisted arms, formation of white lesions, a deflated appearance, arm loss, necrosis, structure loss, death[6] (**Fig. 1**)
- SSWD cause unknown; studies suggest imbalance in microbial community followed by bacterial infection[7]
- Variability in abundance, diversity, and dominant sea star species varied greatly in 2015. In 2016, abundance trends declined and remained strongly negative across all regions through 2018[5] (**Fig. 2**)

Figure 1. Mottled star with an advanced case of sea star wasting disease. Symptoms shown include white lesions deflated body, and partial loss of limbs[15]

Figure 3. Map of Kachemak Bay. Sample site locations: Red – Jakolof Bay, Blue – Peterson Bay, Green – Land's End, and Yellow – Bishops beach[14]

Materials and Methods

- Agar Plates
- Incubator
- Swabs
- Antibiotic disks: Streptomycin (S10) - 10 µg, Tetracycline (Te30) - 30 µg, Penicillin (P10) - 10 IU/IE/UI, Chloramphenicol (C30) - 30 µg, Neomycin (N30) - 30µg
- Staining bottles
  - Crystal violet
  - Gram's Iodine
  - Decolorizer (acetone/alcohol)
  - Safranin

Field Work

Locations:

- Sites were chosen within Kachemak Bay by criteria: accessible beach, rocky intertidal zones, and areas with tide pools at negative tides
- Sites: Bishops Beach, Land's End, Peterson Bay, and Jakolof Bay (**Fig. 3**)
  - Each of these sites was visited once except Peterson Bay which was visited twice

Taking a swab:

- Sea stars were swabbed where it was, without getting the swab wet with sea water, if possible
- The sea star would be swabbed on both sides, if possible, and transferred to an agar plate
- The agar plate was sealed with parafilm and labeled with location, species, a number, and if there were signs of SSWD
- Type and number of sea stars swabbed: red-banded star (1), leather star (2), little six-ray star (5), ochre star (7), mottled star (7), sunflower star (1), and Stimpson sun star (1) and 1 unknown species (dead)

Lab work

Growing Sea Star Bacteria:

- Agar plates were placed in incubator at 37 ˚C for 72-120 hrs
- The plates were taken out when there were large bacteria colonies to sample, then refrigerated

Gram Staining:

- Gram staining was conducted on the unique and common bacteria colonies
- Gram staining procedure followed from Microbiology Experiments[8]
- Complete slides were examined under a microscope a few days later, using oil (**Fig. 4**)
- The type and color of bacteria were recorded, along with any anomalies (**Table 1**)

Antibiotic Disk Sensitivity Tests:

- Eight bacteria colonies were chosen for antibiotic disk sensitivity tests
- Antibiotic disk sensitivity tests methods followed from Laboratory Applications in Microbiology[9]
- Inhibition zone was measured in mm (**Fig. 5**). The inhibition zone is the circle around the disk (**Fig. 6**)
- The diameter of the inhibition zone will show if the bacteria is sensitive, intermediate, or resistant to the antibiotic. Known inhibition ranges used to try and further identify the type of bacteria [9, 10]

Results

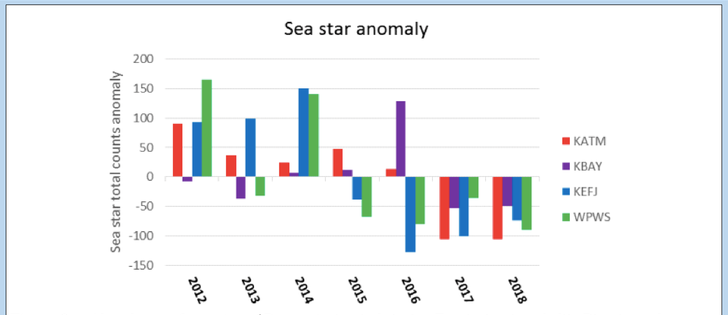

Figure 2. Abundance of sea stars (*Dermasterias imbricata*, *Evasterias troschelii*, *Pisaster ochraceus*, and *Pycnopodia helianthoides*) in four study areas spanning the northern Gulf of Alaska. WPWS (2007, 2010–2018), KEFJ (2008–2018), KBAY (2005, 2009, 2011–2018), and KATM (2006, 2008–2010, 2012–2018).[5]

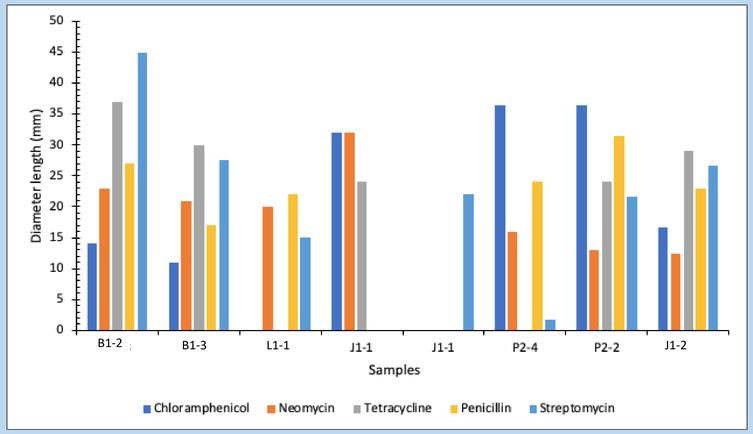

Figure 5. Diameter length (mm) of inhibition zone from antibiotic disk agar plates. The colored bars represent the antibiotic type. Samples were taken from Bishops Beach (B1), Land's End (L1), Jakolof Bay (J1), the first trip to Peterson Bay (P1), and the second trip to Peterson Bay (P2). The second number shown on the sample axis represents the agar plate from that location.

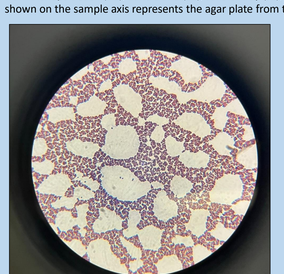

Figure 4. Microscope slide showing gram-positive (purple) and gram-negative (red) cocci bacteria.

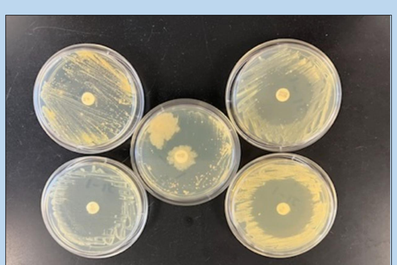

Figure 6. Agar plates showing antibiotic disks (S, N, P, T, and C) with varying levels of antibiotic resistance bacteria.

Table 1. The type of bacteria and gram-staining results. The top row shows location: Bishops Beach (B), Land's End (L), Jakolof Bay (J), the first trip to Peterson Bay (P1), and the the second trip to Peterson Bay (P2). The number after the dash represents the agar plate from that location used. The far-left column presents the bacteria colony chosen from the agar plate. Blank areas were bacteria colonies that were not chosen for gram staining.

	B-2	B-3	L-1	L-5	P1-1	P1-2	P2-1	P2-4	J-1	J-2
1	Purple coccus	-	-	Red bacillus, purple bacillus chains	Purple and clear coccobacillus	-	Purple and pink bacillus	Dark purple coccus and red coccus	Small purple coccus	Purple bacillus
2	Red coccus and purple coccus	-	-	-	Clear and red coccobacillus	Purple coccus	Purple coccus	Small red bacillus	Purple and red coccus	Purple bacillus
3	Purple and red circle coccus chains	Purple coccus and red bacillus	-	-	-	Purple bacillus, red and purple coccobacillus	-	Purple coccus	-	-
4	Purple coccobacillus, purple bacillus, and red coccobacillus	-	-	-	-	-	-	Purple coccus	-	-
5	Purple coccus, pink bacillus, and pink coccus	-	Purple coccus	-	-	-	-	-	-	-
6	Purple bacillus	-	-	-	-	-	-	-	-	-
8	-	-	Purple coccus	-	-	-	-	-	-	-

Discussion

- Antibiotics have been found within intertidal zones[11]
- Our results showed that all ochre sea stars that were abnormal were the only ochre samples to grow any bacteria, the first four plates had no bacteria growth(**Fig. 7**)
- Our study shows some species of bacteria found were resistant to at least one antibiotic
- We found the greatest number of bacteria were gram-positive cocci followed by gram-positive bacilli. There were more gram-positive bacteria than gram-negative bacteria (**Fig. 8**) and cocci was most common shape then bacillus and coccobacillus (**Fig. 9**)
- *Other Studies:*
  - Suggest SSWD is caused by changes in pH, temperature, and precipitation or shifts in microbial community[7]
  - Microplastics can have antibiotic resistant bacteria
  - SSWD negative impacts on other marine organisms[12]
- *Future studies:*
  - Genetic analysis on bacteria to compare among sea star species & healthy vs diseased stars
  - Comparing microbial differences of healthy and diseased sea stars
  - Test our antibiotic resistant bacteria for possible SSWD comparisons
  - Conduct more surveys and sampling in more locations and over a broader period of time

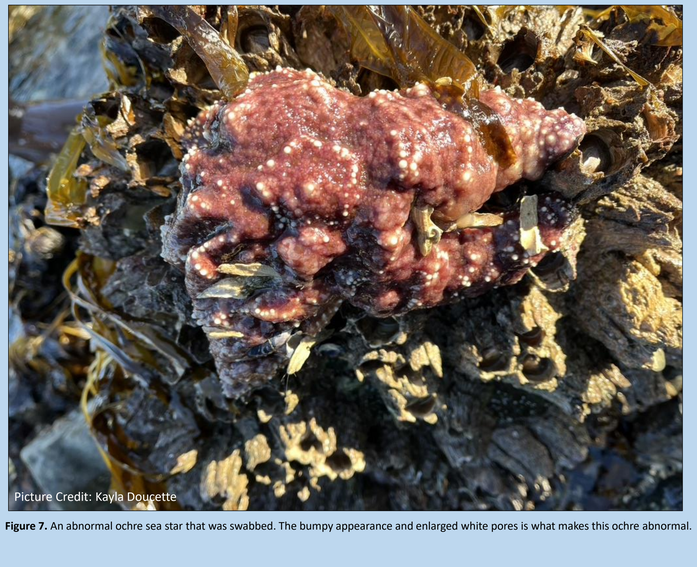

Figure 7. An abnormal ochre sea star that was swabbed. The bumpy appearance and enlarged white pores is what makes this ochre abnormal.

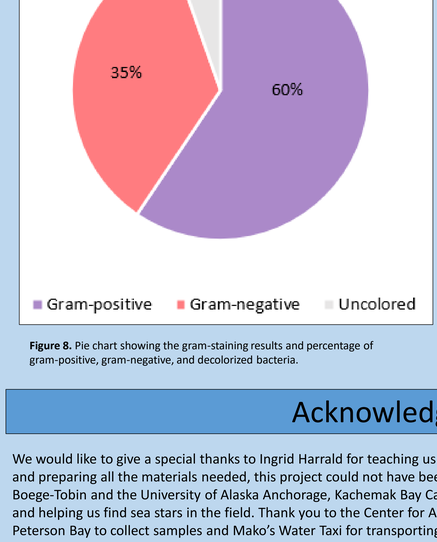

Figure 8. Pie chart showing the gram-staining results and percentage of gram-positive, gram-negative, and decolorized bacteria.

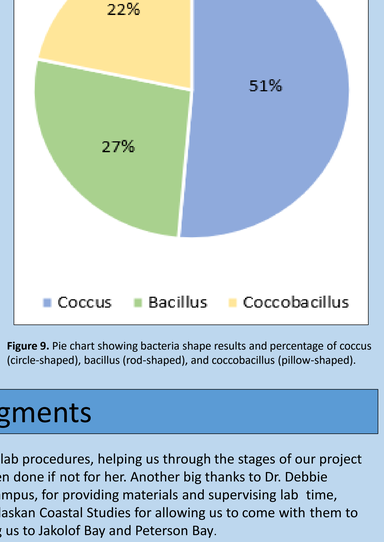

Figure 9. Pie chart showing bacteria shape results and percentage of coccus (circle-shaped), bacillus (rod-shaped), and coccobacillus (pillow-shaped).

Acknowledgments

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Sources: [1]Konar, B., Mitchell, T. J., Iken, K., Coletti, H., Dean, T., Esler, D., Lindeberg, M., Pister, B., & Weitzman, B. (2019). Wasting disease and static environmental variables drive sea star assemblages in the Northern Gulf of Alaska. *Journal of Experimental Marine Biology and Ecology*, *520*, 151209. [2]Mitchell, T. J. (2019). *Wasting disease and environmental variables drive sea star assemblages in the northern Gulf of Alaska* (Master's thesis, University of Alaska Fairbanks). [3]Field and Field. (2016). Beachcomber's guide to intertidal marine invertebrates of Southcoastal Alaska. Northcountry Nature. [4]Bucci, C., Francoeur, M., McGreal, J., Smolowitz, R., Zazueta-Novoa, V., Wessel, G. M., & Gomez-Chiarri, M. (2017). Sea star wasting disease in *Asterias forbesi* along the Atlantic Coast of North America. *PLoS One*, *12*(12), e0188523. [5]Zador and Yasumiishi. (2018). Ecosystem status report 2018 Gulf of Alaska. *National Marine Fisheries Services Report*, 1-194. [6]Fuess, L. E., Eisenlord, M. E., Closek, C. J., Tracy, A. M., Mauntz, R., Gignoux-Wolfsohn, S., Moritsch, M. M., Yoshioka, R., Burge, C. A., Harvell, C. D., Friedman, C. S., Hewson, I., Hershberger, P. K., Roberts, S. B. (2015). Up in arms: immune and nervous system response to sea star wasting disease. *PLoS One*, *10*(7), e0133053. [7]Lloyd, M. M., & Pespeni, M. H. (2018). Microbiome shifts with onset and progression of sea star wasting disease revealed through time course sampling. *Scientific Reports*, *8*(1), 1-12. [8]Kleyn, J., & Bicknell, M. (2007). Introduction to staining of microorganisms. In *Microbiology Experiments: A Health Science Perspective* (5th ed., pp. 32–39). essay, McGraw Hill Higher Education. [9]Chess, B. (2009). Antibiotic disk sensitivity tests for bacterial identification. In *Laboratory Applications in Microbiology - A Case Study Approach* (pp. 529–531). essay, McGraw Hill Higher Education. [10]Reynolds, J. (2021). In *Microbiology Labs 1*. LibreTexts. [11]Zhao, S., Liu, X., Cheng, D., Liu, G., Liang, B., Cui, B., & Bai, J. (2016). Temporal–spatial variation and partitioning prediction of antibiotics in surface water and sediments from the intertidal zones of the Yellow River Delta, China. *Science of the Total Environment*, *569*, 1350-1358. [12]Zhang, Y., Lu, J., Wu, J., Wang, J., & Luo, Y. (2020). Potential risks of microplastics combined with superbugs: Enrichment of antibiotic resistant bacteria on the surface of microplastics in mariculture system. *Ecotoxicology and environmental safety*, *187*, 109852. **Image Sources:** [13]Lower Cook Inlet Management Area image, Alaska Department of Fish and Game. [14]Map of Alaska, Google Maps (2022). [15]Miner, M. Image of sea star with sea star wasting disease, MARINe.