Chapter 4

Case History, Sample Collection, Processing and Shipment

4.1 Case History

A case history is a chronological record of significant events and observations surrounding an outbreak of disease and should be the first entry in a case file for a given outbreak investigation. Detailed field observations during an outbreak and investigation that identify significant events that preceded the outbreak can provide valuable context for interpreting the analytical data. Perceptive, thorough observers are invaluable for an investigation and avoiding preconceptions are imperative if the investigation is to remain unbiased.

Environmental Factors

Environmental changes such as storms, heavy rains, abnormal temperature shifts, changes in water quality can be sources of stress that contribute to outbreaks or mass coral bleaching events. Satellite imagery over the previous months may reveal unusual situations such as large run-off or storm water inputs and should be included as data and information are gathered. It is also important to determine if there were recent industrial or agricultural spills or applications of pesticides or herbicides in the vicinity. Any previous disease outbreaks or die-offs in the area should also be noted.

Estimate Onset of Disease

The timing of disease onset and rate of progression can be estimated for some corals by the degree of bleached tissue or bare skeleton is associated with the lesion and the amount of algal colonization. Note and photo-document algal colonization of affected colonies.

Species and Number Affected

It is important to document which species are affected as well as those that remain unaffected as some diseases appear to infect only a narrow host range while others a much broader range. The incidence or proportion of sick colonies, verses the number of those not displaying lesions is also valuable to the diagnosis.

4.2 Basic Steps

General Considerations

Field observations and data provide a critical link in disease diagnostic work and can significantly affect the outcome of laboratory efforts which depend on the quality of the

samples and the accuracy of the accompanying observations and measurements. The quality of the information depends on a number of factors which include:

- Response Team size, skill and experience
- Response Team organization, interests and biases
- Adherence to clear, detailed protocols, including time-sensitive samples
- Adequacy of planning and suitability of logistics (e.g., type of boat)
- Conducive weather conditions to support safe dive operations
- Care maintained in labeling, processing, stabilizing samples
- Care in adhering to shipping and storage guidelines

4.3 QA/QC Considerations

Minimizing Cross Contamination:

- Visit sites with no signs of disease first
- Sample healthy coral first, then affected/diseased coral
- Use disposable nitrile gloves that are changed between each colony visited
- Gloves will be placed in a 'trash' container underwater and on the surface placed in a second plastic bag where they can be disinfected with bleach and then disposed with garbage
- Use new or decontaminated equipment for each sample.
- On the boat, decontaminate collection equipment by soaking in dilute hypochlorite (5-10% bleach) solution for at least 10 minutes and rinsing in fresh water.

Decontaminating Dive Gear

- Clean dive gear by soaking in decontaminating solution
- Rinse thoroughly in fresh water at the end of each dive.

***Laboratory experiments have been conducted to determine cleaning agents that are effective in disinfection, yet pose little threat to dive gear deterioration. The suggested agent to date is 5% bleach prepared fresh or 3% Lysol™ (diluted according to sanitization strength on packaging and followed by a thorough fresh water rinse).*

4.4 Survey Team- Site Identification and Assessment

The Survey Team will consist of 2-3 members. The primary responsibility of this team is to describe the scene underwater. This includes defining the perimeter of the outbreak, describing the biota, including cover, diversity and presence of other stressors, describing the extent of the outbreak (i.e., determining prevalence: number of species and individuals affected in the context of unaffected individuals), characterizing the lesions, and marking colonies for collection.

4.4.1 Duties of Survey Team

To accomplish the initial site assessment all available divers* should be used to:

- Conduct a survey of the area, (if available, with tow boarding or underwater scooters) to determine the spatial extent of the outbreak

- Count colonies in duplicate (once by each partner) and record the condition of corals within replicate belt transects. Optimally a minimum of two 20 x 1 meter linear transects within the center of the affected area and two transects at the perimeter should be completed (where possible). The dimensions of these transects may vary depending on the size of the affected area, the abundance, diversity and cover of stony corals, depth of the affected area, and size of the team; it is important to capture the diversity of a particular area within chosen levels of statistical confidence. **Note that some areas are more conducive to belt transects and may be the preferred methodology.*

- Characterize affected colonies within a 20 x 1 meter belt (colony size, severity of lesion, genus or species). The level of detail (e.g., type of colony measurements) depends on the available time, size of response team, and level of expertise of the team.

- Assess cover of corals and other major biota

- Record all information on Survey Data Forms

****if numbers of dives per person are restricted, this may be limited to Survey Team members***

4.4.2 Individual Operations within the Survey Team and Their Responsibilities:

- **Videographer-** Video document the site from both planar aspects (at one depth along transect, keeping camera a set distance from the substrate as appropriate for the visibility, relief, cover and size distribution of corals) and pan video to get documentation of general habitat.

- **Cartographer-** Create a generalized map of the area with key landmarks and GPS coordinates noted to allow orientation and ability to triangulate to specific colonies upon follow up visits.

- **Tactical Specialist-** Identify affected individuals and temporarily mark them for sampling. The use of temporary floating chains (plastic chains) to mark colonies is suggested. Record Global Positioning System (GPS) location, depth, and other data designated on the Sample Site Documentation Form. Assign a unique identifier and photo-document each colony marked for sampling.

4.4.3 Survey Approach

Collection of epizootiological data should include, at minimum, the spatial extent of the outbreak; magnitude in terms of the number of colonies affected; and severity, in terms of the percent coral tissue affected or mortality resulting from the outbreak. A standardized disease response should include the following:

- **Broad surveys** to characterize the habitats affected, spatial distribution (e.g, habitats and depths affected) of affliction, and a rapid assessment of potential physico-chemical parameters (depth, water clarity, temperature, nutrient load, etc.), and anthropogenic impacts (pollution, runoff, sedimentation, etc.) that may be linked to the outbreak;

- **Characterization of community structure** in terms of cover of major benthic attributes (substrate, algal abundance and type, and coverage of benthic invertebrates by major phyla or class);

- **Population information** of the scleractinian corals (i.e., abundances, size classes, species diversity, and health status); and

- **Detailed disease assessments** including quantification of susceptible species and the diagnostic features of lesions on individual affected corals including photographic records of the lesions.

The primary survey approaches are described below:

- **MANTA TOW SURVEY:** The method can be used to estimate coral cover, dominant coral types, and broad patterns of disease or mortality. It is not possible to collect detailed diagnostic or quantitative data using this approach.

  A snorkeler (or diver) is towed over the reef by a small outboard motor boat to characterize the major habitats, reef zones, major structural attributes, percent cover of major groups (e.g., stony coral, algae, soft coral, hard bottom), and spatial extent of the disease outbreak, noting areas with the highest prevalence. The snorkeler can drop marker buoys to delineate the area affected by disease. The precision of the manta tow surveys is limited by visibility and depth of the

site, complexity of the reef, and expertise of the observer. One advantage of the technique is that it enables the observer to characterize representative habitats in the context of the entire reef environment.

- **POINT INTERCEPT SURVEY:** Biotic and abiotic components are recorded at certain pre-defined intervals along transects to collect information on cover of various benthic organisms including coral as well as substrate types.

Diver one extends a transect 20 m, parallel to depth gradients, within the approximate center of the affected area. The diver then slowly swims back to the beginning of the line recording the substrate type, and/or organisms to the highest taxonomic resolution possible under the tape every 0.5 m (total of 40 points per line; at minimum, 2 transects should be completed within the outbreak area and two outside of the main affected area). The cover of each component is then determined by dividing the number of points containing the specific category by the total number of points examined (and multiplying by 100). The minimum type of data collected for each point should include:

  - Substrate type: recorded as hard bottom, rubble, sand or dead coral
  - Specific type of algae or invertebrate to highest taxonomic resolution possible. The categories can include:
    - Algal assemblage: recorded as fleshy macroalgae, turf algae, erect coralline algae (e.g., *Halimeda*), crustose coralline algae, and cyanobacteria
    - Stony coral, recorded at minimum to genus
    - Other invertebrate, including sponge, soft coral, gorgonian, anemone, bryozoan, tunicate etc. These organisms should be recorded by major group, and if possible, also include growth form and taxa to highest level possible.

- **CORAL ASSESSMENT SURVEY:** All corals within a predefined area (i.e., 1 x 20 m) are counted and measured and the presence of disease is recorded. This approach will provide detailed data on disease prevalence based on a whole colony assessment, population dynamics, and health status.

Diver two records all colonies (species, maximum diameter, and condition) within one meter of the transect. A 1 m bar marked in 5 cm increments is used to help guide estimation of transect width and to guide estimation of colony size. Only colonies with whose centers lie within the belt transect are recorded; large colonies with their centers (e.g., more than 50% of the colony) lying outside the transect must be ignored.

Colony sizes are preferentially recorded to the nearest 5 cm from a planar view, with measurements only of corals 10 cm or larger in diameter. If the site contains a very large number of colonies, size classes can be lumped into six groups: 10-20 cm; 21-40 cm; 41-80 cm; 81-160 cm; 161-320 cm; and >320 cm. Smaller

colonies should be identified (at least to genus) and counted within the 20 X 1 m belt, lumping them into colonies 0-5 cm and 6-9 cm. If there are large numbers of small colonies, these can be quantified by recording the total number within five 1 m^2 quadrats per transect instead of surveying the entire belt. Quadrats are placed next to the transect tape at predetermined intervals (e.g., 0, 5, 10, 15, 20 m). **Note for certain areas of the Caribbean, colony sizes of 4 cm or greater may need to be included in the assessment.*

- **DISEASE ASSESSMENT:** All colonies with disease or other causes of mortality are identified and counted, and specific detailed diagnostic information is collected for those corals exhibiting signs of the disease under investigation. This approach will provide data on prevalence of all diseases as well as useful diagnostic descriptions for the disease of interest that can assist in determining when the event first occurred, how severe it is, whether it is ongoing, and if it is increasing or declining in severity.

  Diver three identifies every colony within the one meter belt with signs of recent mortality, recording the genus and the common name of the disease or other condition. This includes signs of predation (differentiated into gastropod, fireworm, COTS, or fish bites), disease, bleaching, or compromised health (e.g., algal or invertebrate competition, physical damage etc.). For colonies exhibiting signs of the disease under investigation, the observer should record the genus (or species), maximum diameter, and diagnostic features of the lesion (see section 4.4.3 and Appendix IV). **If time permits accuracy may be improved by having the same diver(s) conduct the community structure surveys as well as the disease assessments.*

  This same diver also identifies corals for sampling and marks them with floating chains and assigns temporary numbered tags. Colonies for sampling should include representatives from all species affected by the disease of interest, as well as different stages in the progression of the disease ranging along a continuum from colonies that appear to be newly infected (small lesions that lack algal colonization) to older well established infections (prominent large lesions with a gradation of algal colonization on exposed skeletal surface).

Depending on the site characteristics, species diversity and abundance, and extent of the disease outbreak, coral assessments or disease assessments may take additional time to complete. As divers finish a task, they can assist the other divers by conducting coral assessment or disease assessment, beginning at the end of the transect and working towards the other divers. If the survey team consists of two divers, one diver would complete point intercept surveys and then begin disease assessment surveys, as the coral assessment may require the most time.

4.4.3.2 Possible Modifications to Consider

Many Indo-Pacific reefs are characterized by high coral cover, a large number of species and colonies, and a dominance of small to intermediate sized corals, making it impractical to measure the size of every coral, especially if dive time is limited and the Response Team is small. It is best to record corals to species, but this may be impractical or not possible on certain high diversity Indo-Pacific reefs and depending on the expertise of the survey team. In this case, divers should record corals to the level of Genus, attempting to differentiate between growth forms when possible (e.g., massive vs. branching *Porites*). The methods described above could be further modified, based on the complexity and size of the affected area, size of the team, and available time.

The minimal survey information that should be collected is an accurate list of all of the genera (or species) and their abundance within the sampling area, along with the numbers of each taxon exhibiting signs of the condition being investigated. A simple data sheet listing all the genera in the first column, a second column to tally the number of healthy corals, and subsequent columns to tally the number of colonies with each type of disease, predation or compromised health. This will provide information on the prevalence of colonies by genus (or species) that are diseased, as well as the prevalence of a particular disease for the entire coral community.

A second level of information could include recording each genera observed within the belt transect, and the numbers of each genera that shows signs of the condition under investigation. The observer could record the maximum diameter of colonies, focusing on measuring only those taxa identified as being susceptible to the particular disease.

A third level of information could involve recording the total numbers of each genera (with and without disease) within particular size classes (e.g., lump all colonies into six categories, <10 cm, 10-19 cm, 20-49 cm, 50-74 cm, 75-100 cm and >100 cm). This could be done for all genera, for a subset of the 12-15 dominant genera, or only for the genera affected by the disease.

4.4.4 Diagnostic Descriptions of Lesions from Gross Observations

For each colony exhibiting signs of the disease under consideration within the survey area, information should be recorded on the affected taxa, its size, and condition (see Appendix IV for assessment form). The lesion should be described in terms of its gross characteristics (tissue loss, skeletal damage, color change, or growth anomaly), the location, lesion pattern, lesion margin, and lesion color (See Fig. 4.4.4), including:

- Location: apical, medial or basal
- Lesion pattern or distribution: linear, annular, focal, multifocal, coalescing, or diffuse
- Lesion size: maximum dimensions

- Lesion margin: condition of disease margin. This should include the thickness, lesion shape (linear, annular or diffuse), and border (smooth, jagged, tissue sloughing).
- Rate of Progression: extent of recent tissue loss and degree of algal colonization, classified as acute, sub-acute or chronic. Colonies exhibiting rapid (acute) disease progress have prominent exposed white skeletal areas with no or minimal algal colonization by turf algae. Moderate (sub-acute) lesions are characterized by large patches of exposed white skeleton along with initial signs of turf and macroalgal colonization on older tissue-denuded skeletal surfaces. Chronic lesions often have a narrow (<1 cm) border of white exposed skeleton adjacent to living tissue, or an absence of recently exposed skeleton; previously denuded skeletal areas are colonized to various degree by turf, macroalgae and crustose corallines.

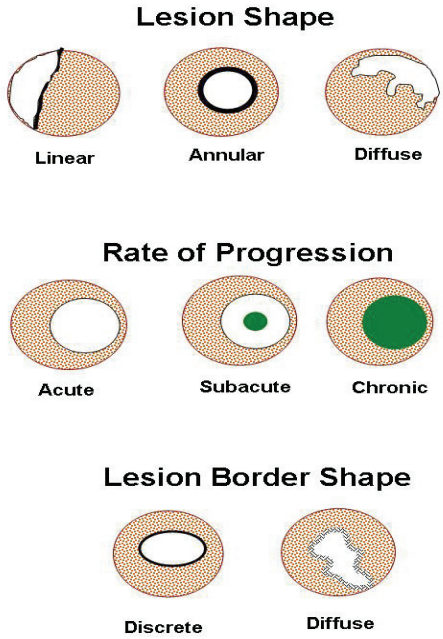

Figure 4.4.4 Diagnostic descriptors for lesions on stony corals. *Modified from (Work and Aeby 2006).*

4.4.5 Field Microscopy

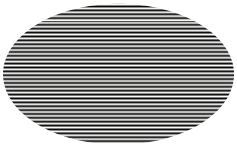

Figure 4.4.5.1.1 Resolution is the shortest distance between objects that allow each to be seen.

4.4.5.1 Introduction

Magnification is the ability to visibly scale up specimens to be able to see more detail than with the naked eye alone. It can be accomplished using a variety of techniques which increases *resolution* which is the smallest distance between two objects at which they can just be seen as two separate and distinct objects.

Magnification of specimens in the field can be accomplished by using simple hand lenses (e.g., 5X magnification) while diving or at the surface or using a simple dissecting microscopes (also called stereomicroscopes) on the boat. Using these simple tools to provide a closer look at field specimens, lesions and disease margins can provide valuable visual details that are not apparent to the naked eye and contribute significantly to the diagnostic process. For example, when observing a brown banding pattern on a coral, it is relatively easy to distinguish between tissue discoloration and a band of ciliates, when 5X magnification is used, whereas visual inspection with the naked eye alone can lead to an erroneous conclusion of tissue discoloration (Figure 4.4.5.2).

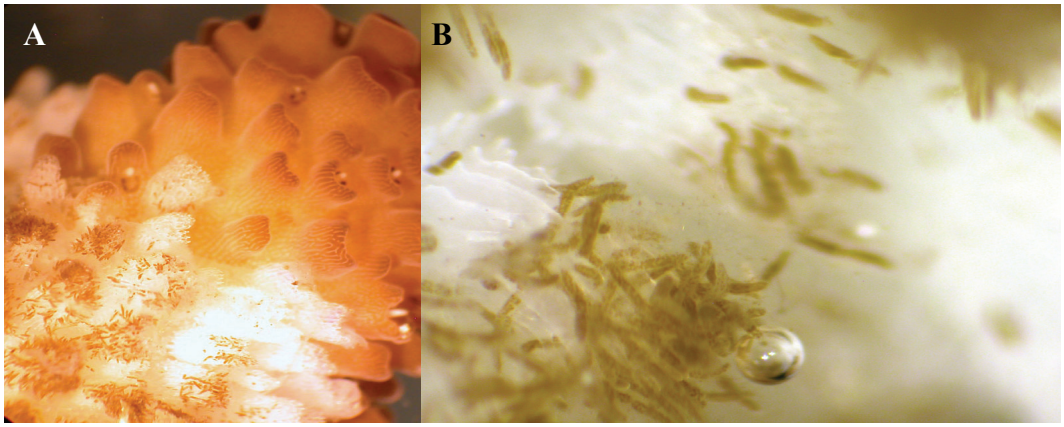

Figure 4.4.5.1.2 *Acropora surculosa*, infected by the Brown band ciliate in a laboratory aquarium tank. A= low power stereoscope B= high power stereoscope *Photo courtesy of Dr. Laurie Raymundo, Univ of Guam.*

4.4.5.2 Relative Sizes

Although visual inspections are imperative during field investigations, closer examinations with a magnifying lens or stereomicroscope is important to consider as a regular part of lesion characterization in order to see its unique physical characteristics (size, shape, motility, color). It is also important to recognize the relative sizes of cells or organisms and to put them in proper perspective and to avoid erroneous descriptions. For example, bacteria are too small to be observed with simple magnifying lenses or even a stereomicroscope since neither instrument has the ability to resolve objects in this size class. Figure 4.4.5.2 illustrates the relative sizes of specimens related to coral reefs and the instruments required to view them.

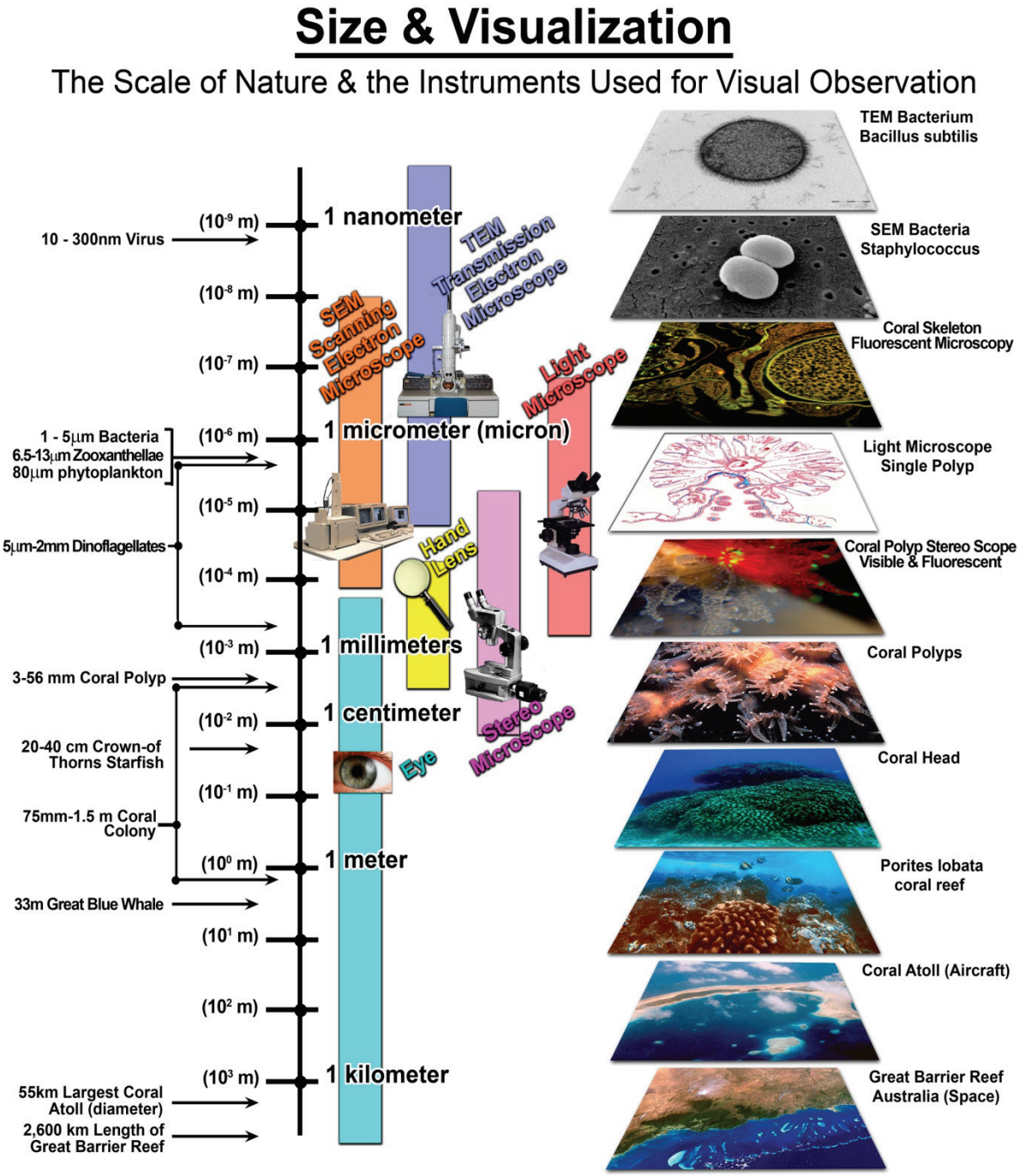

Figure 4.4.5.2 The scale of coral reefs and the instruments used to view them.
Artwork by James Nicholson

4.4.5.3 Use of Stereo Microscope

The American Optical Cycloptic microscope (Figure 4.4.5.3) is a sturdy, easy to use microscope that is available for field investigations.

- To FOCUS the microscope, use the focus knob starting above the good focus level and rack down until the specimen is in sharp focus. This particular microscope is collimated (parallel beams of light) to assure parfocality (stays in focus when magnification is changed) at all magnifications.

- To CORRECT for individual eye differences, first focus the microscope with the right eye. Turn the left eyepiece focusing sleeve counterclockwise until the left image is out of focus, then turn clockwise until the image is in sharp focus with the left eye.

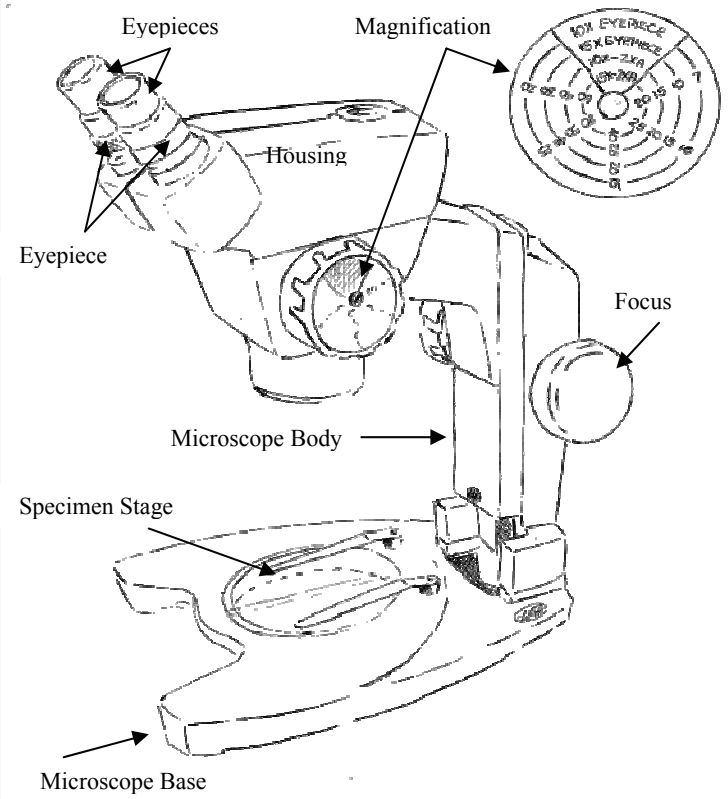

Figure 4.4.5.3 Illustration of an American Optical dissecting scope. *Illustration by Athena Avadanei*

- To adjust INTER-PUPILLARY DISTANCE, grasp the eyepiece housing and adjust the spacing by moving the two until able to view a full single field with both eyes.

- To Change MAGNIFICATION, rotate magnification knob to desired magnification. Magnification values can be read at the indicating dot.

4.4.5.4 Care and Maintenance of a Stereo Microscope

A well-designed stereo microscope requires surprisingly little maintenance. Most problems can be prevented by some simple, common sense, proactive preventative steps. Bear in mind that cleaning optics is inherently destructive over a long period of time so preventing optical contamination is better than cleaning it off. One of the most useful microscope accessories, often unused is the simple dust cover. A microscope should always be covered when not in use. Special consideration should be given to the type of cover where ever there is the possibility of water, chemicals or blowing sand affecting the scope.

Common dust is usually not of concern and if excessive enough to be bothersome is easily removed with a source of air, either commercial canned air, or an ear syringe. The most common type of contamination that requires prompt and thorough cleaning is finger prints. The oils in a finger print can actually etch the optical coatings on the lens. Eye makeup such as mascara can be a chronic problem in the contamination of the eyepieces. The best solution is to discourage the use of eye makeup by personnel using microscopes. Salt spray needs to be removed by the careful use of fresh water cleaning using damp clothes, never liquids that could get into the scope.

Tips for proper cleaning of optics:

1. Have proper materials on hand including good quality lens paper, a source of air and lens cleaner.

2. Always first use air to blow off the optical surface to remove any grit that could scratch the optics during cleaning.

3. Never touch an optical surface with any dry material. Always moisten the cleaning cloth or tissue with lens cleaner or use your breath to fog the lens.

4. Suitable cleaning materials include lens tissue, microcloth, or a well laundered clean handkerchief.

5. Clean in a circular motion without applying excessive force. Make several passes using a clean surface each time.

6. The use of solvents should be carefully restricted to lens contamination such as oil or mounting media that actually requires it. Never apply any solvent directly to a lens but always apply it to lens paper or a cotton swab. Shake off excess liquid before applying to the lens. Materials like oil will require the use of multiple swabs or papers as they must be discarded after each pass. Check all safety instructions for any solvent and make sure you have adequate ventilation, and personal protection as required.

4.5 Collection Team- Sample Collection

The Collection Team may consist of 3 members: the Sampler, Sample Handler, and the Records Diver. However, the tasks can be accomplished by 2 divers, with assistance from a snorkeler on the surface to ferry samples to the boat between each colony. The **Sampler** will physically collect the prescribed samples from coral colonies and photograph the pre- and post- biopsy condition of the affected areas. The **Sample Handler** will assist the sampler in keeping track of the collection bags or tubes, verifying the labeling, securing the samples once taken, and seeing that time intervals are maintained for time-critical samples such that the samples are transported to the surface in the prescribed time. The **Records Diver** is responsible for the Diseased Colony

Collection Form, and will ferry time-sensitive samples to the surface. The Sample Handler may also perform the duties of the Records Diver on the occasion of a two-person collection team.

4.5.1 General Considerations

Specimens are material representative of the problem and suitable for further laboratory analyses. The specimen may be tissues, mucus, environmental samples (e.g., water or sediment) or other flora or fauna that associate with the diseased corals. Photographing lesions and surrounding area provides a record of color, location and appearance of lesions. Both actual size and macro shots should be taken before and after removal of tissue biopsies. It is also important to include a color scale and metric to size and color correct photos.

The primary consideration when collecting diseased tissue is personal safety; universal precautions for potential health hazards should be observed. To avoid transmission of possible disease agents, disposable gloves should be used, and disinfection with a commercial disinfectant or a 5-10% bleach solution (prepared w/in 12 hrs of use and kept out of direct sunlight) should be used to decontaminate collection tools, work areas and dive gear. Observations of these guidelines will minimize transmission and protect team members.

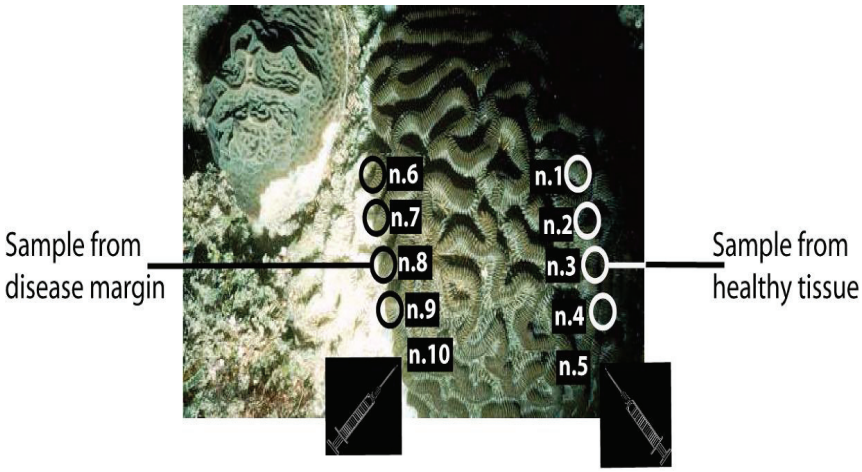

Figure 4.6.1.1 Diagram illustrating disease margin, unaffected areas and possible sampling design. *Illustration by Shawn W. Polson.(Polson et al. 2006).*

"Collection and analysis of samples is the basis of investigation, and the validity of the results and conclusions of any study is totally dependent on the quality of the samples collected" (Wobeser 1994). Properly trained individuals proficient in collection techniques are critical to the proper collection and preservation of samples. Improperly collected or preserved specimens can look the same as a good sample, but if handled improperly or contaminated, it will preclude further analyses and compromise the integrity of an investigation.

4.6 Collection Protocols for Biological Analyses

4.6.1 Labeling Scheme Guideline

- Letter or number designation of the collection site
- Four letter abbreviation for coral species (first letter of genus, first three of species)
- Colony number within site
- Two letter sample type abbreviation

Colony Type	Analyses/Collection Method		Example
Reference	**W**ater	**P**rotein	R-P
Healthy	**S**ediment	**F**ixative	H-F
Unaffected	**M**ucus	**B**acteria	U-M
Diseased	**A**pplicator (Swab)		D-S

ex. Reference Site A= A.*Dstr*.1.R-P
Diseased Site B= B.A*pal*.4.D-F and B.*Apal*.2.U-M

Definitions

- "Reference" - uninfected or 'healthy-looking' colonies from areas where no corals exhibit signs of the disease
- "Healthy" - apparently healthy corals in affected sites
- "Unaffected" - areas of diseased colonies with normal appearance, distant from the lesion
- "Diseased" - margin of the lesion

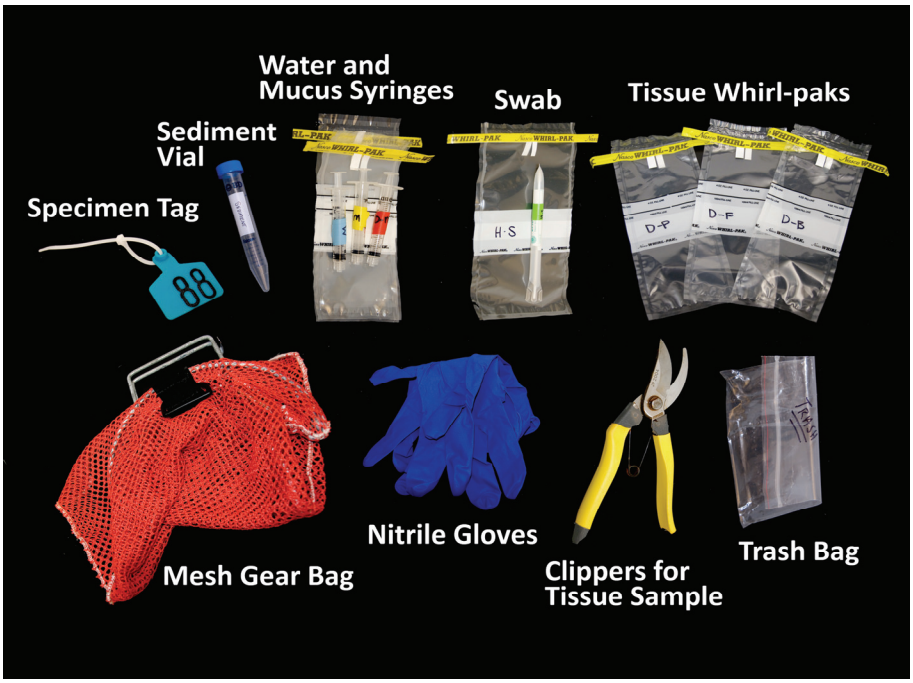

Figure 4.6.1.2 Example of components that may be used in for specimen collections during an outbreak investigation

Due to time sensitivity of some samples, such as the tissue for protein analyses, sampling should adhere to a specific order.

Within each site, samples should include:

- Water
- Sediment
- Applicator/Swab
- Syringe/Mucus
- Core or Clipped Tissue Samples for each analysis planned

4.6.2 Sediment

- Scoop sediment with sterile pre-labeled 15mL conical or similar container

This type of sample is used solely for microbiological sample analyses as a reference for microbes situated in the sediments that may be mobilized from disturbances such as storms.

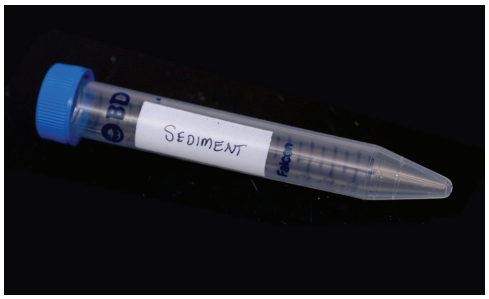

Figure 4.6.2 15cc Falcon tube for microbiology sediment collection

4.6.3 DNA Swab

- Wipe across the area to be sampled three times.

This is currently experimental and may provide less invasive sampling. The swab samples are limited to DNA analyses of surface tissue and mucus.

Figure 4.6.3 Epicenter DNA swabs

4.6.4 Water

- Collect one reference volume for each colony
- Should be equal in volume to mucus sample
- Collect in a 3cc or larger syringe

This sample is used as a reference for microbiological analyses to allow analyst to account for possible water contamination of mucus and tissue samples as well as a comparison for microbes that may be found in surrounding waters, but not primary colonizers of corals.

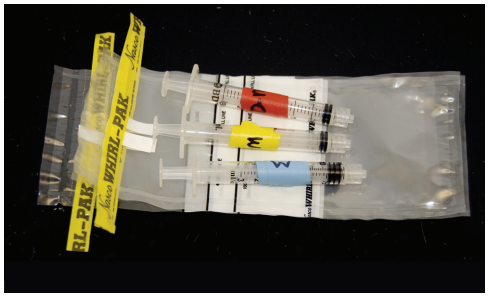

Figure 4.6.4 Syringes for water and mucus collections

4.6.5 Mucus

A sterile syringe without the needle is used to aspirate (draw in) mucus from the surface of the coral. For diseased samples, mucus is collected along the disease margins and unaffected samples across the surface of unaffected areas. If swab samples are collected, this should be done first which should provide the irritation required to obtain mucus. It is important to collect mucus already present on the colony. The diver should avoid initially irritating the colony, as mucus subsequently released by the coral will have a depauperate microbial flora community.

Mucus samples have been one of the primary types of specimens used in culture dependent and independent microbiological analyses. It seems to provide consistency across temporal and spatial sampling for microbial diversity studies. Recent work however has shown different microbial profiles are obtained depending on whether live-ground tissue or mucus is being analyzed. It appears that these two micro-environments contain different microbial communities, with tissue samples having a more diverse and robust community than mucus.

4.6.6 Tissue biopsy

Fragment/Tissue

- Coring technique- 1- 2.2cm diameter uniform disk samples of tissue + skeleton for larger colonies, using two punch sizes. **clay should be inserted after coring to minimize further damage (Roma Plastalina, no 2-from Rex Art, Miami, FL)*
- Clippers/Pliers/Garden Shears- can be used for clipping from branching specimens

Figure 4.6.6.1 Clippers used for sampling branching corals

Figure 4.6.6.2 Tools used for bolder corals. Stainless steel coring tube for histology (A) is 2.5 cm, while leather punch (B) biopsy is 1.5 cm.

Tissue samples are collected for a variety of clinical analyses. Currently available analyses include histology/histopathology, microbiology, cellular diagnostics (primarily

protein chemistry based and includes a suite of various biochemical and cell-based parameters that can be measured for building a diagnosis) and genetic or functional genomic assays.

4.7 Sample Processing for Biological Analyses

Each sample has a predetermined experimental or analytical role, which determines how each will be processed on the boat and back on land. The Sample Technician of the Support Team will do most processing.

4.7.1 SUPPORT TEAM

This team will consist of at least 2 members who will provide topside and field-lab support. The primary job of the Sample Technician is to ensure the proper handling, documentation and stabilization of each sample collected. The Logistics Chief is responsible for all dive gear and collection equipment and assists the Sample Technician.

Figure 4.7.2.1 Collection bags for healthy coral samples for H-F=fixative (histology), H-P=protein H-B=bacteria (microbiology)

Figure 4.7.2.2 Collection bags for diseased coral samples. D-P=protein, D-F=fixative (histology), D-B=bacteria (microbiology)

4.7.2 PROCESS TIME SENSITIVE SAMPLES FIRST

- **Tissue for Protein** (H-P, U-P, D-P) samples should come to the surface in dark bags or covered (e.g., glove) to protect them from light for light sensitive assays. They are time sensitive and need to be processed in a dark or shaded area. Mucus should be rinsed by swishing in seawater, dabbing on Bounty™ paper towel (or lint-free paper towel), and placing in a new, prelabeled Whirlpak™. Since Whirlpak™ bags are prone to shattering at liquid nitrogen vapor temperatures, the bags are wrapped in aluminum foil with an identifying label on the outside and placed immediately into a dry shipper. **Do not write on aluminum foil** as it is not permanent, use labeling tape or cryotags and waterproof marker. The time interval between collection and freezing should be approximately 15 minutes, longer than this will exclude certain cellular diagnostic assays due to creating artifact by changes in the sample.

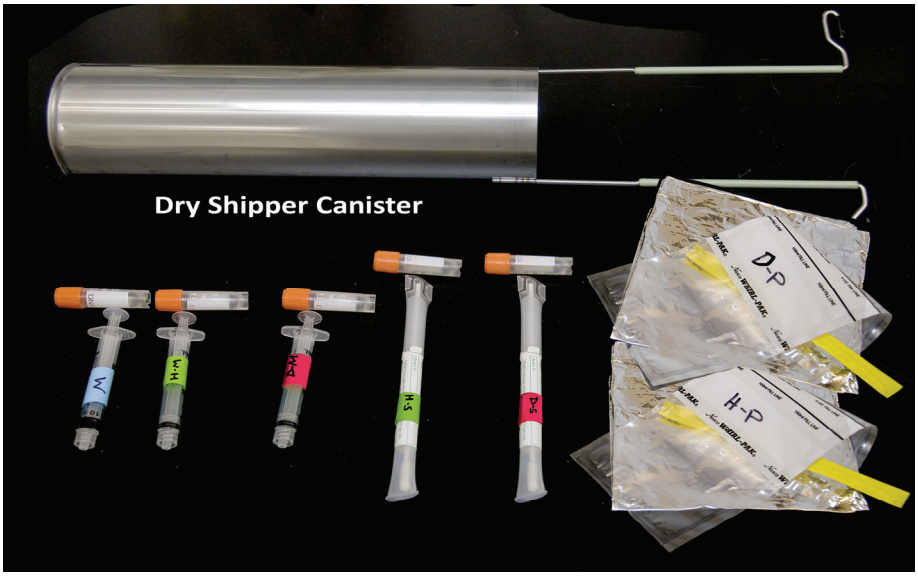

Figure 4.7.2.3 Summary of samples to be frozen showing the packaging used for freezing.

- **Tissue for Histology -** The tissue biopsies collected from **H**ealthy, **U**naffected portion of diseased colony and the **D**isease margin (**H-F, U-F, D-F**) are placed in bags or tubes underwater and on reaching the boat, if transport of fixative is logistically sound, the samples are immediately placed in a 50cc polypropylene tube containing approximately 25 mL of an appropriate fixative for a 2cm punch biopsy or an approximately 2-3 cm branch (if larger, the fixative volume should be increased in proportion). When fixative transport is precluded, histological samples should be stored in bags or a container containing seawater and securely stored to minimize stress until a destination for fixation is reached. We routinely use Z-fix (Anatech Ltd.) diluted 1:4 in sterile artificial sea water (ASW; 35ppt) and held at ~25°C, because of the ability to retrieve intact DNA from the samples for subsequent molecular and immuno-staining. The ratio of tissue to fixative should be at minimum 1:10 (1:20, preferred). **DO NOT FREEZE THESE SAMPLES.**

  Alternatively seawater-buffered formalin can be used for fixation of corals for light microscopy and formalin is generally available at marine labs, hospitals and veterinary clinics. This is prepared by filtering either natural or artificial seawater and diluting formalin stock (37.5% formaldehyde) 1 part formalin to 9 parts filtered seawater. The samples are fixed from 4 hrs to overnight then rinsed in tapwater and stored in 70% ethanol or alternatively can be stored in the 3.75% formalin-seawater.

  For shipping Kim-wipes™ or other lint-free paper is saturated with the preservative (e.g., 70% ethanol) and stuffed into the tube. This stabilizes the samples and keeps them moist, while avoiding shipping tubes filled with a hazardous material.

These samples are planned for light microscopy analyses. Electron microscopy requires different stabilization and processing and is not covered here.

- **Tissue for Microbiology** (H-B, U-B, D-B) should be kept in a Whirlpak™ with sterile 35 ppt artificial sea water added if needed, keep at ambient temperature in a cooler with local seawater. Upon return to shore, homogenize tissue and skeleton with sterile mortar and pestle (with its own mucus), flash freeze half of homogenate, and culture bacteria on marine agar or other desired media, with other half of homogenate.

Figure 4.7.2.4 Equipment used in processing tissue samples for microbiology. These include a mortar and pestle for grinding fresh tissue, device to flame sterilize spreading rods, agar plate for culturing bacteria and cryovial for freezing ground tissue sample.

- **Swabs or Applicators** (H-A, U-A, D-A), if they are Whatman FTA™ type swabs, should be wiped on the card, and then the tip should be broken off and stored in a 15 cc tube or cryovial. Other types of swabs (Epicenter, Madison WI) simply need to be broken off and the tip stored. Cards can be stored in a reclosable food storage bag (e.g., ziploc™) and shipped at ambient temperature; swab tips should go in the cryovial. ***Alternative storage to freezing is being investigated using sodium chloride saturated dimethylsulfoxide (DMSO).*

- **Mucus** samples which were collected in a syringe without needle need to be split: Half should be placed in a cryogenic vial and immediately flash frozen in a liquid nitrogen dry shipper for molecular analyses. The other half should be kept in screw top vials at ambient seawater temperature and cultured on marine agar media as soon as possible for microbiology. (See Support Team Processing Guidelines Form in Appendix V).

- **Surface sediment (**H-S, D-S) - loosen cap and attempt to remove as much water as possible. Leave about a 2 cm gap between sample and cap, cap tightly and freeze in dry shipper.

- **Water** (H-W, D-W) should be split into two samples. Half can be transferred from the syringe to a 2.0 mL cryogenic vial and placed in the dry shipper. The other half should also go in a 2.0 mL vial, but be kept at ambient temperature for culture-dependent methods.

The other roles of the Support Team at this point are to catalog these samples, track and label all samples, label and link digital photos to samples, download GPS coordinates and upload to GIS (if available), and prepare to ship time-sensitive samples.

4.8 Sample Shipment

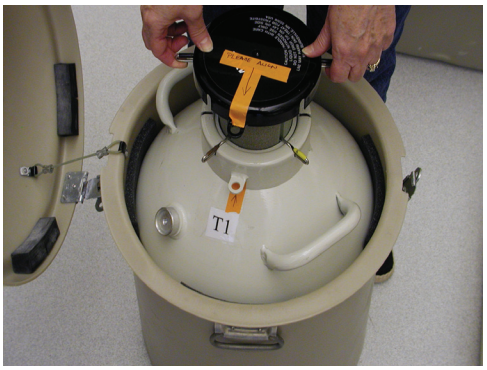

Figure 4.8 Dry shipper. Note lid and canister alignment are critical to ensuring proper seal.

The Planning Chief or Incident Commander, in collaboration with the Regional Coordinator and National Coordinator should make prior arrangements with the appropriate diagnostic laboratories to conduct the analyses, and provide advanced notification with likely dates of sample arrival before conducting the response. These dates should be confirmed before shipping samples. The Incident Commander should also follow up with the lab to ensure arrival of samples to the lab and to the appropriate person.

Samples should be placed in appropriate packaging to ensure safe delivery, avoid leaks, and fines. Each shipment should be labeled as non-regulated material to avoid concerns by the currier or inspection agents (e.g., customs). Dry ice and dry shipper labels should be used where appropriate.

4.9 Permits

It is imperative that all biological samples are collected and shipped under appropriate permits, and relevant documentation is included with samples.

4.9.1 Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Permits

CITES is an international agreement between 166 governments to ensure that international trade in specimens of wild animals and plants does not threaten their survival. Under CITES, a species is listed at one of three levels of protection, each of which has specific restrictions on trade: no commercial trade is allowed in Appendix I species, while trade is allowed for Appendix II species when accompanied by an export permit that indicates the species was legally acquired and trade is non-detrimental. Coral reef species currently listed in CITES on Appendix II are 1) all corals including scleractinian (stony) corals, hydrozoan corals (*Millepora* and *Stylaster*), organ pipe coral (*Tubipora*), and blue coral (*Heliopora*), 2) all antipatharian (black) coral species, 3) giant clams (*Tridacna* and *Hippopus* spp.), 4) queen conch (*Strombus gigas*), 5) all seahorses (*Hippocampus*), and 6) humphead wrasse. For stony corals, permits are required for live corals (whole colonies and pieces with recognizable corallites), skeletons, eggs/sperm/planula, live rock, reef substrate and eroded skeletal fragments greater than 3 cm in diameter. A CITES permit is also required for coral samples less than 3 cm in diameter if the corallites are discernable and allow identification of the coral to genus (or species).

When shipping coral samples from outside of the U.S. and U.S. territories to laboratories located in the U.S., shipments must include a valid export permit issued by the exporting countries Management Authority in the country of origin (or an approved equivalent form issued by equivalent national authority in the case of countries not party to CITES). The U.S. does not require import permits. CITES permits are not required for shipments between the U.S. and our territories (Puerto Rico, U.S. Virgin Islands, Guam, Commonwealth of the Northern Mariana Islands (CNMI) and American Samoa).

When shipping coral samples from the U.S. to international destinations a valid export permit issued by the CITES Management Authority in the U.S. [Division of Management Authority (DMA), U.S. Fish and Wildlife Service] must be included with each shipment; depending on the destination country (e.g., the European Union), a CITES Import Permit is also required.

Importation of fish and wildlife, including corals, must be imported at one of the 14 Designated Ports and must be declared using USFWS Form 3-177. It is important that shipments are clearly labeled as CITES material.

When using international mail or an overnight type courier (e.g., Federal Express or UPS), shipments sometimes bypass USFWS and are delivered directly to the importer. It remains the responsibility of the importer to file the appropriate declaration form; failure to file when required is a violation of the Endangered Species Act of 1973. USFWS has simplified the declaration process by allowing the 3-177 Form to be submitted electronically (eDec) followed by mailing the original CITES permits along with a copy of the eDec confirmation page (https://edecs.fws.gov). If Certificates of Scientific Exchange are used (see below), no original CITES permits need to be sent to USFWS.

As an alternative, the CITES Secretariat has endeavored to streamline the permitting process for scientific samples by encouraging scientific institutions to register for Certificates of Scientific Exchange (COSE). The International Registry of Coral Pathology (IRCP) in Oxford, MD, administered by Dr. Shawn McLaughlin (shawn.mclaughlin@noaa.gov) and the Coral Disease and Health Consortium, administered by Dr. Cheryl M. Woodley (cheryl.woodley@noaa.gov) in Charleston, SC hold COSE permits for exchange of histological materials. To facilitate such exchanges, investigators are urged to work with their local scientific institutions to register for a COSE with the CITES Management Authority of their country (or approved for this purpose by an equivalent national authority in the case of countries not party to CITES). A one-time application and minimal fee (or waived in certain cases) permits exchange of specimens among registered institutions in lieu of filing CITES export or import permits for each shipment. COSE authorizes non-commercial loan, donation, and exchange of legally acquired scientific specimens between any institutions registered for this purpose. In the U.S., application is made by submitting USFWS Form 3-200-39 to the Division of Management Authority (http://www.fws.gov/). Upon approval, the institution is assigned a registration number and added to the list of registered institutions. Fixed and embedded specimens and micro-slides prepared from legally obtained corals may then be shipped from a registered institution to a registered institution without application for additional

CITES permits. Legally obtained Appendix II specimens may be imported into the U.S. by simply entering the registration number of the importer and exporter on the USFWS claim form. This significantly reduces the time, effort, and potential cost spent on obtaining export or import permits for individual shipments (Mc Laughlin et al. Unpublished) however, both the exporting institution and the importing institution must be registered.

4.9.2 Other Collection Permits

Each state, territory and jurisdiction has their own regulations regarding scientific collection permits. In some locations multiple jurisdictions exist, for example the Florida Keys National Marine Sanctuary's boundaries are within the State of Florida's waters and both the State and Sanctuary permits are required for the collection of coral, in others one entity issues permits. To date blanket permits have not been approved for Coral Disease Outbreak Responses, but managers acknowledge the urgency of these cases and have recommended an expedited or emergency permit process. It is important for Response Coordinators to develop a dialogue with permit offices in their region that explains the Response process and oversight provided in determining when sampling is warranted. The coordinators should also include a minimal sampling plan, rationale and projected analyses for samples that are taken during an investigation.

4.10 Types of Laboratory Analyses

4.10.1 Histology

Histological analyses are used to characterize the microscopic morphology of tissue and may help guide further investigations. They provide systematic evaluation of cellular changes that occur in tissues under normal, stressed or diseased conditions. The microscopic evaluation determines which cells or tissues are affected and whether foreign organisms (i.e., bacteria, fungi, metazoa, protistans, viruses) are present. Most

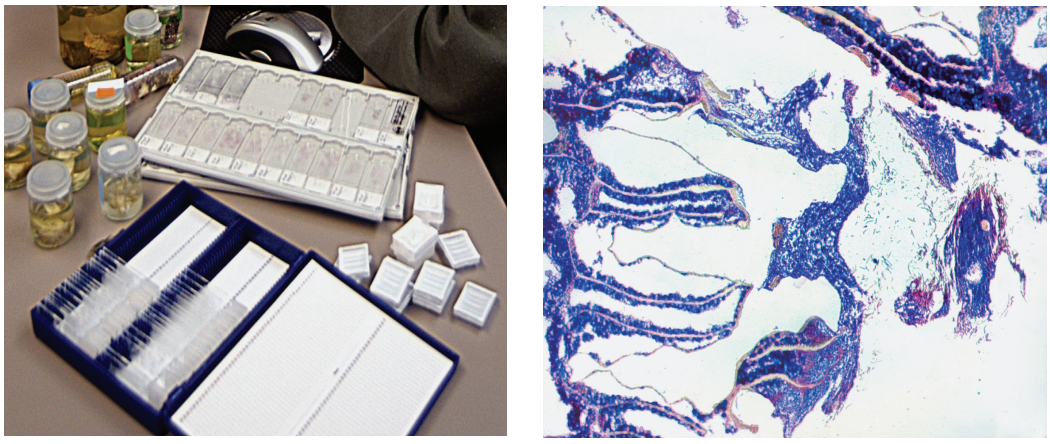

Figure 4.10.1 Histological specimens and slides (left), micrograph of coral tissue (right) *(Photos courtesy of Shawn McLaughlin)*

evaluations begin with light-level microscopy; however transmission electron microscopy can provide evidence of sub-cellular changes that are informative in understanding functional changes associated with a particular pathology.

4.10.2 Microbiology

Microbiology is one of the fundamental disciplines used in clinical diagnostics when an infectious agent has not been excluded. Coral disease research focuses heavily on microbiology ideally combining culture-dependent methods, with DNA-based technologies. While culture dependent methods are useful in identifying specific dominant cultivable microorganisms, culture-independent methods allow examination of the diversity of microbial communities associated with corals and coral mucus and shifts in these communities between hosts, species, seasons, geographic location and when exposed to different stressors. This facilitates investigations in microbial ecology, functional studies of microbial communities and differential analyses of these communities between various health conditions of corals.

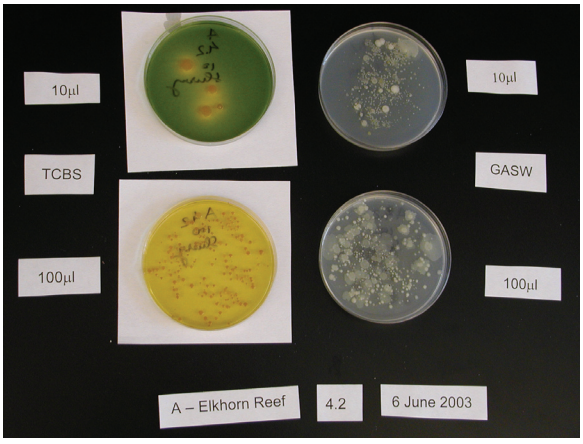

Figure 4.10.2.1 Culture dependent microbiology. Shown are samples plated on selective media (TCBS) and general media (glycerol seawater agar)

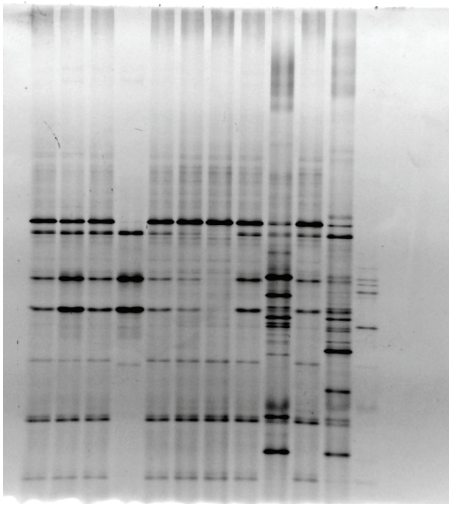

Figure 4.10.2.2 Culture independent microbiology. Example shown is microbial community profile using denaturing gradient gel electrophoresis.

4.10.3 Molecular

Molecular analyses address the formation, structure, and function of macromolecules essential to life, such as nucleic acids and proteins. Biochemical and cellular endpoints are often used in clinical diagnostic assays. Several of these have been adapted to coral and referred to as cellular diagnostics (Downs 2005a). The concept of cellular diagnostics is based on using a systematic approach to defining biomarkers of exposure, effect, and susceptibility and integrating levels of these cellular parameters into a profile that is diagnostic for certain cellular functions and disease states, and provide indicators of overall performance. The selection of cellular parameters is based on known functionality within a cell and knowledge or inference of how alterations in single or sets of cellular parameters affect cellular physiological processes. Many of these processes are involved in key metabolic pathways or cellular structural components that are

essential for cellular function and homeostasis. The behavior of these processes defines the cell's physiological condition. Thus the identification and quantification of pattern changes in the cellular endpoints provides a basis for defining health status (i.e., diagnosis) and providing a prognosis.

Molecular biology and coral functional genomics projects focus on nucleic acid, RNA and DNA. These studies are also vital to improving our understanding of the function and control of coral genes. One of the most urgent applications is to begin identifying more coral genes, understanding their expression patterns and control mechanisms. This will assist in improving our understanding of coral cellular physiology and form a stronger basis for pathology and the development of new diagnostic assays for studying and diagnosing coral disease.

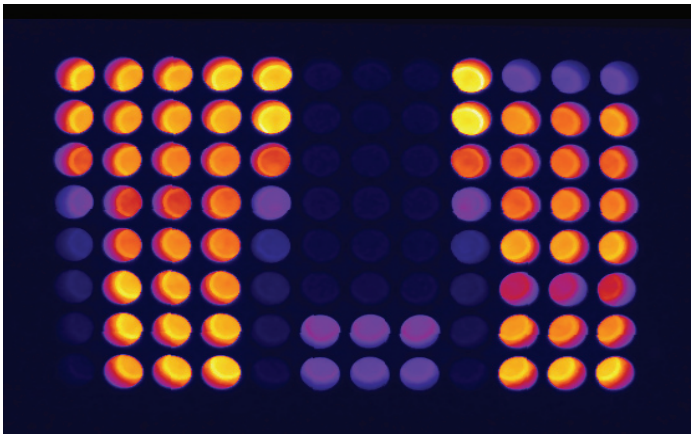

Figure 4.10.3 ELISA assay for measuring cellular diagnostic parameters. *(Photo courtesy of CA Downs)*