Caribbean Marine Research Center

Workshop on

Pathogens and Parasites of Reef Corals:

Field Techniques

2-9 November 1993

Lee Stocking Island, Bahamas

PATHOGENS AND PARASITES OF REEF CORALS: FIELD TECHNIQUES WORKSHOP AGENDA

2-9 November 1993
Lee Stocking Island, Bahamas

Monday November 1	Afternoon	Arrival of charter plane Start Unpack/Set-up
	1800	Welcome/Happy Hour (Dennis and Head)
	1930	Reef Corals in an Entirely Artificial Ocean (Lang)
	2000	Continue Unpack/Set-up
Tuesday November 2	0800	Check out Dive (McAllister) Field dive(s) (Lang)
	1300	Reef Environments and On-going Coral Monitoring/Research at LSI (Lang) Environmental Monitoring at LSI (Dennis)
	1400	Introduction to Coral Disease Research (Peters)
	1530	Comparative Coral History Lab (Peters)
	1930	Susceptibility and Spread of Black Band Disease in the Field (Bruckner)
	2000	Pathogens and Symbionts of Reef Corals and Sponges (Santavy)
Wednesday November 3	0800	Search for Coral Diseases Dive(s), Collect BBD (Santavy, Lang)
	1400	Microbiological Techniques Lab (Santavy)
	1930	Marine Bacteriophages in Chesapeake Bay (Wommack)
	2000	Aquatic Viral Pathogens (Suttle)
Thursday November 4	0800	Quantifying Putative Diseases Dive-1 (Peters, Lang); Collect Sea Water (Suttle)
	1400	Viral Enumeration Lab (Suttle)
	1930	Ridge Mortality of *Diploria* at the Flower Gardens (Zimmerman)
	2000	White Band Disease, Stress Related Necrosis, Other Pathogens and Parasites (Peters)

WORKSHOP AGENDA (continued)

Friday November 5	0800	Quantifying Putative Diseases Dive-2, Collect putative WBD (Peters, Lang)
	1300	Coral Histopathology Lab (Peters)
	1930	Sublethal Measurements of Stress in Response to Pesticide Exposure (Firman)
	2000	Microalgal Invertebrate Symbioses (Trench)
Saturday November 6	0800	Isolation and Culture of Symbiotic Dinoflagellates Lab (Trench)
	1330	Nutrient Recycling Inside the Skeletons of Hermatypic Corals (Ferrer)
	1400	Analytical Techniques for Symbiotic Dinoflagellates Lab (Trench)
	1930	Experimental Studies of Temperature and UV-B on Coral Reef Organisms (Reaka)
	2000	Quantifying Coral Color (Maguire)
	2030	Reef Community Sampling Design and Disturbances on Deep Reefs (Ohlhorst)
Sunday November 7	0800	Generalities Discussion: Research Needs (Lang, Suttle, Trench)
	0930	Specifics Discussion: Questions, Techniques, Resources (Maguire, Santavy, Peters)
	1400	Follow-up Exploratory Research Dives and/or Labs (All)
	2000	Optional Night Dive (All)
Monday November 8	All day	Follow-up Exploratory Research Dives and/or Labs (All)
Tuesday November 9	Morning	Departure of charter plane

Coral Workshop Participants

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INTRODUCTION TO CORAL DISEASE RESEARCH

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Although coral reefs generally appear to be flourishing and healthy, a number of studies conducted during the last three decades have shown that corals and other reef organisms are susceptible to diseases caused by pathogens and parasites as well as to those conditions caused or aggravated by exposures to anthropogenic pollutants and habitat degradation.

"Disease" is defined as any impairment (interruption, cessation, proliferation, or other disorder) of vital body functions, systems, or organs.

Biotic diseases are those in which the etiologic agent is a living organism such as a pathogen or parasite. A variety of organisms normally live in interspecific associations known as symbioses on or within the tissues of other organisms (Amadjian and Paracer 1986). Such associations can range from mutualistic symbioses (beneficial to both organism and host) to parasitic symbioses where the organism derives a nutritional benefit from the host. If a parasite causes disease and death of the host, then it is known as a pathogen. Infectious agents, those that are spread from host to host, include viruses, bacteria, fungi, and protozoans (also known as microparasites), and metazoans such as helminths and arthropods (macroparasites). Many associations of organisms and hosts occur without clinical signs of disease. Infectious host-specific diseases caused by pathogens may weaken or disable individuals so they are more susceptible to predation or stressful environmental conditions. Such diseases may also occur as epizootics, dramatic increases in disease prevalence and mortalities in large numbers of organisms of a single species.

Abiotic diseases are those structural and functional body impairments that result only from exposure to abiotic environmental stresses such as changes in physical conditions (salinity, temperature, light intensity or wavelength, sedimentation, oxygen concentrations, currents) or exposures to toxic chemicals (such as heavy metals and organics like oils or pesticides).

The causal agent of a disease may appear to be either biotic or abiotic, but both types of diseases are often closely interrelated and identification of the the causative factor may be difficult. In some cases a pathogen or true parasite may not harm its host unless the host is stressed by some other biotic or abiotic disease factor. Conversely, an abiotic disease can become complicated by secondary infections from normally harmless microorganisms.

The mechanisms by which changing environmental conditions, toxins, or pathogens, cause disease appear varied and will also differ with the species and individual (for reviews see Sparks 1985, Sindermann 1990). Furthermore, although motile organisms may be able to avoid or limit their contact with pathogens, toxic agents, or adverse physicochemical conditions, sedentary invertebrates, such as scleractinian corals, generally cannot. (They may produce planktonic larvae to escape, however.)

Black Band Disease (BBD) was the first reported disease affecting scleractinian corals from reefs off Belize and Bermuda, but since the mid-1970s it has been found throughout the Caribbean as well as the Indo-Pacific (Rützler et al. 1983, Antonius 1985). Massive brain corals (*Diploria* spp., *Colpophyllia* spp.) and star corals (*Montastraea* spp.) are the most commonly affected members of the Family Faviidae, while elkhorn, staghorn, and pillar corals resist natural infections. The disease results from the invasion of coral tissue by a cyanobacterium, *Phormidium corallyticum*. The disease line appears as a black mat a few millimeters wide composed of fine cyanobacterial filaments that may also contain other bacteria and protozoa. This band or mat moves across the surface of the coral at the rate of a few millimeters per day, leaving behind bare coral skeleton that is eventually colonized by filamentous algae. Healthy corals can become infected with BBD when in contact with an infected colony, but injured colonies are most susceptible. Most studies have found that less than 2% of Caribbean corals are infected with BBD on any given reef area, although there have been recent outbreaks at several sites, including Looe Key, Florida (Peters, 1993). Edmunds (1991) observed that 58% of BBD-infected brain corals (*Diploria strigosa*) on reefs off St. John, U.S. Virgin Islands lost over 75% of their tissues within 7 months.

Also in the mid-1970s, acroporid (elkhorn and staghorn) corals off St. Croix, U.S. Virgin Islands, exhibited tissue sloughing, which started at the base of the branches and moved toward the branch tip at the rate of a few millimeters per day. In contrast to BBD, however, no predators were present and no consistent microorganisms could be found at the junction separating the sloughing tissue from bare coral skeleton. This disease was termed "white band disease" (WBD), "white plague", or "white death", because the sloughing left a broad band of bare skeleton several centimeters wide on the colony that was also eventually colonized by filamentous algae (Gladfelter, 1982; Peters, 1993). These disease signs have since been observed on acroporid species throughout the Caribbean, the Red Sea, and off the Philippines.

The etiology of white band disease is unknown. Unusual aggregates of gram-negative rod-shaped bacteria were found scattered in the calicoblastic (skeleton-producing) epidermis that lined the gastrovascular canals of the porous skeleton in affected acroporids from St. Croix and Bonaire, Netherlands Antilles (Peters et al., 1983). The bacterial aggregates were also found in apparently healthy colonies at St. Croix. Five years later, up to 95 percent of the elkhorn corals there had died. Although these bacteria have not been found in apparently healthy acroporids from other sites, the role of this microorganism in the development of disease has not yet been determined.

Basal tissue sloughing has also been observed in acroporids and other species of scleractinian corals from the field as well as in captivity, but microscopic studies have not found observable microorganisms within their tissues. Because there was an association with adverse environmental changes in some of these cases, Peters (1984) proposed the term "stress-related-necrosis" for cases in which degenerative changes in cell structure are observed in the absence of obvious pathogens, particularly bacterial aggregates, as determined by microscopic examination of fixed, embedded tissues.

Corals also harbor a variety of protozoan and metazoan microorganisms, some of which may be parasites. Gregarine protozoans have been found in Caribbean coral tissues, causing localized adverse host tissue reactions including loss of zooxanthellae and necrosis (Peters 1984). Another such relationship has been examined in Hawaiian corals (*Porites compressa*) that contained the metacercarial stage of a digenetic trematode, *Plagioporus* sp. The host for the final stage of this parasite is probably a coral-feeding fish. Aeby (1991) examined the effects of the parasite on the coral polyps which become pink, swollen nodules, with altered ability of the polyps to retract into their calices. Parasite encystment resulted in reduced growth rates of parasitized corals. Fish fed preferentially on infected polyps, and as a result the altered polyp appearance provided both an enhancement of the parasite's transmission rate and parasite removal from the coral. Healthy polyps then grew back over the feeding scars. Thus, this phenomenon may act as a host strategy of parasite defense.

Another sign of disease in corals is "bleaching." This phenomenon is due to the loss of zooxanthellae, which normally give the coral tissue a brownish coloration, and/or to the loss of photosynthetic pigments from the zooxanthellae. Bleaching of corals, gorgonaceans, alcyonaceans, and anemones has been attributed to exposure to high light levels, increased solar ultraviolet radiation, temperature extremes, salinity extremes, high turbidity and sedimentation resulting in reduced light levels, and other factors. The nature and extent of bleaching varies between individuals of a species and among species at the same location during a bleaching event and have been attributed to different physiological tolerances of the strains (or species) of zooxanthellae and the coral hosts. Chronic partial or widespread loss of zooxanthellae, for whatever reason, signals a disturbance in the normal metabolism of the coral host and can lead to delayed or reduced reproduction, tissue degeneration, reduced growth, and death of the affected tissue (Williams & Bunkley-Williams 1990). Partial and patchy bleaching patterns have also been found in association with the presence of potential parasites, including gregarine protozoans (Upton & Peters, 1988) and trematode metacercariae in *Porites astreoides* from Belize (E. Peters, unpublished).

The increased incidence and prevalence of black band disease (BBD) at some sites during certain times of the year, however, appears to be related to adverse environmental conditions, including warmer than normal temperatures, nutrient loading, increased sedimentation and turbidity, predation resulting in colony injury, and toxics (Peters 1993). In the laboratory, antibiotics can control bacterial colonization of mucus on the surface of corals in BBD. Similar results were obtained for corals exposed to elevated concentrations of crude oil, copper sulfate, potassium phosphate, dextrose, or sedimentation (Mitchell and Chet 1975, Hodgson 1990).

Increased mucus production, while an important defense mechanism for corals and other tropical marine organisms, requires high energy expenditures. Available energy can be limited when nutritional sources are scarce or metabolic processes are altered by exposure to temperature extremes or pollutants, leaving the animal susceptible to attack by pathogens.

A study of corals and octocorals from Biscayne National Park, off southeast Florida (USA) revealed high levels of organochlorine pesticides and heavy metals, similar to those levels used in toxicity tests that led to bleaching and mortality of the same reef-building species of corals in the laboratory. Approximately one-third of coral colonies sampled from this site exhibited lesions and possibly pathogenic microorganisms were found in their tissues, although the presence of the lesions could not be linked to contaminants in this study (Glynn et al. 1989). High tissue burdens of such chemicals resulting from chronic exposures may increase the susceptibility of the organisms to disease when additional physical or chemical stresses are encountered. Similar studies of corals off Australia and elsewhere have detected uptake of pesticides and heavy metals into tissues; however, the presence of biotic or abiotic diseases in contaminated corals in the wild has not been documented.

Biomarkers or biological indicators, defined as "molecular, biochemical, and cellular changes caused by pollutant chemicals which are measurable in biological media such as cells, tissues and body fluids" (McCarthy & Shugart 1990: 457), are being developed to assess the stresses that may be encountered by aquatic organisms in their natural habitats. These biomarkers can provide information on current and potential adverse ecological effects of changes in environmental conditions and may help identify causal mechanisms underlying observed effects at the population or community levels. Biomarkers also permit the simultaneous monitoring of sensitivities to stress, specific responses to exposure to toxicants or other changes in water quality, effects on the health of the organism, and pertinent ecological factors.

Biomarkers under consideration include molecular and biochemical responses such as DNA-adduct formation and cytochrome P-450 system enzyme induction that occurs during exposure to certain pollutants, physiological and behavioral reponses such as respiration rate and changes in swimming patterns, alterations in the immune system as a result of exposure to chronic stress, histopathological changes in cells and tissues, and food consumption or bioenergetics studies that may be indicative of changes in growth and fecundity have also been studied.

Similar biomarkers may be useful in monitoring the condition and health of coral reef organisms. The normal ranges for the various parameters must be carefully examined first (Adams 1990; Huggett et al., 1992) both within and between individuals and species. The study of bleaching in corals and other zooxanthellate organisms has increased to the point that zooxanthellate densities and photosynthetic pigment fluorescence may be useful biomarkers.

Previous investigations of the structure and function of coral reefs and associated soft-bottom habitats (mangrove and seagrass communities) have not considered the role of pathogens and parasites in population and community development and alterations. Not only may these

organisms affect host responses to environmental stresses, thereby altering population size and geographic distribution, but they can also influence intra- and interspecific interactions of species (Kinne 1980). At the population level, disease may either produce acute to chronic progressive mortalities of individuals over varying periods of time or alter the structure or function of the individual in such a manner as to make it more susceptible to predation or environmental stresses. Diseases affecting the gonads, sexual maturation, associated tissues, and mating or spawning behavior may also lead to reduced fecundity of individuals with subsequent reductions in population size or changes in gender and age composition.

Diseases of scleractinian corals change the structure and function of coral reef communities. Loss of live tissue cover not only reduces the number of polyps producing gametes for potential new recruits, but also opens up new hard substratum space for settlement of benthic organisms (Edmunds 1991). White band disease has affected large areas of reef throughout the Caribbean and Florida Keys, with bioerosion of the remaining exoskeletons changing reef structure (Gladfelter 1982) and causing eventual collapse of the reef. Extensive loss of topographic relief has affected fish populations as protective niches and important habitats have been altered. The decline of coral reef fisheries among western Atlantic coral reefs may be related to such structural changes, along with overfishing (Rogers 1985).

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DRAFT—DO NOT QUOTE OR CITE

PREPARATION OF CORAL TISSUE FOR HISTOPATHOLOGICAL EXAMINATION

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Procedures for collecting and processing scleractinian coral tissues for examination by light or electron microscopy are similar to those used for other organisms, including human and mammalian tissues. Thus, general reference books on histological techniques are important sources for information. While the basic steps for tissue dehydration, clearing, embedding, and sectioning have remained the same, with recent and ongoing changes in modern tissue processing equipment and the availability of new microtomy equipment and techniques for achieving thinner tissue sections, some of the following procedures may require modifications for a particular research problem. This information is presented as guidance only—the investigator should seek additional expert histology advice depending on the materials and equipment available. A list of consultants and references is provided.

Coral Collection

> Check to see if coral collection is prohibited or restricted first! Remember to obtain permission for collection of coral specimens prior to conducting the study. Permits from more than one state, local, or government agency may be required. If coral specimens are to be brought into the United States from a foreign county, a CITES permit may also be required.

Colonies for study should be identified and marked in the field prior to collection of samples. For disease studies, both apparently healthy colonies (having a uniform covering of tissue with normal pigmentation and corallite morphology appropriate to the species) and colonies of the same species showing lesions of interest should be included. Whenever possible both types of colonies should be collected from the same vicinity and depth.

- The study location should be mapped and coral colonies to be sampled should be marked with tags and/or small floats on weights or fastened to nails inserted into the substratum.

- Photograph each specimen in situ to have a record of the appearance of the lesion. Record observations on each specimen's condition, locality, size, color, etc.

- Small whole colonies may be collected by breaking the attachment at the base with a masonry hammer and chisel or pneumatic chisel. Portions of colonies may be collected by hammer and masonry chisel or pneumatic chisel, or a pneumatic or hydraulic masonry hole saw or coring drill to collect a uniform disc of coral tissue and skeleton (approximately 2 to 4 inches in diameter). For coring devices, a wooden guide should be used to avoid touching the surrounding tissue to minimize damage to the colony.

- If cores are taken, cement or clay plugs should be inserted in the skeleton. Hudson (1981) contains a description and illustrations of this technique, and the following information is contributed by Harold Hudson (pers. commun., October 1993). To make the plugs, prepare a rich mixture of cement by mixing:

    1 part builder's sand
    1 part Portland Cement

  (Type 2 is preferable, because it does not react with seawater, but it is not easy to find.)

  Pour the mixture into round precision-made plastic, styrofoam, or paper cups, to a depth of 3 to 4 inches. The cups should have tapering, not straight, sides. A nail may be used to inscribe a code in the upper center surface of the plug while the cement is still soft. Allow the plugs to cure and dry for approximately 2 weeks before using.

  (It is *critical* to match the plug diameter to the diameter of the coral core you will be obtaining. Use your drill to drill a sample hole in a cement block and check the diameter of the cup to find the diameter where the plug should meet surface of coral. Then make the upper edge of the mold start approximately 1/8 inch *above* this diameter, so that the plug will fit snugly when inserted. If the correct cup diameter will result in a plug that is longer than 3 inches or so, the bottom of the cup should be filled with builder's sand so the final plug depth is adjusted correctly.)

  After the coral core is removed from the colony, place the plug, code side up, into the hole and tap it in with vertical strokes from the *wooden handle end* of a 4 lb. maul hammer (the metal end will shatter the plug). The plug only needs to be inserted sufficiently snugly to keep it from being easily removed by pulling on it with fingers and to seal the cut, it does not have to be flush with the surface of the colony). If necessary, the plug can be trimmed using a masonry chisel and hammer.

  If the sample core or portion removed is less than approximately 2 inches in diameter, the hole may be sealed by filling it with modeling clay, The hardest grade of clay available will stay put better and easier to work with, it may be shaved off with a knife to trim after pressing it in with the fingers.

- Handle samples at base edges or exposed skeletal surfaces only and place each sample in an individual plastic ziploc bag. Several small holes should be cut into the bottom and sides of the bag prior to the dive. The holes will allow sediment to drain and water to circulate, and permits easy fixation (see below). [**Note**: If exoparasites, algae, or bacteria will be studied, the sample should be placed in a unperforated plastic bag or sterile Whirlpak bag with seawater at time of collection. After other studies are completed the sample can be placed in the perforated bag for fixation]. The bag can contain a prewritten label made by using pencil on a piece of waterproof paper.

- Place bagged samples into a bucket or other container, one layer deep to avoid damage. Lift samples into boat. If a boat trip is necessary before fixation, partially cover buckets or place bagged samples in seawater in an insulated cooler to prevent overheating and sun exposure. Preferably fix samples immediately [or as soon as possible after other studies are conducted].

Fixation

> This step is critical! Samples must be rapidly and completely fixed for proper interpretation of lesions, abnormalities, microparasites, etc.

Choice of fixative will depend on the embedding medium to be used, whether the tissues will be examined by light or electron microscopy, and other factors. Four liters of fixative should be enough for approximately 10-15 25 cm^3 samples in one bucket ($\approx$ 8 L lab bucket with tight fitting cover). The following are preferred:

Modified Helly's Solution (for light microscopy, paraffin embedding)

Basic Solution (for 4 liters): 100 g potassium dichromate
200 g zinc chloride
4 L tap water or seawater

Chemicals can be premeasured and placed in plastic screw cap jars to transport to the field. The zinc chloride is hygroscopic and caustic, so gloves should be worn when handling this chemical. Weigh and place in jar quickly, seal jar with electrical tape or Parafilm. Anytime prior to the dive, place chemicals and water in fixation bucket and stir to mix.

Just before placing the samples in the solution, add:

200 ml of formalin (40% formaldehyde)

The maximum lifespan of this fixative after the formalin is added is 24 hours. After fixation for 16 to 20 hours (smaller or fewer samples may take only 8 to 12 hours), place the bagged samples into running seawater or in several changes of tap water or seawater to remove excess fixative. Wash for 24 hours.

Samples should then be preserved in 70% undenatured ethanol. Either wrap the bags in several layers of cloth saturated with ethanol and place in well-sealed plastic bags or place in sealed jars or buckets, depending on the method to be used for returning the samples to the laboratory.

Upon return to the laboratory, place samples in jars of fresh 70% ethanol and change the ethanol frequently (every few days) until it turns clear. The samples may also be decalcified and processed before the ethanol turns clear (see Barszcz and Yevich 1975).

Bouin's (for light microscopy, paraffin embedding)

Bouin's may also be used for fixation of coral tissues. Premade Bouin's is available commercially or may be mixed following histological procedures.

Coral samples should be fixed for only 6 hours. It is important to note that **all** excess Bouin's must be removed from the tissue by rinsing in 70% undenatured ethanol **prior** to further processing because of the picric acid. The tissue samples should be stored in 70% ethanol.

10% Seawater-Formalin (for light microscopy, paraffin embedding)

If Helly's or Bouin's are unavailable, a 10% seawater-formalin fixative solution may be prepared by mixing 1 part formalin (40% formaldehyde) with 9 parts seawater. Do not use neutral buffered formalin for fixing specimens from seawater.

Specimens may remain in this fixative indefinitely, although transfer to 70% undenatured ethanol is preferred for air shipment of specimens. However, the results are not as good as with either of the above fixatives, especially the final staining of tissue sections.

Zamboni's (for light or electron microscopy, plastic embedding)

Zamboni's is a picric acid-paraformaldehyde-phosphate buffered fixative that may be used for either light or electron microscopy. See Sheehan and Hrapchak (1980:329-330) for the procedure for mixing this fixative and contact Ms. Iliana Quintero for additional information on using Zamboni's with coral tissue (see

below). It is stable at room temperature for one year, is not sensitive to light, and can fix medium-sized tissues quickly. Tissues may be stored in the fixative up to one year.

Miscellaneous Notes

Fixatives for electron microscopy vary widely with the purpose and investigator. The latest literature should be examined in order to select an appropriate fixative for this purpose. It is most important that the final osmolarity of the fixative be close too, or slightly more than that of seawater to ensure proper fixation of the tissues.

- Bags containing the coral samples should be lifted from the bucket or cooler, allowed to drain briefly, then plunged into the fixative-containing bucket. Be sure to let the fixative solution enter the bag through the holes and agitate it a bit to ensure penetration of the fixative into the tissue.

Decalcification and Trimming

> Sample condition and collection information should be recorded in a notebook (logbook) and given a short identification code. This numeric or alphanumeric code is used to track the sample during the following steps.

Following removal of excess fixative, the calcium carbonate exoskeleton must be removed with an acid solution to allow examination of the tissues (unless a special plastic-embedding-grinding-polishing procedure is to be used to examine the tissue/skeleton interface—contact Esther Peters for more details).

- Remove coral samples from bags and place in glass or plastic jars containing the decalcifying solution. Be sure to include an identifying tag, preferably waterproof paper, and label each jar. Commercial decalcifying solutions are available from several vendors. Most of these solutions consist of 1 N hydrochloric acid with a chelating agent, ethylene diamine tetraacetic acid (EDTA). Other procedures, such as citric/formic acid solutions, may also be used, but staining quality may be affected. For preparation of samples for transmission electron microscopy, a gentle EDTA solution must be used (see E.H. Gladfelter 1982).

- Change the decalcification solution daily just until the skeleton is completely removed. The amount of time necessary for complete decalcification will vary with the species, amount of sample, and skeletal structure. Do not allow the tissue to remain in the

solution any longer, because the acid will start degrading the tissues and affect their quality for staining and storage.

- Immediately after decalcification is complete, remove the remaining tissue from the solution, rinse briefly in fresh tap water, and place on a dissection board. Gently separate underlying boring sponge tissue and/or algal filaments from the coral tissue.

- Trim the coral tissue with a sharp razor blade into several pieces to fit into perforated plastic or steel cassettes for processing. Sections should be no thicker than 2 to 3 mm and approximately 1 to 1.5 cm long, and should include portions for examining oral, aboral, and longitudinal views of the polyps (the latter will provide a view of the gonads if sections through the aboral region are not cut deep enough during microtoming later).

- Place one to two pieces of trimmed coral tissue into each cassette with either the oral, aboral, or longitudinal surface face down. Be sure the cassette is appropriately labeled or include identifying tags (this will depend on the type of cassette being used). Snap on top of cassette.

- Place cassettes immediately in a dish with running tap water (a self-siphoning water bath works best) and leave for a full 24 hr in order to remove all traces of the decalcification solution. This is necessary for proper staining reactions.

- After cassettes have washed for 24 hr following decalcification, drain the water from them and place in a dehydrating solution (either 70% undenatured ethanol or, if you are using the Technicon procedure, the dehydrating reagent is S-29). The cassettes may remain in this solution indefinitely prior to processing.

Processing

> Procedures and reagents used in processing depend on the type of tissue processing equipment. A histotechnologist should be consulted to be sure that modifications are made to procedures used for other animal tissues.

During processing, tissues must be dehydrated to remove all traces of water, cleared with a reagent that is miscible with the dehydrating solution and the embedding medium, and finally infiltrated with a liquid that can be hardened sufficiently to allow cutting of thin sections for examination. Processing can be done either manually (a time-consuming, difficult-to-control method) or by using an automated tissue processor that moves the cassettes through the different solutions at the appropriate times. The newest tissue processors have programmable computers and vacuum infiltration. The most important items to note for processing coral tissue are:

- For embedding in paraffin, transfer the cassettes to fresh dehydrating solution in the tissue processor. Suggested solutions and times (may also be done by manually transferring the cassettes from beaker to beaker):

Beaker	Reagent	Time
1[a]	70% ethanol, S-29[b]	60 min or more
2	80% ethanol, S-29	30 to 60 min
3	95% ethanol, S-29	30 to 60 min
4	100% ethanol, S-29	30 to 60 min
5	100% ethanol, S-29	30 to 60 min
6	xylene, UC-670	30 to 60 min
7	xylene, UC-670	30 to 60 min
8	xylene, UC-670	30 to 60 min
9	paraffin[c]	15 to 30 min[d]
10	paraffin	15 to 30 min
11	paraffin	15 to 30 min

[a]With agitation.
[b]Technicon clearing and dehydrating reagents.
[c]Paraplast (Sherwood Medical), melting point 60 °C.
[d]Length of time in paraffin changes depends on thickness of tissue, but should be minimized to prevent cracking and hardening of tissue.

If another embedding medium is used, such as glycol methacrylate (JB-4) or another brand of paraffin, follow instructions provided (or seek expert assistance) for dehydrating, clearing, infiltrating, and embedding the tissue.

- When using liquid paraffin, be sure to embed (place the tissue in the mold and cool) as rapidly as possible to avoid overcooking the tissue.

Sectioning

> Obtain training on the proper and safe use of whatever type and brand of microtome is available. Practice before attempting to cut coral tissue.

Coral tissues may be very easy to section, however, even after decalcification bits of spicules from boring sponges or tougher tissues from worms, barnacles, or molluscs may make sectioning difficult. Again, the type of equipment used here will depend on the embedding medium selected.

- When using paraffin blocks, cool blocks on ice before placing in microtome chuck.

- Use disposable blades whenever possible.

- Cut sections at 6 microns or thinner (some of the newer paraffins or plastics allow sections to be cut at 4 microns or less). Use appropriate procedure to remove wrinkles and air bubbles from the ribbon and allow expansion of the sections to their original size (usually done by floating on a warm-water [45 °C] bath].

- Pick up sections on clean glass slides. For paraffin sections and routine staining, do not use gelatin, albumin, or other adhesive on the microscope slides, but be sure they are absolutely clean (wipe with 95% ethanol if necessary). Plastic sections and staining procedures that may use harsh chemicals or long periods in the solutions may require the use of an adhesive.

- After section(s) are picked up on the slide, drain it briefly and place in a rack in a warm oven (58 to 60 °C) or on a slide warmer for 2 to 4 hours to allow the section to dry and the tissue proteins to adhere to the slide. Be sure the slide has been labeled (the new frosted end slides allow rapid marking with a pencil or solvent-resistant pen, much easier than using a diamond pen).

- After drying, remove the slides from the oven or warmer and place the slides in slide boxes or other suitable storage container. Do not leave exposed to heat for a longer period of time.

Staining

> Staining procedures will vary with the embedding medium used. Be sure to obtain assistance and training in the available staining and coverslipping techniques, particularly the proper differentiation of stains.

Three of the most useful general stains for coral tissue are described below. The solutions and procedures are based on Helly's fixed, paraffin-embedded tissues. Tissues fixed with different solutions may require additional steps (check Luna, 1968; Yevich and Barszcz, 1981; Sheehan and Hrapchak, 1980). Be sure to adjust quantities of staining solutions for the number of slides and size of containers to be used. Modifications of these procedures may be required for plastic-embedded tissues (see Talge, 1991). The use of a microwave oven may reduce staining times (see recent literature). Following staining, sections should be coverslipped using an appropriate mounting medium, such as Permount or one of the newer acrylic compounds.

Hematoxylin and Eosin

This stain is used routinely to examine general tissue and cell architecture and composition. The hematoxylin binds to nucleic acids and basophilic substances, such as mucopolysaccarides, tinting them various shades of blue or purple. Eosin stains acidophilic components, such as cytoplasm and connective tissue, with shades of red to pink. A number of different procedures may be found in reference books (e.g., Luna, 1968). Harris's hematoxylin and eosin requires a differentiation step, in which the hematoxylin is briefly washed out of the tissues with acid alcohol. This is tricky, but if done properly nuclear staining will be crisp and chromatin can be distinguished. There are also commercially available premade hematoxylins, such as Gill's, that may not require the differentiation step.

Modified Heidenhain's Method

This modification of Heidenhain's Azocarmine-Aniline Blue method (Yevich and Barszcz, 1981) is used to distinguish the mesoglea (staining bright blue from the aniline blue), nervous elements and fibers (staining dark against the blue ground substance), and muscular elements, nuclei, and granular cells staining orange to red (or blue to red in the case of the "ductless digestive gland cell aggregates). This stain also allows comparison of the condition of the zooxanthellae. Healthy whole algal cells appear reddish whereas degenerating cells or debris appears as greenish pigment. Although most mucous secretory cells remain clear with this technique, the contents of some of these cells in several species stain light blue. Masson's trichrome procedure or Cason's one-step trichrome procedure may also be used (Cason, 1950).

Solutions:

1% Azocarmine G solution

Azocarmine G	1.0 g
Distilled water	100.0 ml

Bring to boil, cool to 56 °C, filter, cool completely, add 1 ml glacial acetic acid. This solution must be kept in the refrigerator and filtered before each use.

1% Aniline-Alcohol Solution

Aniline	1.0 ml (dangerous, use proper handling procedures)
80% ethanol	100.0 ml

1% Acetic Alcohol

Acetic acid, glacial	1.0 ml
95% ethanol	100.0 ml

5% Phosphotungstic Acid Solution

Phosphotungstic acid	5.0 g
Distilled water	100.0 ml

Aniline Blue

Aniline Blue, water soluble	0.5 g
Orange G	2.0 g
Distilled water	300.0 ml
Acetic acid, glacial	8.0 ml

May be refrigerated after each use and used many times.

Staining Procedure

1. Deparaffinize and hydrate to distilled water.
2. Place in preheated Azocarmine G solution at 56 °C for 15 min.
3. Rinse in distilled water (3 min).
4. Differentiate in aniline-alcohol solution until cytoplasm and connective tissue are pale pink and nuclei are distinct, about 10 to 15 min. Rinse briefly in distilled water and check with microscope to be sure differentiation is correct.
5. Mordant (soak) in phosphotungstic acid solution for 15 min.
6. Rinse in distilled water.
7. Place in Aniline Blue for 15 min.
8. Rinse in several changes of distilled water.
9. Dehydrate in 95% ethanol, 2 changes, 2 min each, then 3 changes each of absolute alcohol and xylene. Coverslip.

Modified Movat's Pentachrome Method

This procedure is essential for observing changes in the mucopolysaccharides of the mucous secretory cells. Changes in molecular structure, composition, and pH are reflected in differential binding of the alcian blue and saffron solutions. Changes in mucous secretions and their cell structures may occur with rough handling, exposure to chemical or physical irritants (such as oil hydrocarbons or sediment), nutritional disorders, or metabolic changes. These changes may or may not be reversible, depending on the circumstances. This modification of Movat's procedure was developed by Yevich and Barszcz (1981). It does not include the stains and steps for elastin, and is more appropriate for marine invertebrates.

Solutions:

Alcian Blue

Alcian blue	1.0 g
Distilled water	100.0 ml
Acetic acid, glacial	1.0 ml

Solution may be save and reused many times.

Alkaline Alcohol

Ammonium hydroxide	10.0 ml
95% ethanol	100.0 ml

Weigert's Iron Hematoxylin

Solution A:

Hematoxylin crystals	1.0 gm
95% ethanol	100.0 ml

Solution B:

Ferric chloride, 29% aqueous	4.0 ml
Distilled water	95.0 ml
Hydrochloric acid, concentrated	1.0 ml

Mix equal parts of Solutions A and B prior to staining.

Woodstain Scarlet

Woodstain scarlet N.S. conc.	0.1 g
Distilled water	95.5 ml
Acetic acid, glacial	0.5 ml

May be saved and reused.

0.5% Aqueous Glacial Acetic Acid

Acetic acid, glacial	0.5 ml
Distilled water	99.5 ml

5% Aqueous Phosphotungstic Acid

Phosphotungstic acid	5.0 g
Distilled water	100.0 ml

Alcoholic Saffron

Saffron	6.0 g
100% Ethanol	100.0 ml

Place in an airtight stoppered glass container to prevent dehydration. Extract the saffron by placing the solution in an oven at 58 °C for 48 hr. May be reused many times (saffron is expensive!).

Staining Procedure:

1. Deparaffinize and bring to absolute (100%) ethanol (3 changes each of xylene and 100% ethanol, 2 min each).
2. Alcian blue, 30 min.
3. Wash in running water, 3 min.
4. Alkaline alcohol, 2 hr (60 min).
5. Rinse in distilled water (2 min).
6. Weigert's hematoxylin, 1 min.
7. Wash in running water, 15 min.
8. Rinse in distilled water, 2 min.
9. Woodstain scarlet, 1 min.
10. Rinse in 0.5% aqueous glacial acetic acid.
11. Differentiate in 5% aqueous phosphotungstic acid solution, 7 to 10 min.
12. Rinse in 0.5% aqueous glacial acetic acid.
13. Absolute alcohol (100% ethanol), 3 changes, 2 min each.
14. Alcoholic saffron, 7 to 10 min, in airtight staining dish (seal edges with tape after inserting slides).
15. Rinse in absolute alcohol, 3 changes, 2 min each, then 3 changes of xylene, 2 min each. Coverslip.

Histopathological Examination

> Always compare normal (apparently healthy) specimens with those showing obvious signs of disease.

Interpretation of lesions and identification of microorganisms should be based on careful comparison of normal apparently healthy tissues and grossly affected tissues, as well as on previous studies on invertebrate and coral histopathology (e.g., Lasker et al., 1984; Peters, 1984b; Sparks, 1985; Peters and Pilson, 1985; Glynn et al., 1986; Peters et al., 1986; Upton and Peters, 1986; Sindermann, 1991; Peters, 1993). Experts in marine histopathology should also be consulted (see Contacts and References below). The reproductive status of each coral sampled should be determined during the histological examinations (see recent literature, such as Chornesky and Peters, 1987).

Examples of normal and diseased coral tissues are archived in the Registry of Tumors in Lower Animals (contact: Dr. John C. Harshbarger, Director, 202-357-2647) and the Department of Invertebrate Zoology (contact: Dr. Stephen D. Cairns, Curator, 202-786-2129), at the National Museum of Natural History, Smithsonian Institution, Washington, DC 20560; and at the Center for Marine and Estuarine Disease Research, U.S. Environmental Protection Agency Environmental Research Laboratory, 1 Sabine Island Drive, Gulf Breeze, FL 32561 (contact:

Dr. William S. Fisher, Pathobiology Branch Chief, 904-934-9394). Interested investigators may examine the coral histoslides and other materials at these facilities or arrange for a short-term loan.

Contacts

For further information and the latest advice on preparing marine invertebrates, including scleractinian corals, for histopathological examination, the following individuals should be consulted:

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A survey of cellular reactions to environmental stress and disease in Caribbean scleractinian corals

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ABSTRACT: Despite growing concern about the demise of coral reefs in many areas of the world, few studies have investigated the possibility that bacteria- or virus-caused diseases may be important agents in the disappearance of living coral tissue from reefs, and that their occurrence and transmission may be influenced by natural or man-made changes in water quality, particularly increased sedimentation and turbidity. One forereef site off St. Croix, U. S. Virgin Islands, and three shallow-water reef sites off Puerto Rico were examined for variations in coral composition, local environmental conditions, and the presence of possible diseases in the stony corals. Visual observations were supplemented with standard histopathological examination under the light microscope of tissues from 257 specimens (representing 9 genera and 13 species), along with additional samples obtained from the Netherlands Antilles, the Grenadines, the Florida Keys and the Smithsonian Coral Reef Microcosm. This procedure proved to be necessary to accurately determine the condition of the colony, to detect the presence of microorganisms, and to correlate tissue health and microparasite infestations with apparent symptoms. These lesions varied with the species and the site. For example, off Guayanilla Bay, three species showed increased or decreased mucosecretory cell development, and another exhibited an unusual microparasite, which may be related to the chronic sedimentation at this site. Although colonies of several species showed signs of "white band disease" at five locations, bacterial colonies composed of Gram-negative rods were present only in acroporid tissues from the relatively pristine St. Croix site and the Netherlands Antilles. The distribution and possible mode of occurrence of these and other diseases and microparasite infestations suggest that acute changes in microhabitat conditions or injuries to individual colonies may be as important to the development of some of these lesions as are chronic adverse environmental conditions over a particular reef.

INTRODUCTION

While accumulating evidence indicates that diseases caused by viruses, bacteria and other microparasites are increasing in many marine invertebrates in environmentally stressed areas (Hodgins et al. 1977; Bang, 1980; Sindermann et al., 1980), few studies have investigated the possibility that such diseases may also be important agents in the disappearance of living coral tissue from tropical shallow-water reefs. Widespread interest in the causes of coral mortalities has only recently developed, notably at the Coral Reef Pathology Workshop held at the Third International Coral Reef Symposium, Miami, Florida (May 1977). Corals have evolved under relatively stable and uniform environmental conditions and are very sensitive to natural or man-made physical and chemical changes in water conditions (Johannes, 1975; Sheppard, 1982). Factors which adversely affect the health of the corals will also affect the condition of the other reef

dwellers, because of the interactions occurring within this complex ecosystem (Odum & Odum, 1955; Goreau et al., 1979).

Mortalities of reef corals have been linked to the decrease in water quality due to increased sedimentation and turbidity with continuing resuspension and transport of sediment. These problems are the result of poor management practices in land clearing, discharge of wastes, dredging and filling of coastal margins, and mining of corals for construction, in the Bahamas, the Florida Keys, Puerto Rico, Samoa, Hawaii, Malaysia, Micronesia, the Seychelles and other locations (Stephenson et al., 1958; Goreau, 1964; Johannes, 1972, 1975, 1976; Voss, 1973; Marszalek, 1981; Ricard, 1981). The presence of suspended sediments may stress reef corals by reducing incident light levels essential for the symbiotic zooxanthellae (Muscatine, 1973; Rogers, 1979). Corals also expend energy operating their epidermal muco-ciliary system to remove sediments and prevent physical abrasion or burial (Hubbard & Pocock, 1972; Schumacher, 1979). Studies by Dodge & Vaisnys (1977), Bak (1978), Sheppard (1980), and Grigg & Dollar (1981) suggest that corals may be more tolerant of short-term sediment loading (providing muco-ciliary action can remove the particles), than of chronic high turbidity, in which reduced light levels and the need for constant sediment removal may exceed the coral's counteractive capacity (Stebbing, 1981) and lead to decreased calcification rates, reduced tissue energy replenishment by the zooxanthellae, or death (Squires, 1962; Dodge et al., 1974; Hudson, 1981a). Sedimentation tolerance also varies with the species (Marshall & Orr, 1931; Roy & Smith, 1971; Loya, 1972, 1976; Bak & Elgershuizen, 1976). However, recent observations suggest that adverse water conditions (due to sedimentation or pollutants) may also increase the surface layer of naturally occurring bacteria. These bacteria utilize coral mucus as an energy source, and can cause coral mortalities from bacterial toxins or anoxia (Mitchell & Chet, 1975; Garrett & Ducklow, 1975; Peters, 1978; Rublee et al., 1980).

Studies by Antonius (1977) and Dustan (1977) have suggested that the presence of "black line disease" (BLD), "shut-down-reaction" (SDR), and "plague" (characteristics like "white band disease" – WBD), in which coral tissues are sloughed off the skeleton within hours to days, might be associated with chronic increased sedimentation and temperature extremes on Florida Keys reefs. Despite recent field and laboratory observations on BLD (Antonius, 1973; Garrett & Ducklow, 1975; Ducklow & Mitchell, 1979), WBD (Gladfelter et al., 1977; Gladfelter, 1982), and SDR (Antonius, 1977), histopathological techniques at the light microscope level have been underutilized in examining the effects of physical injuries, environmental stresses, and the role of microorganisms, in the survival of the stony corals (Rinkevich & Loya, 1977, 1979; Schumacher, 1979; Peters et al., 1981). Histopathology has become an increasingly important tool for investigating the sublethal effects of environmental perturbations and diseases in marine organisms, and is useful in correlating physicochemical and physiological changes with those changes seen at the population and community level (Yevich & Barszcz, 1983).

The objectives of this study were twofold: (1) to survey four Caribbean reefs in order to determine the effect of increased sedimentation and turbidity on coral health and the incidence of these diseases, and (2) to use histopathological techniques to examine corals for the presence of microorganisms which might be associated with diseases or other abnormal conditions, and to determine their effects on the coral tissues (Table 1).

Table 1. Abbreviations for diseases, abnormal tissue conditions, and parasitic infestations of scleractinian corals

Disease or condition	Abbreviation	Citation
"Black line disease" (= "black band disease") bacteria and cyanobacteria	BLD (= BBD)	Antonius (1973) Garrett & Ducklow (1975) Mitchell & Chet (1975) Ducklow & Mitchell (1979) Antonius (1981a) Antonius (1981b) This paper
"White band disease" Gram-negative colonial bacteria	WBD	Gladfelter et al. (1977) Peters et al. (1983) This paper
Similar "white band disease" ovoid basophilic bodies similar to those found in WBD	SWBD	This paper
Beggiatoa and other bacteria – net formation	BN	Mitchell & Chet (1975) Ducklow & Mitchell (1979) This paper
"Shut-down-reaction"	SDR	Antonius (1977) Antonius (1981a)
"Stress-related-necrosis"	SRN	Dustan (1977)? Antonius (1981b)? This paper
Epidermal cell hyperplasia associated with chronic physical irritation	ECH	This paper
Tissue changes and necrosis associated with sedimentation and algal interactions	ASN	This paper
Nematopsis sp. spores	NS	This paper
Coccidean spores	CS	This paper
Unidentified microparasites (ciliates and amoebae)	UMP	This paper
Coral neoplasia	NPL	Lauckner (1980) – review Bak (1983) This paper

MATERIALS AND METHODS

Reef locations

The reefs were first examined in late July 1980 and were revisited in early August 1981. At Tague Bay, St. Croix (17° 45′ 48″ N, 64° 36′ 24″ W), most observations were made on the forereef, seaward and east of the reef crest "Swim Channel" (Fig. 1) in depths of 2–8 m. The Tague Bay reef is considered relatively pristine, without sediment or pollutant inputs, except during severe tropical storms (Rogers et al., 1982). However, many divers visit the reef each year (C. S. Rogers, pers. comm.). Off Puerto Rico, site 1

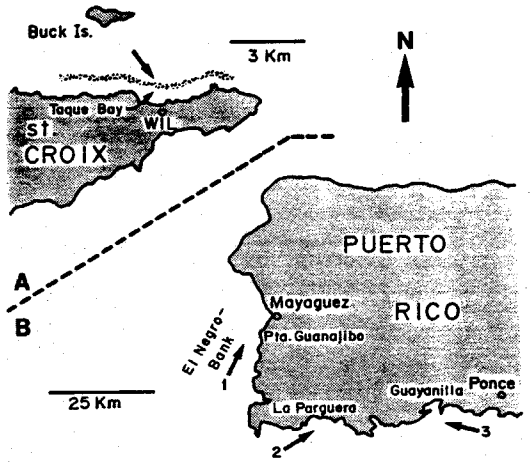

Fig. 1. Sampling sites. (A) Forereef off Tague Bay, St. Croix. (B) Reefs off Puerto Rico: site 1, El Negro-Bank Reef, 6 km west of Punta Guanajibo; site 2, La Parguera, patch reef northwest end of La Media Luna Reef; site 3, forereef west end of Cayo Maria Langa off Guayanilla Bay

was a shallow patch reef on the El Negro-Bank reefs located approximately 6 km west of Punta Guanajibo, south of Mayaguez (18°08'18"N, 67°14'36"W). This area has been little studied, and lacks the extensive reefs found at La Parguera, possibly as a result of river inputs, which may lower salinity and increase turbidity during the rainy season (Loya, 1976; R. J. Zimmerman, pers. comm.). Site 2, at La Parguera, was a patch reef at the northwest end of La Media Luna reef, on the landward side of Cayo Media Luna (17° 56' 24" N, 67° 03' 06" W). The reefs at La Parguera are the most well-developed reefs around Puerto Rico and have been extensively studied (Almy & Carrion-Torres, 1963; Glynn et al., 1964; Morelock et al., 1977; Rogers, 1977). High summer winds and seas prevented examination of the seaward reefs here. Site 3 was located at the western end of Cayo Maria Langa, a reef at the southern seaward entrance to Guayanilla and Tallaboa Bays (17° 58' 00" N, 66° 45' 28" W). This coastal area has been chronically stressed over the last two decades, receiving chemical and thermal effluents from several industries, an oil refinery, and a steam generating plant, and experiences increased turbidity and sedimentation from shipping traffic (Lopez, 1979). Water depths at the Puerto Rico sites averaged 2 to 3 m. Underwater visibility exceeded 15 m at the Tague Bay and El Negro sites. Sediments stirred up by summer winds reduced visibility to 9 m at La Parguera. Underwater visibility at the Guayanilla Bay reef was at most 3 m.

Field observations

At each site, a general survey of the coral composition of the reef was performed by divers during August 1981 with the aid of five 10 m transects at intervals of 2 m parallel

to the reef crest. Each colony underlying the weighted nylon line was identified to species and the portion of the colony underlying the line was measured to the nearest cm for a coverage analysis (Loya, 1972, 1977). The condition of each colony was noted. Healthy colonies were considered to be those having a uniform covering of evenly pigmented tissue over a skeletal morphology appropriate for the species, lacking scrapes or other lesions. Photographs were taken of the reefs, and of healthy and diseased corals.

Histopathology

Small whole colonies or portions of colonies (both apparently healthy and diseased) were removed from the reef with a masonry hammer and chisel, placed in numbered plastic bags with holes cut in them to drain sediment, and collected in a bucket before removing them from the water. Upon returning to shore, colonies were fixed in Helly's solution for 20 h and rinsed in several changes of freshwater for 24 h (Barszcz & Yevich, 1975). Samples were then packed in plastic bags containing diapers soaked in 70 % ethanol for preservation, and shipped to the University of Rhode Island. Subsamples for examination with electron microscopy techniques were removed by chisel from samples taken back to the laboratory, and fixed with a cold glutaraldehyde/formaldehyde solution (Peters et al., 1983).

Photographs were made of each colony using a Zeiss Tessovar low power magnifying camera to record the condition of each coral before decalcification. Tissues were washed in running water for 24 h, and trimmed for processing by standard techniques (Yevich & Barszcz, 1982). After embedding tissues in Paraplast (Sherwood Medical), 6 µm sections of oral, aboral and longitudinal polyp surfaces were cut. Slides were stained with Harris' hematoxylin and eosin (H & E), and Heidenhain's aniline blue method (HAB) for connective tissue. Additional stains used on some sections included modified Movat's Pentachrome, Periodic Acid Schiff (PAS)-alcian blue and PAS-alcian blue-hematoxylin, Fuelgen-picroaniline blue, Taylor's modification of Brown & Brenn's stain for Gram-positive and Gram-negative bacteria, Hirano-Zimmerman method (silver stain), and Grocott's method for fungi (Luna, 1968; C. A. Farley, pers. comm.). Photomicrographs were taken with a Zeiss Photomicroscope III. Samples for electron microscopy were treated and examined as described in Peters et al. (1983). Other colonies of healthy and suspected diseased corals were contributed for diagnosis by these methods from Rincon and Desecheo Islands, Puerto Rico, by V. P. Vicente; from Sandy Island, Grenadines, West Indies, by M. Goodwin; from reefs off Bonaire and Klein Bonaire, by T. van't Hof; from Grecian Rocks, Key Largo Marine Sanctuary, Florida, by J. Halas; and from the Coral Reef Microcosm at the Smithsonian Institution, Washington, D. C., by W. Adey and J. Johnson.

Adverse changes in water conditions were reported only at the Microcosm, including sediment resuspension and high nutrient levels (Adey, 1983). The Key Largo reefs have experienced gradually increasing sedimentation in recent years as well as temperature extremes (Dustan, 1977; Hudson, 1981a, b).

RESULTS

Reef observations

The four sampling sites are pictorially compared in Figure 2 A–D, and the results of the transect surveys for each reef are reported in Tables 2 and 3. Percent cover of coral colonies was highest at El Negro-Bank reef (26.28 %), while the Tague Bay forereef had a greater number of species and colonies per transect. The Tague Bay and La Parguera sites were most similar in number of species per transect and area coverage. All of these statistics were significantly lower at the Guayanilla Bay site. The most common coral occurring on all reefs was *Porites astreoides*, followed by *Agaricia agaricites*, *Montastrea annularis*, *Favia fragum*, and *Stephanocoenia michelinii*.

The most noteworthy damage to corals was found on colonies of *Acropora palmata* on both the Tague Bay and El Negro reefs. At Tague Bay, many colonies had been toppled during Hurricanes Frederic and David of 1979 to form a rubble zone (C. S. Rogers, pers. comm.; Rogers et al., 1982), but others remained upright and appeared healthy. Regrowth of damaged colonies was also evident. Of the living colonies, approximately 20–50 % of the branches exhibited signs of WBD, suggesting a major epidemic was underway. In addition, colonies of *Acropora cervicornis* at the base of the forereef, and one colony of *Agaricia agaricites* had tissue sloughing off the skeleton. Two cases of BLD were also found on widely separated specimens of *Colpophyllia natans* (seen in 1980) and *Montastrea annularis* (seen in 1981). However, in 1981, the disease line was present in 10 colonies of *Diploria strigosa* within a 2 m radius at the reef crest swim channel.

No WBD was observed on any colonies of *A. palmata* at El Negro in 1980, but tissues were sloughing off the base of branches of a few small colonies in 1981. Also, in 1981, there was evidence that most shallow colonies of *A. palmata* had died, but patches of live tissue were rapidly overgrowing the algal-covered skeletons. Deeper *A. palmata* were not affected. Although BLD was present on three widely separated colonies of *D. strigosa* and *M. annularis* in 1980, no examples were found in 1981. The general survey of the La Parguera patch reef did not reveal any BLD, but one colony each of *A. cervicornis* and *Diploria labyrinthiformes* were sloughing tissue off their skeletons in 1981.

A number of colonies on these three reefs were heavily infested with algal mats or lawns, developed by territorial damselfish (principally *Eupomacentrus dorsopunicans*). Colonies of *Montastrea annularis*, particularly the knobby variety, were most often affected, as well as *Porites porites* and *Stephanocoenia michelinii* (Fig. 2 E–F). Algal "tumors" were observed infrequently on *Acropora palmata* colonies. These "tumors" consisted of knobs of coral tissue and skeleton growing around a core of filamentous algae, and were also caused by damselfish.

At each of the four reefs examined, coral tissue on many mound-type colonies of various species, including *M. annularis*, *Diploria* spp., *Siderastrea* spp., often appeared dark, with ill-defined polyps at the edge of the colony, next to accumulations of filamentous and calcareous algae and sediment on the eroded skeleton. The type of algal accumulations could be differentiated by the presence of damselfish, appearance of the colony edge zone, and the location of the algal patch. The only other visible signs of coral distress were the appearance of patches of whitish filaments, composed of bacteria *(Beggiatoa)*, on the surface of two *Porites astreoides* colonies, one at the El Negro site

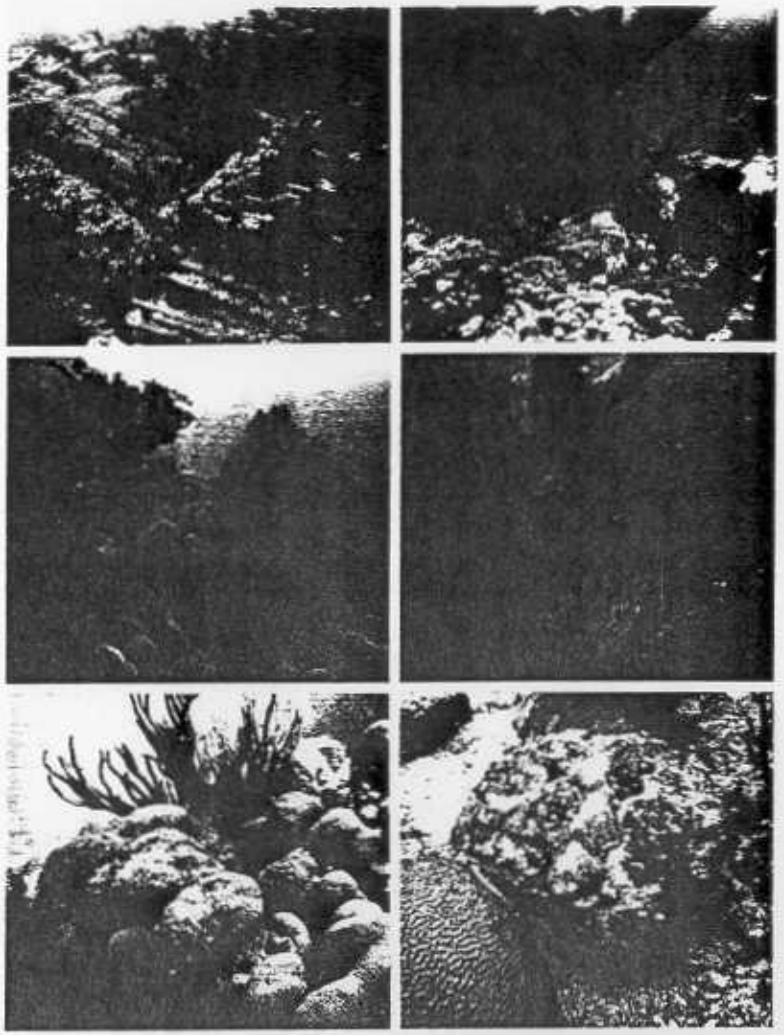

Fig. 2. Reef conditions at the sampling sites. (A) Tague Bay forereef, St. Croix. (B) El Negro-Bank reef shallows. (C) La Parguera patch reef, coarse sediments resuspended by summer winds. (D) Guayanilla Bay, Cayo Maria Langa reef, note turbidity, erosion of dead corals, and scarcity of living corals. (E) Damselfish with territorial algal lawn on *Montastrea annularis* colony, La Parguera. (F) Algal tufts on surface on *Stephanocoenia michelinii* colony, El Negro

Table 2. Statistics calculated for five transects at each of the three reef sites, Puerto Rico, and the Tague Bay forereef, St. Croix, as average values per transect. Standard deviations in parentheses. H'_N = Shannon-Weaver index of diversity. H'_C/H'_{max} = Pielou's evenness index, a measure of the degree of dominance in a sample. (After Loya, 1976)

Location	Total No. species	No. species	No. colonies	Cover %	H'_N	H'_C/H'_{max}
Tague Bay	7	5.00 (1.00)	18.00 (6.04)	13.96 (6.62)	1.316 (0.163)	0.818 (0.033)
El Negro-Bank Reef	5	3.40 (0.89)	15.40 (4.04)	26.28 (11.26)	0.921 (0.293)	0.779 (0.091)
La Parguera	11	4.40 (2.19)	8.40 (4.51)	10.02 (7.22)	1.130 (0.640)	0.629 (0.355)
Guayanilla	4	1.60 (0.89)	2.20 (1.30)	0.90 (0.43)	0.347 (0.491)	0.354 (0.485)

Table 3. Relative abundance of coral species at the Tague Bay forereef, St. Croix, and the three reef sites, Puerto Rico. Index for relative abundance of corals: average number of colonies per 10 m transect is between 0.1–1.0 = rare; 1.1–2.0 = sporadic; 2.1–4.0 = common; 4.1–7.0 = abundant; over 7.1 = dominant; after Loya (1976). —— = not found underlying the transect and not observed within the total transect area. obs. = observed within the total transect area of the reef site

Species	Tague Bay	El Negro	La Parguera	Guayanilla
Acropora cervicornis	–	–	rare	–
Acropora palmata	rare	abundant	–	rare
Agaricia agaricites	dominant	rare	rare	rare
Colpophyllia natans	obs.	–	rare	–
Dichocoenia stokesii	–	–	obs.	–
Diploria labyrinthiformes	–	–	obs.	–
Diploria strigosa	–	rare	rare	obs.
Eusmillia fastigiata	–	–	rare	–
Favia fragum	rare	obs.	rare	obs.
Montastrea annularis	obs.	obs.	common	obs.
Montastrea cavernosa	–	–	rare	–
Porites astreoides	abundant	sporadic	rare	sporadic
Porites porites	common	dominant	–	–
Siderastrea radians	–	–	rare	rare
Siderastrea siderea	rare	obs.	rare	–
Stephanocoenia michelinii	rare	obs.	obs.	obs.

and one at the Guayanilla site (noted in separate years), and on one colony of *Diploria labyrinthiformes* from Tague Bay (seen in 1980).

Histological examinations

This survey revealed several surprises, particularly the presence of microparasites within healthy-appearing, as well as necrotic, coral tissues.

Reproduction

Table 4 contains the observations of gonad development, and/or the presence of planula larvae in the colonies examined for each site. Data for both years were combined, with note made of significant differences between years for some of the samples collected. The largest number of samples of *Acropora cervicornis* and *Acropora palmata* were collected in 1981 for another study (see below), and revealed that not all colonies from this site developed gonads concurrently. Three colonies of *A. palmata* from El Negro (1981) contained planula larvae and were lacking gonads, while others contained ova and sperm. Colonies of this species collected from the Guayanilla Bay site may have been immature, since these colonies were all small (<12 cm high) and all other species collected from this site contained gonads. The colonies of *A. cervicornis* from La Parguera were necrotic. Presence of gonads in both species from Tague Bay did not correlate with WBD symptoms (76 and 38 % of diseased *A. cervicornis* and *A. palmata*, respectively, had developing gonads). The data also suggest differences in gonad development and spawning between different species on the four reefs, and between the reefs, especially for *Porites astreoides* at the Guayanilla Bay site. Differences in the collections from 1980 and 1981 indicate individual variability and effects of seasonal cycles on reproduction.

Coral-algal interactions

Colonies infested by damselfish algal lawns experienced cellular erosion and tissue necrosis as algae invaded the skeleton (Fig. 3 A–B). Although the degeneration of the tissue may have been initiated by the damselfish, in some cases the algae were in direct contact with calicoblast tissue which exhibited a "blistering" necrosis of the cells (ASN). This ASN was observed in colonies of *Diploria strigosa*, *Sidereastrea siderea*, and *S. radians*, as well as in *Montastrea annularis*, *Porites porites*, and *Stephanocoenia michelinii*, from all the reef sites, and in the Grenadines' samples of *M. annularis*, *Diploria clivosa*, *D. strigosa*, *Meandrina meandrites* and *S. siderea*. The darkened edge of tissue (Fig. 3 C) adjacent to sediment-algal accumulations on the skeletons of mound-type colonies of *Colpophyllia natans*, *D. strigosa*, *Favia fragum*, *M. annularis*, *Porites astreoides*, *S. radians*, *S. siderea*, and *S. michelinii*, as well as the branching corals *Acropora cervicornis* and *P. porites*, had changed from the normal epidermal structure (tall columnar cells uniformly interspersed with mucus-secretory cells) to a hyperplasia (ECH) of the columnar epidermal cells (Fig. 3 D). Various colonies from all four locations were affected by this process. Additionally, elongated algal filaments, with a basophilic wall, containing spherical bodies, were found in the tissues of colonies of *Porites* spp. and *S. radians* from El Negro and Guayanilla Bay in 1981 (Fig. 3 E). The coral tissue responded by developing a hyperplasia of columnar cells and fibrocytes in an attempt to wall off these algae.

One unexpected finding was the occurrence of basophilic sheathed structures, which appeared to be associated with the boring green skeletal alga *Ostreobium* sp. (Fig. 3 F). These modified filaments appear to compress the calicoblast layer of tissue (consisting of the gastrodermal cells, mesogloea, and calicoblast epithelium) and distort the normal structure of the tissues. Filaments can also be seen in cross-section as they invade the gastrodermal cells. These structures were found in colonies of *Agaricia*

Table 4. Development of ova (O), sperm (S), ova and sperm (O/S), and presence of planula larvae (L) in colonies examined from each reef site
= total number of colonies of each species examined from each site. * = found in 1980 only. ** = found in 1981 only

Species	Tague Bay					El Negro					La Parguera					Guayanilla				
	#	O	S	O/S	L	#	O	S	O/S	L	#	O	S	O/S	L	#	O	S	O/S	L
A. cervicornis	54**	13	–	21	–						3**	–	–	–	–					
A. palmata	64	5	2	22	–	9	3	–	2	3**						3	–	–	–	–
A. agaricites	1	–	–	–	–	4	1	3	–	–	2	–	1	–	–	2	2	–	–	–
C. natans	5	3	–	–	–						1	–	–	–	–					
D. labyrinthiformes	1	–	–	–	–						4	2	–	–	–					
D. strigosa						5	4	–	–	2*	4	2	–	–	–	11	5	–	–	–
F. fragum						1	–	–	1	–						4	1	–	3	–
M. annularis	10	3*	–	–	–	6	5	–	–	–	9	4	–	1	–	3	1	–	1	–
P. astreoides	1**	–	–	–	–	5	1**	–	–	–	3**	–	–	–	–	11	4**	–	2**	–
P. porites	3	–	–	–	–	4	–	–	–	–	1	–	–	–	–					
S. radians						1	–	1	–	–	1	–	–	–	–	5	1*	2**	–	–
S. siderea	1	1	–	–	–	1	–	–	–	–	3	1*	–	–	–	1	1	–	–	–
S. michelinii	4	2	1	–	–	2	1	1	–	–	2	1	1	–	–	2	–	1	–	–

agaricites, Diploria labyrinthiformes, D. strigosa and *Montastrea annularis* from the Puerto Rican reefs only. Lukas (1973) did not believe that these endolithic algae could penetrate the living coral tissue, although she had observed the filaments to extend to the edge of the coral skeleton. Upon viewing these slides, she could not determine the nature of these algal structures (pers. comm.). Additional studies on this phenomenon are now underway.

Bacteria-associated diseases

The total number of colonies collected for each species is shown in Table 5 along with the number of colonies in which necrosis, tissue changes, and microorganisms were observed in each species and at each location. Colonies of *Agaricia agaricites* and *Diploria strigosa* exhibited increased muco-secretory cell development at the Guayanilla reef site (Fig. 4 A–B). *Favia fragum* from El Negro and Guayanilla showed similar responses of the epidermis, but were more variable. Changes were also seen in coral tissues from the Coral Reef Microcosm (Fig. 4 C). Three of these colonies possessed abundant active mucosecretory cells, but other samples exhibited loss of mucus cells from the epidermis (Fig. 4 D). Although tissue on all of these colonies had appeared superficially normal at the time of collection (except for sloughing of tissue around the base of each colony or branch), all of the tissues were in varying stages of degeneration and necrosis. This same condition was found in colonies of *Agropora palmata, A. cervicornis* and *Diploria labyrinthiformes* collected from La Parguera and El Negro. None of these necrotic corals contained visible microorganisms, and only the specimen of *D. labyrinthiformes* showed developing gonadal tissue.

In contrast, all of the colonies of *Acropora palmata* and 80 % of the *A. cervicornis* colonies examined from the Tague Bay reef, including those that appeared healthy without signs of WBD (Fig. 4 E), contained ovoid basophilic bodies up to 40 µm or more in diameter, surrounded by a refractile wall (Fig. 4 F). These Gram-negative bodies were found throughout the tissue remaining on the skeleton, scattered in the mesenteries and calicoblast tissue of the gastrovascular canals. Examination of these bodies by electron microscopy revealed that they are composed of rod-shaped bacteria in a colony, surrounded by coral cells (Peters et al., 1983). Microscopic counts of colonies per area of tissue showed that the number of bacterial colonies was significantly higher in diseased corals, although numbers in both obviously diseased and apparently healthy corals overlapped (diseased range 0.1 – 1.8, $\bar{x} = 0.62$, versus healthy range 0.0 – 0.8, $\bar{x} = 0.37$, $p < 0.005$, $n = 25$, two sample t-test). There was no correlation between presence or absence of gonads and number of bacterial colonies per unit of tissue. These bacteria were also found in samples of *Acropora* spp. from Bonaire (with indications of disease), the Grenadines and the Florida Keys. Similar ovoid basophilic bodies were also found in *Agaricia agaricites* in which tissue was sloughing from the base of the colony, and in one-third of the apparently healthy colonies of *A. agaricites* and *Stephanocoenia michelinii*, from widely separate locations on the four reefs. Two-thirds of the *Porites astreoides* examined contained similar ovoid basophilic bodies in the mesogloea of the tentacles. These bodies did not appear to be associated with any necrosis and differed from the WBD-associated bacterial colonies in that the "rods" within the body are larger (Fig. 5 A), crescent-shaped, and break apart from one another very easily in these

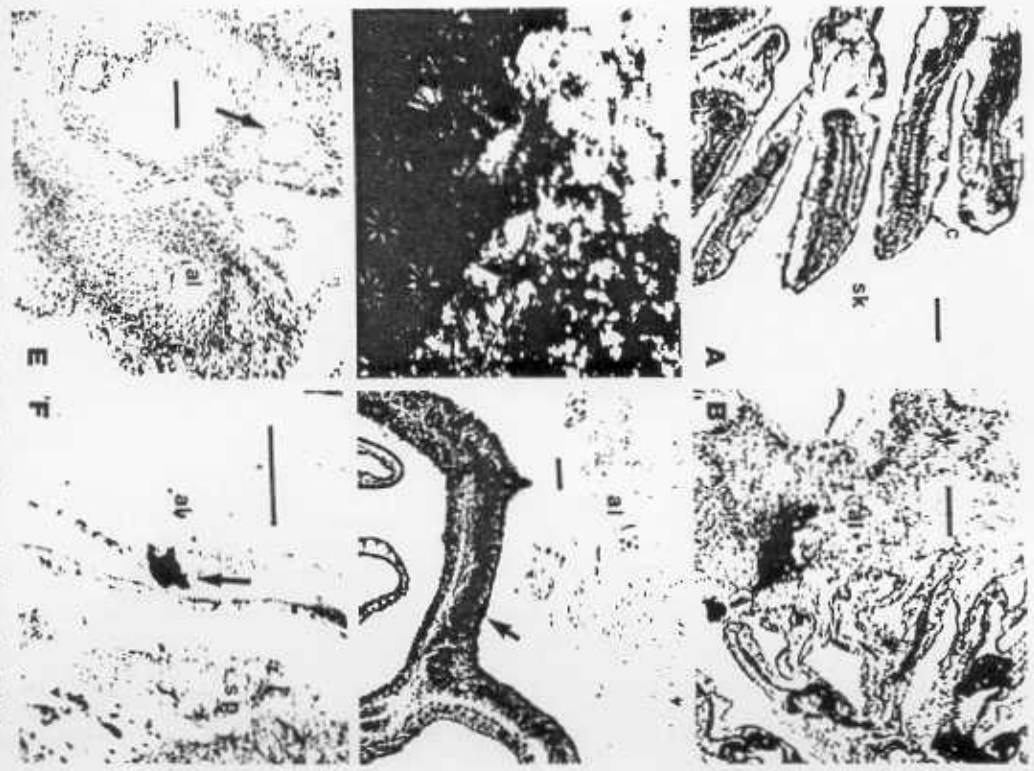

Fig. 3. Coral lesions associated with physical injuries from algae and sedimentation. Scale bars = 50 μm (A) Cross-section of portion of healthy polyp of *Stephanocoenia michelinii*, stained with H & E, La Parguera. (B) Cross-section of polyp damaged by algal erosion. H & E. La Parguera. (C) Gross appearance of sediment-algal border of *Montastrea annularis* colony, note darkened tissue. Tague Bay. (D) Photomicrograph of epidermal cell hyperplasia next to sediment-algae accumulation. *M. annularis*, H & E. El Negro. (E) Fibrocytic hyperplasia in tissue of *Siderastrea radians* chronically irritated by algae. H & E. Guayanilla Bay. (F) Unidentified endolithic algae-associated structure invading gastrodermis of *Diploria strigosa* through calicoblastic epithelium. H & E. Desecheo Island. al = algae; c = calicoblastic epithelium; e = epidermis; g = gastrodermis; sk = former position of skeleton; sp = spermary

Table 5. Necrosis, tissue changes and microorganisms observed in coral tissues, listed by species (in which numbers refer to the total number of colonies found to be affected) and by location (listing the number of colonies of each species affected). # = total number of colonies of each species examined from all sites. See Table 1 for abbreviations. UMP# = unidentified microparasite of the type indicated. The final category is for unidentifiable spherical basophilic bodies

Species and location	#	SRN	tissue changes	WBD	SWBD	BLD	BN	NS	CS	UMP #1 ciliate	UMP #2 ciliate	UMP #3 amoeba	unid. bas bodies
A. cervicornis	57	1		44									
A. palmata	76	3		64							1		
A. agaricites	9		2	3									
C. natans	6					2							
D. labyrinthiformes	1						1						
D. strigosa	20		11			2			1				
F. fragum	5		3										
M. annularis	28	1				1							3
P. astreoides	20		1		13		1		6	15			
P. porites	8							4		7			3
S. radians	7											3	
S. siderea	6											1	
S. michelinii	10			2									
Tague Bay		1 *Ma*		44 *Ac* 64 *Ap* 1 *Aa* 1 *Sm*	1 *Pa*	2 *Cn* 1 *Ma*	1 *Dl*	2 *Pp*	1 *Pa* 3 *Pp*	1 *Pa*	1 *Ap*		1 *Ma* 2 *Pp*
El Negro		3 *Ap*		2 *Aa*	4 *Pa*	2 *Ds*	1 *Pa*	2 *Pp*	1 *Ds* 3 *Pa*	3 *Pa* 4 *Pp*			1 *Ma* 1 *Pp*
La Parguera		1 *Ac* 1 *Dl*		1 *Sm*	2 *Pa*					2 *Pa*			
Guayanilla Bay			2 *Aa* 11 *Ds* 3 *Ff* 1 *Pa*		6 *Pa*				2 *Pa*	9 *Pa*		3 *Sr* 1 *Ss*	1 *Ma*

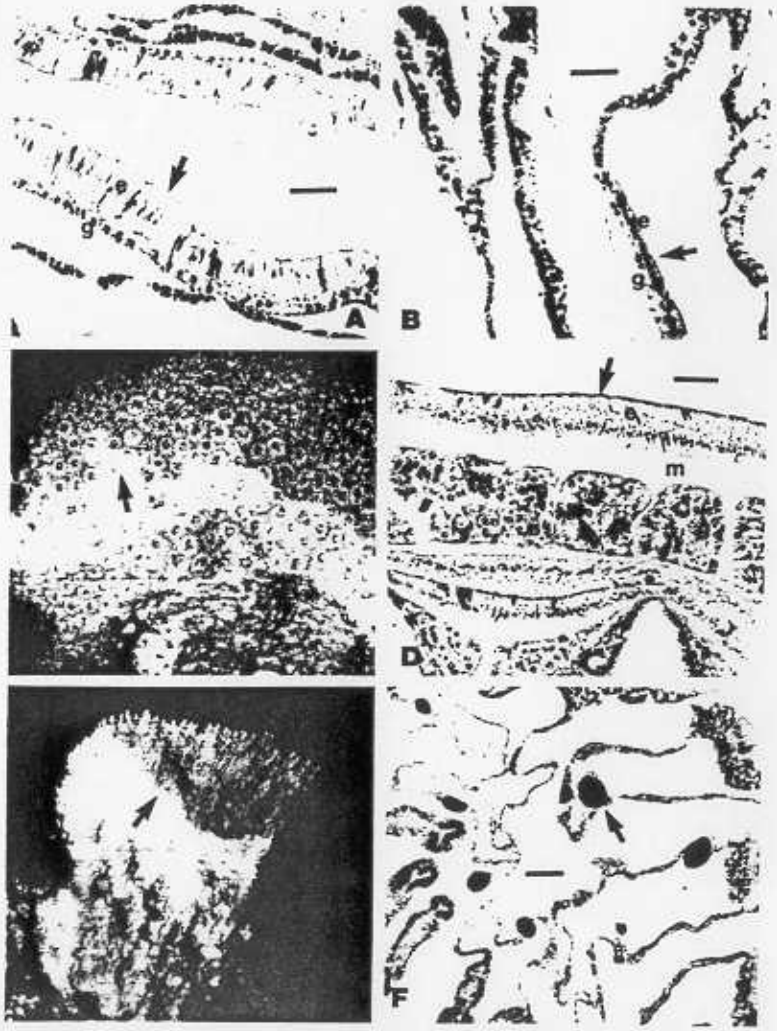

Fig. 4. Tissue changes and necrotic conditions. Scale bars = 50 µm. (A) Photomicrograph of healthy tissue from *Agaricia agaricites*, H & E, El Negro. (B) Epidermal erosion of *A. agaricites*, H & E, Guayanilla Bay. (C) Healthy appearing *Montastrea annularis* with tissue sloughing off the base of the colony, after fixation, Coral Reef Microcosm. (D) Photomicrograph of abnormal tissue from similarly afflicted *M. cavernosa*, H & E, Coral Reef Microcosm. (E) Sloughing of tissue from branch of *Acropora palmata* in "white band disease", Tague Bay. (F) Ovoid Gram-negative bacterial colonies in tissues of diseased *A. palmata*, H & E, Bonaire. e = epidermis; g = gastrodermis; m = mesogloea

preparations. They have not yet been examined by electron microscopy to determine their true nature.

Colonies of *Colpophyllia natans*, *Diploria strigosa* and *Montastrea annularis*, from the four reef sites and Key Largo, were collected in order to examine the effects of BLD microorganisms on the coral tissue (Fig. 5 B). Necrosis of tissue occurred only at the disease line, which contained a variety of microorganisms, including ciliates, cyanobacterial filaments, spiral and rod-shaped bacteria (Fig. 5 C). The remainder of the coral tissue was healthy, with gonads and planula larvae present (Table 3). However, the *M. annularis* sample from Tague Bay showed patches of necrotic tissue over the entire surface of the sample.

Other microparasites

Besides the bacteria, a number of protozoans were found in otherwise apparently healthy coral tissue. Thick-walled ovoid capsules (14 µm × 17–20 µm) were found in the calicoblast epithelium of *Porites porites* colonies (Fig. 5 D). One sample of this species from the Coral Reef Microcosm was especially infested. These have tentatively been identified as a sporozoan, *Nematopsis* sp. (C. A. Farley, pers. comm.). These spore capsules stain intensely red with HAB, lightly with PAS, and not at all by the Fuelgen technique (although this stain may not have worked properly due to decalcification of the coral tissue). These sporozoans appeared to cause an hypertrophy of the calicoblast epithelial cells, with an increase in eosinophilic intracellular granules. They are classified in the family Porosporidae, with a life cycle previously reported to include crustacean and mollusc hosts (Kudo, 1966). Another type of spore was observed in the lobes of the mesenterial filaments in *Diploria strigosa* and *Porites astreoides* in 1981 from the Tague Bay, El Negro, and Guayanilla sites (Fig. 5 E), and also found in mesenterial filaments of *Montastrea cavernosa* and *Meandrina meandrites* from Rincon, Puerto Rico. These latter spores, 6–7 µm × 12 µm, with 3 or more basophilic bodies in each, are Fuelgen positive, and have been tentatively identified as coccidean spores (F. Kern and C. A. Farley, pers. comm.). No other stages of these sporozoan life cycles were observed.

Large ciliates were often found feeding in association with necrotic tissue, but also occasionally found on the surfaces of healthy corals (Fig. 5 F). Colonies of *Porites astreoides* and *P. porites* contained a ciliate (C. A. Farley, pers. commun.), in the gastrodermal cells lining the gastrovascular canals in these species (Fig. 6 A). This ciliate exhibits one macronucleus and one or more micronuclei, and is most easily seen with the HAB when the cytoplasm stains light blue. Another ciliate was found in the epidermis of one colony of *Acropora palmata* from Tague Bay, which was originally collected to look at a scraped lesion on its surface. The entire epidermis of this sample was necrotic and sloughing, with these ciliates embedded in the cell layer (Fig. 6 B). Amoebae were found in the calicoblastic epithelium of both *Siderastrea* spp. from Guayanilla Bay. The concentration of amoebae obliterated the calicoblast cells (Fig. 6 C). One colony of *Colpophyllia natans* from the Grenadines contained worm-like organisms (P. Yevich, pers. comm.) embedded in a layer of enlarged necrotic gastrodermal cells (Fig. 6 D). Additionally, spherical basophilic bodies were occasionally observed in the mesenterial filaments of *M. annularis* and *P. porites* colonies from all locations except La Parguera. More recent observations on the ciliates (UMP#1 and UMP#2, Figs 6A and 6B) indicate that these structures are probably nematocysts in various stages of development (#1) or degeneration (#1 and #2).

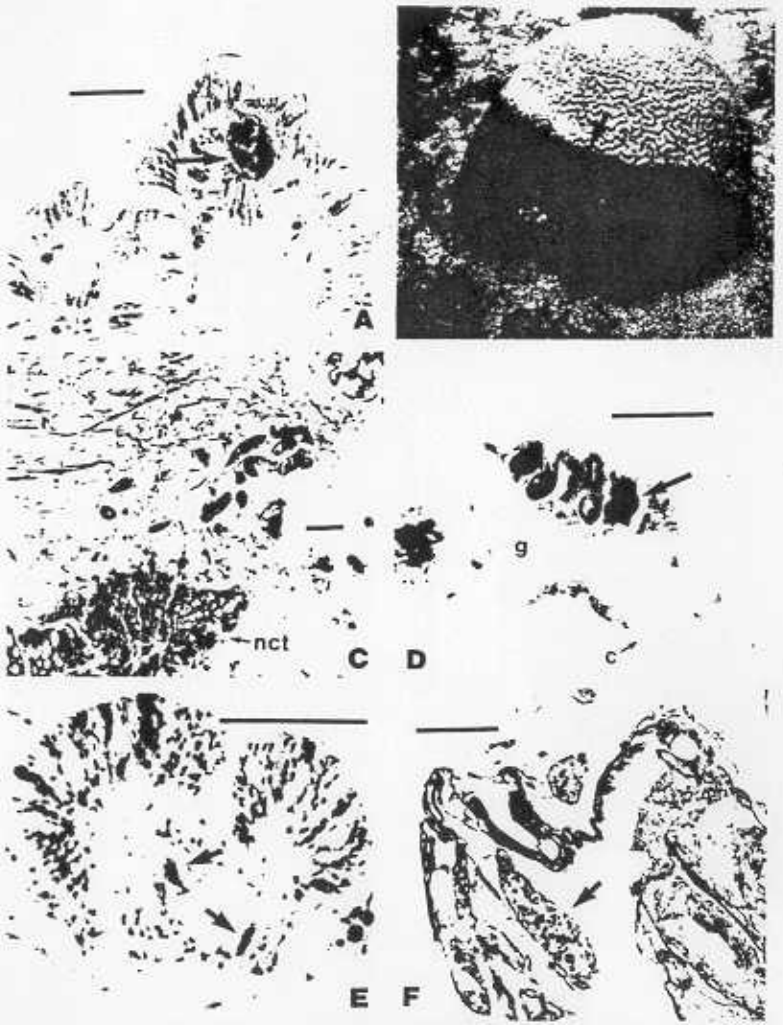

Fig. 5. Coral diseases and microparasites. Scale bars = 50 µm. (A) Basophilic body similar to that found in "white band disease" coral tissue from colony of *Porites astreoides*, HAB, La Parguera. (B) "Black line disease" on colony of *Diploria strigosa*, El Negro. (C) Microorganisms of "black line disease" and necrotic coral tissue, *D. strigosa*, HAB, El Negro. (D) *Nematopsis* sp. spores in calicoblastic epithelium of *P. porites*, HAB, El Negro. (E) Unidentified coccidean spores in mesenterial filaments of *P. astreoides*, H & E, El Negro. (F) Ciliates associated with necrotic *D. strigosa* tissue, H & E, Coral Reef Microcosm. c = calicoblastic epithelium; g = gastrodermis; nct = necrotic coral tissue

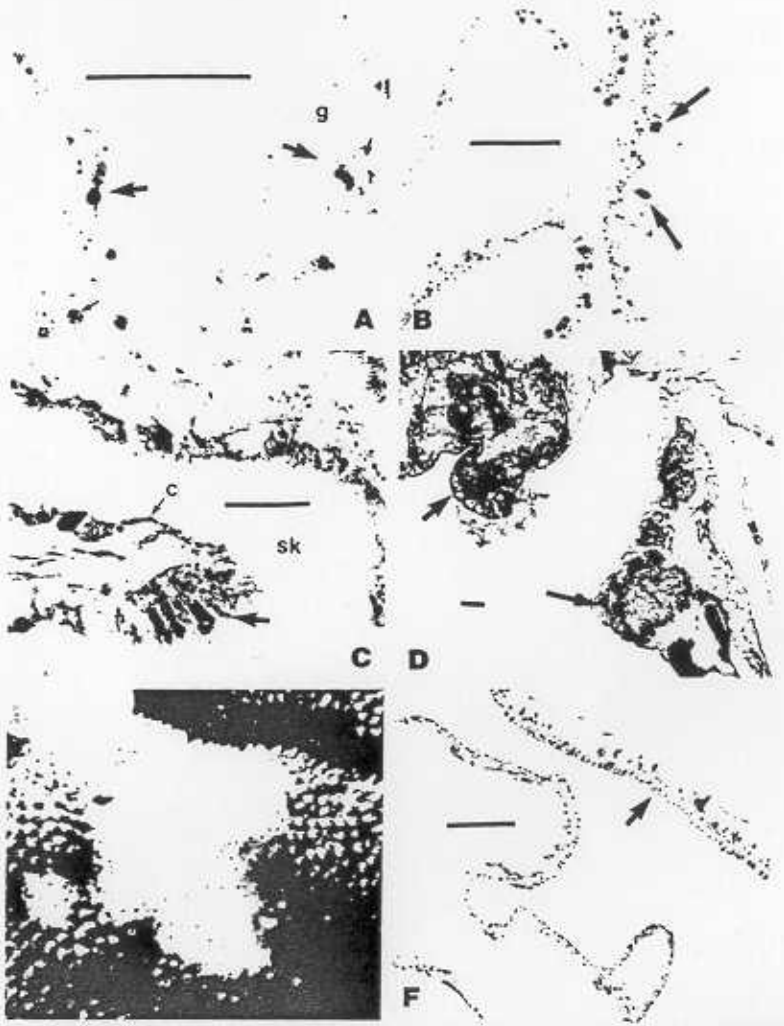

Fig. 6. Additional microparasites of corals, and skeletal abnormalities. Scale bars = 50 µm. (A) Unidentified ciliate (#1) in gastrodermal cells of *Porites porites*, HAB, El Negro. (B) Unidentified ciliate (#2) destroying epidermis of *Acropora palmata*, HAB, Tague Bay. (C) Unidentified amoebae (#3) infesting calicoblastic epithelium of *Siderastrea radians*, H & E, Guayanilla Bay. (D) Endoparasitic worm in gastrodermal tissue of *Colpophyllia natans*, H & E, Grenadines, (E) Specimen of *A. palmata* with skeletal tumor, Key Largo Marine Sanctuary. (F) Photomicrograph of *A. palmata* tumor tissue. Note lack of zooxanthellae in gastrodermis, and changes in tissues surrounding the gastrovascular canals, HAB, Key Largo. c = calicoblastic epithelium; g = gastrodermis; sk = former location of skeleton

Coral tumors

Skeletal abnormalities were found in samples of *Acropora palmata* collected from Grecian Rocks, Key Largo (Fig. 6 E). Histopathological examination of the tissues revealed rapid proliferation of the calicoblast layers of the gastrovascular canals, producing a bulging porous skeleton overgrowing the polyps. Although an epidermal cell layer covers the surface of this area, the cells differ structurally and zooxanthellae are absent from the gastrodermis in all areas of this growth (Fig. 6 F). Because of the appearance of uncontrolled growth, destruction of polyps, changes in cell structure and function, and spread of the abnormal skeletal growth along a branch, this condition appears to be a true neoplasia of the coral (Cheney, 1975; Bak, 1983; J. C. Harshbarger, pers. comm.). These tumors will be discussed more fully in a future paper.

DISCUSSION

Except for an earlier note (Peters et al., 1983), this is the first report of bacterial colonies and protozoal microorganisms living within coral tissues, and the first observations that some of these microorganisms may be harmful to the corals. Although Lauckner (1981) reported no studies on viral or bacterial diseases in the cnidarians, he believed such diseases would be found, especially since Burkholder & Burkholder (1958) could not demonstrate antimicrobial activity in tissues of *Acropora palmata*, *Porites porites* and *Montastrea* sp. Commensal and parasitic protozoans have been reported from other cnidarians (Lauckner, 1981; T. Jacques, pers. comm.), but the only previous reports of parasites in corals were on damage to tissues of the Pacific *Porites* spp. by trematode metacercariae (Cheng & Wong, 1974), and endoparasitic copepods in a number of Indo-Pacific and Caribbean species (Stock, 1975; Butter, 1979; Herriott & Immerman, 1979; other literature reviewed in Lauckner, 1980).

In a review of coral pathology, Antonius (1981a) suggested that the tissue sloughing observed in WBD was a physiological reaction to some stress. He could not find any microorganisms which might be removing the tissue, the disease could not be spread by inoculation techniques, and the coral did not recover when treated with antibiotics (Antonius, 1981b). The histopathological examinations performed here demonstrated that unusual Gram-negative bacterial colonies were present in *Acropora* and *Agaricia* spp. which show signs of WBD. These bacteria were also found in apparently healthy colonies on the same reefs. Whether these bacteria are responsible for this disease has yet to be proven, but, if they are, it is possible that a threshold concentration in colonies is necessary before the tissue begins sloughing. These bacteria may be impervious to traditional antibiotics, or, when the disease finally becomes apparent, the tissue damage is irreversible. This might explain Antonius' observations. Other colonies of *Acropora palmata* and *A. cervicornis*, as well as colonies of *Diploria labyrinthiformes* and *Montastrea annularis* in this study did not have observable microorganisms within their tissues, yet necrosis was evident. Similar sloughing of coral tissues from several species of Microcosm corals revealed degenerative changes in cell morphology and function, which might be related to the high nutrient or sedimentation levels in the tank. Antonius (1981b) also reported tissue sloughing in colonies of *Montastrea annularis* and *Diploria strigosa*, and Dustan (1977) observed this in colonies of *Mycetophyllia ferox*, which they correlated with environmental stress. However, the corals were not examined histologically.

I propose the term "Stress-Related-Necrosis" (SRN) to differentiate cases in which corals show signs of WBD and in which degenerative changes in cell structure are observed in the absence of obvious pathogens (as determined by microscopic examination of the fixed and embedded tissues), from WBD, in which ovoid Gram-negative bacterial colonies are found in the coral tissues. However, future studies utilizing electron microscopy or other techniques may find a virus or bacteria associated with this disorder. Dustan (1977) reported successful inoculations of apparently healthy colonies of *Mycetophyllia ferox*, *M. larmarkiana* and *Colpophyllia natans* with tissue from "plague"-infected colonies of *M. ferox* and *C. natans*, suggesting species-specific differences in resistance, or perhaps different pathogens responsible for the necrosis and sloughing of tissue. SRN may be a reversible condition. Antonius (1981b) noted that it was rarely lethal for the whole colony in *Diploria strigosa*, *Montastrea annularis*, and *M. ferox*. Gladfelter et al. (1977) noted an occasional reversal of tissue sloughing in *Acropora palmata* afflicted with WBD, with tissue regrowth. These colonies may not have been infected with the bacteria, or may have recovered from the infection. In all these cases of sloughing tissue, tissue is lost first from the basal edge of the colony. This tissue may be metabolically older, or may be more shaded and less likely to be in a favorable position for zooxanthellar nutrient acquisition. Thus, necrosis and sloughing of the tissue occurs first in this region.

During this study, no visible signs of diseases were found on the shallower reefs of Guayanilla Bay. However, there had been a noticeable loss of living coral cover and erosion of reef structure. Both were dependent on the tolerance of individual colonies, as well as the species, to chronic turbidity and sedimentation (Morelock, 1979). Histopathological examination of the corals at this site revealed signs of stress, including increased mucus-secretory activity and epidermal cell erosion. Only one possible case of bacterial colonization, indicated by a whitish film of *Beggiatoa* filaments, was seen. Laboratory studies by Ducklow & Mitchell (1979) and Rublee et al. (1980) indicated that mucus production and surface bacterial populations increased with increasing turbidity or other irritants, leading to the development of anoxic conditions on the surface of the coral, and the appearance of the sulfur-reducing bacterium *Desulfovibrio*. Finally, heterotrophic sulfide-oxidizing *Beggiatoa* filaments form a net over the necrotic tissue (Mitchell & Chet, 1975). Another unusual finding was the presence of amoebae that destroyed the calicoblastic epithelium in four colonies of *Siderastrea* spp., found only at this site. While skeletal growth may be lower here because the turbidity-reduced light levels are adversely affecting the zooxanthellae-coral calcification system, the protozoans may also interfere with this process. Although weakened by constant heavy mucus production and microparasites, it appears that the corals remaining at Guayanilla Bay are still able to meet their metabolic energy expenditures. All species, except the *Acropora palmata*, contained developing gonads (although the viability of any planula larvae produced is unknown). Rinkevich & Loya (1977) and Peters et al. (1981) reported reduced or absence of gonad development in corals exposed to chronic oil pollution, and suggested that other environmental stress conditions might inhibit reproduction in corals.

As found in this study, reefs under chronic sedimentation stress may not be totally

destroyed (Roy & Smith, 1971). The presence of young *Acropora palmata* indicates that recruitment from adjacent reefs may occur. However, Morelock et al. (1979) noted that the substrate here has been covered by algae and sediment accumulations, which reduces the potential settling sites for planulae, and also damages established colonies. The histopathological observations reveal that edge zone coral tissues develop an epidermal cell hyperplasia, probably due to chronic irritation (Cheville, 1976). This is influenced by colony morphology as well as sediment loading. Colonies are constantly sweeping particles which fall on their surfaces to the edge of the tissue, where they may be removed by water currents. If the sediment is not removed, it may become a substrate for algal colonization. Walker & Ormond (1982) noted that increased algal growth may increase the sediment load experienced by corals, by trapping sediment on enhanced filamentous algal growth. Schumacher (1979) found that the number of functioning mucus cells decreases with increasing sediment load and are not replaced. Eventually, the cells at the colony border may die. Hughes & Jackson (1980) noted that partial colony mortalities are difficult to detect in situ, unless time-series photographs are examined, because skeleton which appears as a result of the death of coral tissue is readily overgrown by algae. As this phenomenon also affected colonies on the other reefs examined in this study, along with *Beggiatoa* nets and outbreaks of disease and microparasites, these observations suggest that perhaps localized changes in water quality may also be important in controlling the health of corals on reefs. Furthermore, the interaction of pathogens and microparasites with physical stress and injuries may be as important in regulating natural populations of corals as in other invertebrates (Anderson & May, 1981), and may also be influenced by species-specific susceptibility to invasion by a pathogen.

For the reefs studied here, it appears that daily and seasonal variations in sedimentation and turbidity are more likely to occur than acute salinity or temperature perturbations. For example, sedimentation rates are increased, on the deeper reefs of the El Negro-Bank (Loya, 1976), by rainy season river inputs of sediments which do not accumulate on the shallower reefs. However, winter storms associated with northwesterly fronts may resuspend these sediments, and may have contributed to the death of *A. palmata* colonies in 1980. Corals were actively shedding sediment at the La Parguera patch reef during this study, but high winds and seas, common on summer days along the southern coast of Puerto Rico (Almy & Carrion-Torres, 1963), are reduced in the winter. The amount and duration of stress experienced by an individual coral colony during these infrequent episodes of sedimentation and turbidity, as well as during intense tropical storms (Endean, 1976; Connell, 1978), will be affected by surrounding irregularities in reef topography and sediment deposits. These habitat variations change with time, and with the growth, death, and erosion of neighboring colonies (Dana, 1976).

The effects of these small-scale microhabit disturbances are easily seen in coral-algal interactions. Besides the development of epidermal cell hyperplasia in response to sediment-algae accumulations along the margins of colonies, a major cause of loss of living coral tissue from the Tague Bay, El Negro and La Parguera reefs was the widespread development of territorial algal lawns by damselfish (Brawley & Adey, 1977; Kaufman, 1977; Lobel, 1980). These fish continuously remove coral tissue, to provide a bare substrate which is colonized by algae. Normally, tissue may regenerate to cover small lesions very quickly (Bak & Steward-van Es, 1980). Sammarco & Williams (1982)

found that damselfish increased the numbers of rarer corals such as *Favia* because this species is more resistant to algae. From his studies of Pacific damselfish, Potts (1977) concluded that the algal mat caused corals to expend energy to keep surfaces clean, and death followed the exhaustion of metabolic reserves during periods of minimum nutrient availability.

Fish and other organisms which prey on corals (Robertson, 1970; Ott & Lewis, 1972; Antonius, 1973; Glynn, 1973; Endean, 1976; Brawley & Adey, 1982) may be responsible for the spread of some of the diseases and microparasites. In particular, microorganisms caught in the mucus layer of the fishes' mouths could be spread as they feed on coral tissue (D. Spoon, pers. comm.). Gladfelter et al. (1977) and Frydl (1979) noted that parrotfish feed on *Porites astreoides, Acropora palmata, Diploria strigosa* and *Montastrea annularis*. All of these species are afflicted by parasites, WBD and BLD.

BLD is rarely encountered (1–2 % incidence) on reefs throughout the Caribbean and off Bermuda (Antonius, 1973; Garrett & Duckow, 1975). The high incidence of this disease at the reef crest swim channel at Tague Bay may have been due to heavy wave surge sediment resuspension, and natural predators, as well as from coral injuries due to diver traffic, thus supporting observations that environmental stress (Antonius, 1977; Dustan, 1977; Ducklow & Mitchell, 1979) and tissue injuries (Antonius, 1981b) may increase the occurrence of this disease. WBD was first reported by Gladfelter et al. (1977) from St. Croix reefs well before the hurricane damage, but it appears that physical injuries and stress may also influence this disease. Knowlton et al. (1981) observed that hurricane-damaged *A. cervicornis* off Jamaica that may have been killed by a disease (symptoms of WBD), which they believed may have been present on the reef, and its spread was accelerated by stress. In experiments off Curaçao, Bak & Criens (1981) fragmented *Acorpora* spp. and found that despite an initial increase in cover, all fragments came down with a disease in which tissues sloughed off the skeleton three months later. Fragmented colonies of *Madracis mirabilis* were unaffected. The histopathological observations in this study indicate that the WBD-associated bacterial colonies may be present in corals for some time before signs of the disease are visible. In addition to finding the amoebae in *Siderastrea* spp. at Guayanilla Bay, the occurrence of the colony of *P. porites*, which was most heavily infested with *Nematopsis* sp. spores in the Coral Reef Microcosm suggests that environmental stress may likewise affect the infestation of some microparasites. Anderson & May (1981) noted that occult infections of pathogens or parasites, which can exist in apparently healthy hosts but increase in numbers and cause mortality or decreased reproductive potential when the host organism is stressed, are little understood.

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SYMBIOSIS TO PATHOLOGY: ARE THE ROLES OF MICROORGANISMS AS PATHOGENS OF CORAL REEF ORGANISMS PREDICTABLE FROM EXISTING KNOWLEDGE?

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ABSTRACT

Despite the importance of pathogens as regulators of populations in the marine environment, few studies have investigated these relationships in tropical coral reef organisms. Variations in microbial symbioses and recent cases of biotic pathosis in interspecific associations are examined in this paper. More multidisciplinary research is needed to determine the etiology of diseases of tropical marine organisms. Analysis of the potential impact of pathogens on populations and the environment should further our understanding of the complex reef ecosystem and adjacent habitats.

INTRODUCTION

The pathology of marine organisms has been described as "the wonderful world of unknowns" (P. P. Yevich, pers. commun.). Observations of tissues under the microscope have revealed a variety of viruses, rickettsiae, chlamydiae, mycoplasmas, bacteria, fungi, algal cells and protozoans living in interspecific associations on and within the tissues of other organisms. Further sleuthing has revealed that these relationships can be classified along a continuum from mutualistic symbioses, beneficial to both microorganism and host, to parasitic symbioses, where the microorganism derives a nutritional benefit from the host. If it impairs the vital functions of its host, the parasite can cause disease and death of the host, and is then known as a pathogen (Ahmadijan & Paracer 1986).

Kinne (1980:1) noted that "Diseases affect basic phenomena of life in oceans and coastal waters: for example, life span, life cycle, abundance, distribution, metabolic performance, nutritional requirements, growth, reproduction, competition, evolution, as well as organismic tolerances to natural and man-made environmental stress. In short, diseases are a major denominator of population dynamics." However, most studies on the effects of symbionts and how their associations may be mediated by nutritional disorders, various types of pollutants, or even diel and seasonal cyclic changes in environmental conditions, have dealt with commercially gathered species in temperate climates (Andrews 1976, Sieburth 1979, Kinne 1980, 1983, 1984, 1985, Johnson 1984, Sparks 1985, Jangoux 1987).

Our understanding of the intimate relationships of microorganisms with tropical marine flora and fauna, particularly those parasites causing disease, remains "the world of the very unknowns". Until recently, few laboratories with adequate facilities and equipment for disease studies were located adjacent to coral reefs, and there has been a scarcity of trained scientists and funding prospects. The magnitude of these deficiencies and the value of understanding disease phenomena have become apparent during recent mass mortalities of sponges, corals, sea urchins and fish.

Participants in the Coral Reef Pathology Workshop held at the Third International Coral Reef Symposium in Miami, Florida, May 1977, initiated efforts to examine processes that cause disease in reef organisms. The goals of this mini-symposium at the Sixth International Coral Reef Symposium are:

(1) To review recent investigations of variations in symbiotic associations and the pathogenesis of diseases caused by microorganisms (prokaryotic and non-algal eukaryotic cells, i.e. excluding zooxanthellae) in tropical marine environments.

(2) To discuss new approaches and techniques for the study of these associations.

(3) To encourage scientists working on coral reefs to pursue studies in microbiology, histopathology, and pathophysiology, and to identify how such studies can benefit a variety of different disciplines.

(4) To help identify resources, facilities, and personnel to assist with such studies.

GAPS IN OUR KNOWLEDGE

The importance of research on diseases has been demonstrated by studies showing that any change in the environment which adversely affects the health of any population of reef organisms can also affect other reef organisms (e.g. Loya & Rinkevich 1980, Glynn 1983, Pastorak & Bilyard 1985). However, many basic questions remain about the interactions of microbial pathogens and their hosts.

First, there are difficulties in establishing what type of association the microbe has with its host Many viruses, bacteria, and other microorganisms can be present on or in organisms in the absence of clinical signs of disease. Mutualistic symbioses of chemoautotrophic bacteria that enable their hosts to live in potentially toxic environments or to subsist on nutritionally limited diets have been discovered in tropical marine organisms. Sulfur-oxidizing bacteria are found in the gills of the bivalve Lucina floridana and under the cuticle of the gutless oligochaetes Phallodrilus spp. living in the sulfur layer of calcareous sediments (Fisher & Hand 1984, Giere & Langheld 1987). There are nitrogen-fixing bacteria associated with the gastrointestinal tract of Caribbean sea urchins (Guerinot & Patriquin 1981).

Second, marine symbiotic relationships are not static. A commensal or mutualistic symbiont under normal conditions does not harm the host, but if environmental or physiological changes occur, the delicate balance of the association will change. Abiotic factors, such as changes in temperature, salinity, illumination, or exposure to pollutants, can cause disease when the organism cannot maintain its physiological functions. The stresses of captivity and inappropriate diets can also alter the relationships of microorganisms and their hosts. Often, if the host is stressed, the microorganism can multiply and cause disease, or an abiotic disease may be complicated by secondary infections from microorganisms that are not normally pathogenic. This has been experimentally demonstrated for the bacterial populations found on coral mucus (Ducklow & Mitchell 1979).

Finally, little is known about the regulation of symbiont populations by the host, i.e. what mechanisms or factors control the abundance and physiological state of the symbiotic association with the host. The susceptibility or relative resistance of the host can change, depending on the size of the population of microorganisms present, changes in host defense mechanisms and their effectiveness, and the stage of development of disease. How a pathogen causes disease in its host is also largely unknown. The symbiont may induce abnormal cellular physiology and metabolism in the host, resulting in associated structural anomalies of cells and tissues, either locally or systemically. This may occur by secretion of toxins, utilization of scarce resources, or disruption of cytoskeletal components or biosynthetic processes. There is often a high degree of host specificity related to susceptibility. High levels of infection of unidentified species of Perkinsus suggest that these protozoans cause mortalities in some species of Great Barrier Reef bivalves. However, these protozoans have also been found at low levels of infection and not associated with mortalities in other bivalves, indicating that there may be species-specific susceptibilities or variations in the prevalence of pathogenic strains of Perkinsus (Coggin & Lester 1987). There is now concern that species introduced into new environments (particularly inter-ocean transfers, often for mariculture purposes) may carry symbionts that are pathogens for species in the new region.

SYMBIOSIS AND PATHOSIS IN SPONGES

Many species of sponges harbor various bacterial symbionts within the intercellular matrix. These associations often involve mutualistic unicellular or multicellular phototrophic cyanobacteria which may provide nutrition for their sponge hosts (Wilkinson 1980, Lauckner 1980), and/or other bacteria utilizing host metabolic wastes (Wilkinson 1978a,b). For many of the bacteria found in symbiotic associations with the sponges, we are unsure of their relationship with the sponge, or what they may provide to their host.

There have been few reports of sponge mortalities caused by microorganisms. Commercial sponges in the Caribbean suffered heavy mortalities apparently from a fungal infection (Smith 1941). Bacterial diseases in sponges have been noted, but the accuracy of the diagnoses have been questioned because of the presence of probable mutualistic bacteria and secondary infestations from seawater populations (Lauckner 1980). In the phototrophic associations, the host sponges may be able to control the populations of symbionts to maximize the benefits of the relationship to the sponge (Wilkinson 1978b). Conversely, Rutzler (in review) described a disease apparently caused by the inability of the mangrove demosponge, Geodia papyracea, to control the quantities of the cyanobacterial symbionts within its tissues. When the cyanobacteria multiply faster than the sponge archeocytes can remove the excess, the host sponge tissue is destroyed. In defense, the sponge establishes spongin barriers and the decaying tissue is sloughed off. Also, globular pseudogemmules of spongin microfibrils form around the cyanobacteria trapped inside the archeocytes, and are expelled when the decomposing sponge cortex breaks apart.

This species of sponge is also known to occur without the cyanobacterium in other localities. The significance of the psuedogemmules is unknown at this time, but if the phagocytic archeocytes could regain control of the cyanobacterial populations perhaps new sponge colonies could be formed. Could this be a newly evolving mutualistic relationship in which the host's control mechanisms are just developing (Jeon 1987)? Does this cyanobacterium really contribute any nutritional benefit to the sponge, or is it actually only a pathogen in a very slowly developing disease? This fascinating relationship should lead to further contemplation of the interactions of bacterial symbionts and their host sponges.

BACTERIAL DISEASES OF CORALS

Recent studies indicate that corals can show similar signs of disease but may be afflicted with different pathogens or abiotic diseases. A cyanobacterium, Phormidium corallyticum, has been identified as the causative agent of Black Line or Black Band Disease (BBD) in Caribbean faviid stony corals and gorgonia (Rutzler & Santavy 1983, Rutzler et al. 1983, Taylor 1983). This disease also occurs on faviid species of corals from Indo-Pacific reefs (Antonius 1985a). BBD owes its name to the black mat of fine filaments separating bare coral skeleton from living coral tissue. The cyanobacterium is believed to cause necrosis of the tissue by a toxic exudate. Although usually found only on widely scattered coral heads, a recent outbreak of BBD within the Looe Key National Marine Sanctuary has prompted further studies on the etiology of this disease and treatment of affected corals (B. Causey, pers. commun.).

The appearance of BBD on a particular reef may be influenced by adverse environmental conditions (Dustan 1977, Antonius 1985b). Taylor (1983) noted that the development of a pathogenic microcommunity on coral tissues was dependent on abnormal physiological stress or trauma that lowered coral resistance and released potential substrates for the cyanobacteria. Antonius (1981b, 1985b) and Rutzler et al. (1983) demonstrated an experimental association between physical injury to the coral tissue and the development of BBD. Antonius (1981a,b, 1985a,b) noted that the appearance of bare white skeleton, signaling the sloughing of coral tissue at the rate of a few millimeters per

day, was the natural starting point for the attack of the BBD microorganism. The loss of tissue could occur either at the base of the colony or adjacent to the abandoned holes of boring organisms on the coral surface. Because he could not find any microorganisms which might be removing the tissue, he proposed that the tissue sloughing was caused by a toxic shock to coral tissues in contact with unidentified species of algae or other epibenthos, or resulting from the toxic interactions of interspecific coral aggressions.

The rapid loss of coral tissue (variously known as "white band disease" = WBD, "white plague", "white death") has been applied generally to the sloughing of tissue in all species of corals, when it may actually represent the end (or beginning) stages of different diseases. Tissue sloughing was first recognized and termed WBD in Caribbean acroporid corals (all three species) in the mid-1970's (Gladfelter et al. 1977, Gladfelter 1982), and has since been reported to occur throughout the region (Rogers 1985). These disease signs also occur in nine species of acroporids in the Red Sea and the Philippines (Antonius 1985a). Tissue sloughs off the exoskeleton from the base of the branches and proceeds to the tips, and is distinguishable from predator damage. Because the acroporids are major reef framework builders, there has been concern about the presence of this disease on reefs. At most sites, only a few colonies of Acropora spp. are affected. However, the A. palmata at Tague Bay and nearby Buck Island National Monument, St. Croix, U. S. Virgin Islands, have been decimated by this disease over the last decade. There have also been reports of extensive losses of A. cervicornis colonies elsewhere, possibly due to this disease (Bak & Criens 1981, Knowlton et al. 1981, van Duyl 1983).

The etiology of WBD is unknown. Peters et al. (1983) discovered unusual Gram-negative rod-shaped bacteria living in colonies ("ovoid basophilic bodies") within the degenerating tissues of afflicted Acropora spp. from Tague Bay and Bonaire. These bacteria were also found in 25 samples of apparently healthy A. palmata and in 16 out of 25 samples of apparently healthy A. cervicornis from Tague Bay (sampled in August 1981). There were more bacterial colonies per unit area of tissue in those colonies showing signs of the disease than in the still apparently healthy colonies at Tague Bay (Peters 1984). Such bacteria were not found in healthy acroporid samples from Puerto Rico or elsewhere. A new survey at Tague Bay in October 1986 revealed that up to 95% of the A. palmata colonies were dead. There has not been any correlation of the presence of the disease with any adverse man-induced or natural environmental factors (Gladfelter 1982, Peters 1984). However, whether this microorganism is actually responsible for the loss of living coral tissue, or is a secondary pathogen, has not yet been determined.

Using histopathological techniques, Peters (1984) also examined several acroporid, poritid, and faviid field-collected corals and coral colonies from the Coral Reef Microcosm at the Smithsonian Institution, Washington, D. C., which exhibited basal tissue sloughing similar to that found in WBD. Although tissue on all of these colonies had appeared superficially normal at the time of collection, the tissues were actually in varying stages of degeneration and necrosis. However, there were no observable microorganisms within their tissues. The Microcosm corals experienced adverse changes in water conditions, including sediment resuspension and high nutrient levels. Peters (1984) proposed the term "Stress-Related-Necrosis" (SRN) for cases in which corals show signs of WBD and in which degenerative changes in cell structure are observed in the absence of obvious pathogens (determined by microscopic examination of fixed embedded tissues). She defined the WBD state as that in which the external signs of tissue sloughing are present and ovoid Gram-negative bacterial colonies are found in the coral tissues. Future studies may reveal a virus or bacterium associated with SRN. Dustan (1977) reported that inoculations of apparently healthy colonies of Mycetophyllia ferox, M. lamarkiana and Colpophyllia natans with tissue from "plague-infected" colonies of M. ferox and C. natans caused "plague" in the healthy colonies. SRN may be a reversible condition. Antonius (1981b,1985b) noted that it was rarely lethal for the whole colony in faviids. Gladfelter et al. (1977) reported an occasional reversal of tissue sloughing in Acropora palmata afflicted with WBD, with tissue regrowth. These colonies may not have been infected with the bacteria, or may have recovered from the infection.

Further histopathological examinations of a variety of anthozoans have revealed other "ovoid basophilic bodies" living within the tissues of these organisms (Peters, unpub.). Most occur very sporadically in the epidermis and have not been examined by electron microscopy, but those that have been identified as bacteria represent widely different morphologies. Some of these relationships may have existed on reefs for some time. At the turn of the century, microscopic observations by Duerden (1902) revealed ovoid colonies of unidentified microorganisms in A. cervicornis (plate I, fig. 5,6) and P. astreoides (plate IV, fig. 36) similar to the bacterial colonies now found. Protozoans have also been found within coral tissues, where they may cause localized adverse host tissue reaction (Peters 1984, Upton & Peters 1986), and a fungus has been associated with a BBD of Venezuelen corals (Ramos-Flores, 1983). Combined efforts by microscopists, pathologists and microbiologists should prove fruitful in determining the types of microbial symbiotic relationships within the corals and reveal new associations.

WHAT HAPPENED TO THE DIADEMA?

The long-spined sea urchin Diadema antillarum suffered documented mass mortalities from January 1983 through the summer of 1984. This phenomenon was first reported on reefs off Panama and subsequently around the Caribbean (Lessios et al. 1984a,b). Populations were reduced by 85% to 100% of their former numbers (Bak et al. 1984, Lessios et al. 1984a,b, Hunte et al. 1986). Apparently, juveniles were not as affected, and urchins remained healthy at a few isolated sites. In all areas, this was the only species of echinoid affected. No abnormal fluctuations in tidal levels, sea surface temperatures, rainfall or salinity were noted. Some recruitment of juveniles has occurred in some areas (Hunte et al. 1986, Williams et al. 1986), but there has been no recovery

of the urchin populations in Jamaica after three years (Hughes et al. 1987). Thick mats of fleshy and filamentous macroalgae now cover some reefs, with declines in coral species, crustose coralline algal cover, and clionid sponges. The herbivorous fish have not been able to continue removals of the algae that the urchins formerly grazed (Ruyter van Steveninck & Bak 1986, Hughes et al. 1987).

The wide geographical spread (certainly the most extensive epizootic ever reported for marine invertebrates) and species-specificity suggested a water-borne pathogen as the most likely causal agent (Lessios et al. 1984a). The pattern of the mortalities followed major Caribbean currents, reminiscent of that seen during the sponge disease epizootic of the late 1930's. Diseased urchins died within four days (Bak et al. 1984, Lessios et al. 1984b). Some urchins apparently survived the disease; they were found later with regenerating distal ends of spines and healed skin lesions on the surfaces of the tests. The lesions observed were similar to those seen in "bald sea urchin disease" (possibly caused by bacteria) that results in widespread mortalities in ten species of temperate urchins (Jangoux 1987).

Only a few urchins were collected and fixed for histopathological examination during the 1983 die-off. These specimens were in poor condition and further processing and examination were not attempted (R. E. Scheibling, pers. commun.). Bauer & Agerter (1987) reported the isolation of three strains of Gram-positive anaerobic sporeforming rods identified as Clostridium perfringens and C. sordellii from two specimens of Diadema antillarum which had died in September 1983 while in a flow-through seawater aquarium at the University of Miami, Florida. Subsequent exposure of healthy D. antillarum to cultures of these bacteria caused death in 10 hrs to 6 days, depending on the water temperatures. There were no mortalities in unexposed control urchins. These bacteria were also isolated from the tissues of experimentally infected urchins, although they were unable to detect these bacteria in the aquarium sediments or in healthy field-collected urchins during July-August 1984 or January-February 1985. They speculated that the clostridial isolate may have been responsible for the mass mortalities of D. antillarum in the Caribbean. In particular, since C. perfringens has been associated with water pollution, there were suggestions of the introduction of the pathogen via passing ships and/or other human activities (Bak et al. 1984, Bauer & Agerter 1987). Unfortunately, Bauer & Agerter's (1987) results are questionable because of the presence of possible contaminants and the lack of identification of all the isolates. They also did not describe signs of the disease leading to the deaths of the two urchins, and no histopathological examinations were performed.

Another die-off of D. antillarum was observed on reefs off St. Croix in September of 1985. Urchins representing four stages of the disease (from apparently healthy to severe spine and epidermal tissue loss, similar to the lesions seen during the first mortality event) were fixed and processed for histopathological examination. While most tissues of the apparently healthy animals were in good condition, Gram-positive micrococci (0.5 - 0.8 m in diam) were evident in the mucoid cells of the glandular crypts of the esophagous and in connective tissue and muscle bundles of the peristome, spines, and ampullae. With loss of spines, more bacteria, atrophy and necrosis of all tissues were evident. No micrococci were found in the gonads (E. C. Peters, R. C. Carpenter, & R. E. Scheibling, in prep.).

In this case, no samples were available from which microbiologists could try to isolate and characterize the bacteria present. Because the bacteria were found in "apparently healthy" specimens, it is possible that these urchins had already been infected, or some of the bacteria may occur naturally in the urchins (Bauer & Agerter 1987). Or perhaps the primary pathogen was a virus (Williams & Williams 1987). Williams et al. (1986) reported localized mortalities of the urchins Astropyga magnifica and Eucidaris tribuloides off the northwest coast of Puerto Rico in the winter of 1984-1985, but no samples were obtained for microscopical or microbiological examination. Much remains to be learned about the susceptibility of tropical urchins to pathogenic microorganisms and the identification of factors influencing these disease outbreaks.

CONCLUSIONS

"The wonderful world of unknowns" in the study of diseases in coral reef organisms is clearly illustrated by the above examples. The interaction of pathogens and microparasites with physical stress and injuries can alter the structure of these populations as in other groups of plants and animals (May 1983), and may also be influenced by species-specific susceptibility to pathogenic invasions. As we learn more about the associations of microbial symbionts in the lower latitudes, it will be beneficial to explore some of the recent theories on parasitic and pathogenic control of community composition and functions (May & Anderson 1983, Holt & Pickering 1985).

We need more basic research on these interspecific relationships, and a rapid response plan for investigating disease phenomena as they arise in tropical areas (Williams & Williams 1987). Field scientists need to be aware of resources to contact for assistance in these studies. The Caribbean Aquatic Animal Health Laboratory has recently initiated efforts to act as a clearinghouse of information on disease phenomena and scientists studying diseases of marine organisms in the Caribbean region (contact: Dr. Ernest H. Williams, Jr., Caribbean Aquatic Animal Health Laboratory, Department of Marine Sciences, University of Puerto Rico, Box 5000, Mayaguez, Puerto Rico 00709-5000). Other such networks may be developing in the Indo-Pacific. As more scientists participate in collaborative studies, there will be more opportunities to share information and to explore funding sources.

It is important to integrate studies of symbioses and diseases with existing knowledge of reef dynamics. There is also a need to consider how the increase in human activities in the tropics will alter the normal environments of these organisms, and possibly change parasite/pathogen relationships (Sindermann, 1983). Such research will provide information to aid efforts in the preser-

vation and conservation of tropical marine environments (Salvat 1981, Rogers 1985).

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Comparison of Microbial Community Assemblages

and Characterization of Marine Bacteria

using BIOLOG Gram Negative Microplates

Deborah L. Santavy

INTRODUCTION

Changes in bacterial community structure from different reef habitats will be characterized by metabolic fingerprinting using a rapid method which does not require cultivation of individual organisms. This system can be utilized for several other useful applications. It was originally designed to characterize and identify individual organisms which can be cultured, primarily of clinical importance. Another useful application is use as an aid in developing culture medium for symbionts, by profiling metabolic capabilities. The BIOLOG@ (Hayward, CA.) system utilizes a redox system incorporating a tetrazolium based dye to produce unique metabolic profiles of communities or isolates as a measure of comparison. The system employs 95 unique carbon sources with a basal medium to produce colorimetric patterns of sole carbon source utilization when bacteria are added.

MATERIALS:

BIOLOG GN Microplate (store at 4°C, do not freeze)
Sterile saline diluent (2% NaCl) in 20 ml test tubes
Sterile cotton swabs
Micropipettor or Multichannel Pipettor and sterile tips (150ul)
Spec 20
Turbidity Standards
Parafilm
Petri Dish
Scoring Datasheet
Sharpie Marker

SOLUTIONS

Diluent (2%NaCl) 1 liter
20 g NaCl
1000 ml Mille-Q H_2O

Add NaCl and H_2O to acid washed Erlenmeyer flask, adjust pH to 7.8-8. Autoclave. Dispense in sterile disposable plastic 20 ml tubes. (Tube will be read in spec).

Turbidity Standards

Use Phillips Milk of Magnesia (81 mg/ml $Mg(OH)_2$), dilute 48 ul (Lo) and 54 ul (Hi) into 20 ml water and mix thoroughly.

PROTOCOL

FOR CULTURED MARINE BACTERIA

1) Grow isolate on solid medium to log phase, (usually overnight to 24 hours, some slower growing isolates may take longer) using optimum growth conditions. Use a sterile cotton swab to remove cells from plate by rolling swab over colonies rather than sliding across them to prevent clump formation. Immerse swab in diluent and twirl against inside surface of tube. Gently mix to obtain uniform suspension, allowing large clumps to settle to bottom.

2) Warm microplate to incubation temperature.

3) Measure the absorbance of the Hi and Lo Standards on the Spec 20. First blank the Spec with a tube containing uninoculated diluent. Adjust the inoculum density within the Hi and Lo Standards range, by adding more saline or more cells. Bacterial cell concentration should be in range of 10^7 to 10^8 cells/ml.

4) Inoculate cells into microplate promptly. Some isolates lose metabolic activity if held too long in saline (longer than 10 minutes). Pour cell suspension into Petri dish, dispense 150 ul per well aseptically, taking care not to carry over chemicals or splash from one well to another.

5) Place lid on plate, label the plate, and wrap the sides closed with Parafilm. Place in incubator (we will use cooler in this lab) and record results every 12 hours on the microplate template. Most environmental isolates will require approximately 48 hours to completely develop. Keep in hydrated chamber to eliminate any dehydration of the outer wells, a plastic container lined with dampened paper towels works well. Wash the chamber daily with EtOH to minimize mold contamination.

6) Score the results as a colorimetric change compared to the control well (A1) on the provided datasheet. Often the purple color will settle to the bottom of the well. Or if you have access to a microplate reader which measures absorbances, use it to record the results. Absorbance values for each well on each plate should be recorded at 4, 24, and 48 hours on a Perkin Elmer Microtiter Plate reader using a 590 nm filter.

FOR ENVIRONMENTAL SAMPLES

ADDITIONAL MATERIALS

Cycloheximide Stock (5mg/ml) (Eukaryotic Metabolic Inhibitor)
Sterile Tissue Homogenizer
Sterile 2% NaCl pH=5.5-7.0
50ml sterile centrifuge tubes
0.2um filtered seawater
10% pyrophosphate
10ml sterile pipets and bulb
tissue homogenizer

SOLUTIONS:

Cycloheximide (5mg/ml)
50 mg Cycloheximide
1 ml 100% EtOH
9 ml Mille-Q H_2O

Dissolve cycloheximide in EtOH, then add H_2O. Filter sterile through a 0.22 um membrane. Store at 4^oC.

PROTOCOL

1) Each type of environmental sample will require different preparation:
   a) *Sediment sample*:
      Remove approximately 10ml soil from core, place in 50ml centrifuge tube, add 20ml 0.2um filtered seawater. Sediments and high particulate samples are treated with 0.1% pyrophosphate (PP), to enhance desorption of bacteria from the particles. Add PP to sample, vortex, or shake vigorously, collect supernatant. Remove 10ml of supernatant, add 10ml of diluent and 10ul of cycloheximide. Use this solution as inoculum.

   b) *Water column sample*:
      Concentrate 2L-500 ml seawater sample (dependent upon cell concentration in water column) to 10 ml using 0.2um filtering chamber. Add 10ml of diluent and 10ul of cycloheximide to sample. Use this solution as inoculum.

   c) *Black band sample*:
      Remove material and homogenize gently with loose fitting pestle in Dounce tissue homogenizer in the saline diluent. Add 20 ul of cycloheximide (stock solution=5 mg/ml) to 20ml inoculum if eukaryotic cell death is desired. Use this solution as inoculum.

   d) *Coral mucus sample*:
      Centrifuge coral mucus sample in sterile centrifuge tube. Decant the seawater above the pellet. Resuspend the pellet in 20ml of diluent, add 20ul of cycloheximide. Use this solution as inoculum.

2) Proceed with steps 2-6 as above.

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PATHOGENS AND PARASITES OF REEF CORALS: FIELD TECHNIQUES
Lee Stocking Island, Bahamas
1-9 November 1993

Microbiological Techniques
Deborah L. Santavy
U.S. EPA, Gulf Breeze Environmental Research Lab.

Seminar: Pathogens and Symbionts of Reef Corals and Sponges

Field Collection by SCUBA

1) Collect material using aseptic or sterile technique as possible, with concern for potential sources of microbial contamination (ie. skin, collecting apparatus, collection vessel, etc.).

2) Collect seawater from water column first from boat, before any divers enter water. Pump seawater to surface using pneumatic pump into sterile cubacontainers.

3) Collect material from Black Band diseased coral, using sterile syringe. Dispense into sterile whirl pacs with minimal seawater.

4) Collect sediment in sterile 60ml centrifuge tubes, transport back in cooler to lab.

5) Collect mucus sheet from *Porites astreoides*, or other coral surface with syringe.

6) Store all material in cooler with ambient seawater (no ice) until return to laboratory for processing.

Microbiological Techniques Lab

1. Culture of microorganisms from different environments: band of black band disease, *Porites astreoides* coral mucus, sediments, and seawater column. Use several different types of media, to illustrate different components of microbial community resulting from culture conditions.

2. Metabolic Profiles of Different Microbial Communities, using same environments as above.

3. Microscopic examination of samples from each of the four environments as above.

The lab participants will be divided into 4 working groups. Each group will process the material from a single environment using the three approaches listed above. Exchange between the working groups is encouraged.

Lab demonstrations prior to start include:
Preparation of dilution tubes and culture plating
Inoculation of BIOLOG plates
DAPI staining

Enumeration of Bacteria by DAPI Staining

INTRODUCTION

DAPI (4, 6-diamidino-2-phenylindole) specifically stains dsDNA, and is used to visualize bacterial cells from environmental samples. It will stain the nucleus of eukaryotic cells. Phototrophic microorganisms containing chlorophyll autofluorescence red when viewing with DAPI filter sets. Acridine orange is a fluorochrome which stains nucleic acid, both DNA and RNA, it can also be used to visualize bacterial cells. Size ranges for microorganisms you may see:

Bacteria 0.2um < 2.0um, very rare up to 6um
Flagellates 2 - 18um
Ciliates > 15um
Cyanobacteria (Blue Green Algae):
    coccoid 1um - 5um
    filaments 1.5 um(diameter) x tens of um long
Zooxanthellae 2-8um

EQUIPMENT:

25mm diameter filtering tower and fritted base filtration unit with vacuum source
Epifluorescent microscope fitted with filters for viewing DAPI

MATERIALS:

0.2um pore black stained 25mm diameter polycarbonate filters (Nucleopore)
0.45um pore 25mm diameter Millepore GS filters
Formalin filtered through 0.2um polycarbonate filter
0.2um filtered seawater (particle free)
1.5 Microfuge tubes
Micropipets and tips (P200, P20)
10% EtOH in filter MilliQ water
10 ml syringe and disposable 0.22um filter unit
Microscope slides with coverslips
Low viscosity, low fluorescence immersion oil (type A or DF)
25ml sterile pipets and bulb
Sharpie marker

SOLUTIONS:

DAPI

Prepare 1 mg DAPI (SIGMA)/ ml 0.2 um filtered Milli-Q stock solution. Store in amber vial, @ 4^{o}C and in dark. Preparation of small volumes for immediate use prevents fading problems with prolonged storage of dye. Prepare working solution by making a 1:10 dilution from stock bottle (final concentration 100 ug/ml).

PROTOCOL:

1. Preserve cells by adding filtered formalin to a final concentration of 2% to the sample in seawater.

2. Stain all samples except sediment sample by adding DAPI to final concentration of 5 ug/ml (50 ul working solution DAPI to 1 ml sample) of sample in microfuge tube. Stain for minimum of 5 minutes. Optimum concentration is 10^7 cells/ml. For open reef water, use 5 ml. Stain concentration can be adjusted higher or lower as necessary. Sediments and high particulate samples can be treated with 0.1% pyrophosphate (PP), to enhance desorption. Add PP to sample, vortex, collect supernatant and stain as above.

3. Set up 25 mm filtering tower with fritted base. Place a 0.45 um millepore GS or comparable filter on the fritted base. Place a 0.2 um pore size pre-stained black polycarbonate filter (you can stain your own with 1 g Irgalin Black in 500 ml Milli-Q + 10 ml glacial acetic acid) on top of the GS filter. Prewet filters with 10% ethanol in Milli-Q delivered by 0.2 um syringe filter (wash solution). If sample volume is small, leave 1 ml wash solution in tower. Add sample to tower, filter under gentle vacuum (< 15 mm Hg). Remove filter, allow to air dry briefly, then transfer to a clean pre-labeled glass slide. Add a small drop of low viscosity, low fluorescence immersion oil (type A or DF), and place a cover glass over the filter. Press down on cover glass to squeeze out all excess oil.

4. To count bacteria use an epifluorescence UV illumination with mercury bulb. Count a minimum of 10 random fields or 200 cells (whichever is less) using a square grid. If there are lots of cells, 1-2 rows of the grid can be counted per field. The minimum 10 fields should cover the filter in a cross pattern to incorporate variation in the dispersion of cells on the filter. Additional fields should also cover the entire filter surface. Chose fields randomly by not looking into the scope while moving the stage (watch the objective position over filter). Determine the grid average for each grid sample from the same filter. Multiply the grid average by the grid area (grid area must be determined by use of a stage micrometer for each microscope)/filter area (filter area is area measured from the bottom of the filtering tower) ratio, and divide this value by the volume of the sample (in ml) to obtain the number of cells/ml.

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ISOLATION and CULTIVATION PROCEDURES FOR MARINE BACTERIA

FOR SAMPLES FROM CORAL TISSUE, CORAL MUCUS,

SEDIMENT AND SEAWATER

MATERIALS:

Latex gloves
95% EtOH Bottle
Parafilm
Petri plates with 3 types of media
Sterile seawater dilution tubes (9ml/tube)
Sterile 50ml centrifuge tubes
Sterile 1ml pipets
Sterile 10ml pipets
0.2um filtered seawater
Pipet bulb
10% pyrophosphate in water
0.2um filtering 500ml filtering chamber
Loose fitting pestle and Dounce tissue homogenizer
Gas source
Sharpie Marker
glass hockey stick
turntable
sterile toothpicks

PROCEDURES TO USE WITH ALL ISOLATIONS

1) Conduct all these procedures using aseptic technique. Handle all specimens with latex gloves, EtOH sterilize all tools used. Rinse all tools, etc. with EtOH and sterile seawater prior to use.

2) Parafilm all plates shut to minimize contamination. Keep the incubation coolers upright and dry. Do not store uninoculated plates with inoculated plates.

PREPARATION OF SAMPLES:

1) Each type of environmental sample will require different preparation prior to inoculation:
   a) *Sediment samples*:
      Remove approximately 10ml soil from core (or obtain a weighted aliquot), place in 50ml centrifuge tube, add 20ml 0.2um filtered seawater. Sediments and high particulate samples are treated with 0.1% pyrophosphate (PP), to enhance desorption of bacteria from the particles. Add PP to sample, vortex, or shake vigorously, for about 1-2 minutes. Collect supernatant. Use supernatant as inoculum.

b) *Water column samples*:

Concentrate 2L-500 ml seawater sample (dependent upon cell concentration in water column) to 10 ml using 0.2um filtering chamber. Use this solution as inoculum.

c) *Coral tissue sample*:

Remove material and homogenize gently with loose fitting pestle in Dounce tissue homogenizer. Dilute with sterile seawater if necessary. Use this solution as inoculum.

d) *Coral mucus samples*:

Obtain a quantitative volume of coral mucus from a known area of coral surface. Coral mucus and tissue are best estimated by volumetric measure per unit of coral surface area. Depending on amount of water in the mucus sample, either directly use the collected sample as the inoculum after vigorously vortexing or shaking the sample. Or if the sample is very dilute, centrifuge coral mucus in sterile centrifuge tube, decant the seawater above the pellet. Resuspend in filtered seawater. Use this solution as inoculum.

INOCULATION OF MEDIA

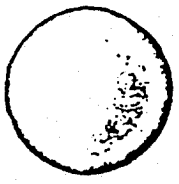
1) Prepare dilution tubes by aseptically making the dilutions detailed in step 2. Be sure to use a new sterile pipet for each dilution, flame the tube prior to pipetting, and close the top prior to making next dilution.

2) Label 4 dilution tubes, each with one of the following dilution information: 10^{-1}, 10^{-2}, 10^{-3}, or 10^{-4}. Label Petri plates with type of environmental sample, medium, and dilution. 3 types of media are to be used: Modified Marine 2216 Agar, Fortified Seawater, and Solidified Seawater.

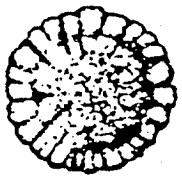
3) Prepare pipet, unscrew cap, flame cap, obtain 1ml slurry from tube and pipet in 9ml sterile dilution tube (10^{-1}). Flame new tube top, close cap tightly, vortex or roll tube between hands 5-10 times to evenly distribute the sample. Obtain new pipet. Repeat this procedure using aseptic technique to do the following transfers. Pipet 1ml from 10^{-1} tube into another 9ml dilution tube (10^{-2}), 1ml from 10^{-2} tube into another 9ml dilution tube (10^{-3}), 1ml from 10^{-3} tube into another 9ml dilution tube (10^{-4}), and 1ml from 10^{-4} tube into another 9ml dilution tube (10^{-5}). Only do one dilution series for each sample.

4) Plate each of following on the three different media types for corresponding dilutions using the spread plating technique: Remove 100ul of solution from original homogenate or dilution tube with a pipet and place on plate, this is 10^{-2} plate dilution. Repeat using all of the dilution tubes, noting that the plate dilution is two orders of magnitude less than the designated tube dilution. Using a hockey stick dipped in EtOH, flamed, and cooled, spin plate on platform and spread the liquid evenly over the surface of the medium. Repeat this procedure on all media using the spread plating technique.

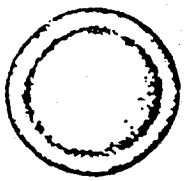
5. Incubate plates upside down after they are dry in empty coolers. After 1-4 days pick colonies from them with sterile toothpicks and transfer to other plates of similar medium used for isolation. You can divide the plate and add multiple isolates on one plate (6/plate) if they are not swarmers. Make sure you label all the isolates. Give them a unique number and record the following information in your notebook or data sheet: date of isolation, station, type of sample, dilution number, medium of isolation.



1. Round



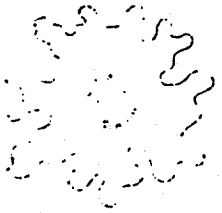
2. Round with Scalloped Margin



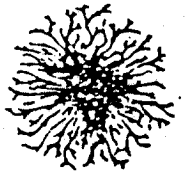
3. Round with Raised Margin

4. Wrinkled

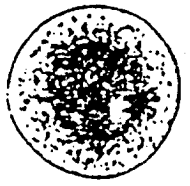
5. Concentric



6. Irregular and Spreading



7. Filamentous



8. L-Form

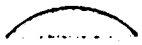
9. Round with Radiating Margin

10. Filiform

11. Rhizoid

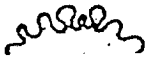
12. Complex

CONFIGURATIONS

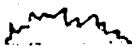


1. Smooth (Entire)

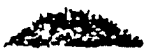
2. Wavy (Undulate)



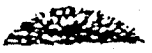
3. Lobate



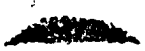
4. Irregular (Erose)



5. Ciliate



6. Branching



7. Wooly

8. Thread-Like

9. "Hair-Lock"-Like

MARGINS

1. Flat

2. Raised

3. Convex

4. Drop-Like

5. Umbonate

6. Hilly

7. Ingrowing Into Medium

8. Crateriform

ELEVATIONS

SANTAVY'S CULTURE COLLECTION OF
MARINE BACTERIA

Field Number: _____ Family: _____ Genus: _____

Host Field Number: _____ Host Genus: _____

H2O Sample Collected: _ THIS IS H2O SAMPLE DATA: ___ Substrate: _____

ISOLATION DATA

Isolation Medium: ___ Isolation Dilution: __ Isolation Replicate: _

Liquid N2: _ Freezer No.: __ Box No.: ___ Position: ___ No. Vials: _

PHENOTYPIC DATA

GN Rxn: ___ Oxidase: ___ Catalase: ___ Glucose Ferm.: ___ Sucrose Ferm.: ___

Pigmented: _____ Mucoid: ___ Colony Size: _____ Colony Shape: _____

Agar Digester: ___

LOCALITY DATA

Country Collected: _____ Location: _____

BIOACTIVITY

Bioactivity Tested: _ V. algin.: ___ Staph.: ___ Bacillus: ___ Candida: ___

Remarks: _____
_____
_____
_____

Maintance Vial: ___ Data Entered: _____

CONCENTRATION AND ENUMERATION OF VIRUSES FROM SEAWATER FOR THE PURPOSE OF ISOLATING PATHOGENS OF CORALS

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Introduction

There is considerable interest in identifying and isolating pathogens of corals and other reef organisms. The methods described here can be used to concentrate, enumerate and isolate viruses and bacteria from seawater. Similar protocols have been used to isolate viruses from seawater which infect marine phytoplankton and bacteria (Suttle et al. 1991, Suttle 1993), and the techniques should be equally useful for isolating naturally-occurring viral and bacterial pathogens which infect corals and other reef organisms. Many of the procedures are similar to methods that were originally developed for other scientific and medical uses. This paper will briefly describe methods for concentrating viral and bacterial pathogens from seawater using ultrafiltration, and for counting these natural communities using epifluorescent microscopy. These concentrates can be archived and used as a library from which previously unknown pathogens can be isolated.

Concentration of Natural Virus Communities From Seawater

Background

Successful isolation of a viral or bacterial pathogen depends on bringing host and pathogen together so that infection of the host and amplification of the pathogen occurs. Unless a pathogen is abundant the chances of isolating one directly from seawater is slim; the probability is increased if a larger volume of water is screened. Also, keep in mind that viruses will propagate most efficiently at high host densities. Screening a relatively large volume of water can be accomplished in some cases by growing the potential host to high density in the water to be assayed, or by exposing the potential host to natural bacteria and virus communities concentrated from seawater by ultrafiltration. Additional information on recovering viruses from the natural environment can be found in Berg (1987).

Equipment:

- Pump for vacuum and pressure filtration
- Two 142 mm stainless-steel filter holders
- Stainless-steel reservoir for pressure filtration
- Peristaltic pump (several liters per min) for ultrafiltration
- 30,000 MW-cutoff spiral ultrafiltration cartridge (e.g. Amicon S1Y30) and accessories to connect to the peristaltic pump

Supplies:

- 142 mm-diameter glass-fiber filters (MFS GC50, 1.2 µm nominal pore-size) and low-protein-binding "Durapore" membrane filters (Millipore, 0.22 or 0.45 µm pore-size)
- Assorted tubing and tubing connectors to set up the filtration systems
- 0.1 N NaOH
- 50 mM phosphoric acid

The probability of host-virus encounter is increased by concentrating the natural virus community by ultrafiltration, and adding aliquots of the concentrate to potential host organisms. Using this method a large number of potential hosts can be screened expeditiously. As well, the concentrates serve as a library of natural virus communities that can be screened for other pathogens. The disadvantages of the approach are that prefiltration removes viruses (*e.g.* Paul *et al.* 1991) and that ultrafiltration is somewhat expensive and time consuming.

Collect 20 to 250 l of the water from which the viruses are to be concentrated. The larger volumes are appropriate for very oligotrophic waters that are frequently associated with coral reef communities. Dispense as much of the water as possible into a pressure vessel. Stainless steel pressure vessels can often be obtained from soft drink wholesalers for a modest deposit. Pressure filter the water (<130 mmHg) through 142 mm-diameter glass-fiber and Durapore membrane filters, connected in series and held in place by stainless-steel filter holders. The 0.45 µm pore-size filters let bacteria through and make ultrafiltration slower, but the 0.22 µm pore-size likely excludes many larger viruses. The 0.22 µm pore size is suitable for smaller viruses. If the desire is to concentrate the bacterial community as well, eliminate the filtration step through the 0.22 or 0.45 µm pore-size filters. This will make ultrafiltration slower and it may be necessary to reduce the volume of water that is processed. Refill the pressure vessel as often as is necessary. Concentrate the filtrates 100-1000 fold using a 30,000 MW ultrafiltration cartridge (Amicon). Refer to the manufacturer's instructions for setting up the ultrafiltration system. Flow rates at a back pressure of 1000 mmHg are about 850 $ml \cdot min^{-1}$. Frequently replace the tubing on the peristaltic pump which is prone to catastrophic failure. The cartridge and tubing are cleaned after

use by flushing with 2 l of 0.1 N NaOH heated to 45° C. The cartridge is stored refrigerated in 50 mM phosphoric acid; we have found that long-term storage in NaOH as recommended by the manufacturer may weaken the membrane. Prior to reuse flush the cartridge with 0.1 N NaOH followed by 7 l of deionized-distilled water. The NaOH appears to reduce the charged surfaces in the membrane and reduce binding of viruses to the membrane.

The concentrated natural virus community should be stored at 4 °C, in the dark and screened as soon as possible for the presence of pathogens. Aliquots should also be preserved in 1 % glutaraldehyde (final concentration) and stored refrigerated for subsequent enumeration by epifluorescence (see below) or transmission electron microscopy (Suttle 1993).

Enumeration of Viruses by Epifluorescence Microscopy

In this section I detail methods for enumerating DAPI (4', 6-diamidino-2-phenylindole)-stained viruses by epifluorescence microscopy. Three other methods that are used for enumerating viruses in aquatic environments are plaque assays, most-probable-number assays (MPNs) and transmission electron microscopy (TEM); these are described in detail elsewhere (Suttle 1993). Each method tells us something different. Plaque assays and MPNs are for quantifying the abundance of infectious units which cause lysis of a particular host. Obviously these assays require the host of interest to be culturable. TEM is typically used for enumerating the number of virus-like particles (VLPs), either in whole water or in culture medium, while epifluorescence microscopy has been used for quantifying virus-sized particles containing double-stranded DNA. The procedure used depends on the question being addressed and the accuracy and sensitivity required.

Epifluorescence microscopy has the advantages that fluorescence is an objective criterion and a modest amount of equipment is required compared to TEM. The method also has a number of disadvantages including that i) the viruses must be concentrated before counting; ii) the organisms that the viruses infect and the composition of the viral community remains unknown; iii) only double-stranded DNA viruses can be visualized by DAPI staining; iv) some small viruses stain poorly; v) samples that are prefiltered to remove bacteria, can also remove a large proportion of viruses; vi) small bacteria are difficult to distinguish from large viruses; vii) individual viruses cannot be distinguished from small clumps of viruses.

Viruses can be concentrated and enumerated in a variety of ways. The simplest way is by filtration onto 25 mm Anotech or stained polycarbonate filters (*e.g.* Hara et al. 1991). Viruses can also be concentrated to $> 10^8$ ml^{-1} using ultrafiltration (as described above) and enumerated directly on glass slides (*e.g.* Suttle *et al.* 1990, 1991, Paul *et al.* 1991). This method is too time consuming to use for routine virus counting of natural samples. Small-volume centrifugation

concentrators potentially provide an alternative means of concentration, but more research is required to increase the efficiency of recovery of natural virus communities using these systems.

Equipment:

- Epifluorescent microscope with a 100 watt Hg bulb, 334-365 nm excitation and > 420 nm emission filter sets, and an ocular quadricule divided into 100 grid squares.
- 20 µl and 1 or 5 ml adjustable pipettes
- 25 mm polysulfone filter funnel (Gelman)
- Microcentrifuge for 500 µl centrifuge tubes

Supplies:

- Polypropylene, polycarbonate or high-density polyethylene bottles for sampling
- 5 µg ml^{-1} solution of DAPI (4', 6-diamidino-2-phenylindole) (Sigma D 1388)
- 0.2 M solution of $Na_2HPO_4 \cdot 2H_2O$ (M.W. 178.05)
- 0.1 M solution of Citric acid$\cdot H_2O$ (M.W. 210.14)
- 0.015 M solution of NaCl (M.W. 58.44)
- Acetic acid ($C_2H_4O_2$; M.W. 60.05)
- DNase I (Sigma D 4527)
- 0.2 µm pore-size, 25 mm, polycarbonate membrane filters (Poretics)
- 0.03 or 0.02 µm pore-size, 25 mm, polycarbonate or ceramic membrane filters (Poretics or Anotech, respectively)
- Irgalan black (Ciba-Geigy) if 0.02 µm pore-size polycarbonate membrane filters are used
- Formaldehyde or 25 % EM-grade glutaraldehyde
- 500 µl centrifuge tubes
- Microscope slides
- 22 x 22 mm cover slips
- Low-fluorescence immersion oil

DAPI and Irgalan Black Solutions:

Make up a 5 µg ml^{-1} solution of DAPI in McIlvaine's buffer (pH = 4.4). To make the buffer dissolve 3.561 g of $Na_2HPO_4 \cdot 2H_2O$ in 100 ml of distilled water (Solution A) and 2.101 g of citric acid$\cdot H_2O$ in 100 ml of distilled water (Solution B) and combine 8.82 ml of Solution A with 11.18 ml of Solution B. Store at 4 ^oC in the dark as DAPI is very light sensitive.

If 0.03 µm pore-size polycarbonate filters are going to be used they must be made non-fluorescent, by soaking them for several hours at 90 ^oC in a solution of 2 g Irgalan black dissolved

in 1 l of 2 % acetic acid. After staining rinse the filters in filtered distilled water. For larger orders stained 0.03 µm pore-size filters are currently available from Poretics Corp. (Livermore California, USA). It is not necessary to stain the Anotech filters.

DNase Solution:

The proportion of DAPI-positive particles that are DNase-sensitive is usually small, however, the use of DNase can make counting easier by reducing background fluorescence. To make a stock solution of DNase I, dissolve 10,000 Kunitz units in 1.0 ml of ice cold 0.15 M NaCl. Aliquot 50 µl subsamples into 500 µl microfuge tubes and freeze at -80 °C. This should keep for many months, but it should be assayed periodically for activity per the manufacturer's instructions.

Enumeration of Filtered Samples:

Filter a freshly collected water sample through a 0.2 µm pore-size polycarbonate filter to remove most bacteria and larger DNA-containing particles. To a 2 ml subsample add 50 µl of stock DNase solution (500 Kunitz units) and incubate for 30 min at room temperature. After DNase treatment the sample can be fixed in 1 % glutaraldehyde or Formalin. Add 0.2 ml of the DAPI stock solution (1 µg ml^{-1} final concentration) and incubate in the dark for 30 min. The fluorescence is less if higher concentrations of DAPI are used (Coleman *et al.* 1981). Filter the entire sample through a 25 mm, 0.03 µm pore-size, irgalan-black stained polycarbonate filter or a non-stained 0.02 µm pore-size, ceramic filter (vacuum 200 mmHg). These filters are very fragile and must be handled with care. They should be laid over a pre-wetted 0.45 µm pore-size nitrocellulose filter for even filtration. The filtration may take > 30 min if a polycarbonate filter is used; the porosity of ceramic filters is higher and filtration is more rapid. Do not rinse the filter as the DAPI binding is reversible. Turn the vacuum off as soon as the filter drys. If the filter is too wet or dry the slide will be poor. Lay the filter over a small drop of low-fluorescence immersion oil on a microscope slide. Place three tiny drops of oil on a cover slip and gently lay this over the filter, trapping the oil between the cover slip and filter. The filter should be of even color, with no wrinkles, dry spots, or emulsion of water and oil.

At 1000 x magnification count 20-100 DAPI-positive particles in each of 20 random fields. It can be very difficult to achieve a good quality image and a low background. Much depends on the optics of the microscope and the optical filters that are used. Calculate the number of viruses per ml (N_V) from:

$$N_V = P_f * \frac{A_S}{A_f} * \frac{1}{V_S} * \frac{V_S + V_e + V_g + V_D}{V_S} * 1000$$

where:

P_f = number of fluorescent particles per field

A_S = filtration area of filter (μm^2)

A_f = area of field (μm^2)

V_S = Volume of sample to which DNase, fixative and DAPI added (μl)

V_e = Volume of DNase added (μl)

V_g = Volume of glutaraldehyde or Formalin added (μl)

V_D = Volume of DAPI added (μl)

For most applications counting a minimum of 20 fields containing at least 200 DAPI-positive particles represents a reasonable compromise between accuracy and effort. Assuming a Poisson distribution this yields upper and lower 95 % confidence limits of 174 and 230 or an error of about 15 %. Doubling the counts to 400 decreases the 95 % confidence limits to about 10 %. As increases in accuracy are a function of the square root of the number counted it is necessary to count much larger numbers of viruses to get any appreciable increase in accuracy. If desired, 95 % confidence intervals can be estimated from the following formulae (Lund et al. 1958):

$$\text{Lower} = n - 1.96 * \sqrt{(n + 0.5)} + 1.42$$

$$\text{Upper} = n + 1.96 * \sqrt{(n + 1.5)} + 2.42$$

where n is the number of DAPI-positive particles counted.

Enumeration of Samples with High Virus Concentrations:

If the virus concentration is about 10^9 to 10^{10} ml^{-1}, DAPI-stained viruses can be counted directly on glass slides. The method is particularly well suited for counting viruses in laboratory experiments where further concentration is frequently not required. However, these concentrations are considerably greater than in most natural water samples (ca. 10^7 ml^{-1}); hence, the viruses must be concentrated from natural waters before they can be counted by this protocol.

If quantitative counts are desired for natural communities, the efficiency of concentration should be checked using an internal standard such as 50 nm fluorescent beads, labelled with fluorescein isothiocyanate (FITC, excitation/emission = 458/540 nm), or by adding a trace addition of a marine bacteriophage to one of a pair of duplicate samples and titering the replicates by plaque assay before and after concentration (Suttle 1993).

Transfer 20 µl of the virus sample to a microfuge tube. Dilute 10 µl of stock DNase solution with 90 µl of ice cold 0.15 M NaCl, and for each 20 µl sample place 5 µl of DNase I (5 Kunitz units) on the inside of each microfuge tube. Briefly centrifuge the sample (*ca.* 15,000 x g for 3 s), vortex, re-centrifuge and allow to incubate for 30 min at room temperature. DNase will

dissolve free DNA that could interfere with the counting method, but will not interfere with DNA protected by a protein coat. If required the samples can be fixed at this point with 1 % Formalin or glutaraldehyde. Do not add excess glutaraldehyde as it imparts background fluorescence and makes counting difficult. To stain, add 5 µl of DAPI stock solution (1 µg ml^{-1} final conc'n), centrifuge and vortex, as above, and incubate in the dark at 4° C for at least 30 min.

Pipette 3-5 µl on a clean glass slide and cover with a clean 22 x 22 mm cover slip. Sometimes the quality of the glass slides can be variable, resulting in an unacceptable background fluorescence. If so, a much better quality image can often be obtained by pipetting the stained viruses onto a cover slip and covering the drop with another cover slip. The sample, sandwiched between the two cover slips, can then be laid directly on top of a glass slide and viewed.

At 1000 x magnification count 20-100 DAPI-positive particles in each of 20 random fields. Use the appropriate number of grid squares in the quadricule. The viruses will tend to adsorb to both of the glass surfaces, so be careful to count both planes of focus. Calculate the concentration of viruses per ml (N_V) using the following formula (Confidence intervals can be calculated as outlined above):

$$N_V = P_f * \frac{A_c}{A_f} * \frac{1}{V_c * C_f} * \frac{V_s + V_e + V_g + V_D}{V_s} * 1000$$

where:

P_f = number of fluorescent particles per field

A_c = area of cover slip (μm^2)

A_f = area of field (μm^2)

V_c = Volume of sample under the cover slip (µl)

C_f = concentration factor of the sample

V_s = Volume of sample to which DNase, fixative and DAPI added (µl)

V_e = Volume of DNase added (µl)

V_g = Volume of glutaraldehyde or Formalin added (µl)

V_D = Volume of DAPI added (µl)

Isolation of Viral or Bacterial Pathogens

The first step is to increase the probability of host-pathogen encounter by introducing aliquots from a concentrated natural virus or bacteria/virus community into growing cultures of the potential host organism. Obviously, the exact culturing protocol will depend on the organism being

screened (for zooxanthellae see another section in this volume). Compare the viability of the treated cultures to cultures which have received heat-treated concentrate (autoclave or heat to 100 °C for 30 min) and to which nothing has been added.. Culture lysis or severely suppressed growth rates is good evidence for the presence of a pathogen. Propagate the infection several times by introducing an aliquot from an "infected culture" into a previously untreated culture in order to confirm that the inhibitory agent is a pathogen. If the pathogen can be propagated the next step is to determine if it is a virus or bacterium. Test if the infective agent can be removed by filtration, whether it is sensitive to antibiotics and whether it is host specific. Finally, use TEM to confirm the presence of VLPs.

Cloning of the pathogen can be accomplished in a number of ways. If the host is amenable to growth on solid substrate the simplest way to clone the virus is by plaque purification. Different dilutions of an infected or lysed culture are added to a series of "lawns" of the host organism, and the pathogen allowed to amplify. A single well-separated area of clearing (plaque) where lysis of the host cells have occurred is removed from the lawn. The plaque is eluted in medium overnight and the eluent used for another dilution series and plaque assay. This procedure should be repeated several more times to be sure that the virus has been cloned.

For hosts that will not grow on solid medium cloning must be accomplished by amplifying a single infectious unit in a liquid culture. The first step is to determine the titer of a culture lysate by a most-probable-number assay. Based on these results a dilution series is set up and 0.2 of an infectious unit is added into each of 20 exponentially-growing cultures of the host. The probability of a culture receiving a single virus is 0.164 and therefore would be expected to occur with a frequency of 3.27 out of 20 cultures. The probability of a culture receiving 2 or more viruses is < 0.02 (Cottrell and Suttle 1991). Hence, by repeating this procedure twice one can be very confident that the virus has been cloned.

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Robert K. Trench

METHODS FOR THE ISOLATION, CULTURE AND CHARACTERIZATION OF SYMBIOTIC MICROALGAE

INTRODUCTION

Many symbiotic microalgae occur inside the cells (**intracellular**) of their respective hosts. This is the case in forams, some sponges, coelenterates. and some flatworms. In some sponges, flatworms, ascidians, and in the clams (e.g. *Tridacna*, *Corculum*, *Fragum*), the algae are not inside animal cells, and are **intercellular**. In cnidaria, although the algae are predominantly in the endodermal cells, in certain groups (e.g. *Palythoa*, the jellyfishes *Cassiopeia* and *Mastigias*), they may also occur in "amoebocytes" in the mesoglea and/or in ectodermal cells.

It is often difficult to determine the taxonomic affiliations of symbiotic microalgae by examination of the algae *in hospite* because some of them undergo major changes, having entered the hosts' cells. For example, the prasinophyte *Tetraselmis convolutae* symbiotic with the flatworm *Convoluta roscoffensis* loses most of its free-living morphological characteristics on establishing the association. Determination of its specific identity came only after the algae were brought into culture and analyzed, in this case, by ultrastructure. Similarly, dinoflagellates are known from their "free-living" dinomastigote morphology (Fensome et al. 1993); but dinoflagellates symbiotic with corals etc. occur as coccoid cells in their hosts and do not show these traits, until isolated and cultured.

In the procedure of isolation and culture of symbionts (whatever they may be) it is very important that Koch's postulates be satisfied, i. e. that it be determined unambiguously that the entity in culture is indeed the same that was in the host. Sources of potential error are contaminants adhering to host surfaces (particularly coral skeletons not covered with tissue). The culture medium that one chooses to employ may also select one contaminant genotype which may outgrow a symbiont genotype.

Having established that the isolate is the symbiont, one can make use of a range of characteristics (some of which can be used to test Koch's postulates) to determine the affiliation of the symbiont. These include (i) analysis of the morphology of all stages of the life history (if they are polymorphic)(see Banaszak et al. 1993), (ii) analysis of stable characteristics of the photosynthetic apparatus (see Iglesias-Prieto et al. 1991, 1993), (iii) genetic analysis (e.g. SSU rDNA sequence analyses, or sequence analysis of any gene) (see Rowan, 1991). A caveat that needs to be stated is that no single approach is without its potential flaws, and ultimately, a combination of several approaches is probably best.

ISOLATION OF SYMBIOTIC MICROALGAE

The technique chosen will be dictated by the nature of the host. For soft hosts (jellyfishes, anemones), the animals are blended, after washing quickly in fresh water, and the algae sedimented by centrifugation at low speed, after passage through gause or plankton netting. For stony corals, use of a WaterPic to remove tissue works well.

Samples of the freshly-isolated algal cells should be stored frozen (for subsequent biochemical analysis (see below), and/or fixed for examination at the ultrastructural level. The algae are then inoculated into an appropriate sterile culture medium at low (ca. $0.5.10^3.ml^{-1}$) in screw cap culture tubes. If algae are to be compared, then it is probably best that they should all be grown in the same medium. Several growth media have been successfully employed. These include Provasoli's ES (enriched sea water, Provasoli 1968), F/2 and ASP-8A (Ahles 1967, Blank 1987). The latter is the medium routinely used in my laboratory. Maintenance growth conditions are 26-27° C, and about 80 μmol quanta . m^2 .sec^{-1} PAR on a 12:12 photoperiod, for tropical species.

With an apropriate inverted microscope, the culture tubes can be examined without disturbing the culture. It is very often useful to add antibiotics (e.g. AM9, Pinter and Provasoli 1968) to discourage bacterial (and cyaobacterial) growth, unless of course, the microalga being cultured is a cyanobacterium. In some circumstances, fungistats may also become necessary. It is possible to plate symbiotic dinoflagellates on agar and then isolate individual cells by standard microbiological dilution procedures. Germanium dioxide is often useful to control diatoms contaminating dinoflagellate cultures, but the concentration is critical as GeO_2 can also retard dinoflagellate growth.

METHODS FOR ASSESSING THE IDENTITY OF THE ISOLATED ALGAE

As indicated above, it is essential that the algae isolated and cultured be compared with the initial population taken from the host. This can be accomplished by (i) ultrastructural analysis, (ii) comparison of isoenzyme patterns, (iii) comparison of isoelectric focusing patterns of sPCP and (iv) comparison of gene sequences.

Ultrastructural analysis

Standard techniques have been repeatedly described (see Schoenberg and Trench 1980b, Trench and Blank 1987; Banaszak et al. 1993). TEM is important in comparing coccoid cells of symbiotic dinoflagellates in culture to coccoid cells *in hospite* (freshly isolated). SEM important in determining the taxonomic affiliation relative to other free-living members of the group (e.g. determine whether symbiont is Gymnodiniales, Amphidinium, Prorocentrales, Peridiniales, etc.).

Isoenzyme patterns

Various isoenzymes have been detected in symbiotic dinoflagellates (freshly isolated and after culture). These include: the esterases, carbonic anhydrase, malate dehydrogenase, malic enzyme, the superoxide dismutases, glucose phosphate isomerase, hexose kinase, alcohol dehydrogenase, glutamate dehydrogenase acid and alkaline phosphatases, and several peptidases (see Schoenberg and Trench, 1980a, Colley, 1984, Matta et al. 1992). In comparing cultured and freshly-isolated algae, one should be aware of the possibility of animal tissue contamination in the former, and include the appropriate controls. In comparing different isolates by this method, it is important that all the isolates be grown in the same culture medium under the same growth conditions and be harvested at approximately the same phase of population growth. It would not be appropriate, for example, to compare a stationary or senescent pahse culture with one in log phase growth.

Isoelectric focusing of sPCP

In dinoflagellates, the water-soluble peridinin-chlorophyll *a*-proteins (sPCP) provide a powerful tool for confirming Koch's postulates. All photosynthetic organisms possess light-harvesting pigment-protein complexes associated with the photosynthetic apparatus. Among dinoflagellates, these pigment-protein complexes are readily extracted and can be separated by isoelectric focusing methods (Chang and Trench 1982, Iglesias-Prieto et al. 1991). Different algae produce different patterns, and these are stable characters. The one disadvantage is that one needs a rather large sample of algae.

In principle, the proteins migrate through the polyacrylamide gel until they achieve a pH value in the gradient that is equivalent to their isoelectric point, when the net charge on the protein is zero. Here they accumulate. It appears that many of the various iselectric forms of sPCP are coded separately in genomic DNA. They appear not to be products of post-translational modification.

GENETIC ANALYSIS

High molecular weight DNA can be extracted from microalgal symbionts. With the appropriate use of oligonucleotide primers (Medlin et al. 1988), particular genes can be amplified by the polymerase chain reaction (PCR), and these can be sequenced either directly as PCR products or after cloning into *E. coli* (Rowan and Powers 1991a,b, 1992, Sadler et al. 1991). Most such analyses have taken advantage of the large data base available for the small subunit ribosomal RNA gene (SSU rDNA), which has the characteristics of being highly conserved across taxa, and is large enough to be informative (in dinoflagellates approximately 1,800 base pairs). Computer methods for analyzing the sequences include (but are not restricted to) (i) Wagner Parsimony (PAUP, Swofford 1989), (ii) Fitch and Margoliash's distance (PHYLIP, Felsenstein 1990, 1993) and (iii) DNA Maximum Likelihood (Olsen et al. 1993).

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MICROALGAL-INVERTEBRATE SYMBIOSES: A REVIEW

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Key words: acclimation, adaptation, Bacillariophyta, cyanobacteria, Chlorophyta, Dinoflagellata (Pyrrophyta), diversity, evolution, mutualism, phylogenetics, symbiosis.

Summary: The field of microalgal-invertebrate symbiosis has gradually been undergoing some significant conceptual changes. The concept of low genetic diversity among symbiotic microalgae and high diversity among animal hosts has been challenged, and shown to be inaccurate. An increasingly high taxonomic diversity of microalgae are now recognized as forming symbioses with a high diversity of invertebrates, in marine and freshwater habitats, in benthic and pelagic communities. Information from a wide range of sources indicates that there is symbiont-host specificity in these symbioses at the species level, a realization that is completely in agreement with the patterns observed in other mutualistic and in parasitic symbioses. There has been a general misunderstanding that the less restrictive specificity observed under laboratory conditions is equivalent to that occurring in the natural environment. The mechanism of selectivity underlying the expressed specificity of microalgal-invertebrate symbioses is unknown, but evidence supporting the concept of molecular signalling between symbionts and hosts is rapidly accumulating. Such molecular "cross-talk" between symbionts and hosts has now been documented in bacteria-legume symbioses, and has also been implicated in interactions between plants and bacterial pathogens. Many aspects of the physiology, biochemistry and ecology of microalgal-invertebrate symbioses are better appreciated with the realization that functional diversity probably parallels genetic (taxonomic) diversity, and many of the apparently controversial aspects of the biology of microalgal-invertebrate symbioses can be reconciled by embracing this idea. Based on recent new evidence on the structure and composition of the photosynthetic apparatus of symbiotic dinoflagellates, it is apparent that some of the paradigms of functional, and ultimately, ecological aspects of the symbioses involving these algae will have to be modified. Of the various subdisciplines of the field, the most controversial, and indeed confusing, is that pertaining to the acquisition and utilization of nitrogen and phosphorus.

I have attempted to review the various aspects of microalgal-invertebrate symbioses pertaining to their taxonony, genetic diversity and evolution, morphology, biochemistry and physiology, as concisely as possible. As I believe that the field lacks an overall general theory that would guide future research directions, I conclude the article with a speculative discourse which hopefully may serve as a focal point. I believe that most of the ideas expressed, though not always supported by tangible evidence, are cast in the form of potentially testable hypotheses.

1. INTRODUCTION

The phenomenon of symbiosis between microalgae (used here to include cyanobacteria) and invertebrates has been recognized for over a century. Following the realization by de Bary (1879) that lichens were symbioses between microalgae and fungi, the early studies of Brandt (1881, 1882), Geddes (1882), Just (1884), and Klebs (1892) established the principle that the entities (the microalgae) they described were not of the same cellular nature as the invertebrate hosts in which they were found. In many instances however, the microalgae were regarded as parasites (e.g. Hovasse 1922, 1923, 1924). A plethora of names, almost equivalent to the numbers of different authors, were initially employed to identify the algae (e.g. Winter 1907, Rhumbler 1909, Pascher 1911), many of which fell into disuse, leading to the situation generally accepted in the 1960's that "zoocyanellae" referred to symbiotic cyanobacteria, "zooxanthellae" referred to golden-brown symbiotic microalgae, and "zoochlorellae" to green ones (McLaughlin and Zahl 1966; see also review by Blank and Trench 1986). None of these terms had any taxonomic significance, and indeed encompassed distinct taxa. For example, "zoochlorellae" included *Tetraselmis convolutae* (the symbiont in the acoel flatworm *Convoluta roscoffensis*) and *Chlorella* (the symbionts of *Hydra* and *Paramecium*), and "zooxanthellae" encompassed dinoflagellates in corals and *Tridacna* (among others) and the diatom *Licmophora* sp. symbiotic with *Convoluta convoluta*. For several years, probably beginning with the recognition of amphidinioid dinoflagellates as symbionts (D.L. Taylor 1971a), a great deal of research effort has been placed on understanding the taxonomic diversity of symbiotic algae. Although there were several distinct motivations underlying such studies, a significant quest has been the elucidation of the cellular and molecular basis for specificity in microalgal-invertebrate symbioses. The concept of specificity itself is predicated by the recognition of symbiont diversity, which was initially believed to be low (D. L. Taylor 1984, Law 1985, Smith and Douglas 1987, Smith 1993), but is now appreciated as being much higher (Trench and Blank 1987, Blank and Huss 1989, Trench 1992, Rowan 1991), even though our views on the extent of this diversity are in a constant state of flux. Nonetheless, it is now apparent that symbiotic microalgae are represented among six Divisions, including cyanobacteria, rhodophytes, chlorophytes, prasinophytes, diatoms and dinoflagellates. The reader is referred to volumes and articles such as Goff (1982), D. L. Taylor (1984), Blank and Trench (1986), Smith and Douglas (1987), Rowan (1991) and Trench (1988, 1992) for more comprehensive overviews of different interpretations of the subject.

The phyletic diversity of invertebrate hosts that harbour microalgae is also very high, ranging through the Phyla Protozoa, Porifera, Cnidaria, Platyhelminthes, Mollusca and Urochordata. If one regards these symbioses as "autotroph-heterotroph" associations, then adding the chemotrophic and methanotrophic bacteria-invertebrate symbioses (Fisher 1990, Feldbeck and Distel 1991, Childress and Fisher 1992) to the picture, increases the phyletic distribution of the phenomenon to include the Vestimentifera, Pogonophora and Annelida. In many review articles, it has been implied in "comprehensive lists" of examples presented, that most instances of microalgal-invertebrate symbioses are described. On the basis of personal experiences, I would venture to state that this is almost certainly untrue. For example, having observed in the field in Belau several marine ciliates associated with what appeared to be dinoflagellate symbionts, subsequent consultation with the literature illustrated the paucity of information available on marine ciliate-dinoflagellate symbioses. Again, from time to time I have been

made aware of "obscure" publications referring to algal symbioses involving invertebrate taxa not generally known to be symbiotic. For example, among the bivalves, the tridacnids are obvious examples of symbiosis, studied repeatedly since the pioneering studies of Yonge (1936), but two genera, *Fragum* and *Corculum* in the subfamily Fraginae (Kawaguti 1983), and one in the family *Trapeziidae* (Morton 1982) also harbour microalgal symbionts, in the latter case, algae of unknown taxonomic affiliation. Recently, Anderson and Matsuoka (1992) described an unidentified microalga as symbiotic in the radiolarian *Dictyocoryne truncatum.*

In this article I shall attempt to provide as current a discussion as possible of the biology of microalgal-invertebrate symbiosis. Some areas of the subject, for example, nutrient relations and flux between symbionts, and intercellular interactions (recognition), and specificity, have undergone assessment recently (Cook 1983, McAuley 1988, Rahat and Reich 1988, Reisser 1988, Smith 1988, Trench 1987, 1988, 1992). The reader is referred to those papers for a more historical perspective.

2. SYSTEMATICS AND TAXONOMIC DIVERSITY OF SYMBIOTIC ALGAE

Tables 1 and 2 list symbiotic microalgae of which I am currently aware, and their respective hosts. This list should not be construed as being exhaustive. The term symbiosis is used here in its broad sense as discussed in Lewis (1973), Stanier et al. (1977) and Smith and Douglas (1987). All the examples given do not necessarily involve intracellular mutualistic symbioses. For example, since foraminiferans and radiolarians may feed on microalgae, it is often difficult to distinguish whether all the various algae found in these organisms are items of food, or are symbionts (Leutenegger 1977). Again, Spindler and Hemleben (1980), Gains and Elbrächter (1987) and Spero and Angel (1991) have described dinoflagellates in the genera *Gymnodinium*, *Gyrodinium* and *Amphidinium* which are free-swimming and use the external environment of the sarcodines as a microhabitat, the associations possibly being "commensal" ones.

Comparison of Tables 1 and 2 with, for example, the species list of D. L. Taylor (1984), will re-emphasize three points; (i) that symbiotic microalgae are taxonomically highly diverse; (ii), that there is no correlation between symbiont and host taxa, similar (but not identical) symbiotic algae can be found in phylogenetically distantly ralated hosts (Schoenberg and Trench 1980a, Rowan 1991, McNally et al. 1993), and closely related hosts may harbour phyletically distantly related symbionts (e.g. *Convoluta roscoffensis* and *C. convoluta*); (iii) that dinoflagellate symbionts are also taxonomically diverse, and are not restricted to the genera *Symbiodinium* and *Amphidinium.* Recognition of this last point, combined with the remark made above, should be enough to eliminate the continued use of the trivial term "zooxanthellae" when referring to symbiotic dinoflagellates (Trench and Blank 1987, Banaszak et al. 1993, McNally et al. 1993). This point becomes significant in the context of the differences observed in aspects of the physiology, biochemistry and ecology of these algae (discussed below).

The high phyletic diversity of symbiotic microalgae, viewed along with the high phyletic

Table 1. Non-dinoflagellate microalgal taxa symbiotic with invertebrates.

Microalga	Host	Authority

1. Cyanobacteria		
Cyanocyta korschikoffiana	*Cyanophora paradoxa* (Sarcomastigophora)	Hall & Claus 1963
Skujapelta nuda	*Glaucocystis nostochinearum*	Hall & Claus 1966
Synechococcus sp.	*Paulinella chromatophora* (Sarcomastigophora)	
Prochloron didemnium	*Didemnum carneolentum* (Ascidiacea)	Swift 1989
	Diplosoma virens (Ascidiacea)	Alberte 1989
Aphanocapsa feldmanni.	*Ircinia ramosa* (Porifera)	
A. raspaigellae	*I. variabilis* (Porifera)	
Synecocyctis tridemni	*Ulosa funicularis* (Porifera)	
Oscillatoria spongeliae	Several sponges	Wilkinson 1992
2. Rhodophyceae		
Porphyridium sp.	*Peneroplis planatus* (Foraminifera)	Leutenegger (1977)
Porphyridium sp.	*Spirulina arietina* (Foraminifera)	Lee 1980
3. Chlorophyceae		
Chlorococcum sp.	*Hydra magnipapillata* (Hydrozoa)	Rahat & Reich (1985)

Chlorella vulgaris is reported as symbiotic with the ciliates *Coleps hirtus*, *Paramecium bursaria*, *Euplotes daideos* and the flatworm *Phaenocora typhlops* (Eaton & Young 1975, Esteve et al. 1988, Reisser et al. 1988).

Chlorella sp.	*Hydra viridis* (Hydrozoa)	Oschman 1967
Chlorella sp.	*Stentor polymorphus* (Ciliata)	Reisser 1981
Chlorella sp.	*Spongilla* sp. (Porifera)	Reisser 1984
Chlorella sp.	*Anodonta* sp. (Bivalvia)	Pardy 1980
Chlorella sp.	*Anthopleura xanthogrammica* (Actiniaria)	O'Brien 1978
Chlamydomonas hedleyi	*Archais angulatus* (Foraminifera)	Lee 1983
C. provasolii	*Cyclorbiculina compressa* (Foraminifera)	Lee 1983
4. Prasinophyceae		
Tetraselmis convolutae	*Convoluta roscoffensis* (Turbellaria)	Parke & Manton 1967
Pedinomonas symbiotica	*Thallassolampe margarodis* (Radiolaria)	Cahon & Caram 1979
P. noctilucae	*Noctiluca scintillans* (Dinophyceae)	Sweeney 1976
5. Bacillariophyceae		
Licmophora sp.	*Convoluta convoluta* (Turbellaria)	Apelt 1969
Fragilaria shiloi	*Amphistegina lessonii* (Foraminifera)	Lee et al. 1982
Nitzschia panduriformis	*Heterostegina depressa* (Foraminifera)	Lee et al. 1982
Nitzchia sp. ?	*Prianos melanos* (Porifera)	Cox & Larkum 1983

diversity of their hosts, is consistent with the concept of a polyphyletic origin of microalgal-invertebrate symbioses (Trench 1992, Banaszak et al. 1993, McNally et al. 1993). Although many of the microalgal groups (e.g., the cyanobacteria and the rhodophytes) involved in symbiosis may be of ancient lineages, it is not currently possible to determine when in their respective histories symbiotic algae and

invertebrates initiated their associations. Despite the many recent advances in molecular phylogeny, there is at present no unambiguous way to determine the time of initiation of microalgal-invertebrate symbiosis. It is also not possible to resolve whether two taxa currently existing in symbiosis have remained together throughout, or if there have been occurrences of dissolutions of symbioses, followed by subsequent reassociations involving different taxa. The view is generally held that symbioses between dinoflagellates and scleractinian corals were initiated in the mid Triassic (Wells 1956, Stanley 1981), and in at least one scheme of dinoflagellate evolution (Loeblich 1984) symbiotic dinoflagellates in the genus *Symbiodinium* (referred to as *Zooxanthella*) are represented as having evolved in the Triassic. There are no other invertebrate or microalgal groups where available evidence permits even the most guarded flights of fancy (Lee et al. 1979, Lipps 1983).

Although few symbiotic cyanobacteria have been identified, the morphological differences perceived in published micrographs suggests that their diversity is higher than is currently appreciated. In the case of *Prochloron*, the green cyanobacteria symbiotic with didemnid ascidians and purportedly some other invertebrates, there is morphological (Swift 1989), physiological and biochemical evidence (Alberte 1989) which suggests that the genus may not be monospecific. Although these cyanobacteria have not been cultured, it should be possible to apply molecular techniques such as restriction fragment length polymorphism (RFLP) analyses of the complete genome, or sequence analyses (Rowan 1991) of individual genes in order to assess their taxonomic diversity and geographic distribution, as well as their phyletic affiliation.

Despite the concerted taxonomic attention that the *Chlorella* group, symbiotic with fresh-water ciliates, hydroids and sponges, has received (Reisser 1984, Reisser et al, 1988), our understanding of the species diversity and host distribution of these algae remains rather confused. However, it seems reasonably clear from the studies of Kessler (1984) and Reisser et al. (1988) that the genetic diversity of symbiotic *Chlorella* may be higher than previously appreciated, but in general, these studies were unable to elucidate interspecies relationships (Huss et al. 1989). Although phylogenetic reconstruction inferred from analyses of the small subunit ribosomal RNA gene (SSU rDNA) sequences has been conducted with some members of the genus (Huss and Sogin 1990), only free-living representatives were employed. Hence it remains unclear whether the *C. vulgaris* identified by Reisser et al. (1988) as symbionts are the same genetic entities identified as *C. vulgaris* and analysed by Huss and Sogin (1990). It is also apparent from the studies emanating from D. C. Smith's laboratory (Douglas and Smith 1984) and those from Muscatine's laboratory (Muscatine et al. 1975) that the *Chlorella* harboured by the English *Hydra viridis* and that by the Florida *H. viridis* are probably not identical. It is also possible that the English and Florida "strains" of *H. viridis* are not conspecific.

Excluding the dinoflagellates, the other two microalgal groups with diverse symbiotic representatives

Table 2. Known dinoflagellate taxa symbiotic with marine invertebrates

Microalga	Host	Authority
6. Pyrrophyta		

Gymnodiniales		
Gymnodinium béii	*Orbulina universa* (Foraminifera)	Spero (1987)
G. vertebralis	*Marginopora vertebralis* (Foraminifera)	Lee (1980)
Gyrodinium sp.	*Globigerinoides ruber* (Foraminifera)	Spindler & Hemleben (1980)
Aureodinium sp.	*Globigerinoides sacculifera* (Foraminifera)	Anderson & Bé (1976)
Symbiodinium microadriaticum	*Cassiopeia xamachana* (Scyphozoa)	Freudenthal (1962)
S. goreauii	*Ragactis lucida* (Actiniaria)	Trench & Blank (1987)
S. kawagutii	*Montipora verrucosa* (Scleractinia)	Trench & Blank (1987)
S. pilosum	*Zoanthus sociatus* (Zoanthidea)	Trench & Blank (1987)
S. corculorum	*Corculum cardissa* (Bivalvia)	Banaszak et al. (1993)
S. meandrinae	*Meandrina meandrites* (Scleractinia)	Banaszak et al. (1993)
S. pulchrorum	*Aiptasia pulchella* (Actiniaria)	Banaszak et al. (1993)
S. cariborum	*Condylactis gigantea* (Actiniaria)	Banaszak et al. (1993)
S. bermudense	*Aiptasia tagetes* (Actiniaria)	Banaszak et al. (1993)
S. californium	*Anthopleura elegantissima* (Actiniaria)	Banaszak et al. (1993)

Various *Symbiodinium* species apparently occur in varied taxa such as ciliates, sponges (e.g. *Cliona*), nudibranch molluscs, etc., but neither their taxonomy nor functional aspects of these associations have been studied in detail.

Amphidinium klebsii	*Amphiscolops langerhansi* (Turbellaria)	D. L. Taylor (1971)
A. belauense	*Haplodiscus* sp. (Turbellaria)	Banaszak et al. (1993)
Amphidinium sp.	*Peneroplis* sp. (Foraminifera)	D. L. Taylor (1974)
Amphidinium sp.	*Orbitolites* sp. (Foraminifera)	D. L. Taylor (1974)
Peridiniales		
Scrippsiella velellae	*Velella velella* (Pacific) (Hydrozoa)	Banaszak et al. (1993)
S. nutricula	*Collozoum inerme* (Radolaria)	Banaszak et al. (1993)
S. chattonii	*V. velella* (Mediterranean)(Hydrozoa)	Banaszak et al. (1993)
Phytodiniales		
Gloeodinium viscum	*Millepora dichotoma* (Hydrozoa)	Banaszak et al. (1993)
Prorocentrales		
Prorocentrum concavum	*Amphiscolops* sp. (Turbellaria)	Yamasu (1988)
Gonyaulacales		
Pyrocystis fusiformis	*Hastigerina pelagica* (Foraminifera)	Alldredge & Jones (1973)

are the prasinophytes and the bacillariophytes. Although more detailed analyses of these taxa need to be conducted in order to determine their specific identities and their host and geographic distribution, the information currently available also indicates no correlation between the algal taxa and that of their respective hosts (Lee 1980).

The status of our understanding of symbiotic dinoflagellates indicates that, contrary to past views of two genera of symbiotic dinoflagellates (*Symbiodinium* and *Amphidinium*), there are at least seven

genera (Table 2) of endosymbiotic dinoflagellates (*Amphidinium*, *Aureodinium*, *Gloeodinium*, *Gymnodinium*, *Prorocentrum*, *Scrippsiella* and *Symbiodinium*) representing at least four orders (Trench 1992, Banaszak et al. 1993). The taxonomic position of *Amphidinium* is ambiguous (Lenaers et al. 1991, McNally et al. 1993). If "commensal" dinoflagellates (*sensu* Spero and Angel 1991) are included, then the phyletic distribution of symbiotic dinoflagellates is even broader (Alldredge and Jones 1973, Gaines and Elbrächter 1987). There are in addition several dinoflagellate symbionts whose specific identities remain obscure. Thinh et al. (1986) described the alga symbiotic with the zoanthid *Zoanthus* sp. from Australia as *S. microadriaticum*. However, this alga does not conform in its morphology *in hospite* to the type species *S. microadriaticum*, most notably in having two pyrenoids per cell, and a pattern of microtubules beneath the continuous cell wall of the coccoid (non-motile) cell. The mastigote stage is unknown. The stacked chloroplast thylakoids ("grana") are not taxonomically significant, as this feature has been observed in *S. microadriaticum* (A. Mandura and Trench, unpubl.) and *S. californium* (Banaszak unpubl.) as a response to growth in culture under high illumination, and in *S. velellae* (Banaszak et al. 1993) grown in culture under blue light. The identity of the undescribed symbionts of the jellyfish *Linuche unguiculata* (Kremer et al. 1990) is also unresolved.

3. MORPHOLOGY, BIOCHEMISTRY AND PHYSIOLOGY

The morphological characteristics of the genus *Symbiodinium*, and of the type species *Symbiodinium microadriaticum* have been established (Freudenthal 1962, Kevin et al. 1969, Trench and Blank 1987). Briefly, the coccoid cells *in hospite* or in culture are limited by a continuous cellulosic cell wall (Markell et al. 1992), which is external to the plasmalemma. The single chloroplast is lobed, with one pyrenoid and two stalks, and no invasive chloroplast thylakoids (as are present in the genera *Amphidinium*, *Scrippsiella* and *Gloeodinium*). Chromosome numbers vary with species (26-97). In culture, the motile (mastigote) stage is typically gymnodinioid, with the epicone slightly larger or of similar dimension to the hypocone, with very thin thecal plates in vesicles and associated microtubules. The transverse flagellum is ribbon-like, and the longitudinal flagellum lacks mastigonemes.

There has been for several years an implicit misunderstanding among many investigators of symbioses involving symbiotic dinoflagellates in the genus *Symbiodinium*, that the algae differ in their morphology, cell cycle and life history attributes *in hospite* and in culture. In culture, the algae occur as coccoid cells, limited by a continuous cell wall, and divide mitotically in the coccoid state only. Two mitotic divisions in rapid succession produce tetrads (Fig. 1). Cells may transform to the motile dinomastigote stage either following karyokinesis and cytokinesis, or without mitosis, producing one (Fig. 2), two or four (Fig. 1) motile cells (Fitt et al. 1981, Fitt and Trench 1983). In all these stages of the life history, the chromosome numbers remain constant for each species analysed (Blank and Trench 1985, Trench and Blank 1987), and despite reports to the contrary (Freudenthal 1962, D. L. Taylor 1973, 1974), there is neither evidence of meiosis nor of sexual recombination. *In hospite*, the algae occur predominantly as coccoid cells limited by a continuous cell wall, and divide mitotically in the coccoid state. Each algal cell is contained in a vacuole, the symbiosome, and following algal cytokinesis, each daughter cell is allocated to an individual symbiosome. Evidence that algal cells may produce their

motility apparatus (basal bodies and flagella) within the symbiosome was first reported in the zoanthid *Protopalythoa* sp. by Schoenberg and Trench (1980b), and has since been observed elsewhere (Trench 1981, Thinh et al. 1986). Tetrads have also been observed in *Zoanthus* sp. (L. V. Thinh, pers. commun.) and in *Tridacna maxima* and *Xenia* sp. (Trench, unpubl.).

INSERT FIGS. 1, 2 & 3 HERE

Both *Scrippsiella velellae* and *Gloeodinium viscum* (Banaszak et al. 1993) occur as coccoid cells *in hospite*, and mitosis occurs in the coccoid state. In culture both algae alternate from coccoid to motile stages, but as far as it has been determined, mitosis only occurs in the coccoid state. In *S. velellae* the motile cells are typically peridinioid, and transformation from the mastigote to the coccoid state involves lateral ecdysis (Morrill 1984). The mastigote stage of *G. viscum* occurs very infrequently, and motile cells are found in such low numbers as to make their study very difficult. *Gloeodinium marinum*, a free-living congener of *G. viscum*, is typically found in the plankton as mucilaginous aggregates (Bouquaheux 1971, F. J. R. Taylor 1976), and *G. viscum* in culture demonstrates the same characteristic. In the symbiotic *Amphidinium* species, be they intercellular (D. L. Taylor 1971) or intracellular (Trench and Winsor 1987), the algae *in hospite* retain their free-living morphology, including the flagellar apparatus, and are not coccoid. Both of these examples are different from *Tetraselmis convolutae* which, on establishing a symbiosis with its host *Convoluta roscoffensis*, loses its flagella, cell wall and eyespots (Parke and Manton 1967), and from the diatom *Licmophora* sp., which loses its silica "shell" after becoming associated with *C. convoluta*.

Our perception of the structure of the photosynthetic apparatus of *Symbiodinium* has recently undergone major modification. The water-soluble peridinin-chlorophyll *a*-proteins (sPCP) that were thought to be the major light-harvesting complexes of dinoflagellates (Prézelin 1987) are now known to represent only a small proportion of the cellular chlorophyll (Chl.)*a* and peridinin (Iglesias-Prieto et al. 1993). sPCP occurs as different supramolecular complexes in different dinoflagellate species. In *S. kawagutii*, there are two distinct spectral forms of Chl *a* molecules, each associated with two dimeric peridinin molecules and a monomeric apoprotein with apparent molecular mass (M_r) of 35 kDa. In *S. pilosum*, there are two distinct spectral forms of Chl *a*, each associated with two dimeric peridinin molecules and a dimeric apoprotein with subunits of M_r 15 kDa. Both of these supramolecular complexes coexist in *S. microadriaticum* (Iglesias-Prieto et al. 1991), but *S. californium* may also possess in addition to the 35 kDa complex, a complex with two spectroscopically distinct Chl. *a* molecules associated with two dimeric peridinin molecules and one "silent" peridinin, and a dimeric apoprotein, a situation analogous to that reported by Siegelman et al. (1977) for *Amphidinium carterae* (Ply 450). These data are consistent with the analysis of crystallized sPCP from *A. carterae* by Steck et al. (1990), where the estimated molecular mass of the complex is about 39 kDa. Similar complexes from a variety of dinoflagellate species are in the size range of about 37-40 kDa (Chang and Trench 1984, Prézelin 1987). The only report that is at variance is that of Gerberding et al. (1991) who proposed a native tetramer of sPCP of 120-140 kDa in *Symbiodinium* sp. from the coral *Acropora formosa*, based on electron microscopy of an isolated isoform, but provided no corroborating evidence. It is very likely that the structures observed are artifactual aggregates. sPCP of symbiotic dinoflagellates occurs as

several isoelectric forms that are species specific (Chang and Trench 1982, 1984), and the isoelectric points correlate well with the molecular size of the apoproteins (Iglesias-Prieto et al. 1991).

The major light-harvesting pigment-protein component in dinoflagellates is a Chl *a*-Chl c_2-peridinin-protein complex (acpPC) (Iglesias-Prieto et al. 1993). This complex, isolated by thylakoid solubilization with n-dodecyl-ß-d-maltoside and sucrose density gradient centrifugation, contains Chl *a*, Chl c_2 and peridinin in a molar ratio of 1:1:2, and represents 45%, 75% and 70% of the cellular Chl *a*, Chl c_2 and peridinin, respectively, in *S. microadriaticum*. The acpPC apoproteins possess an Mr value of 19-20 kDa, similar to analogous proteins in diatoms and brown algae. Antibodies against sPCP failed to cross-react with any of the thylakoid-associated complexes, as did antibodies against Chl*a*-*c*-fucoxanthin apoprotein complexes from diatoms. The acpPC appears to be associated with the P_{680} reaction centre (photosystem II).

The core of photosystem I, possibly associated with a light-harvesting complex, contains 12% of the cellular Chl *a* and all the P_{700} activity in preparations from *S. microadriaticum*. This complex is spectroscopically similar to analogous preparations from different taxonomic groups, but demonstrates an unique apoprotein composition. Antibodies against the P_{700} apoprotein of plants did not cross-react with the photosystem I complex from *S. microadriaticum* (Iglesias-Prieto et al. 1993). Similar results were obtained with other symbiotic dinoflagellates, and with the non-symbiotic dinoflagellate *Heterocapsa pygmaea*. The evidence currently available (Iglesias-Prieto and Trench 1994) suggests that excitation energy absorbed by sPCP and by acpPC is transferred to the P_{680} reaction centre II, and ultimately to the P_{700} core of reaction centre I.

The mechanism of photosynthetic carbon fixation has been studied fairly extensively in *Symbiodinium* species, and has been reviewed recently by Muscatine (1990). Many species appear to fix CO_2 via the Benson-Calvin pathway, often referred to as the C_3 pathway, and produce ^{14}C-labeled 3-phosphoglycerate as the first product of $^{14}CO_2$ fixation. Consistent with this is the immunocytochemical localization of ribulose-bisphosphate-carboxylase-oxygenase (RubisCO) in carboxysome-like structures, and in the plastid stroma and pyrenoid of the chloroplast of *S. kawagutii* (Blank and Trench 1988). Gerberding et al. (1990) claimed to have isolated RubisCO from several *Symbiodinium* species and, using antibodies against RubisCO from silver beet in immunoblot analyses, they estimated the molecular size of the large subunit as approximately 35 kDa. In most photosynthetic organisms the large subunit of RubisCO is 50-60 kDa.

In addition to RubisCO, *S. kawagutii* grown in axenic, unialgal culture demonstrate (i) 10-25 times higher activities of phosphoenol pyruvate carboxylase (PEPcase) than several other species of symbiotic dinoflagellates, (ii) NAD- and NADP-dependent malate dehydrogenase (MDH) (Ting 1976, Trench and Fisher 1983), and probably most significantly, (iii) pyruvate-Pi dikinase activity (Tytler and Trench 1986). All these enzymes are characteristic of the β-carboxylation or "C_4" pathway of photosynthetic carbon fixation (Zelitch 1971, Appleby et al. 1980). Consistent with the presence of these functional enzyme systems, this alga demonstrates ^{14}C-malate as one of the earliest stable products of $^{14}CO_2$ fixation. However, these enzymes have not been detected in some other *Symbiodinium* species, and dinoflagellates do not demonstrate the spatial and temporal separation of C_3 and C_4 fixation as do

tropical grasses and crassulacean plants. Therefore the debate on whether symbiotic dinoflagellates demonstrate C_3 or C_4 photosynthesis is moot. Different species of *Symbiodinium* in culture do demonstrate different sensitivities in photosynthesis under conditions of different O_2 and CO_2 tensions (Trench and Fisher 1983).

In estimates of the productivity of symbiotic dinoflagellates, a significant consideration is the existence of photorespiration. One of the early essential enzymes of the photorespiratory pathway, phosphoglycolate phosphatase, has been demonstrated in *Symbiodinium* species, (Randall 1976, Trench and Fisher 1983), but glycolate oxidase has never been demonstrated, and evidence for glycolate dehydrogenase is at best equivocal. These two enzymes may follow phosphoglycolate phosphatase in the photorespiratory pathway (Raven and Beardall 1981). Catalase has been demonstrated by Tytler and Trench (1988) and Matta and Trench (1991), as have the superoxide dismutases (SOD) (Matta et al. 1992). The potential for photorespiration does exist in *Symbiodinium*, and freshly isolated or cultured algae demonstrate ^{14}C-glycolate as a product of photosynthetic $^{14}CO_2$ fixation, and release glycolate to the medium (Muscatine and Cernichiari 1969, Trench 1971a, b, Trench and Fisher 1983). Photorespiration by algae *in hospite* has not been demonstrated, but there are reports indicating CO_2 limitation (Weis et al. 1989, Weis 1991), and high O_2 tension (D'Aoust et al. 1976, Dykens and Shick 1982) in symbiotic associations. These are the precise conditions that promote photorespiration (Benedict 1978).

Symbiodinium species are known to release a significant proportion (25-60%) of the total photosynthetically fixed radiolabelled carbon as small molecular weight metabolites (glycerol, glucose, alanine and organic acids) when freshly isolated from their respective hosts and incubated in the presence of an homogenate of the host's tissues (Muscatine 1967, Muscatine et al. 1972, Trench 1971a, b, reviewed in Cook 1983, and Muscatine 1990). Symbiotic *Amphidinium* species do likewise (Trench, unpubl.). In general these algae only release a small proportion of their photosynthetically fixed carbon as small metabolites when in culture, except *S. kawagutii* (Chang et al. 1983). However, it has recently become apparent that symbiotic dinoflagellates release significant quantities of glycoconjugates in culture (Markell et al. 1992, Markell and Trench 1993), and preliminary immunocytochemical localization studies using antibodies against released glycoconjugates indicate that the phenomenon also occurs *in hospite* (Markell and Trench, unpubl.). Consistent with these observations, O'Conner and Muscatine (pers. commun.) have found ^{14}C-labelled essential amino acids in hydrolysates of the tissues of *Aiptasia pulchella* after incubation of the intact association in $^{14}CO_2$ and subsequent removal of the algal cells. As these glycoconjugates contain virtually all the *essential* amino acids, and assuming host assimilation, they could indicate that intact symbioses involving these algae are closer to being truly autotrophic than previously appreciated. It is also apparent that the algae may be more than a source of "junk food" (Falkowski et al. 1984) to their hosts, and this will have to be given serious consideration in future calculations of the quantity and quality of the contribution of symbiotic algae to the nutrition of their hosts.

Nitrogen and phosphorus metabolism in microalgal-invertebrate symbioses continues to be an area of controversy, and I shall not discuss phosphorus here. There are three recent reviews that the interested reader should consult; Miller and Yellowlees (1989), D'Elia and Wiebe (1990) and Falkowski

et al. (1993). Nitrogen is potentially available from the environment to the symbiosis as NH_4^+ or as NO_3^-. Traditionally, the view has been that the algae are responsible for the assimilation of inorganic nitrogen, either as NH_4^+ or NO_3^- from the environment or as NH_4^+ from the hosts' pool of end-products of protein catabolism (Lewis and Smith 1971). This idea has been the corner stone of the concept of nutrient recycling within microalgal-invertebrate symbioses, and has also been the basis of the concept that in nitrogen depauperate oligotrophic environments, the animal hosts regulate the growth rates of the algal population by controlling the availability of nitrogen to them. Depletion of NO_3^- by several corals and a foraminiferan has been demonstrated (see review by Muscatine 1980), but the anemone *Aiptasia pulchella* and the jellyfish *Mastigias* sp. do not take up NO_3^- (Muscatine and Marian 1982, Wilkerson and Muscatine 1984). All *Symbiodinium* species tested to date assimilate NO_3^- in culture (Wilkerson and Trench 1985), but only after the supply of NH_4^+ is depleted, an observation that is consistent with the inhibition of nitrate reductase by NH_4^+, and its induction in the absence of NH_4^+ and the availability of NO_3^-. The explanation for the estimated low rates of algal population growth *in hospite* has been that the algae are nitrogen limited (Cook and D'Elia 1987). However, when natural populations of corals are experimentally placed in reduced levels of illumination, the algae photo-acclimate by synthesizing *de novo* pigment-protein complexes (Falkowski and Dubinsky 1981), a process that is severely inhibited in dinoflagellates experiencing nitrogen limitation (Prézelin and Matlick 1983).

Empirically determined specific growth rates (μ = divisions d^{-1}) of *Symbiodinium* spp. in log phase culture in saturating light ranges from 0.22 to 0.43, depending on the species (Chang et al. 1983). Values of μ estimated for algae *in hospite*, usually based on mitotic index data, range from a high of 0.39 for the algae in the nudibranch *Pteraeolidia ianthina* (Hoegh-Guldberg et al. 1986) to a low of 0.009 (Muscatine et al. 1983, 1984) (see Wilkerson et al. 1983, Cook et al. 1988). However, specific growth rates of algae during repopulation of *Aiptasia* spp. are 0.38 (Schoenberg and Trench 1980c) and 0.40 (Berner et al. 1993). In each case, following attainment of a characteristic population density, algal growth achieves an asymptote, the level of which depends on the algal species, implying that some unknown density-dependent factor becomes limiting.

Determination of the carbon:nitrogen ratio of symbiotic dinoflagellates in log phase culture grown under saturating light produced mean values of 5.4-7.6 depending on the species assayed (Chang et al. 1983). The values for freshly isolated symbionts from field-collected *Aiptasia pallida* was 7.9 (Cook et al. 1988), suggesting no nitrogen limitation *in hospite*, and 9.66 in the jellyfish *Mastigias* sp. (Muscatine et al. 1986), which does suggest nitrogen limitation. It has also been demonstrated that the division rates of algae *in hospite* increase when the NH_4^+ levels in the medium are experimentally increased (Cook and Fitt 1989, Muscatine et al. 1989), and when the host is provided with a surfeit of organic nitrogen as *Artemia* nauplii (Muscatine et al. 1989, Fitt and Cook 1989). Most reports indicate that in marine coelenterates, "excess" algae are not expelled by the host, but voiding of viable symbiotic algae from the hypertrophied siphon through the rectum and anus has been documented in *Tridacna maxima* (Trench et al. 1981). Symbiotic cnidarians do not excrete NH_3, but aposymbiotic and non-symbiotic ones do. C. E. Taylor et al. (1989), in a computer simulation study of the control of algal population density in

Hydra, indicated that the phenomenon is complex, and that a combination of factors, including nutrients other than nitrogen and phosphorus, may play a role. The significant point was that alleviating a limitation resulted in a new equilibrium level becoming established. All the available data, when viewed together, are consistent with the principles encompassed by Leibig's Law of the Minimum, and therefore the phenomenon of algal growth *in hospite* can be reconciled by the application of either chemostat or turbidostat models, or a combination of these, and the concept of the cell quota (Droop 1983).

The controversy over the mechanism of nitrogen acquisition by symbiotic associations appears to revolve around which partner in the association, the alga or the host, is responsible for the uptake of NH_4^+. Basically, algae are believed to incorporate NH_4^+ via the glutamine synthase (GS)-glutamine oxaloglutarate amino tranferase (GOGAT) system, and via the glutamate dehydrogenase (GDH) system. Animal hosts are believed to use the GDH system (Miller and Yellowlees 1989). Several *Symbiodinium* spp. have been shown to possess several isoenzymes of GDH (Colley 1984, Dudler and Miller 1988), and evidence for a functional GS system comes from the studies of Muscatine (1980) and Summons and Osmond (1981), but to my knowledge, GOGAT has never been demonstrated. However, release of NH_3 by symbiotic corals when exposed to azaserine has been interpreted as evidence of a functional GOGAT system (Rahav et al. 1989). Although it has been pointed out by Rees (1987) and Miller and Yellowlees (1989) that animal tissues possess higher specific activities of GDH than their algal symbionts, it is the affinity of the enzyme for the substrate that is significant, and not the activity of the enzyme. The affinity of the algal GS is at least an order of magnitude higher than that of animal hosts GDH (Rahav et al. 1989).

4. SPECIFICITY AND SELECTIVITY

On the basis of the information presented in Tables 1 & 2, it is very evident that microalgal-invertebrate symbioses demonstrate specificity (Trench 1988). As far as it has been determined, in the natural habitat, *Velella velella* is always symbiotic with *Scrippsiella velellae* (Pacific) or *S. chattonii* (Mediterranean). The possibility that the two host populations may not be conspecific has been raised by Banaszak et al. (1993). Again, *Convoluta roscoffensis* is always symbiotic with *Tetraselmis convolutae*, *C. convoluta* with *Licmophora*, and *Cassiopeia xamachana* with *Symbiodinium microadriaticum* (Trench 1988, Rowan 1991). Even in cases where a given host harbours simultaneously two species of algae, as in the case of *Haplodiscus* sp. (Trench and Winsor 1987), the algae, *Amphidinium belauense* and *Symbiodinium* sp. co-occur throughout the geographic range of the host. Based on the information currently available, it is reasonably clear that different *Symbiodinium* species are symbiotic with different species of invertebrates, with the known exception of *S. microadriaticum* that is symbiotic with *Cassiopeia xamachana* and *C. frondosa*. Therefore, the basic tenet of the argument that in marine symbioses, symbiont diversity is low (Law and Lewis 1983, Law 1985, Smith and Douglas 1987, Smith 1993) is not substantiated. Without the realization of high symbiont and high host diversity, the concept of specificity in microalgal-invertebrate symbiosis is without foundation.

A rather large volume of literature exists on hypotheses, and attempts at testing hypotheses, of the mechanism(s) involved in producing specificity. Much of this literature has been reviewed (Trench

1988). At that time, the field seemed content with the premises that: (i) in their interactions during the re-establishment of symbioses, microalgae and hosts demonstrate a great deal of selectivity, (ii) that initial uptake of algae by host cells did not imply the formation of a stable association, (iii) that under conditions of laboratory experimentation, there appeared to be less specificity (i.e. a given host could form symbioses with different algae) than observed in natural populations, (iv) that the expressed specificity in the natural habitat was the end result of a multi-step process, on which forces of selection acted, resulting ultimately in one alga being best fit to form a stable symbiosis with a given host (e.g. Trench et al. 1981). In those hosts that directly transmit their algal or bacterial symbionts from one sexual generation to the next, the basis of specificity is apparent. The explanation why specificity in the laboratory seems less stringent than that expressed in the field is that the selective forces that may act on an association in the natural habitat do not operate under controlled laboratory conditions.

In those symbioses wherein the algae are faithfully transmitted from parent to offspring during sexual reproduction in a "closed system", it is evident how the specificity of the association is maintained. It is in those symbioses with an "open system" of inheritance that the process of selection is best manifested. It also bears reiteration that non-symbiotic species of corals or anemones can not be rendered symbiotic by exposure to symbiotic algae obtained from their symbiotic relatives (Trench 1987).

An hypothesis that was the foundation of the concept of selectivity, was that specific molecules (ligands) on the surface of algae, associated with cell walls, interacted with receptors on the plasmalemma of animal cells, and that selection was based on recognition by the host of specific algae that displayed the appropriate molecular signals. An opposing viewpoint, advanced by Rahat and Reich (1988) was that microalgae are "preadapted" to existence in a "characteristic" host cell environment, and that selectivity was based on the alga's ability to survive in a given host cell milieu, and not on any molecular recognition phenomenon. This latter notion is not consistent with the ever expanding evidence derived from analyses of analogous symbioses such as *(Brady)Rhizobium*-legume associations (Lerouge et al. 1990, Sánchez et al. 1991), and pathogenic bacteria-plant interactions (Dixon and Lamb 1990, Regensburg-Tuink and Hooykaas 1993). The cellular processes of phagocytosis (the mechanism whereby symbiotic algae enter hosts' cells [McNeil et al. 1981, Colley and Trench 1983, 1985]) involves ligand-receptor interactions and various signal transduction events. Other processes in the establishment of specific associations, such as inhibition of phago-lysosome fusion, in all likelihood also involve ligand-receptor interactions The molecular aspects of such interactions in microalgal-invertebrate symbioses have not yet been elucidated.

Evidence for the existence of macromolecules associated with the cellulosic cell walls of *S. microadriaticum*, *S. kawagutii* and *S. pilosum*, comes from the report of Markell et al. (1991), who found that, after extraction of isolated cell walls, polypeptides ranging in mass from 14-200 kDa could be resolved by SDS-PAGE. There was no obvious correlation between the polypeptide patterns from the different algae and compatibility with the scyphistomae of *C. xamachana* It was not determined whether the polypeptides were constituitive components of the cell wall or were molecules in transit through the walls.

Perhaps more significant than the presence of macromolecules associated with the cell walls was the observation that symbiotic dinoflagellates exude an heterogeneous mixture (M_r range, 14-200 kDa) of macromolecules in culture (Markell et al. 1992, Markell and Trench 1993). Several species of *Symbiodinium* tested all demonstrate the same phenomenon. In culture, the algae export 40-50 pg . $cell^{-1}$ after 30 days of growth. Analysis of the exudates indicates that they contain protein, neutral sugars, sugar amines and uronic acids. The protein:neutral sugars ratios range from 0.3 to 4.9, depending on the algal species. Neither the sugar, sugar amines nor relative protein content showed any correlation with the alga's ability to infect, and form an association with *C. xamachana*. However, all three species of *Symbiodinium* that infect *C. xamachana* under laboratory conditions, demonstrated significantly higher uronic acid content of the exudates than those species that do not infect. A net negative charge on algal surfaces has been implicated as a factor in the recognition process (McNeil et al. 1981). Antibodies prepared against a large molecular weight fraction (>50 kDa) of the exopolysaccharides from *S. microadriaticum* (anti-SmXuL) cross-reacted with exudate from *S. microadriaticum* only, in immunoblot assays. It is not currently known whether the antibodies recognize the carbohydrate or the protein components of the exudate.

Evidence that the release of exopolysaccharides by symbiotic dinoflagellates also occurs *in hospite* comes from the preliminary observations that, in electron microscopic immunocytochemical localization studies employing anti-SmXuL, components that cross-react with the antibodies were detected in the host tissues (Markell and Trench unpubl.), including a select group of host cell nuclei. Of interest was the observation that, in the process of transport from the algae to the host amoebocytes and then to the host tissues, there was no evidence of vesiculation of the glycoconjugates.

5. PHYLOGENETICS AND EVOLUTION

The wide phyletic distribution of symbiotic microalgae, the wide phyletic distribution of their hosts, and the absence of correlation between symbiont and host taxa, taken together are indicative of a polyphyletic origin of microalgal-invertebrate symbiosis (Trench 1992, Banaszak et al. 1993, McNally et al. 1993). The same can be said of the symbioses between sulfur oxidizing and methanotrophic bacteria and invertebrates (Fisher 1990, Feldbeck and Distel 1991). Despite the recent advances in molecular phylogeny, many aspects of the phylogenetic relationships among microalgae remain unresolved. Similarly, many details of the phylogenetics of invertebrates also remain obscure. There are two invertebrate groups in which the phylogenetics is reasonably clear, the Foraminifera (Lipps 1983) and the Scleractinia (Wells 1956, Veron 1986). Both groups demonstrate the principle that there is no correlation between host taxa and phylogeny, and symbiosis with microalgae (Trench 1987).

In any attempt to understand phylogenetic relationships among symbiotic dinoflagellates, the symbiotic species must be placed in the context of their non-symbiotic (free-living) relatives. The evolution and phylogenetics of dinoflagellates have been discussed at length by Dodge (1984), Loeblich (1984), F. J. R. Taylor (1987), and more recently, Fensome et al. (1993).

A reconstruction of protozoan phylogeny inferred from analyses of SSU rDNA sequences, indicates that dinoflagellates, ciliates and apicomplexans share a common ancestry (Johnson et al. 1990,

Gajadhar et al. 1991, Barta et al. 1991, Sadler et al. 1992, McNally et al. 1993). The evidence presented by Ariztia et al. (1991) and Bhattacharya et al. (1992) and McNally et al. (1993) indicate that dinoflagellates are distantly related to the chromophyte-oömycete lineage. This conclusion is consistent with the observations of Iglesias-Prieto et al. (1993) that the pigment-protein complexes of the light-harvesting and core reaction centres of dinoflagellates are not homologous with those of diatoms.

In most phylogenetic considerations, either the prorocentroids (F. J. R. Taylor 1987) or an *Oxyrrhis*-like (Loeblich 1984) dinoflagellate has been proposed as being most closely related to the ancestral form. In the study conducted by Sadler et al. (1992), *Crypthecodinium cohnii* rather than *Prorocentrum micans* appeared to be the dinoflagellate most closely linked to the apicomplexans. However, the study of McNally et al. (1993) emphasized that, given the data base of dinoflagellate SSU rDNA sequences (either complete or partial) currently available, it is not possible to resolve which dinoflagellate group represents the ancestral form. Whether *C. cohnii* is gonyaulacoid (F. J. R. Taylor 1987) or peridinioid (Dodge 1984) appears to be reconciled by molecular phylogenetics which places *C. cohnii* and *Alexandrium tamarense*, an undisputed gonyaulacoid, in very close juxtaposition, and indicates a distant relationship to the peridinioids *Heterocapsa* and *Thoracosphaera* (McNally et al. 1993). It is significant that prorocentroid (*P. concavum*), gonyaulacoid (*P. fusiformis*) and peridinioid (*S. velellae*) dinoflagellates are recognized as symbionts (see Table 2).

INSERT FIG. 4 HERE

The analyses of McNally et al. (1993) indicated that the genera *Symbiodinium* and *Gymnodinium* are closely related, and both are distantly related to *Amphidinium* (Fig. 4). The latter observation is consistent with the results of Lenaers et al. (1991) who inferred the phylogeny of dinoflagellates employing partial sequences of the large subunit of the rRNA gene.

Among the symbiotic dinoflagellates in the genus *Symbiodinium*, the three species *S. pilosum* (from the zoanthid *Zoanthus sociatus* from Jamaica, W.I.), *S. meandrinae* (from the massive form of the scleractinian coral *Meandrina meandrites* from Jamaica) and *S. corculorum* (from the bivalve *Corculum cardissa* from Belau [Palau], Western Caroline Is.) were found to be very closely related based on the similarity of the complete SSU rDNA sequences. Based on analyses of partial SSU rDNA sequences, employing hypervariable regions V2 and 4 of the rRNA gene, *S. bermudense* (from *Aiptasia pallida* [= *A. tagetes*] from the Caribbean) and *S. pulchrorum* (from *Aiptasia pulchella* from Hawaii) were also very closely related, but distant from the three species mentioned above. The molecular evidence was found to be completely consistent with evidence from biochemistry, physiology and morphology. Morphologically, *S. pilosum*, *S. meandrinae* and *S. corculorum* all demonstrate characteristic mucocysts and a pilose surface (Figs. 1 & 3) in the coccoid and motile stages in culture, but show differences in the thecal plate patterns in the mastigote stage. Biochemically, all three species possess the dimeric 15kDa form of the water-soluble peridinin-chlorophyll *a*-apoprotein (sPCP) (Govind et al. 1990). Although *S. pulchrorum* and *S. bermudense* are morphologically and genetically closely related, the former possesses the 35kDa monomeric form of sPCP, while the latter possesses the 15kDa dimeric form. Neither of these two species possess mucocysts or a pilose surface. Blank and Huss (1989) based on DNA hybridization analyses, concluded that *Symbiodinium microadriaticum* and the symbionts from

Condylactis gigantea (*S. cariborum*) were conspecific, the latter being a subspecies of the former. However, Govind et al. (1990) showed that the biochemistry of the two algae, with regard to the structure of the sPCP apoproteins were different.

Many aspects of the evolution of dinoflagellate-invertebrate symbioses remain unresolved. One very fundamental problem pertains to the time of initiation of these associations. Resolution of this problem, aggravated by the high diversity of algal symbionts and their hosts, requires secure evolutionary histories of both partners. Unfortunately, such historical information is not currently available Although it is often assumed that the 260 million years of evolution of scleractinian corals reflects the time frame of their symbiosis, there is no good evidence indicating that those coral taxa currently existing in symbiosis have been associated with dinoflagellates for that entire period. The same can be said for the foraminiferans. These two invertebrate groups possess probably the best known fossil record; in other groups, such as the actiniarians, alcyonaceans, the acoelous flatworms and the bivalves, the fossil record provides very little information.

Another problem pertains to the rates of evolutionary change in symbionts and hosts. Despite the advances in molecular phylogeny, it is still very difficult to determine the time of divergence among most microalgal groups. It is often assumed that the intracellular environment that many microalgal symbionts occupy is such a "stable" one than evolutionary change would proceed very slowly. A corollary of the specificity of microalgal-invertebrate symbiosis is that symbionts and hosts co-evolve. There is very little direct evidence to support either view. At present there is no evidence for any exchange of genes between microalgal symbionts and hosts.

The only evidence on aspects of the rates of genetic change in symbiotic microalgae can be extracted from the phylogenetic analyses of Rowan and Powers (1991a, b, 1992) and McNally et al. (1993). Comparison of the sequences of the rDNA from *Symbiodinium bermudense* and *S. pulchrorum*, symbiotic with *Aiptasia tagetes* and *A. pulchella*, respectively, indicates that they are very closely related, but not identical. The former association is Caribbean, while the latter is Indo-Pacific. Similar analyses of sequences of rDNA from *S. pilosum*, *S. meandrinae* (Caribbean) and *S. corculorum* (Indo-Pacific) indicate the close genetic similarity of these algae, which are symbiotic with a zoanthid, a stony coral and a bivalve, respectively. Using the estimated time of the last physical isolation of the Caribbean and Indo-Pacific regimes as about 3-3.5 million years ago (Keigwin 1982, Coates et al. 1992), it is possible to interpret the data in two opposite ways. First, that little genetic change has occurred in the algae mentioned above, regardless of the host taxa with which they are associated. Second, the difference in SSU rDNA sequences between *S. pilosum*, *S. meandrinae* and *S. corculorum* is about 0.6% (McNally et al. 1993), which, when placed in the context of the 1% change per 50 million years estimated as the average rate of change of eukaryote SSU rRNA genes (Ochman and Wilson 1987), would translate to a substitution rate equivalent to approximately 1% per 6 million years, which, if speciation was coincident with the separation of the Atlantic and Indo-Pcaific, is quite rapid. It is very likely that diversification within the genus *Symbiodinium* was already well underway before the separation of the two oceans, and so the 3.5 million year date may be an underestimate of the time of divergence. This might be significant in light of the fact that, with the exception of *S. pilosum* in *Z. sociatus*, which represents a "closed

system" of inheritance (*sensu* Trench 1987) and is therefore more isolated, all the others represent "open systems". Fixed rates of nucleotide subsitution across diverse taxa should be interpreted with caution, as it is clear that within some groups (e.g. the apicomplexans) certain genera such as *Plasmodium* demonstrate apparently more rapid change than other members of the group (Sadler et al. 1992), and among the dinoflagellates, *Alexandrium tamarense* and *Crypthecodinium cohnii* demonstrate a similar phenomenon (McNally et al. 1993).

6. ACCLIMATION, ADAPTATION AND ECOLOGY

All phototrophic organisms respond to changes in the light regime. As the light field decreases, phytoplankton (Prézelin 1987) and symbiotic microalgae (Trench 1987) respond by increasing portions of the photosynthetic machinery responsible for harvesting the radiant energy. This process, when occurring in the short term is called photo-acclimation, distinguished from long-term genotypically fixed responses termed photo-adaptation (*sensu* Berry and Raison 1981, Björkman, 1981). In all aquatic environments, photon flux density decreases exponentially with increasing depth. Because of the three-dimensional topographic complexity of coral reefs, invertebrates symbiotic with microalgae occupying habitats at the same depth may experience different levels of illumination (Chang et al. 1983). Adjustment of their light-harvesting capabilities results in the algae being able to maintain their contribution of photosynthetically fixed carbon to their hosts (Muscatine et al. 1984).

Approaches to analyses of the mechanism(s) of photo-acclimation of photosynthesis in symbiotic associations has been based on experiments carried out on intact associations and on symbiotic algae in culture. Although the latter approach has been criticized as not being relevant to symbioses, estimating the functional aspects of the algae in intact associations requires a series of assumptions, some of which may not be entirely valid. In natural populations, the symbiotic algae may, or may not be nitrogen limited (as discussed above), and interpretations of photosynthetic performance based on net O_2 evolution by the association, assuming constant animal respiration rates may not be justified (Chang et al. 1983, Edmunds and Spencer-Davies 1988). Analysis of the symbionts in log-phase growth in culture, as is done with phytoplankton, obviates the problems of nutrient status of the algae and possible influences of the animal hosts. This latter approach can be regarded as an approximation of the maximum potential for photo-acclimation that a given species of symbiotic alga may demonstrate. That species may, or may not realize the same potential *in hospite*.

Only a few studies of photo-acclimation in microalgae (symbiotic or planktonic) have directly measured changes in the characteristics of the photosynthetic unit (PSU), or have measured simultaneously both reaction centres (RCI and RCII) (Larkum and Barret 1983). With specific regard to dinoflagellates, Dubinsky et al. (1986) demonstrated than in *Prorocentrum micans* in culture, there was a 1.6-fold increase in RCI in cells grown at 70 µmol quanta $m^{-2}.s^{-1}$ relative to those grown at 600 µmol quanta $m^{-2}.s^{-1}$. This result would be consistent with an increase in PSU number. By contrast, the symbiotic dinoflagellate from the coral *Pocillopora verrucosa* showed no difference in RCII, but those collected from depths 45 meters indicated a 1.5-fold increase in Chl *a*-P_{680} relative to those collected at 20 meters (Zvalinskii et al. 1980). Similarly, the algae from *Stylophora pistillata* taken from habitats of

high and low levels of illumination demonstrated the same RCI content, but the algae from low-light corals had a 4-fold higher ratio of Chl*a*:P_{700} (Falkowski and Dubinsky 1981). These observations are consistent with changes in PSU size. Since these reports, it has been generally assumed that all symbiotic dinoflagellates photo-acclimate to low levels of illumination by increasing PSU size.

The first report to suggest that symbiotic dinoflagellates may not all photo-acclimate to reduced levels of illumination by increasing PSU size was that of Chang et al. (1983) who, employing the algae from *Tridacna maxima* (*Symbiodinium* sp.), *Aiptasia pulchella* (*S. pulchrorum*) and *Montipora verrucosa* (*S. kawagutii*) in log-phase culture, demonstrated different patterns of changes in pigment composition and different photosynthesis versus irradiance (P vs. I) relationships. Employing the theoretical model developed for phytoplankton (Prézelin and Sweeney 1978), they concluded that the three algae acclimated by altering PSU size, PSU number, or the activities of photosynthetic or electron transport enzymes, respectively. It is now recognized that the theoretical model employed, assuming fixed PSI:PSII stoichiometries, is not consistent with empirical observations (Falkowski and LaRoche 1991).

In a recent study employing *Symbiodinium microadriaticum*, *S. kawagutii*, and *S. pilosum* grown at 40 or 250 μmol quanta $.m^{-2}.s^{-1}$, and measuring P versus I relations, photosynthetic pigments, P_{680} and P_{700} contents, Iglesias-Prieto (1993) found that the three species of algae responded differently to low levels of illumination, but that the changes involved differential increases in both the sizes and numbers of the PSU. In *S. microadriaticum*, P_{680} increased 1.3-fold, while P_{700} increased 2.2-fold; in *S. kawagutii*, P_{680} increased 1.5-fold, while P_{700} increased 1.2-fold; in *S. pilosum*, P_{680} doubled, while P_{700} increased 1.5-fold. In *S. microadriaticum*, photo-acclimation also involves differential changes in sPCP; low light cells showed a 4-fold increase in the cellular content of the dimeric form, while the monomeric form remained unchanged. Consistent with the changes indicated, the Chl. *a* content of the various Chl-protein complexes, RCII, acpPC, and RCI, also changed. Perhaps the most interesting observation in terms of the significance of photo-acclimation to the daily net production by the algae was that, compared to the other two species, *S. microadriaticum* was capable of maintaining high cell-specific P_{max}, suggesting that this alga is adapted to efficiently utilize variable levels of illumination. This interpretation raises the possibility that different species of *Symbiodinium* may be genetically adapted to different light regimes (*sensu* Björkman 1981), and that the vertical distribution of symbiotic invertebrates may be a reflection of the adaptive capabilities of their respective algal symbionts.

There are very few reports of analyses of the acclimatory or adaptive capabilities of symbiotic associations to other environmental parameters such as temperature and salinity. The problems of elevated pO_2 and ultraviolet radiation have been studied by Lesser and Shick (1989) and Lesser et al. (1990) and Matta and Trench (1991). Recent concern over the phenomenon termed "coral bleaching", wherein coral reef-dwelling invertebrates lose their algal symbionts in response to elevated temperature (Glynn and D'Croz 1990, Goreau and Hayes, 1993) and may ultimately die (Glynn 1993), has led to an interest in the response of dinoflagellate-invertebrate symbioses to thermal stress. In analyses of the response of symbiotic systems to environmental extremes, it is necessary to determine which of the two components in the association perceives and responds, or whether they both do. Hence, it would be

ideal to establish how the animal hosts and the algae respond separately, as well as together, to any given stimulus.

Gates et al. (1992) analysed the response of the anemone *Aiptasia pulchella* and the coral *Pocillopora damicornis* to low (12° C) and elevated (32° C) temperatures. They observed that under either condition of thermal stress, the animals released intact endodermal cells containing algae, and suggested that thermal stress resulted in animal cell adhesion dysfunction, the implication being that the perception of the stimulus, and the response, is an animal function. Iglesias-Prieto et al. (1992) analysed the response of *S. microadriaticum* in culture to elevated temperatures, and found that photosynthesis was severely impaired at temperatures above 30° C and was completely inhibited at 34-36° C. Analysis of whole cell fluorescence over the same temperature range indicated the same phenomenon, consistent with the interpretation that high temperature results in uncoupling of light energy capture and photochemistry, probably because of phase transition (increased fluidity) of the thylakoid membranes. This process ultimately leads to photodestruction of photosynthetic pigments. The major point here is that the algae also perceive and respond to elevated temperature. Unpublished observations in my laboratory indicate that different species of *Symbiodinium* are adapted to different temperature regimes, and show species-specific acclimatory responses to temperature, as they do to light. Animal hosts and their respective algal symbionts may not have the same temperature tolerance. The progression of the bleaching response by symbiotic invertebrates in many cases, is initiated by reduction in algal photosynthetic pigments (Hoegh-Gulberg and Smith 1989, Porter et al. 1989), and ultimately, reductions in algal cell densities.

7. SPECULATIONS TOWARDS A UNIFYING THEORY

The most fundamental aspect of microalgal-invertebtate symbiosis that needs to be explained is the high diversity of the interacting species. It is apparent that many microalgal taxa and many invertebrate taxa are involved in such associations, and it is abundantly clear that there is no correlation between microalgal taxa and host taxa. What is the basis of the diversity of the interacting species, and of the specificity that these associations demonstrate? Cyanobacteria occur as symbionts in protists, sponges and ascidians; dinoflagellates are symbiotic with invertebrates across a wide phyletic range; bacillariophytes and prasinophytes are associated with protists, sponges and flatworms. What is the explanation for the apparently random distribution of these symbioses? It should be restated at this point that among those coelenterates that harbour *Symbiodinium* species, the distribution is also random; of the three species of *Anthopleura* on the West Coast of the the United States, two are symbiotic , and one, *A. artemesia* is not. Several families of corals (e.g. the Caryophyllidae and Dendrophyllidae) are predominantly non-symbiotic (ahermatypic), but have a few symbiotic genera (Trench 1987). The anemone *Entacmaea medusivora* (Fautin and Fitt 1991) is non-symbiotic even though it feeds on the symbiotic jellyfish *Mastigias papua* (Hamner and Hauri 1981). When intracellular symbioses are regarded as interactions between phylogenetically distinct organisms, then one wonders if the basis of the associations is that somehow, the self-nonself recognition system of the invertebrate hosts has been compromised (Trench 1992). The notion of a poorly developed immune system in invertebrates (D. L. Taylor 1973) has given way to a concept that invertebrates **can** distinguish between self and nonself

(Hildemann et al. 1980, Scofield et al. 1982).

My hypothesis is that the mechanism that underlies the establishment, and maintenance of microalgal-invertebrate symbioses is the continuous movement (before, during and after the formation of the association) of molecular signals, the synthesis of some of which may be induced after the establishment of the association. This situation would be directly analogous to the situation in symbioses such as the legume root nodule (Nap and Bisseling 1990, Brewin 1991). Many types of cells (Kapeller et al. 1973), and many microalgae synthesize and release complex glycoconjugates (Markell and Trench 1993) or exopolysaccharides (Alldredge et al. 1993). The immunological characteristics of these macromolecular complexes from symbiotic dinoflagellates indicate a high level of specificity, in that antibodies against a major glycoconjugate fraction from one species do not cross-react with glycoconjugates from another. If it is assumed that some of these macromolecules are structurally similar to products of invertebrate histocompatibility (MHC) gene complexes, then each potential invertebrate host species will perceive these different macromolecules from different algae and discriminate between them. Those algae that are acceptable then, are the ones that the hosts do not recognize as foreign. Closely related algae, such as some *Symbiodinium* species, may release signals that are similar enough to permit the infection, under laboratory conditions, of a host other than that in which they are normally found. Taxononically dissimilar algae may, by chance, release signals that are compatible with closely related, but not identical, hosts. Two different algal taxa may release signals that are analogous to different products of the MHC gene complex of a given host, allowing them to co-occupy that host, as seen in *Haplodiscus* (Trench and Winsor 1987).

The continued flow of extracellular macromolecules from symbiotic algae to their hosts is essential to the maintenance of the relationship. Any perturbation that interrupts the release of macromolecular signals in an association results in the animals perceiving the presence of a foreign genetic entity within themselves, and hence its elimination, usually by exocytosis, or the reverse process of acquisition. As different host species may harbour different symbiont species, each combination with its own range of tolerance to different environmental stimuli, then different symbioses would react differently to the same stimulus, as a function of its level of sensitivity. It has been demonstrated that in plants, elevated temperature disrupts the synthesis and export of glycoproteins from Golgi cisternae (Chrispeels and Greenwood 1987). A similar phenomenon may occur in symbiotic algae under thermal, and other forms of "stress". Other metabolic functions of these associations may also independently, or associatively (a domino effect) result under thermal and other forms of "stress".

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Figure legends

Figure 1. *Symbiodinium corculorum* isolated from the bivalve *Corculum cardissa.* Transmission electron micrograph of a tetrad in culture, illustrating the continuous pilose mother cell wall (cw) surrounding the four daughter cells which have already produced flagella (arrowheads) prior to becoming motile. These cells possess mucocysts (arrow) in both the motile and coccoid states in culture. Asterisk represents extracellular glycoconjugates. Scale bar = 1 μm. Photograph by Abdulla Mandura.

Figure 2. *Symbiodinium goreauii* from the sea anemone *Ragactis lucida.* A coccoid cell in culture reverting to the mastigote stage without prior mitotic division. Arrowheads indicate profiles of the flagella. The continuous mother cell wall is still intact. Scale bar = 1 μm.

Figure 3. *Symbiodinium pilosum* from the zoanthid *Zoanthus sociatus.* A coccoid cell in culture illustrating the characteristic mucocyst. These algae also possess a pilose continuous cell wall. Scale bar = 1 μm.

Figure 4. A phylogenetic tree based on the 50% majority rule concensus topology of 600 bootstrap resamplings of the data set of partial rDNA sequences (480 sites) using Wagner parsimony. The symbiotic dinoflagellates represented are: *Symbiodinium pilosum, S. meandrinae, S. corculorum,*

S. pulchorum, *S. bermudense*, *Gloeodinium viscum* and *Amphidinium belauense*. The outgroup species (not shown) employed were the apicomplexans *Sarcocystis muris* and *Theilaria annulata*. The scale bar represents 10 changes per 100 nucleotides.