

Complementary approaches to diagnosing marine diseases: a union of the modern and the classic

Colleen A Burge^{1*}, Carolyn S. Friedman², Rodman Getchell³, Marcia House⁴, Kevin D Lafferty⁵, Laura D. Mydlarz⁶, Katherine C. Prager^{7,8}, Kathryn P. Sutherland⁹, Tristan Renault¹⁰, Ikunari Kiryu¹¹, Rebecca Vega-Thurber¹²

Supplemental Material

Critical types of data and techniques are needed to make a correct disease diagnosis using both classic and modern approaches. Consider the following a hypothetical situation concerning a moribund sea star found on the seashore and the steps one might take to diagnosis a potential etiology:

One could record: SPECIES: Pisaster ochraceus SIZE/APPEARANCE: small, at 6 cm avg. arm length, orange color morph, LOCATION: in the wild, low tide from transect B, North side of Caution Point, Friday Harbor, San Juan Island, WA USA, UTM, Zone 10 U, 499488.38 m E, 5377094.62 m N). ENVIRONMENTAL CONTEXT: Rocky intertidal with boulders and crevices, with near 100% algal cover. Site is a marine reserve. Recent seawater (13 °C) and air (26 °C) temperatures warm during daytime low tides, no recent rain. Salinity at 30.5 PSU. Several sea stars of several species observed dead or dying in the sampling location. Additional samples include samples of other species of sea star.

Disease observations should include metadata (data about data), such as information about sample collection [date, location, collection method, name of collector(s)], organisms collected [species, size, condition, tissue type(s)], processing of the collected specimens [fixation and preservation, method of processing, name of processor(s)], and infectious agent(s) (e.g., parasite abundance).

*For our example sea star, in addition to the environmental and demographic data, the following notes would also be useful. Samples taken: Histopathology, EM, molecular, and culture. Case # EIMD-15-1 Cross section of sea star arm next to the leading edge of the lesion including both epidermis, tube feet, pyloric caeca, gonads, and radial canal in histology cassette # EIMD-15-1-1 and placed into Invertebrate Davidson's solution for 24 hours followed by 70% ethanol (care should be taken to use appropriate pen or pencil for marking histopathology cassettes). Additionally, tube feet and epidermis fixed and post-fixed followed by tissue embedding for Transmission Electron Microscopy. Molecular samples, tube feet, epidermis, and coelomocytes were flash frozen in liquid nitrogen. Tissue swab of sterile cut into epidermis on Marine Agar 2216 and Gram-stained. Tissue imprints of leading edge of epidermis taken. Histopathology samples also taken from normal *P. ochraceus* case # EIMD 15-2-1.*

For each sample type, both pictures and a written description are useful to understand the context of the sample.

Collection and processing of samples from live organisms for tissue, cellular, and gene-based technologies is an important consideration. We've created a generalized flow chart of appropriate sample types to take based on typical hosts you might encounter in the field (Supplemental Figure 1). At current time, there is no centralized processing of samples for marine disease, but for certain diseases, the World Organization for Animal Health (OIE) has expert laboratories for sample diagnoses. For other diseases, there are a number of laboratories and experts in the field with diagnostic capabilities. There are a number of manuals or guides, primarily for sampling, fixation, and microscopic analysis of tissue of marine organisms (e.g. (1-7)), as well as specific guidelines for diagnoses set by the OIE for both development of diagnostics and for notifiable diseases (8). Special care should be taken to understand both the fixation and preservation methods based on the organism type and type of fixative.

Invertebrate	Vertebrate										
<p>Common organs for sample collection*</p> <p>Gills, heart, muscle and/or foot, gastro-intestinal system (may be simple digestive gland or cecae depending on organism), reproductive organs or tissues, epidermis, esophagus, mantle, antennal gland, hematopoietic tissue, brain; as present (see 2,3,4,6, 7)</p>	<p>Common organs for sample collection</p> <p>Hematopoietic tissue, spleen, thymus, heart, thyroid, gills, kidney, gastrointestinal system, GI contents, reproductive organs, brain and specialized sensory and endocrine organs, eye, pseudobranch, urine, bile, adrenal glands, liver, gallbladder, choroid rete, musculoskeletal system, and skin; as present (see 1, 2, 5)</p>										
<p>Sample Preservation Methods</p> <table border="1"> <tbody> <tr> <td>Histology:</td> <td>modified Davidson's or 10% buffered formalin</td> </tr> <tr> <td>Electron Microscopy:</td> <td>2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate</td> </tr> <tr> <td>Genomics (DNA):</td> <td>freezing (-20 °C , -80 °C, or flash frozen) or ethanol</td> </tr> <tr> <td>Transcriptomics (RNA):</td> <td>RNA stabilization solution or flash freezing</td> </tr> <tr> <td>Culture</td> <td>appropriate media or flash freezing for some viral isolation</td> </tr> </tbody> </table>		Histology:	modified Davidson's or 10% buffered formalin	Electron Microscopy:	2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate	Genomics (DNA):	freezing (-20 °C , -80 °C, or flash frozen) or ethanol	Transcriptomics (RNA):	RNA stabilization solution or flash freezing	Culture	appropriate media or flash freezing for some viral isolation
Histology:	modified Davidson's or 10% buffered formalin										
Electron Microscopy:	2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate										
Genomics (DNA):	freezing (-20 °C , -80 °C, or flash frozen) or ethanol										
Transcriptomics (RNA):	RNA stabilization solution or flash freezing										
Culture	appropriate media or flash freezing for some viral isolation										
<p>Metadata</p> <p><u>Environment:</u> date, location (latitude/ longitude or GPS coordinates), time of collections, habitat/ substrate type, weather conditions, tidal conditions, recent storm events, tidal zone, air temperature, water temperature, salinity, dissolved oxygen levels, visibility, phytoplankton blooms, water clarity, presence of contaminants, recent human activity (i.e. fishing), etc.</p> <p><u>Population:</u> species (impacted and also unaffected), associated community members, number of animals involved, wild and or aquaculture species</p> <p><u>Organism:</u> size, age, reproductive condition, abnormal behavior (lethargy, erratic swimming/ movement, respiratory distress), gross signs (discoloration, lesions, hemorrhaging, excessive mucus production), if outward disease signs present: severity & prevalence</p> <p><u>Other important data:</u> Names, agencies and contact information for sample collectors and investigators, detailed sample information on collection containers including fixative, possibly chain of custody forms to accompany samples, photo documentation</p> <p>*organs vary in form and structure in invertebrates and in some cases whole animals or simple cross sections may be sufficient, particularly in clonal animals or small specimens</p>											

Supplemental Figure 1. Field guide to collection of marine disease samples

1. Mumford S, Heidel, J., Smith, C., Morrison, J., MacConnell, B., Blazer, V. . Fish Histology and Histopathology. 5/10/2015. Report No.
2. Miwa S. Sampling Methods of Histopathology for Fish and Shellfish, National Research Institute of Aquaculture, : Fisheries Research Agency; 2015 [cited 2015 5/10/2015]. Available from: http://nria.fra.affrc.go.jp/AAHD/histological-methods_e.html.
3. Howard DW, Lewis, E.J., Keller, B.J., Smith, C.S. Histological techniques for marine bivalve mollusks and crustaceans. NOAA Technical Memorandum 52004.
4. Elston R. Health Management, Development, and Histology of Seed Oysters. Baton Rouge, Louisiana: World Aquaculture Society; 1999.
5. The Marine Mammal Anatomy & Pathology Library University of Santa Cruz [cited 2015 August 10 2015]. Available: <http://www.mmapl.ucsc.edu/>
6. Chia F, Koss R. Asteroidea. Microscopic anatomy of invertebrates. 1994;14:169-245.
7. Johnson P. Histology of the blue crab. *Callinectes sapidus*. 1980:190-1.
8. OIE (World Organization for Animal Health). Manual of Diagnostic Tests for Aquatic Animals. 2015. Available: <http://www.oie.int/international-standard-setting/aquatic-manual/access-online/>