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# Some Preservation Techniques for (Deep Water) Coral Samples for Subsequent Molecular Studies: A Special Supplement from Harbor Branch Oceanographic Institute

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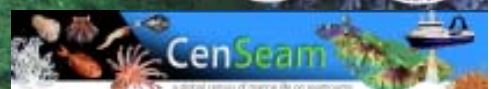
# DEEP-SEA CORAL COLLECTION PROTOCOLS

*A synthesis of field experience from deep-sea coral researchers,  
designed to build our national capacity to document deep-sea coral diversity*

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U.S. Department of Commerce  
National Oceanic and Atmospheric Administration  
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NOAA Technical Memorandum NMFS-OPR-28  
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**NOAA Technical Memorandum NMFS-OPR-28  
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National Oceanic and Atmospheric Administration  
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Under Secretary for Oceans and Atmosphere

National Marine Fisheries Service  
William T. Hogarth, Assistant Administrator for Fisheries



The sea pen *Pteroeides* sp., 9-320 m

## Forward

Gary C. Williams, California Academy of Sciences

Around the time that the thirteen original Atlantic colonies were fighting for independence from Britain, there existed little agreement among naturalists as to the nature of corals. Were they inanimate (stones), plants, animals, or intermediate between the latter two (zoophytes)? This diversity of definition and opinions undoubtedly produced considerable confusion and disagreement among naturalists interested in such things. The symbiotic nature of algal cells in the tissues of some corals was also not well understood. It was not until the Darwinian period in the nineteenth century that little doubt remained, and therefore it was generally agreed, that corals were actually animals – heterotrophic living organisms that prey on other organisms for nutrition and do not produce their own food.

In the past fifty years the basic goals and tenets of deep-sea coral collection, curation, and taxonomy have changed little. On the other hand, the techniques and tools of this particular avenue of research have changed significantly. Regarding the collection of material in the field, some aspects remain fundamentally the same. The use of research vessels, bottom trawls, and naturalist's dredges are still frequently used for deep water research. In shallow water collecting, improvements in SCUBA diving equipment and new innovations, such as Trimix gas and Nitrox diving, have allowed divers to work at greater depths with longer bottom times. Pressure independent dive suits have permitted researchers to attain depths not possible in traditional wet or dry suits. In the past four decades, advances in optics, electronics, and robotic technology have allowed for a rapid sophistication and a broader scope of possibilities regarding manned submersibles, remotely operated vehicles (ROV's), and more recently, autonomous underwater vehicles (AUV's). Great strides have been made since the early 1990's in the technological aspects of the collection and photography of the deep water benthos.

Concerning the techniques and tools of research in the laboratory, the scanning electron microscope has for several decades provided a valuable tool for documenting surface details of sclerites. Newer electron microscopes and digital technology have negated the necessity of using photographic film and images can now be unloaded directly on to computers for processing. Relatively recently, the fields of phylogenetics, molecular biology, and natural products biochemistry have been applied to more traditional modes of research in the field of octocoral systematics.

This document on deep-sea coral collection protocols stands as a valuable resource, because it provides a standardized procedure for researchers during a time of rapidly changing technology regarding exploration of the deep-sea benthos. Stated another way, the protocols give us an instruction manual for research procedures in deep-sea benthic fieldwork. Technological improvements coupled with workable field-tested procedures (such as provided by the present volume) can in the long run, only help to expand and improve our base of knowledge concerning octocoral diversity.

The synthesis of results from research endeavors both old and new, and opportunities allowed by a constantly improving technological scene, provide for a positive potential for the future in the fields of octocoral diversity and systematics.

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## Introduction

Most of our knowledge about corals comes from shallow water tropical reefs and laboratories. These studies showed us that corals are diverse, delicate, slow-growing, and long-lived. They showed us that reefs are vulnerable to nutrient input, climate change, and destructive fishing gear, but healthy coral reefs can protect coastal communities, and provide habitat for numerous associated species. Yet, corals are not restricted to tropical reefs. Corals grow in many types of seafloor habitats ranging from the shallows to the abyss, and from the equator to the poles (Williams, 1990). Not surprisingly, corals from “the other 90%” of the ocean, the deep-sea (>200m), are still very poorly known and poorly understood. Most of what we know is garnered from early oceanographic expeditions that dragged the seafloor with trawl nets. Dried broken fragments fill museum drawers and scientific literature, but *living* coral communities from the deep-sea still remain largely unexplored.



Fig. 1. Septa of cup coral, *Desmophyllum* sp.

Things are changing rapidly. Worldwide, ocean exploration activity is increasing. Interest in deep-sea coral and seamount habitats is also increasing. New technologies are taking us deeper, longer, with better tools. Around the globe, researchers are discovering rich benthic communities, and abundant habitat. As a result, pressure is growing on scientific institutions to curate and identify deep corals and other invertebrates. The scientific community is poised for new understanding, using new sampling techniques, and new database technologies that associate myriad data types with physical samples; including maps, still and moving images, and DNA barcodes. New standards will be necessary. Marine invertebrate sample collection protocols for deep-sea organisms are well known within some institutions but, until now, there were no publicly available, multi-institutional, peer-reviewed methodologies describing how to collect deep-sea coral samples to meet archival standards.

NOAA's Office of Ocean Exploration commissioned these collection protocols to facilitate greater understanding, and to increase our national capacity to document deep-coral diversity in federal waters. This document is designed to partly to provide useful background information, and partly to build consensus among researchers, but mostly to guide collections by non-specialists and non-biologists. Marine archeologists, geologists, fishermen, oilmen, and divers can all make useful scientific collections given proper tools and techniques, so we try to share those techniques here. Each aspect described herein will not always be possible or necessary, so we endeavor to provide simple collection methods that will bring useful biological specimens to a broad spectrum of scientific disciplines.

In reading, you will encounter a variety of techniques and approaches from field scientists across the country in a variety of disciplines. Consensus was not entirely possible within this group, due to the different needs of different disciplines, so we list alternatives wherever we can. The reader must choose what works best for their goals, with equipment at hand. Collectively, the assembled authors provide good background information on deep coral biology, morphology, and habitat. The document is unique for its attention to octocorals. The authors cover important technical aspects of coral collection in some

detail: including **documenting** your specimen *in-situ*, **collecting** your specimen to insure scientific value, **preserving** your specimen to enhance long-term study, **labeling** your specimen to insure accurate transcription, and **shipping** your specimen to accredited scientific institutions. We hope you find the information useful.

### What is a deep-sea coral?

In this document, we refer to deep-sea corals as a loosely defined paraphyletic assemblage of hexacoral, octocoral, and calcified hydroid families in the phylum Cnidaria with species known to grow deeper than 200m, beyond the boundaries commonly attributed to zooxanthellate shallow water hermatypic scleractinian corals. These are also called cold-water corals and deep-water corals (Cairns, *in press*). Fishermen call gorgonians “trees”, and ahermatypic corals “spiders”. The majority of the families described in this document are octocorals (Anthozoa: Alcyonaria: Alcyonacea) but some are hexacorals (Anthozoa: Zoantharia: Antipatharia and Scleractinia).

Deep-sea corals come in many forms, ranging in size from 2 mm rice grain-sized solitary corals, to fist-sized soft colonies, feathery sea pens, bushy black corals, 2 m long whip corals, large flabellate sea fans (gorgonians), and massive bank framework scleractinians. Deep-sea corals are diverse and beautiful, and they range over of a greater part of the coastal United States than the more familiar zooxanthellate tropical corals. Many species have complex branching morphologies and grow large enough to form structure for associated species. Other species, like sea pens, have simple pinnate forms but they grow in abundance, and provide important ecological habitat in an otherwise flat and featureless abyss. Some coral colonies are long-lived. They function as archives of climate change, and they are uniquely adapted to extreme environments. Deep-sea corals are an exciting and fast growing field of research, and an integral part of NOAA’s agenda for deep-sea exploration.

A complete taxonomic list of 48 families of hexacorals, octocorals, and calcified hydroids with species known to occur deeper than 200m is provided in **Appendix 1- Classification of Deep-sea Corals**. This list describes the families for which these protocols will be most useful.



**Fig. 2.** Bamboo coral (*Isidella* sp.) polyps.



**Fig. 3.** Living bamboo coral (*Isidella* sp.) colony



## Deep-sea coral habitat and range

by Peter Etnoyer

When most people think of coral reefs, they think of shallow water tropical reefs. However, deep coral communities of some type lie off the shore of most coastal nations and states, as deep as 6000m. Contrary to popular opinion, both the hard scleractinian hexacorals (the zoantharia) and softer gorgonaceous octocorals (the alcyonaria) have vertical ranges exceeding 6000m. They range worldwide. The higher level taxa (families) are generally cosmopolitan, with some worldwide genera, and some strictly regional genera.

Perhaps the deepest known coral taxon is a sea pen — genus *Umbellula*, from 6620m

(Williams, 1999). Primnoids occur as deep as 5850m (S. Cairns, *pers. comm.*) and thrive in abundance as deep as 3000m (A. Baco-Taylor, *pers. comm.*). The deepest known bamboo coral *Keratoisis profunda*, at 4851m (Cimberg, 1981) near the Northwest Hawaiian Islands. Hydrozoan ‘lace corals’ have a worldwide distribution but are most common near islands and seamounts. Lace corals have a vertical range from 0.5 to 2,787m (Cairns, 1992). Ahermatypic ‘thickets’ of *Lophelia pertusa* in the Gulf and the US South Atlantic bight are generally found between 200 and 1000m (Schroeder et al. 2005.) Most of the coelenterate families that contain deep-sea corals include shallow-water species, too.

Deep-sea corals can occur in many assemblages, including monotypic scleractinian bioherms (e.g. *Lophelia sp.* in the Atlantic), monotypic octocoral *fields* or *meadows* in mixed substrates on low relief mounds (e.g. *Callogorgia* and *Acanella* in the Gulf of Mexico) or diverse rocky reefs of octocorals, anemones and sponges (e.g. New England and Gulf of Alaska Seamounts, Aleutian and Hawaiian



Fig. 5. Stony corals and whip corals on hardbottom habitat.

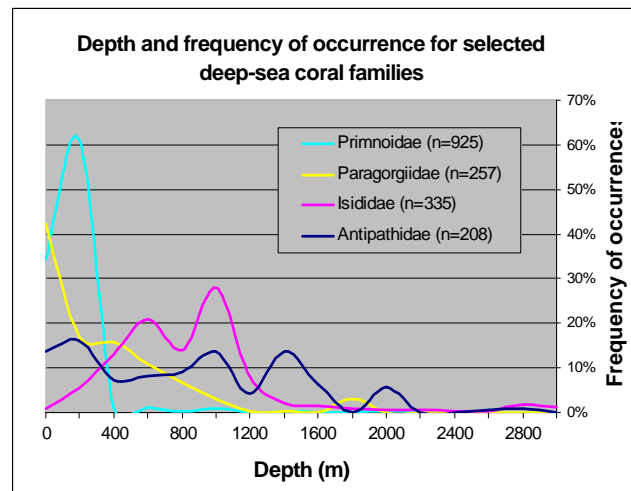


Fig. 4. Depth and frequency for some Northeast Pacific octocoral families (from Etnoyer and Morgan 2005).

Islands). Beginning in the North Atlantic and traveling clockwise around the country, we find *Paragorgia sp.* bubblegum corals are common to 1600m on the New England seamounts (L. Watling, *pers. comm.*). Rare and fascinating octocorals like the beautiful *Metallogorgia* and *Iridogorgia* bright stony corals like *Enallopsammia* pictured here are also found there (Watling and Auster, 2005). South

Atlantic Bight is known for some large banks of *Oculina varicosa* and lithoherms of *L. pertusa* that provide habitat for more than 58 species of fish, and almost 142 species of benthic invertebrates (Reed et al., 2006). Some of these lithoherms have been impacted by destructive fishing gear, but are now coming under protection. The Gulf of Mexico is known for deep thickets of *L. pertusa* (Schroeder et al. 2002), though several species of octocorals occur there. *L. pertusa* colonies can encrust much of a sixty-year-old shipwreck (at 550m) with the largest thickets 6m tall and 3.5m wide (W. Schroeder, *pers comm.*). Images and videos from the shipwrecks can be viewed at the Past Foundation website: <http://www.pastfoundation.org/DeepWrecks/Video03.htm>. *L. pertusa* also encrusts oil rigs.

In the North Pacific, colorful hydrocoral banks like the *Stylaster* mound pictured on the cover are found 25m and deeper as far south as Baja California. These banks are well photographed, and very popular with sport divers and fishermen. Large black, bubblegum, primnoid, and bamboo corals across can be found deeper than 100m on the continental shelves of California, Oregon and Washington states. Coral forests in these regions are thought to play important roles as habitat for Atka mackerel and rockfish (Heifetz 2002, Krieger and Wing 2002). Shallow seamount peaks in the Gulf of Alaska host similar species. The Aleutian Islands of Alaska are incredibly rich. Submerged banks and peaks in the Hawaiian islands are famous for black, gold, and precious corals once harvested for the jewelry trade (Grigg, 1976). Several species are now listed as endangered.

Some solitary cup corals (e.g. *Desmophyllum*) thrive on small ledges and overhangs, while others (e.g. *Balanophylla*) settle on cobbles and pebbles. Gorgonians thrive in rocky habitats with strong currents. Currents facilitate settlement onto clean swept surfaces, increase food availability and capture and, therefore, growth rate and survivorship (Patterson 1984, Sponaugle and LaBarbera 1991). Octocorals have been used as indicators of flow velocity on seamount peaks (Genin et al. 1986), orienting perpendicular to prevailing currents (Grigg, 1972) but some species, like the *Acanella* bamboo corals and the sea pens are also adapted to muddy and mixed substrates of low-relief (Smith et al 2002, Williams 1995). Interestingly, small differences in relief (e.g. boulders ~1m) also provide deep coral habitat in an otherwise featureless plain.

**Fig. 6.** Galatheid crab nestled in outstretched octocoral polyps



## Branching patterns in octocorals

by Juan A. Sánchez

Octocorals are one of many organisms that grow producing multi-branched tree-like colonies. Interestingly, recent molecular phylogenies of octocorals show how branching can evolve independently multiple times from non-branching hypothetical ancestors involving different axial materials (Sánchez et al., 2003). Consequently, a particular branching pattern should not be considered taxon- or taxa-specific. There are remarkable cases of convergent evolution (i.e., analogy without common ancestry) such as nearly identical pinnate morphologies in multiple octocorals, as in the case of *Pseudopterogorgia* (Gorgoniidae), *Plumigorgia* (Ifalukellidae), *Pteronisis* (Isididae), and *Plumarella* (Primnoidae; see **Fig. 7**), which differ in their modular (e.g., polyp and calyx) and ultrastructural (e.g., sclerites) nature. This suggests caution when using branching morphologies as diagnostic characteristics but on the other hand calls to attention the remarkable plasticity in the evolution of colony form in octocorals. **Figure 8** shows a sample of the diverse branching patterns and other colonial morphologies observed in octocorals.

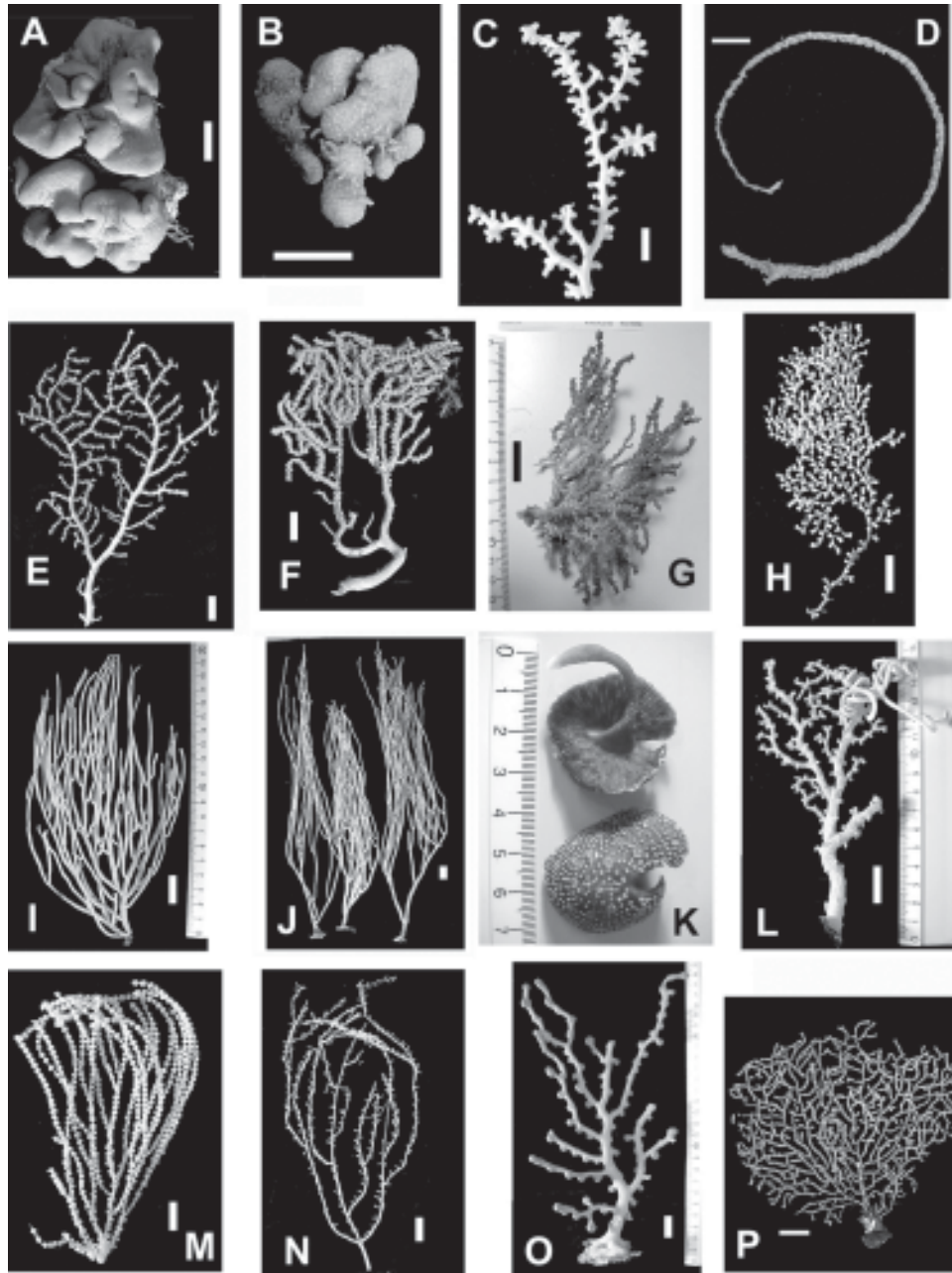


**Fig. 7.** Examples of pinnate branching patterns in octocorals. Left: *Pteronisis incerta* (from Alderslade, 1998) and *Pseudopterogorgia bipinnata* (Colombia, Caribbean).

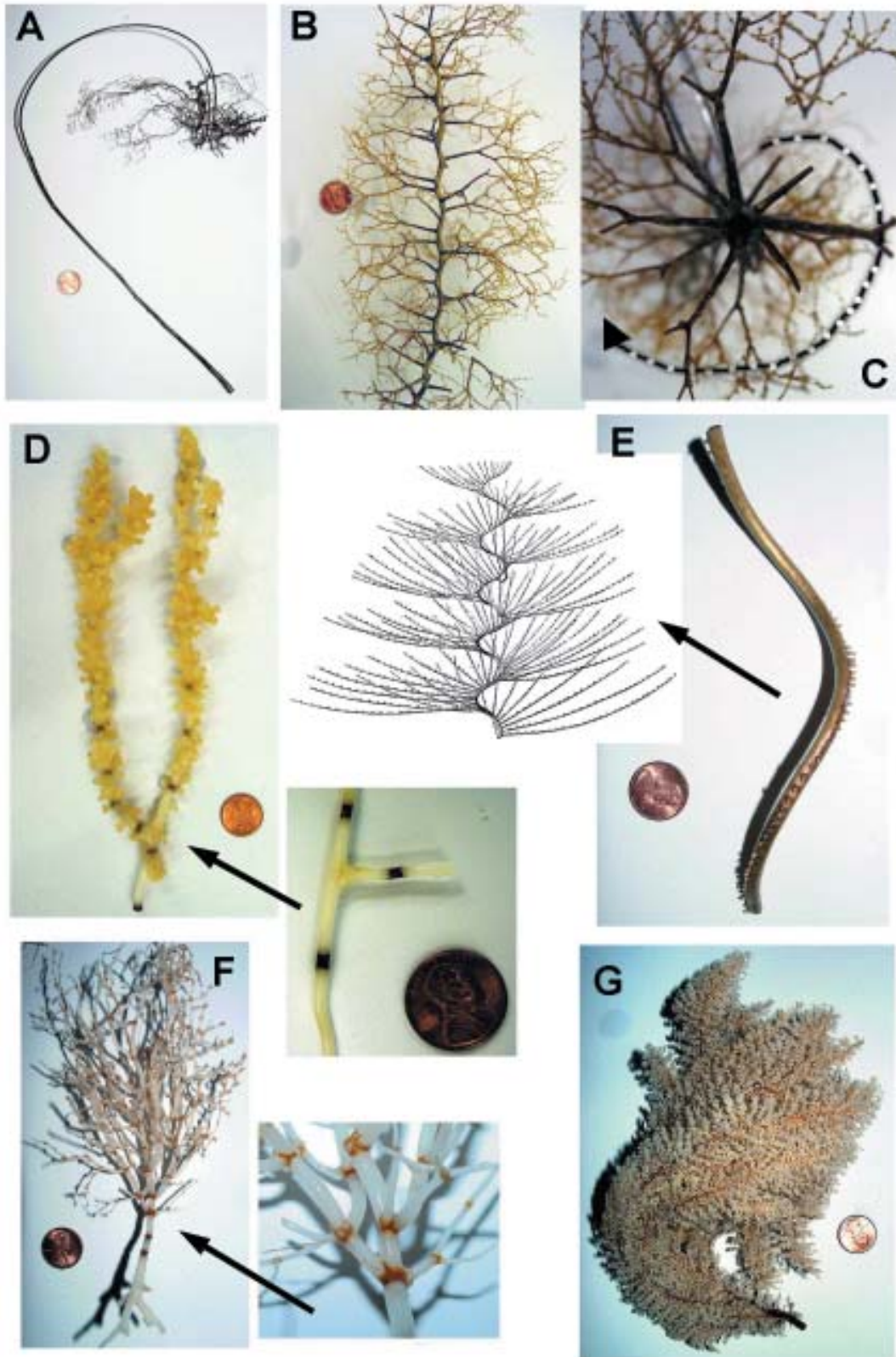
Taxonomists recognize up to 28 terms for the description of octocoral colony shapes and branching patterns (Bayer et al., 1983), which can be redundant or related in some cases. In order to understand the branching pattern of octocorals it is important to know how they grow and branch. Branching in most octocorals is a subapical process (i.e., just below the tip) where some branches, “mother branches,” produce new branches, “daughter branches,” at roughly fixed distances or internodes (Sánchez et al., 2004).

The extreme example of this is pinnate pattern where a single mother branch retains multiple daughter branches (**Fig. 7**). This morphology is a kind of monopodial branching, of which the opposite extreme example is only one “daughter” allowed for every “mother branch” producing a sympodial branching rarely found in octocorals (*Chrysogorgia* sp. in Figures 7h and 8b). This implies that the colony has simple branch set points (i.e., one daughter branch at the time) whereas pinnate branching would have double set points.

Simple branching seems to be the most common in octocorals, generating pseudo-dichotomous (e.g., sea candelabrum) and pectinate/lyrate (e.g., *Ctenocella pectinata*) morphologies all growing in one plane or uniplanar. If the tips of daughter branches from uniplanar morphologies join, or *anastomose*, that creates reticulate branching, examples of this weblike pattern are the common sea fans (e.g., *Gorgonia*, *Pacifigorgia*, *Subergorgia*, etc.).



**Fig. 8.** A. *Sarcophyton glaucum* (Quo and Gaimard), Great Barrier Reef, Australia, Western Pacific, shallow water (United States National Museum catalog number-USNM: 79445, at NMNH, Smithsonian Institution, Washington D.C., USA). B. *Alcyonium digitatum* Linnaeus, off New Jersey, USA, Western Atlantic, 85 m in water depth (USNM 60336). C. *Anthothela nuttingi* Bayer, Hawaii, North Pacific, 1010 m (USNM 94435). D. *Lepidisis olapa* Muzik, Hawaii, North Pacific, 400 m (USNM 56717). E. *Anthomuricea tenuispina* Nutting, Hawaii, North Pacific, 517 m (USNM 98801) F. *Paramuricea hawaiiensis* Nutting, Hawaii, North Pacific, 345 m (USNM 58385). G. *Muricea fruticosa* Verrill, Panama, Eastern Pacific, 60 m (USNM 57102) H. *Chrysogorgia chryseis* Bayer and Stefani, Hawaii, North Pacific, 1010 m (USNM 100848). I. *Lophogorgia alba* Duchassaing and Michelotti, Panama, Eastern Pacific, intertidal (USNM 57091). J. *Leptogorgia virgulata*, Gulf of Mexico, USA, Western Atlantic, 6 m (USNM 49690). K. *Renilla reniformis* (Pallas), Puerto Rico, Caribbean, 2 m (USNM 79448). L. *Corallium ducale* Bayer, Hawaii, North Atlantic, 1205 m (USNM 94458). M. *Narella dichotoma* (Verluyt), Hawaii, North Atlantic, 1218 m (USNM 98822). N. *Isidella elongata* (Esper), Italy, Mediterranean, 350 m (USNM 57457). O. *Paragorgia dendroides* Bayer, Hawaii, North Pacific, 1018 m (USNM 98789). P. *Acanthogorgia* sp. Hawaii, North Pacific (USNM 100340). Scale bar = 2 cm. (from Sanchez et al., 2003a).



**Fig. 9.** Examples of deep-water corals from New Zealand (NIWA collection). A. *Metallogorgia melanotrichos*; B. *Chrysogorgia* sp.; C. axial view of B.; D. *Keratoisis* sp. and branching set point detail; E. *Iridogorgia* branching pattern and axial sample; F. *Acanella* sp. and close-up detail. G. *Thouarella* sp. (scale is a US Lincoln penny).

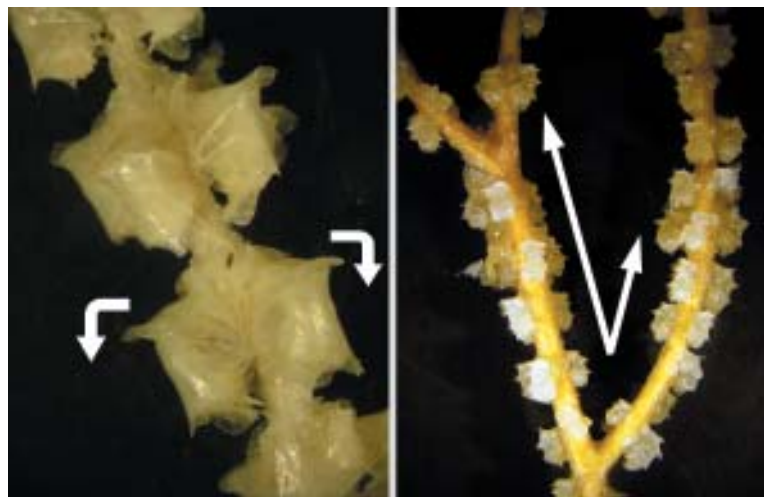
Other colonies can modify and twist their axes to branch in multiple planes such as the spectacular *Iridigorgia* colonies, which have lateral simple branching in a spiralized axis (**Fig. 9E**). Other double branching morphologies, besides pinnate, are alternate branched colonies, which generate pseudo-dichotomous morphologies in the simpler cases (e.g., uniplanar) or bottlebrush colonies in more complicated spiral arrangements (e.g., *Chrysogorgia*: **Fig. 9B**).

Pinnate colonies can also produce branches in multiple directions or bottlebrush morphologies (e.g., *Thouarella*: **Fig. 9G**). In most cases, you will find that many species exhibit combined and more disorganized branching patterns creating simple bush-like colonies (e.g., *Acanella*: **Fig. 9F**). Some combined morphologies present remarkable shapes such as *Metallogorgia melanotichos*, where the colony is a long whip-like mother branch that commences branching on multiple sympodial fronts (e.g., **Fig. 9A**). In summary, octocoral branching patterns are a complex combination and continuum of features such as branching set point (simple lateral/alternate or pinnate), planes of branching (uniplanar, multiplanar, spiral, bushy, etc.) and generational relationships of branches (single mother branch: monopodial to multiple mother branches: sympodial).

It is important to point out certain considerations for getting complete branching information from collections of deep-water octocorals. Undoubtedly, deep-water octocorals exhibit the most interesting and elaborated branching morphologies (e.g., **Fig. 9**). Nonetheless, their sampling is more delicate and samples are more difficult to preserve (e.g., **Fig. 9E**). For closely-related species distinctions, branching patterns often offer important characters, information which should particularly be collected for Chrysogorgiidae, Primnoidae and Isididae. Branching set points are highly important for Isididae where branching from the node or internode could differentiate *Keratoisis* from other Keratoisidinae (e.g., **Fig. 9D, F**).

The orientation of the polyp armatures with respect to the branching can also be of importance in Primnoidae, where some can be upward, outward or downward (e.g., downward, *Narella* sp.

**Fig. 10**). If the material cannot be collected in its entirety it is important to take photographs and details of branching points, nodes, daughter branch detail, and of course the whole colony. An ideal situation for remotely operated vehicles would be to take 3-D stereo-pairs of the colonies at various distances.

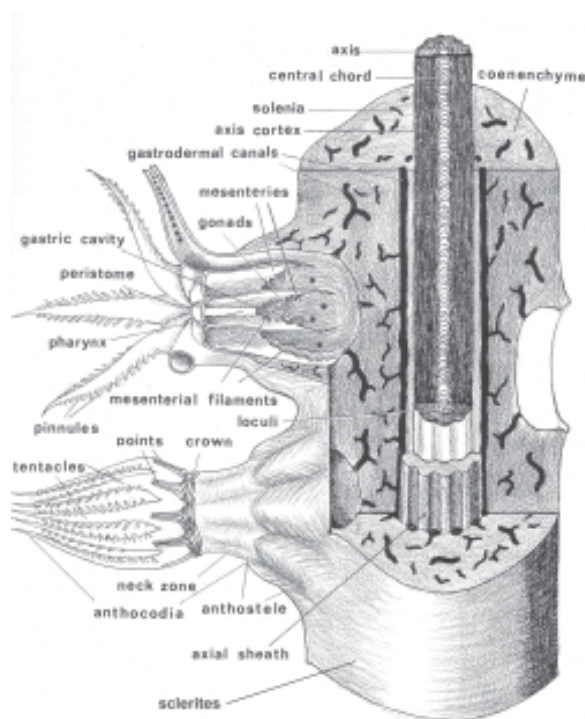


**Fig. 10.** *Narella* sp. (New Zealand, NIWA collection) with arrows showing downward orientation of polyp sclerite armature.

## Sclerites in octocorals

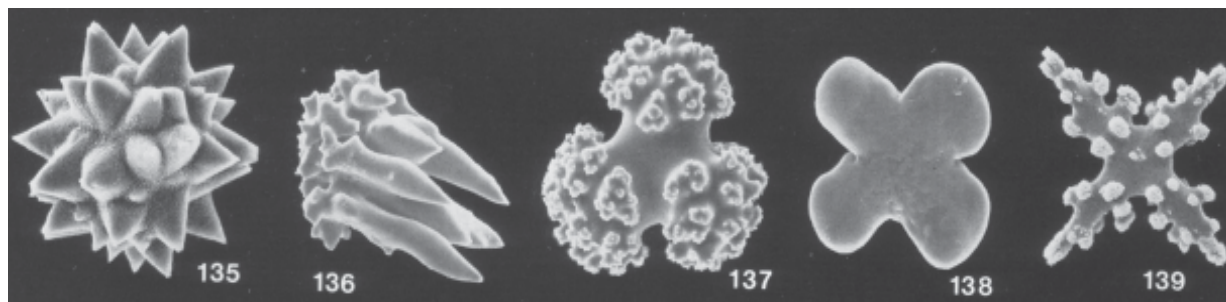
by Stephen D. Cairns

Although branching morphology, corallum size, and color are valuable hints for the identification of octocorals, and are often the only characters available for field identifications, the single most important character in octocoral identification is the microscopic calcareous sclerite (Bayer, 1956). Sclerites, also called “spicules”, are found in almost all 2800 octocoral species. They usually occur in great numbers in every colony, 10’s of thousands not being unusual in a modest sized colony. Even a small fragment may contain thousands of sclerites. Each sclerite is a small piece of calcitic calcium carbonate ranging in size from 20  $\mu\text{m}$  to 5 mm; they are found in the polyps of the colony as well as in the coenenchymal tissue between the polyps, each giving a small amount of support and structure to the tissue and polyps.



**Fig. 11a.** An illustrated octocoral axis and polyps.

Sclerites occur in many shapes and sizes, the first attempt at classifying them was the illustrated trilingual glossary edited by Bayer, Grasshoff, & Verseveldt (1983), which defined, synonymized, and illustrated 57 sclerite types, some with the whimsical, yet descriptive, names of: caterpillar, crutch, finger-biscuit, hockey-stick, rooted head, wart club, capstan, and opera-glass. A species may have only one type and size of sclerite or a variety, each type sclerite type occurring in a particular part of the colony and serving a slightly different function.



**Fig. 11b.**

It is easy to examine the sclerite types from a colony. One simply cuts a small piece containing a polyp and branch from a larger colony, place it on a microscope glass slide, and add a drop of common household bleach. Wait a few minutes, or until the bubbles burst, place a cover slide over the preparation (optional), and examine at x200 with a compound microscope, or as little as x50-100 with a dissecting microscope. SEM of sclerites done at x400-500 or higher magnifications is usually not necessary for routine identifications but does provide excellent illustrations that can be used to describe species.

For cleaner and more permanent preparations one should rinse the sample with successive solutions of hydrogen peroxide, tap water, distilled water, 70% ethanol, and 95% ethanol (see Fabricius & Alderslade, 2001: 38-39). It is important not to preserve octocorals in formalin, because sclerite dissolution will occur over a period of several weeks to months. Specimens may be fixed for a short time, (less than 24 hours) in a weak (~ 4%) formalin solution that is highly buffered.

The ability to identify the suite of sclerites of a specimen using Bayer, Grasshoff & Verseveldt (1983) and using the key to the genera (Bayer, 1981) is an excellent start to the identification of any octocoral, exclusive of pennatulids. As knowledge of sclerites is so important for the identification of octocorals, it is not recommended to ask an octocoral specialist to identify a specimen based on a picture or video, because his invariable response will be to ask for the specimen, if only a tiny fragment containing a few sclerites.

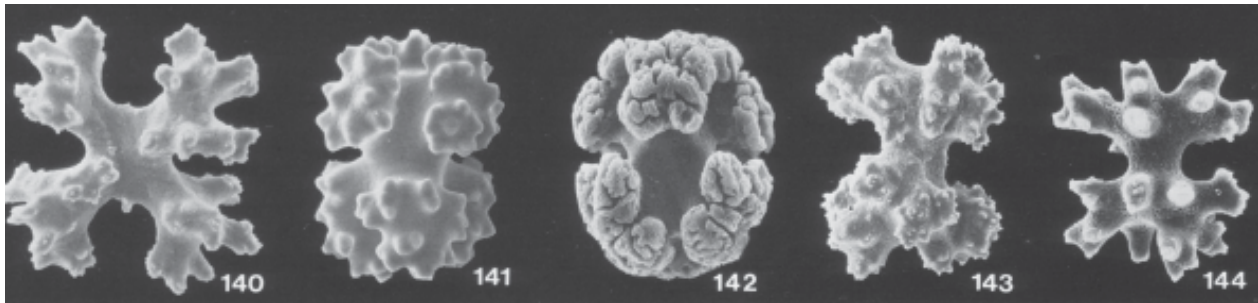


Fig. 11c.

Fig 11b-11c. Types of sclerites found in octocorals:

- |                                  |  |
|----------------------------------|--|
| 135. Spiny ball.                 | <i>Asterospicularia randalli</i> Gawel. x625.  |
| 136. Unilaterally spiny spheroid | <i>Mopsella</i> sp. x250.                      |
| 137. Triradiate.                 | <i>Titanideum ftauenfeldii</i> Kolliker. x350. |
| 138. Cross.                      | <i>Cavernulina</i> sp. x300.                   |
| 139. Cross.                      | <i>Ellisella</i> sp. x375.                     |
| 140. Cross.                      | <i>Gersemia rubiformis</i> Ehrenberg. x375.    |
| 141. Six radiate.                | <i>Corallium borneense</i> Bayer. x375.        |
| 142. Six radiate.                | <i>Paragorgia arborea</i> Linnaeus. x750.      |
| 143. Six radiate.                | <i>Epiphaxum micropora</i> Bayer & Muzik. x375 |
| 144. Six radiate.                | <i>Alcyonium digitatum</i> Linnaeus. x500.     |

**Figures 11a-c** are reproduced from the *Illustrated Trilingual Glossary of Morphological and Anatomical Terms Applied to Octocorallia* edited by Frederick Bayer, Manfred Grasshoff, and Jakob Verseveldt and published by E.J. Brill, The Netherlands, 1983.

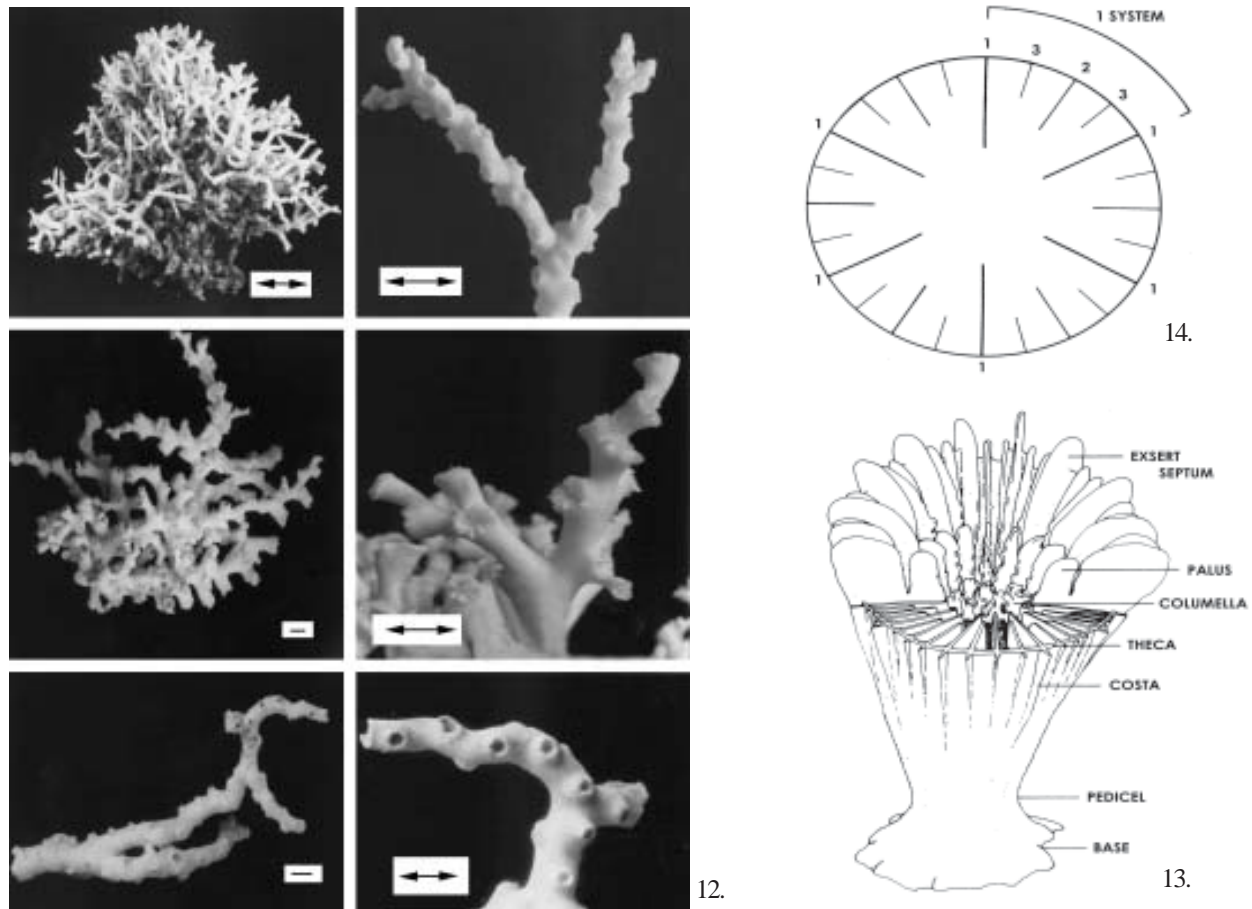


## Ahermatypic scleractinians of the western Atlantic

by John K. Reed

### Western Atlantic Deep-Water Coral Reefs

The dominant corals forming deep-water reefs in the western Atlantic region off southeastern US and Gulf of Mexico are *Oculina varicosa*, *Lophelia pertusa*, and *Enallopsammia profunda* (Reed 2002 a,b, Schroeder et al. 2005). Other branching colonial scleractinia also occur, including *Solenosmilia variabilis*, *Madrepora oculata*, and *Madracis myriaster*, as well as numerous solitary coral species (Cairns, 1979). The deep-water *Oculina* bioherms form an extensive reef system at depths of 70-100 m along the shelf edge and are only known off central eastern Florida. These reefs are comprised of numerous pinnacles and ridges, 3 to 35 m in height. In comparison, deep-water reefs of *Lophelia pertusa* and *Enallopsammia profunda* corals often occur together at depths of 500-850 m from Florida to North Carolina and the Gulf of Mexico. These form reef structures from 10 to 150 m in height. Deep-water *Lophelia* reefs also occur worldwide and extensively off Norway and Scotland. The structure of both the *Oculina* and *Lophelia* reefs are often similar. Each pinnacle may consist of a bank of unconsolidated sediment and coral debris that is capped on the slopes and crest with living and dead colonies of coral.



**Fig. 12.** Colony and branch tip for : top - *Oculina varicosa* (80m); middle - *Lophelia pertusa* (490 m); bottom - *Enallopsammia profunda* (585 m). Scale lines = 1 cm; top left fig. scale = 5 cm. From Reed, J. K. 2002.

**Fig. 13.** Morphological features of coral corallite. From Cairns, S. 1981.

**Fig. 14.** Septa patterns of a coral calice. From Cairns, S. 1981.

## *Scleractinian Coral Descriptions*

*Oculina varicosa* Lesueur, 1820: In deep water (>60 m), *O. varicosa* forms spherical, dendroid, bushy colonies that grow to 1 to 2 m in diameter and height (**Fig. 12, top**). Individual corals may coalesce forming linear colonies 3-4 m in length or massive thickets of contiguous colonies on the slopes and crests of the deep-water reefs (Reed, 1980; Reed 2002 a,b). The branches average 3-6 mm in diameter and frequently anastomose. Corallites are distributed spirally around the branches, and calices are generally 2-3 mm in diameter with 3 cycles (24) of septa which extend as costae or ridges on the outside of the calices. The deep-water form lacks zooxanthellae, whereas in shallow water *O. varicosa* is usually golden brown with the algal symbiont. The shallow-water *O. varicosa* colonies average <30 cm in diameter, with thicker branches, but do not form thickets or coral banks like the deep-water form. *O. varicosa* ranges from the Caribbean to Bermuda at depths of 2-152 m (Verrill, 1902; Smith, 1971; Reed, 1980). The deep-water *Oculina* reefs, however, are only known from 27° 32' N and 79° 59' W to 28° 59' N and 80° 07' W, and at depths of 70-100 m.

*Lophelia pertusa* (Linnaeus, 1758) (= *L. prolifera*): Similar in gross morphology to *Oculina*, this coral also forms massive, dendroid, bushy colonies, 10-150 cm in diameter, with anastomosing branches (**Fig. 12, middle**). Branch tips are slender whereas the base may be several centimeters in diameter. Terminal branches have opposite, alternately arranged corallites which may be variable in shape from round to elliptical and somewhat flared or flower-like. Calices may be up to 15 mm in diameter and number of septa is variable with 7 to 11 primary septa which generally extend beyond the lip of the cup as costae. The columella, at the bottom of the corallite cup, is rare but may consist of 1 to 3 small rods. Its distribution ranges in the western Atlantic from Nova Scotia to Brazil and the Gulf of Mexico, and also in the eastern Atlantic, Mediterranean, Indian, and eastern Pacific Oceans at depths of 60-2170 m (Cairns, 1979).

*Enallopsammia profunda* (Pourtalès, 1867) (= *Dendrophyllia profunda*): This species also forms dendroid, massive colonies up to 1 m in diameter (**Fig. 12, bottom**). Branches are about 1 cm in diameter and less at the tips. Characteristic of this species are the prominent cone-shaped corallites which are 3-4 mm in diameter and generally arranged alternately on opposite sides of the branches. There are generally 3 cycles of 24 septa that do not extend beyond the rim of the cup. A small columella occurs at the base of the corallite as a spongy mass. It is endemic to the western Atlantic and ranges from the Antilles in the Caribbean to Massachusetts at depths of 146-1748 m (Cairns, 1979).

These three common deep-water reef species may be distinguished by the characteristics described above. They also may be differentiated from other bushy colonial species that may co-occur on western Atlantic deep reefs. These include *Solenosmilia variabilis*, which occurs to 2165 m and is most readily identified by its intratentacular budding. Thus the branch tips often consist of a pair of calyces in various stages of splitting apart. *Madracis myriaster* occurs to 708 m and is distinguished by only having 10 primary septa and usually a strong style projects from the center of the columella. *Madrepora carolina* is another colonial that occurs in the Gulf of Mexico. **Fig. 13 and 14** illustrate features of the corallite, and septal patterns of a coral callice.

## Reproduction in deep-sea colonial corals

by Sandra Brooke

As coastal species become more depleted, increased effort will be displaced into deep-sea fisheries, which pose the greatest threat to deep-sea coral habitats. Recovery of a damaged ecosystem is dependent on the ability of the keystone species to re-colonize affected areas. Corals can do this via asexual fragmentation, which can clearly be seen in deepwater coral habitats, but the effectiveness of this mechanism is limited, because fragments cannot travel far from the parent colony. Although colony growth and fragmentation can compensate for local small-scale damage, re-colonization of large areas requires larval recruitment, which is determined by larval lifespan and behavior, local currents, and post-larval survival. Knowledge of reproductive strategy, fecundity, and larval biology will enable us to assess the potential of deep-sea corals to recover from natural and anthropogenic impacts.

Scleractinians do not have true reproductive organs; eggs and sperm develop within the acellular mesogleal layer of the mesenteries (**Fig. 16**). Gamete development begins when primordial germ cells appear within the mesenterial gastrodermis (Szmant-Froelich et al. 1980, 1985, Delvoe 1982, Wyers 1985). These cells proliferate and migrate from the gastrodermis into the mesogloea of the mesenteries, where they undergo meiosis to form primary oocytes. As oocyte development proceeds, yolk is laid down and the cytoplasmic volume increases (**Fig. 16**). Male germ cells originate in the same way as females. Primordial germ cells enter the mesogloea from the gastrodermis, the spermatogonia then differentiate into primary spermatocytes, which undergo meiosis to form secondary spermatocytes then spermatids (Delvoe 1982). Mature gametes are released through the mouth, (spawning cues are unknown), and embryogenesis and larval development take place in the water column.



**Fig. 15.** Living polyps of *Lophelia pertusa*

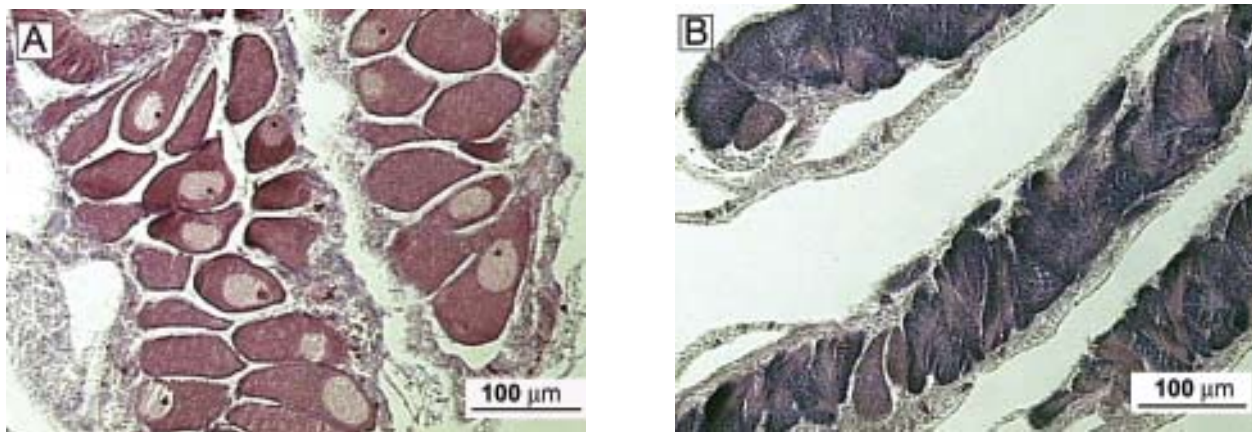
A study of reproduction in deep-sea corals from New Zealand showed that four colonial species, *Enallopsammia rostrata*, *Goniocorella dumosa*, *Madrepora oculata* and *Solenosmilia variabilis* were all gonochoristic: each colony was either male or female. Broadcast spawning was the probable mode of reproduction, with spawning inferred in all species for late April or May. There was a high level of synchrony between species in the seamount localities studied. High fecundities were estimated for *E. rostrata* (>144 oocytes per polyp), *G. dumosa* (>480 oocytes per polyp) and *S. variabilis* (>290 oocytes per polyp), with a negative correlation between oocyte size and fecundity for all three species (S. Burgess, *pers. comm.*).

*Oculina varicosa* is a gonochoristic broadcast-spawning species with an annual reproductive cycle. Gametogenesis begins in early spring and terminates in a protracted spawning season that lasts from late July until early September. Fecundity level varies from ~2000-5000 oocytes per cm<sup>2</sup> of skeletal surface area. There is evidence for male-induced spawning in females, but again, information regarding

spawning patterns and cues is limited as spawning was only observed in the laboratory. Larvae are very temperature tolerant and probably last between 14 and 21 days in the water column, which infers a high dispersal potential. Larval biology has not been studied for any deepwater coral except *O. varicosa* (Brooke and Young 2003).

The gametogenic cycle of *Lophelia pertusa* has been studied in samples from various locations; the northeast Atlantic (Waller and Tyler in press), Norwegian Fjords (Brooke and Jarnegren in prep) and the Gulf of Mexico (Brooke in prep). *Lophelia pertusa* is a pseudo colonial species, but has similar reproductive traits (gonochoristic broadcast spawner) to the deep-water colonial species. In the North Atlantic, the maximum oocyte diameter was 140 $\mu$ m with a fecundity of 3146 oocytes per polyp. Gametogenesis began in the summer (June-August) and spawning was inferred from histological samples to occur between January and February. Samples from the Trondhjem Fjord in Norway showed a similar pattern of gametogenesis and spawning to those in the Northeast Atlantic, with spawning probably occurring in February when the maximum average oocyte diameter (106 $\mu$ m) was observed. The samples from the Gulf of Mexico however show a completely different gametogenic schedule. Vitellogenic oocytes (average diameter 80 $\mu$ m) were observed in July, and samples collected in early October show no signs of any gametogenic material, indicating that spawning occurs sometime in late summer. The factors driving deepwater coral reproduction are still unknown.

Common traits can be observed in all the species studied: they are all gonochoristic broadcast spawning species that reproduce seasonally. Deep-water corals live at depths beyond the photic zone (except for *O. varicosa*, which may experience low light levels during the summer months) and rely exclusively on planktonic food. Reproduction therefore may be driven by seasonal changes in food availability or by changes in temperature, tidal regime, or some other environmental parameter. This is an interesting avenue for further investigation, particularly since the reproductive cycles of *L. pertusa* are different in samples from different locations.



**Fig. 16.** Light microscope images of *Oculina varicosa* mesenteries showing A) late vitellogenic oocytes; B) late stage testes packed with spermatids.

Although the focus of this chapter is reproduction in the deepwater scleractinia, members of the gorgonacea, antipatharia and stylasterina also fall under the definition of colonial corals, and all may comprise significant components of deepwater coral ecosystems. Apart from the stylasterina, these orders all have the same basic bauplan, with simple polyps that contain the gametogenic material.

A study of *Antipathes fiordensis* from New Zealand (Parker et al 1997) showed that like many colonial scleractinians, this black coral species is gonochoristic, with an approximate 1:1 sex ratio and a seasonal reproductive cycle resulting in broadcast spawning of gametes. The gametes originate in the endoderm (gastrodermis) of the mesenteries but, unlike scleractinians, the gametes do not migrate to the mesoglea to continue development. An investigation into gametogenic cycles of 6 common gorgonian species in Biscayne Bay Florida (Fitzsimmons-Sosa et al. 2004) showed that all species were gonochoristic, five showed reproductive seasonality and sex ratios were either 1:1 or 1:2 male: female. As with the hexacorals, gametes of these octocorals also develop within the mesenteries of the polyps (**Fig. 16c**).

The order Stylasterina (phylum Cnidaria, class Hydrozoa) are a group of calcareous encrusting or branching colonial species often referred to as hydrocorals. Unlike scleractinians, there is no information in the literature indicating that the survival and reattachment of branchfragments (fragmentation) is a mechanism of new colony formation in hydrocorals. The male and female reproductive structures or gonophores develop in epidermally-lined cavities called ampullae, which are usually present as superficial hemispheres, but occasionally they are completely submerged (internal) in the coenosteum. They are generally smaller and more numerous in male colonies. Stylasterines are gonochoristic, with internal fertilization and embryonic development. Planular larvae are released via efferent ducts, which are usually seen as small irregular pores on or near the ampullae. A study of reproduction in Alaskan hydrocorals (Brooke 2004), showed a range of developmental stages in all species studied. This implies either continuous reproduction or an extended non-synchronous developmental season. Reproductive strategy has implications for habitat recovery potential; for example, a species with a longer planktonic phase may be more able to recover from large scale damage than one with a very short larval phase, or those that can form new colonies from fragmented branches may recover quickly from small scale physical damage.



**Fig. 16c.** Eggs at the base of a bamboo coral polyp



**Fig. 16d.** Longitudinal section of *Metallogorgia melanotrichos* polyp with developing oocytes

To fully describe gametogenic cycles, samples should be collected monthly over multiple years. However, the logistics and expense of working in the deep sea often prohibit such rigorous sampling regimes. Small fragments (10-20 polyps) should be taken from several different colonies, preferably at least 10. In a gonochoristic species with equal sex ratios, this will provide approximately 5 replicate females, which is usually sufficient for statistical analysis, but more samples would give better statistical power if logistics permit. Males can be used to describe stages of gonad development, but female oocyte size frequency distribution is easier to quantify and is generally used to describe the gametogenic cycle. Samples should be fixed while alive and in good condition. Stressed corals often produce mucous, which can inhibit fixation so ideally, a cold room would be available to allow the corals to recover from the stress of collection and exposure to warm surface water. If not, then fix the samples as soon as they come on board. Dying or decaying tissue is not useful.

There are many different fixatives that can be used; the most readily available effective fixative to use is 10% formalin in seawater, which is made by diluting full-strength formaldehyde (37%) in a 1:10 ratio with seawater. This is not recommended for fine cytological examination, but works sufficiently well to determine gametogenic status. Tissues should be left in this fixative for at least 36 hours before thoroughly rinsing in distilled water and transferring to 70% ETOH for storage. Hellys solution (Barszcz and Yevich 1975) is a better fixative for corals but is a little more complicated. A stock solution of 100 gm potassium dichromate and 200 gm zinc chloride in 4 L seawater can be made prior to sampling, but the addition of the formaldehyde (200ml) should only be made when needed since the full mixture is only good for 24 hours. The samples should remain in the solution for 16-20 hours then thoroughly rinsed in freshwater and transferred to 70% ETOH for storage. Another alternative is 'Z-fix' (Anatech Ltd.), which also contains formaldehyde, and is simply mixed with seawater prior to use.

Corals need to be decalcified before further processing. A solution of 10% hydrochloric acid with EDTA buffer takes between 1 and 12 hours to decalcify, depending on the sample. An acid change may be needed for heavily calcified skeletons. After rinsing thoroughly in running freshwater, tissues are dehydrated through a series of ethanol concentrations (70-100%) and then transferred to a clearing agent such as toluene. The tissues are then embedded in paraffin wax, cut into 8 $\mu$ m sections with a microtome blade and stained using Mayer's Haematoxylin/Eosin B before mounting on microscope slides. This combination of stains will show lipids (vitellogenic oocytes) as bright pink and DNA (primordial germ cells as testes) as purple. Oocyte diameter can be measured using a compound microscope with an ocular micrometer or images of oocytes can be taken using a digital camera and subsequently measured with image analysis software. There will be some shrinkage of the oocyte during the histological processing, so all measurements are "relative". A minimum of 50 (preferably 100) oocytes should be measured per polyp; however this is not always possible. This basic process can be applied to any of the deepwater colonial corals, but for those species with large yolky eggs, paraffin penetration can be improved by using a series of toluene and paraffin solutions.

A full treatise on histological techniques is beyond the scope of this chapter but recommended further reading includes Carson, 1997, Kiernan, J.A. 2003.

## Genetic methods for species identification, evolution, and ecology

by Amy R. Baco-Taylor

Genetic methods may be used to enhance taxonomic studies, to gain understanding of evolutionary relationships, and to elucidate the dispersal patterns of deep-sea corals. Emerging molecular methods may also be used to better understand deep-sea coral physiology. The key to molecular studies of any taxon is to obtain informative markers, genes or segments of DNA that vary enough between individuals or species to address the research hypotheses. Many informative phylogenetic and population genetic markers have been developed for invertebrate taxa. Mitochondrial genes (particularly COI, 16S, cyt b, and others) are commonly used. Unfortunately for coral biologists, many of these markers show little or no variation in hexacorals or in octocorals (Van Oppen et al. 1999; France and Hoover 2002; reviewed in Shearer and Coffroth 2004). Recent efforts have focused on new genes and microsatellite markers. Some of these are reviewed below.

### *Phylogenetics and Systematics*

DNA sequencing and phylogenetic analyses enhance taxonomic studies based on morphology.

Phylogenetic analyses may be used to determine whether two specimens are the same species, or different species. They may be used to identify cryptic species—species that are nearly indistinguishable based on morphology but show clear differences in their DNA sequences.

Phylogenetic analyses also help us understand the evolutionary history of species, helping clarify how coral families are related or whether scleractinians evolved before octocorals, for example. In

phylogenetics, genes are sequenced in multiple individuals, and the resulting sequences are compared. Differences in sequence composition are used to construct phylogenetic trees, which are used to determine evolutionary relationships.



Fig. 17. *Enallopsammia* sp. on Manning Seamount.

The primary markers used in phylogenetic studies of invertebrates are the nuclear gene ribosomal 18S RNA and the mitochondrial genes ribosomal 16S RNA and cytochrome oxidase I (COI). 18S rRNA has been used within the class Anthozoa (Berntson et al. 2001) and for higher-level systematics in octocorals (Berntson et al. 1999; Sanchez et al. 2003a). Mitochondrial 16S rRNA has also been used to study systematics in shallow and deep-sea scleractinian corals (Romano and Palumbi 1996, 1997; Romano and Cairns 2000) and in octocorals, (France et al. 1996). Mitochondrial 16S and COI have low variability in scleractinian corals and octocorals compared to other invertebrates (reviewed in Shearer et al. 2002). However, 16S has two insertion/deletion regions (INDELs, France and Hoover 2001). These INDEL regions have been used to separate species of bamboo corals (Smith et al. 2004) and may be useful for higher-level coral systematics (Sanchez et al. 2003b).

Because Anthozoans have low variability in the genes typically used for phylogenetics, a number of other genes have been tested in octocorals, including mitochondrial genes in the NADH group (ND2, ND3, ND4, and ND6). Octocorals also have a mutS gene homolog in the mitochondrion. A portion of this

gene has been shown to be the most variable of all octocoral mitochondrial genes sequenced to date (France and Hoover 2001; Sanchez et al. 2003b). This gene is likely to be useful for phylogenetic studies of octocorals but has not yet been found in scleractinians.

Other genes that are being tested for applicability in phylogenetics and population genetics for both octocorals and scleractinians include the mitochondrial DNA control region, internal transcribed spacers (ITS), and several introns (see Van Oppen et al. 2000 and reviewed in Shearer et al. 2002). Of these, ITS is the most variable, but can be difficult to align. It has been widely used in studies of shallow-water scleractinians (Medina et al. 1999; Fukami et al. 2004; Vollmer and Palumbi 2004) and has been used in one study of hybridization in shallow octocorals (McFadden and Hutchinson 2004). However, Sanchez and colleagues (2003b) found that ITS sequences were not alignable across families of octocorals, and they suggest that this gene may not be useful for octocoral phylogenetics.

### ***Population Genetics***

Population-genetic studies are often used to infer dispersal patterns in organisms for which movement of larvae or adults cannot be tracked or inferred. These methods should therefore be particularly useful in understanding the ecology of deep-sea organisms but have rarely been applied in deep-sea settings.

The low variability in mitochondrial genes that plagues coral systematists is even more of a trial for population geneticists. Mitochondrial genes such as COI and 16S rRNA are often used in population-genetic studies of other invertebrates but may be invariable at the genus level in corals. Early population-genetic studies of shallow-water corals used allozyme markers (Abbiati et al. 1993; Burnett et al. 1995; Hellberg 1994, 1995; Benzie et al. 1995; Ayre and Duffy 1994; McFadden 1997). Allozymes are allelic forms of enzymes that can be separated using electrophoresis. They are inexpensive and can be informative for population-genetic studies but do not have the same resolution as the other methods discussed here. DNA fingerprinting of individual organisms using amplified fragment length polymorphisms (AFLPs) has also proven useful in coral population genetics (Barki et al. 2000); however, this method has not been widely applied.

Microsatellites are the most promising markers for coral population-genetic studies. Microsatellites are segments of DNA that consist of repeated units of short (di- or tri-nucleotide) sequences. Microsatellites are highly variable and are codominant, meaning that both alleles at a locus can be scored, facilitating the estimation of population-genetic parameters (Roderick 1996; Palumbi 1996; Beaumont and Bruford 1999; Chakraborty and Kimmel 1999; Barki et al. 2000). Microsatellites have been shown to be sufficiently variable in corals to be useful for population-genetic studies, and they are currently in use in many studies of both shallow and deep-sea corals (Maier et al. 2001; Gutierrez-Rodriguez and Lasker 2004; LeGoff-Vitry et al. 2004b; Magalon et al. 2004; Shearer and Coffroth 2004; Baco and Shank 2005; Baco et al. in prep). Though useful, microsatellite loci must be acquired for each species under study, and the loci are rarely usable across taxa, even within a genus. In addition, microsatellites require considerable startup time and funds to develop.

### ***Sampling Requirements for Genetic Analyses***

Phylogenetics: 1-3 individuals of each species of interest, plus an outgroup species.

Population Genetics: 30 or more individuals of the species of interest from as many different sites or populations as possible.



## Handling procedures adopted by the Mountains-in-the-Sea Research Group

by Les Watling



**Fig. 18a and 18b.** The Mountains-in-the-Sea Research Group in action



The Mountains-in-the-Sea Research group got its start in the canyons of

the northeastern United States, where we were first confronted with octocorals of various kinds, but most of which were pretty hardy. During the course of our subsequent expeditions to the New England seamounts of the North Atlantic, we have come to recognize that certain of the octocoral families have to be handled differently from others. Handling issues are especially important when bringing specimens from the cold deep sea through warm temperate or tropical surface waters. Deepwater temperatures can drop to 2 °C. However, the collection and handling procedures outlined below can be modified somewhat when dealing with surface waters that are approximately the same as those at the collection depth. We do not yet know much about the temperature tolerance of octocorals and antipatharians living below normal scuba diving depth, so our guiding principle has been use methods that cause as little shock to the organism's tissues as possible.

### *Collection Procedures*

*Manipulator arm.* We found that if octocorals and black corals (and perhaps other delicate specimens as well) are the only items to be collected with the manipulator arm of the submersible or ROV, the standard three-fingered claw should be replaced by a cutting and gripping style claw. The one designed for us by the Institute for Exploration can be seen on NOAA's Ocean Explorer web site. It consists of two stainless steel angle-iron pieces with two Nalgene tubing pieces arranged so that as the sharpened edges of the opposing angle-irons cut the coral stem the tubing acted to grip the cut piece.

*Collection boxes.* When there is a possibility that the specimens will be recovered through surface waters that are more than a few degrees warmer than the temperatures experienced at the collection site, we try to use tightly sealing insulated bio-boxes. In some cases we have not been able to have all insulated boxes, so we have experimented with which corals put in the non-insulated boxes

and still be useable when recovered. Bamboo corals are the least likely to be useful for any morphological or genetic work if they spend much time at temperatures much above those where they were collected. Note here also that recovery times for the submersible or ROV can be quite long. For example, after Alvin enters the warm surface waters, another 45 minutes can pass before the sub is recovered and safely secured and you have access to the specimen boxes. With ROVs, this time can be shortened somewhat, but can still seem excruciatingly long.

*Oxygenation.* Some ROV dives are as long as 17 hours or more. In that case the lid of the bio-box should be opened every 2 hours or so in order to oxygenate the water in the box. Remember that some deep-sea waters are low in oxygen as it is, so specimen deterioration can occur in the box. In a few cases we have seen that associates of the corals will abandon the host when conditions get bad in the box, but for the most part the associates hang on to the end.

*Mucus production.* Many octocorals secrete copious amounts of mucus when stressed. As a result we try to be careful about what families of octocorals and black corals go in a bio-box together. For example, antipatharians go into a box of their own whenever possible. Bamboo corals, large primnoids (such as *Primnoa resedaeformis*), and the very large *Paragorgia* spp. are pretty good mucus producers. Small primnoids, chrysogorgiids, plexaurids, and other small octocoral species do fine together in one box.

*Video data collection.* Before collection we take very close video of the specimen (using lasers for scale), especially of the colony base which is often not recovered. Longer term close-up video also can be used to show the tentacle pinnules (which can often be counted if the light is right), and to see how any associates living on the coral might be behaving. In practice, we have not been able to get more than 15 minutes of close-up video, however, most likely because the photons from the lights begin to heat the polyps and they start to close or show other signs of being stressed. Video of the surrounding habitat, including small-scale geological features, surficial substrate type, macro-associates and other sedentary fauna should also be recorded, as described in the following section

*Record keeping.* Every place where the ROV or submersible stops is given a station number and the specimen(s) collected are assigned individual specimen numbers. For example, in our system, KEL 408-1, is interpreted to mean Kelvin seamount, 4<sup>th</sup> dive, 8<sup>th</sup> station, 1<sup>st</sup> specimen. With this system we can determine exactly which video we need to view to see the collection of the specimen. We also note the bio-box into which the specimen was put and make a “map” of each bio-box with tentative specimen names, small drawings of the specimen showing its shape, and the order in which the specimens were put into the box. These details will be very important in sorting out the 15-20 specimens in the boxes when everything gets to the surface.

### ***Shipboard Procedures and Other Considerations***

*Immediate storage.* While the ROV or submersible is in the water, a sufficient number of 5-gallon buckets of seawater are chilled to the at-depth temperature. Each bucket is labeled for a single specimen. As soon as the specimens can be removed from the bio-boxes, they are put into the buckets and taken for storage in the cold room.

*Specimen deterioration.* We have seen that specimens in the different coral families deteriorate at very different rates. Bamboo corals often break down very quickly and are handled first, followed by plexaurids such as *Paramuricea* sp. and its close relatives. Chrysogorgiids last for a moderately long time (although many polyps die extended and often with tentacles extended as well) as do *Paragorgia* and its relatives. Deterioration of bamboo corals can be seen in the tendency for the polyps to begin to slough off the axial skeleton. In *Paramuricea* and relatives, the tentacles begin to turn black within about 20 minutes of being put into the buckets.

*Fixation.* There has been a lot of discussion on various lists about using formalin, especially with respect to deterioration of sclerites. While there are many drawbacks and health issues associated with this fixative, there are many benefits, the most important of which is the ease with which delicate tissues can be rapidly fixed. Fortunately bamboo corals have easily-penetratable tissues, which may also be why they deteriorate so rapidly, even when handled with the best of procedures. As a result we have adopted the following quick fixation procedure to be used for all octocorals with especially delicate tissues, such as the bamboo corals and chrysogorgiids. One or two buckets of 4% formalin-seawater are prepared and stored capped in a fume hood. After the specimen has been tagged with its unique identifier, it is put into the formalin bucket for about 12 hours. Following the formalin immersion it is rinsed in another bucket containing seawater and then transferred to a bucket or bag of 70% ethanol. The result is specimens that will be useful for taxonomic studies for a long time. We've seen that these formalin fixed specimens show little contraction of the calyx and the relationship of the sclerites to the coenenchyme is preserved, at least macroscopically.

*Other data collection.* Fixation issues aside, once the specimens are safely stored in the cold room, each specimen goes through the following procedure before being finally stored for later use. The order in which the first three of these items actually occur depends largely on the family of coral being dealt with, the length of time since collection, and etc.

1. *Labeling.* Each specimen is labeled by writing its identifier, collection date, and tentative name, onto a water-proof heavy paper which is tied to the specimen with monofilament line.
2. *Genetic sampling.* Before the specimen is handled by too many pairs of hands (all equipped with suitable gloves), pieces are set aside for genetic analysis. Usually we try to have a small ice-filled tray in the lab into which can be set plastic dishes (such as sandwich containers available from any supermarket) containing cold seawater. The genetic samples can be stored in these small dishes until they can be dealt with.
3. *Photography.* The entire specimen with its label and a ruler for scale is photographed, usually on a black cloth, but we have also used a gridded board. Close-up photos of special features or associates are also taken. We use a 5-megapixel camera with close-up capability. Several pictures are taken to be sure that at least one could be used for publication of the morphological description of the species.

4. *Reproduction.* Sections of the coral for reproductive analysis are then removed. Details of the methods for fixing and preserving tissue for reproductive analysis will be dealt with elsewhere, or interested persons can contact me.

5. *Associates.* All associates are then removed, if possible, and fixed appropriately. The container(s) in which these animals are stored is given the same identifier as the coral from which they were taken. Associates are not removed where excessive breakage of the coral specimen will result. Fixation using formalin (4%) or alcohol follows normal techniques for the various phyla. For example, polychaetes should be fixed for 24 hours in formalin, then rinsed and stored in 70% ethanol, whereas ophiuroids, if they are to be used for taxonomy, should never be formalin fixed. I suspect, however, that as with the octocorals, very short fixation with a weak formalin solution will probably not ruin the carbonate structures needed for taxonomic analysis. We've used other fixation protocols, depending on specific uses of the organisms.

*Final storage.* After all of the various bits and pieces of the specimen have been dealt with and the specimen has been properly fixed, it is prepared for storage aboard ship and made ready for shipping. Specimens are handled according to their special characteristics. Small fan-shaped species are stored in ethanol in heavy-duty zip-loc bags. A group of 10 or so of these bags is then put into tightly sealing chemical storage boxes purchased from an industrial supplier. If the bags develop micro leaks, which is frequent, especially with small primnoids and many of the bamboo corals, the box will contain the leaked alcohol. Some specimens are double-bagged. Larger specimens are loaded into larger bags and stored flat in larger boxes, but it is not possible to get really large (about 44 inches long) boxes with tightly sealing lids. In these cases we have often soaked the specimen in alcohol in a box or bucket for a day or so, then put the specimen in a bag with a small amount of alcohol and removed as much air as possible. (As part of another project in Alaska a vacuum system was used but we haven't evaluated the results yet. Gradually reducing the amount of alcohol facilitates shipping as only small quantities are now allowed.) Very large specimens are stored in their own buckets with sealable lids. These buckets are maintained full of alcohol until shipment.



**Figs. 19a and 19b.** Roundnose fish and *Paragorgia* sp. on a New England seamount in the eastern North Atlantic.

## Documenting deep-sea coral collections

*In situ* video and photography is often available to modern deep-sea explorations. Most submersibles and ROVs are equipped with 3 chip digital video cameras and high resolution still cameras. Video and photography are particularly useful for sharing information between scientists, for tentative species identifications, and for public outreach efforts. Video documentation has the additional benefit of recording community structure, e.g. target species density along a transect, and associated commensal species. Still photo documentation is most useful for tentative species identifications and for publications.

High quality images can depend on several things. The most important are proper exposure, and proper resolution. Too much light (often from a flash) can result in over-exposure. Too little light will result in underexposure, and a grainy image with poor detail. Angle the lights 45 degrees downward to avoid backscatter from plankton. Try and use your available light judiciously, and get as close as possible to your subject. Still image captures from video are limited to the scan resolution of the video and the software. Some video captures are very low resolution, e.g. 72 dpi. This is suitable for internet distribution. Print quality (300 dpi) in situ images may require a still camera, or a “still photo” setting on the DV Camera.

Separate video and photo protocols are provided. The video section will discuss camera, lights, and media format. The video section will also present some proven video transect methods and some recommended camera angles. The photo section will discuss “life cycle” photo documentation so that your in-situ photo stills can be matched to dried specimens on deck, and to live polyps under the microscope.

### Video protocol

#### 1. Camera Quality:

- a. 3 chip (CCD) video cameras are superior to one chip video cameras.
- b. Low light (SIM) cameras are grainy, and poor. HDTV cameras are the best available.

#### 2. Lights:

- a. Quality: All lights have a different color temperature, and the camera setting must match that temperature using the *white balance*. Daylight is 5200 K, tungsten light is 3600 K.. Xenon lights are approximately 4200 K.
- b. Quantity: 1000 watts of Xenon light are best
- c. Position: Lights positioned behind armatures cause shadow, and/or underexposure because they reflect light into the camera iris. Position lights high and to the side, angled downwards.

#### 3. Archival Media Quality:

- a. HD, DVCam, Digibeta, Beta SP, and Mini-DV are archival quality video tape formats.
- b. These are magnetic tape mediums, and will degrade within 10-15 years.<sup>1</sup>

#### 4. Archival digital resolution:

- a. Broadcast quality digital video (.mov, .mpg, .mp4) is 720 x 480 pixels

#### 5. Distribution digital resolution:

- a. Medium: 320 x 240 Compressed video is most suitable for internet distribution
- b. Small: 160 x 120 is commonly referred to as “postage stamp size”, very small

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<sup>1</sup> Temperature and humidity are the most important factors to maintaining footage for extended time spans. If you plan to start a collection, or have an aging one, consider dubbing tapes five to seven years old to a contemporary tape medium, such as Beta SP, then to a digital medium, such as DVD. If you have ¾” Sony tapes older than ten years you may wish to consult a restoration facility, such as SPECS Brothers in Ridgefield Park, NJ. [www.specsbros.com](http://www.specsbros.com)

## Video protocol (cont.)

### 6. Data Burn in

- a. Ideally, data will be burned into one camera only, including dive number, depth, and geolocation. A second camera should have clean, high resolution images for broadcast and distribution.
- b. In many cases a second camera may not be available. Some recorders can turn the overlay information 'off or on'. Harbor Branch Oceanographic Institute (HBOI) uses the overlay 'on' initially to document the overall habitat while they verbally describe a sample, and then turn the overlay 'off' for highest quality video of the specimen.

### 7. Camera angles and recommendations

- a. Wide, medium, tight
  - i. Record a wide view of the community
    1. How dense is the community?
    2. Is it monotypic or diverse?
    3. Are there conspicuous fish or invertebrates nearby?
  - ii. Record a medium view of the colony from base to tip
    1. To what substrate is the base attached?
    2. Do you notice any cryptic species or epibionts?
  - iii. Record a close-up view of polyps, branching morphology, and commensal species
- b. Reasons for getting close
  - i. Pro - better light
  - ii. Pro - less apparent movement in a wide lens
  - iii. Pro - better view of polyps and commensals
  - iv. Con - potential for turbidity
  - v. Con - potential for damage to colony
- c. Reasons for keeping your distance
  - i. Pro - Minimally invasive
  - ii. Pro - Greater range of possibilities for wide, medium, tight shots.
  - iii. Con - Less light, grainier image.
  - iv. Con - More apparent camera movement when "zoomed in"



Fig. 20a. Wide community shot, 6 ft. sponge!

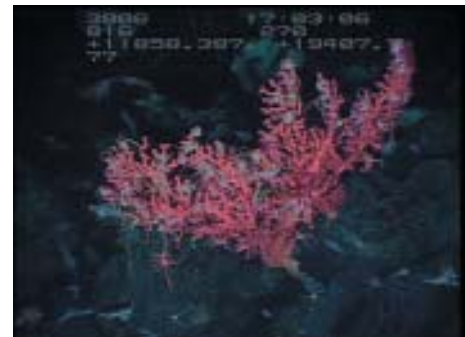


Fig. 20b. Wide colony shot with burn in.

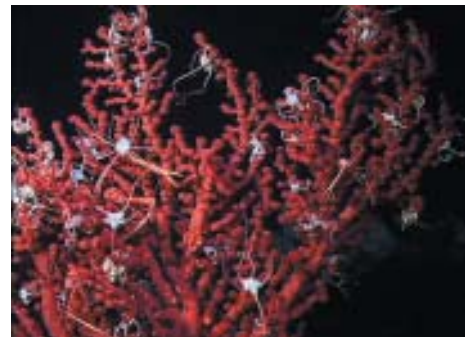


Fig. 20c. Medium shot. Note associated species.

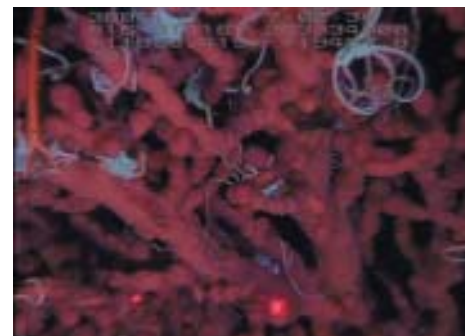


Fig. 20d. Close up with associated species and 10 cm red laser reference for scale.

## Video protocol (cont.)

### Video transect types<sup>3</sup>:

#### *Line (Strip) transect-*

Vertical line transects (Fig 21a) are best for rapid biological assessments of large features, such as seamounts. These start at the base of the feature, and almost guarantee dramatic changes in environmental conditions.

Vertical line transects can be repeated around a feature. They also help discern vertical zonation patterns, and the results are readily quantifiable.

#### *Grid Transect-*

Grid transects (Fig 21b) can be very time consuming, but they can also cover a large area systematically. The results are readily quantifiable. The length of the transects, and the offset are usually predetermined.

Grid transects may be oriented parallel to particular isobaths, or perpendicular to those isobaths. This technique is very powerful when combined with an opportunistic sampling methodology to verify video identifications.

#### *Radial transect-*

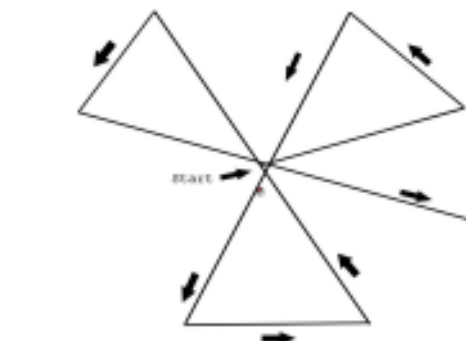
Radial transects (Fig 21c) start from an arbitrary or randomly selected center. These are best for smaller features, or low relief mounds. A radial transect can help determine the limits of a particular community when the size of that community is unknown. All axes need not be the same length.



Vertical Line Transect



Grid Transect



Radial Transect

**Fig. 21a-c.** Video transect types

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<sup>3</sup>The analysis techniques described here are modified from those developed for the Great Barrier Reef in Australia (after Osborne, K., and W. G. Oxley. 1997.)

## Still image protocol

Digital still cameras provide much better resolution than video still frames. These two mediums are not interchangeable. Print quality images are 300 dpi. Video still frame resolution is derived from the number of horizontal lines in the video image. On a 3-chip camera, this amounts to about 72 dpi. This is good enough for internet distribution, but not for print. Newer High Definition (HD) video cameras can produce frame grabs at high resolutions more suitable for print. Unfortunately, these cameras are not yet widespread. Plan to take your best pictures with a digital still camera, rather than depending on video ‘frame grabs’.



**Fig. 22.** Scale is difficult without clues, like laser points, or the crabs shown here. This primnoid colony is ~ 4 feet tall.

Small extended lenses of modern digital cameras perform nicely with a microscope. This procedure takes time. Polyps can remain retracted for hours, shocked by temperature and lighting differences. Some polyps may appear in a bucket of seawater as it sits in a dark cooler for a while. To complete the photo cycle, photograph the entire colony again with a scale reference, so that you can compare the appearance *in situ* to the appearance on-deck. You may wish to pack some *duvatine*, a light absorbing black fabric, and some 2' x 3' dark colored felt material to use as a plain background. Some colored thin foam mats are water repellent.

**Fig. 23.** The dried skeleton of *Keratoisis flexibilis* photographs nicely on a neutral grey background. Scale is again absent, but the image is perhaps better for it. Image ~ 25cm.



The most useful method of documenting biological collections is called “living specimen photography”. First, document the specimen *in-situ*, (**Fig. 20**) with wide shots, medium shots, and close-ups of polyps, before you collect it. Once you bring the colony on-deck, place it in refrigerated seawater and cut a small branch from the colony to examine and photograph the extended polyps through the oculus of a dissection microscope. Then photograph the colony. Sample numbers and scales can be useful additions to the image.



John Reed's August 22 article on Stetson's Reef from Islands in the Stream 2002 expedition has some terrific images, as does the entire 2003 Life on the Edge Expedition, courtesy of Art Howard from NAPRO Communications. Notice that these examples have plain, appealing backgrounds and sharp focus (**Fig. 22**). A tripod and good lighting will improve your depth of field, your camera stillness (unless your boat is rocking!) and your focus. Small flat plastic rulers are important for scale. Coins are also useful (see **Fig. 9**). Simple labels, like a dive number or sample number, should be included. Place these labels so they can be easily cropped out of the image if necessary. You can study these examples of some high quality digital photography at NOAA's Office of Ocean Exploration website:

<http://oceanexplorer.noaa.gov/>

### 1. Image Quality

- a. Printed images or CDROMs of still images may accompany a sample
- b. Print Quality Digital Files: 300 - 600 dpi .tiff, .png, or .jpg image files
- c. Distribution Quality: 72 dpi .jpg image files
- d. Low resolution still images may be collected *ex post facto* from video

### 2. Camera Framing and zoom control (see insets on page 18)

- a. **Community Image**- the widest shot, includes organism, substrate and associated species
- b. **Wide Shot**- frame the entire colony as best you can. (**Fig. 21**)
- c. **Medium Shot**- branching morphology and associated species
- d. **Close-Up Shot**- epiphytes
- e. **Reference** – Any reference for size and scale, e.g. ROV claw or laser scale

### 3. Camera angle

- a. For transects to document habitat cover (%), you can have the camera angled at 90 degrees to the bottom (plan view) or 30-40 degrees ahead for better estimates of colony height. Try to keep parallel lasers in view for scale. ALVIN uses two laser pointers set 10cm apart.

### 4. Data Burn In

- a. include date and time, dive number, depth, and geolocation in metadata
- b. alternatively, enter this data into the image itself

### 5. Practice Living Specimen Photography



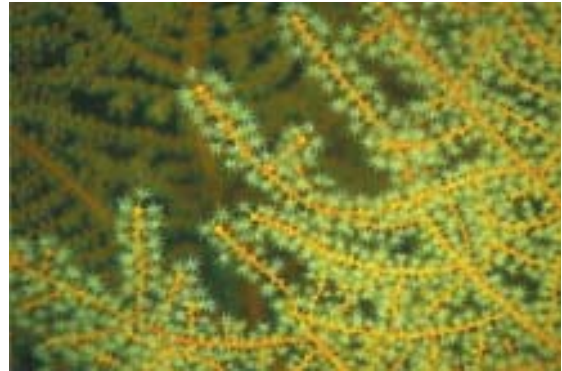
**Fig. 24.** Alaska's Aleutian Islands have diverse and colorful coral gardens deeper than 300 m. Commercial bottom trawl fisheries can damage these gardens

## Living specimen photography

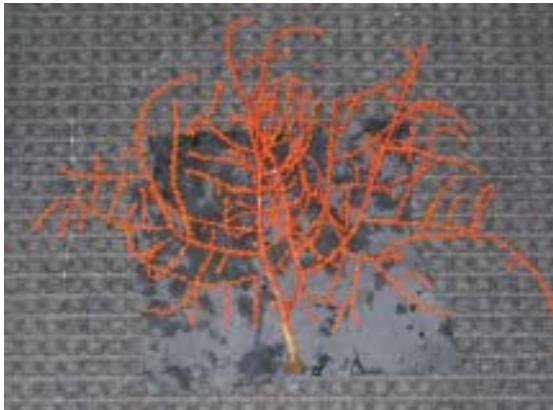
Living specimen photography helps increase our national capacity to document deep-sea coral diversity. The resulting images can be used by subsequent expeditions to identify coral colonies *in-situ*, reducing the need to collect new specimens, reducing time and effort for future expeditions, and facilitating broader scale habitat characterization efforts. The method photo-documents the specimen *in-situ* with a wide shot, a medium shot, and a close up of the polyps, collects a branch or colony, then reproduces these shots on deck with a scale, and photographs polyps in the wet lab with a microscope. This is a time consuming process, but the results are useful. Pictures show *Swiftia* sp. in the Gulf of Mexico.



**Fig. 25a.** *In-situ* wide shot. Nearby colonies evident.



**Fig. 25b.** *In-situ* medium shot. Polyps extended.



**Fig. 25c.** On-deck wide shot on a measured grid



**Fig. 25d.** On-deck medium shot. Polyps retracted



**Fig. 25e.** Dissecting scope medium shot (10x)



**Fig. 25f.** Dissecting scope close shot (40x)

**Fig. 25.** Example of the sequence of photographs documenting a deep-sea coral. Photographs are taken from the deep sea (a,b), on the deck of the boat (c,d) and under a microscope (e,f).1

## Collecting

There is a broad range of opinion about how much coral to collect, depending upon the intended purpose of the sample. Most museums would prefer an entire colony, especially if the specimen is a new species, or intended for display. These can be large, though, and difficult to ship. For age and growth studies, the holdfast of a large colony is most important. For species identifications based on gross morphology, a small branched segment will often suffice, as described in the chapters by Sanchez and Cairns. For molecular studies, a small amount of preserved tissue is most useful, particularly if tissue can be collected from several colonies.

One thing deep-coral researchers can agree upon is that photographs and videos alone are not particularly useful for species level identifications. Photographs can help, of course, especially when accompanied by a physical specimen, and referenced with a biogeographic database. Photographs are often suitable for genus and family level identifications.

There is also a broad range of opinion about how to preserve a sample, and this is discussed on following pages. For some corals, like bubblegum and primnoid corals, dry samples can be useful. Dried skeletal materials of ahermatypic scleractinia, antipatharians, and bamboo corals are also useful for radiocarbon analyses. Some biologists feel that dry samples should be made only when absolutely necessary; for example, when you don't have room to store the whole specimen. Wet samples collected by the Mountains in the Sea Research Group are not small, on purpose, because they don't want to lose information that only wet samples can provide. Large wet samples will benefit the most future studies, but 6-10" branched segments are usually sufficient for species identification.

Your study organism may have the opportunity for regrowth if you leave the organism in place, and take only a branch. This is particularly relevant to cases where repeated samples of a single species are being collected. Selective clippings are the most responsible way to perform good science without impacting a localized population. Try to take only a clipping from the largest specimen. The largest colonies may be the most productive, and should be left alive in situ. It is preferable to collect your sample from a dense aggregation. Solitary colonies may be pioneers that facilitate more settlement, and therefore more future habitat.

## Permits

Orders Scleractinia and Antipatharia, and Family Stylasteridae are listed on CITES Appendix II. Collection from U.S. waters and subsequent transport within the U.S. does not require a CITES permit, transport of these corals across international borders requires an export permit from the CITES Management Authority in the country of origin. Collections in state waters (up to 3 miles) need a state permit, those in Federal waters need a Letter of Acknowledgement of scientific research, which is available from regional NMFS offices. To collect from Marine Reserves, you need special permission from the reserve management authority. These permits are granted by regional fisheries science centers. A list of these centers is available here: <http://www.noaa.gov/fisheries.html>.

## Preserving

When brought aboard, octocorals can exhibit a foul odor. Be prepared. Always work with a ventilation hood. Field systematics can be performed with a careful review of the polyp and sclerite morphology under a microscope, in reference to morphological and photographic keys. Further laboratory analyses (e.g. SEM microscopy and molecular classification) will require well preserved tissue samples. The preservation medium depends on your research intentions. These vary in toxicity from 95% Ethanol (grain alcohol, a preservative) to Formalin (a highly toxic, carcinogenic, all purpose fixative). Tissue fixation is not absolutely necessary for species identification. Preservation in 70- 95% ethanol is usually sufficient. To *fix* specimens (i.e., change their structural properties) for molecular work, histology, or long-term preservation you need a fixative.

These protocols for preservation have been established largely with Gorgonacea, *branched octocorals*, in mind. The techniques outlined in the section on handling procedures by Watling all work on both black corals and zoanthids. The preservation method you choose depends upon your purposes, most of which are outlined here.

### *Dried Museum Specimen*

- a. Before drying a specimen, preserve reference tissue from a mid-size branched segment.
- b. Branches range from delicate/fleshy (black coral) to thick/spongy (*Paragorgia* sp.) to bony/oozing (bamboo coral). Some corals clean up nicely, e.g. bamboo corals.
- c. Hang the specimen to dry—outdoors, if possible — in a splash free environment. Avoid laying specimens on deck due to safety concerns. If you must lay specimens on deck, secure the area, obtain permission, and take appropriate precautions.
- d. If you must dry a specimen, especially because of size, you may soak the specimen in formalin over night, then stand upright in a fume hood for two days before wrapping in plastic for storage. Putting the whole specimen in a cardboard sleeve (made from boxes) will protect against breakage.

### *Tissue Fixation*

- a. A general comment on fixing tissue for analysis, these pieces should be small enough to allow full penetration of the preservation medium - no more than 2cm thick. As with any fixative, use approximately a 1:10 ratio of tissue to fixative.
- b. Formalin is readily available and cheap and can be used at 5-10 percent. Full strength formalin is a 37 percent solution of formaldehyde. To make a 10 percent solution, dilute the 37 percent formaldehyde (formalin) 1:10 (not 1:3.7).
- c. To reduce calcium dissolution, formalin can be buffered by using seawater as the diluent and/or addition of borax crystals. Samples can be held indefinitely in formalin, but calcified samples (even if buffered formalin is used) should be rinsed briefly and post-fixed in 70% ETOH after 24 hours.
- d. You can ship small quantities of ethanol (<30ml) without special paperwork. Some samples can be wrapped in an ethanol-soaked paper towel in an airtight container. On reaching the lab, specimens can again soak in 70% ETOH or back into 10% formalin, depending on their ultimate purpose. The use of neutralizing solutions, which are commercially available from scientific catalogues, can solve waste disposal problems.

### *Tissue preservation*

- a. Do not let specimens dry. Keep them cool in native seawater.
- b. We recommend 70-95% ethanol or analytical absolute alcohol to preserve tissue, not denatured alcohol (which is about 100% but it has some nasty stabilizers).
- c. Formalin is not universally recommended for preserving octocorals. Formalin is toxic, carcinogenic, difficult to transport, and degrades DNA. As above, formalin can be used briefly as a fixative to get good tissue preservation without degrading sclerites.
- d. The smallest samples should be in 30 ml vials (e.g. blue-tip vials from Fisher). For molecular work, use 12ml and 3ml plastic vials with color coded caps.

### *SEM Analysis*

- a. In the particular case of octocorals, samples for SEM analysis are usually digested in bleach (e.g., Clorox) and then coated with gold. You should preserve material in ethanol or let it dry. It is easiest to dry the material, but preserved tissue will provide DNA.
- b. Glutaraldehyde is also used as a fixative for SEM preps, and tissues are transferred to buffer after an hour in fixation. Glutaraldehyde is also very toxic material.

### *DNA Analysis*

- a. DNA samples need to be fixed as soon as possible after collecting. Do not let specimens dry before wet fixation.
- b. Do not use your hands to collect branches for DNA preservation. Even if you wear gloves, use forceps and a scalpel to collect the branches for DNA preservation. Using a clean kimwipe, blot excess water from the tissue just before adding it to preservative. Clean (with ethanol) or change the forceps and scalpel after every collection.
- c. Preservation Options for DNA Analysis:
  1. Frozen samples
    - i. Samples frozen in liquid nitrogen work best, as long as they can be kept frozen
    - ii. An ethanol/dry ice bath should also be sufficient
    - iii. Store at -20 to -80 degrees Celsius
    - iv. Secure against power failures and minimize freeze/thaw episodes in the lab.
  2. Ethanol (95 percent)
    - i. Ethanol will suit both tissue and DNA preservation but may not be ideal for either.
    - ii. Use of ethanol reduces the number of solutions brought on board.
    - iii. DNA degrades in ethanol-preserved samples after 5-10 years.
    - iv. An ethanol concentration of 70 percent may be sufficient but is not recommended. We recommend 95 percent ethanol if available.
    - v. In an emergency, ethanol is available as Everclear in liquor stores. Some twentieth century Caribbean marine biologists used rum for their choice of preservative!

### 3. Dimethyl sulfoxide (DMSO; 95%)

In a population genetic study with a gorgonian in the Caribbean, DMSO worked 90% of the time versus 50% of the time using ethanol. DMSO is non-flammable and non-toxic but it penetrates the skin, so take precautions against contact with contaminants or toxics that may use the DMSO medium to penetrate your skin. DMSO is often used in medicines as a way to slowly release an active component (sticky patches for sea sickness, bladder infection treatments). This saturated saline solution is preferred for fixing DNA, but this is a difficult concoction.

- i. DMSO: 250 ml 0.5M EDTA pH=8.0 100 ml DMSO (final concentration 20%)
- ii. 105 g NaCl ddH<sub>2</sub>O for about 400 - 450 ml, Stir a couple of hours, add 10 g of NaCl and stir overnight
- iii. Next day complete volume to 500 ml and let the excess salt to precipitate.
- iv. 1.5 ml for 0.5 cm<sup>3</sup> of sample, use gloves and prevent contact with skin

There is some question about our ability to get good DNA out of samples that have been stored longer than 5 years in DMSO. Some researchers at the prefer DMDM Hydrantonin as a non-toxic, non-flammable, long-term solution for tissue preservation.

#### *RNA Analysis*

- a. RNA is much less stable than DNA. Various RNA specialty companies market buffers.
- b. Although immediate freezing (but not DMSO) can adequately protect RNA, many researchers use “RNAlater” (Ambion, Austin Texas, 512-651-0200) to store tissue samples for subsequent lab work.
- c. RNA will degrade after < 1yr, even if stored at -80. if you want to use RNA down the line, RNA Later is essential.



Fig. 26. Primnoid corals in the genus *Narella*, from Hawaii's deep coral beds.

## **Some preservation techniques for (deep water) coral samples for subsequent molecular studies: a special supplement from Harbor Branch Oceanographic Institute**

by J.V. Lopez

Tissue Storage for Molecular Experiments: To store field collected animals for molecular biology work, it is important to store the sample such that the DNA is protected from endonucleases, which can quickly degrade the DNA once harvesting occurs. Gloves further protect samples from degradation.

**One option is to immediately plunge the sample into a deep freeze immediately**, and in the field this entails liquid nitrogen (LN2) or an ethanol/dry ice bath. Samples then can be transported in LN2 dry carriers, which though convenient are expensive and must be charged after 1-2 weeks with more LN2. At destination, most samples can be stored indefinitely in a freezer (-20 to -80oC) for subsequent DNA analyses. Alternatively, place the sample in DMSO (dimethyl sulfoxide) storage buffer, or place the sample on a dessicant. Frost-free freezers stay frost-free by going through freeze-thaw cycles, which is bad for DNA and RNA samples.

### DMSO storage buffer (Adapted from Seutin and White, 1991)

20% DMSO  
250 mM EDTA  
dH<sub>2</sub>O (to final volume)

DMSO buffer is SATURATED with NaCl. In practice this means about 210g or more per liter. It takes a while to go into solution. Make up a 0.5M EDTA (pH 8.0) solution first, then add DMSO and NaCl to half of this, adjusting the pH to 8.0 as you go - it will help dissolution. Final pH should be 8.0. Place the sample in a wide mouthed tube (with a lid that seals water tight, such as a 15 or 50 ml conical polypropylene centrifuge tube; this depends on the size of your tissue specimen), and add enough DMSO storage buffer to cover the sample. Samples can be transported in this solution and stored at room temp for up to 2 weeks, then should be placed in a 4°C refrigerator. These samples can then be stored for several months in the fridge. Dawson et al (1998) gives an excellent evaluation of various other storage techniques, but supports the DMSO solution as the best for DNA studies.

For subsequent RNA studies, using the frozen tissue protocol will often suffice, or tissue samples can be placed in “RNALater”, specifically designed for RNA preservation when used with the manufacturer’s directions (Ambion Inc -[www.ambion.com](http://www.ambion.com); Austin Texas).

For more details see Baker et al. 1997, France and Kocher 1996, and Lopez et al. 2002.

## Standard Deep-sea Coral Preservation Protocols Tear Sheet

1. Care should be taken not to contaminate samples. Use rubber gloves and change them periodically. Use non-serrated forceps to avoid tissue build up in serrations. Clean forceps between samples.
2. Maintain the specimen in native seawater as long as possible.
3. Transport the specimen to cold storage for handling (if available).
4. Use clean scissors to clip a branched segment with extended polyps into a petri dish of the coral's native seawater.
5. Light and photograph extended polyps under the dissecting scope
6. Fix the remaining colony in refrigerated 4% buffered formalin seawater for ~12 hours.
7. Prepare duplicates of each sample, i.e. two vials for each coral collected, one for a curator and one for research.
8. The size of the jar or vial and the choice of preservative depends on your needs and your preferences
9. Prepare **50 ml vials** with **70-95% ethyl alcohol** or **33-50% isopropyl alcohol** solutions made with fresh water, not seawater
10. Prepare **15 ml** polypropylene centrifuge tubes with **95% DMSO**
11. Place pencil marked label of archival paper inside each vial
12. Use clean scissors to clip one 4 -5 cm branched segment and another piece of tissue 1-3cm
13. Place 4-5 cm branched segment in each 50ml vial
14. Place 1-3 cm of tissue in each 15ml vial for genetics
15. Change alcohol after two days.
16. Additional material may be dried or frozen.
17. Dried or frozen samples should be bagged in plastic with internal and external label, and then stapled, taped, or zip-locked shut.

**\*Modify accordingly. These are only recommendations.**

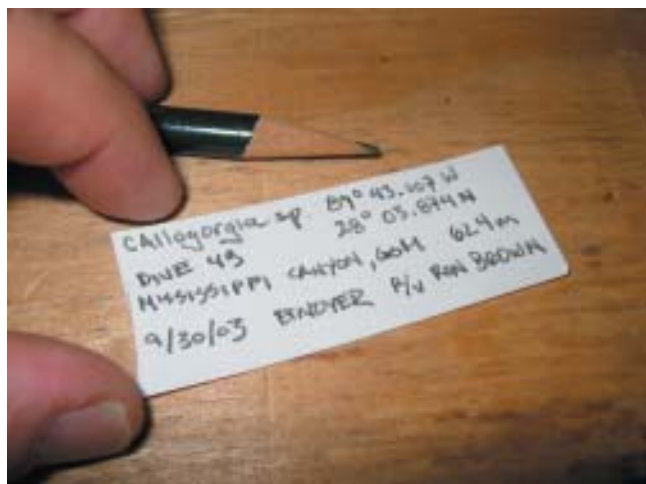


## Labeling

The significance of proper labels cannot be underestimated. If you are sending your samples to a scientific institution or laboratory, all the hard work of collection may be lost to dissolved ink or illegible handwriting. Furthermore, improper labels can be frustrating to those trying to help you. Researchers at the Smithsonian Institution note that externally labeled vials tend to lose their markings. **The best labels are on paper inside the vial, in the preservative, clearly written in pencil.** You may wish to keep a sample log, and reference each entry to the label in the vial. Wet samples should be related to dry samples. **Vials should also be labeled on the outside on the cap and the body.** Use only solvent resistant markers for the exterior label. These vials should be wrapped in parafilm before shipping.

### Label Information

1. Use pencil on a small strip of paper approximately ½" by 4"
2. Paper should be either 100% rag bond, Nalgene (TM) or Resistal (TM) contact: University Products 517 Main Street, P.O. Box 101, Holyoke, MA 01041-0101.
3. Write the date, cruise name, ship, researcher, station number, and sample number in clear block letters include geoposition (longitude and latitude), place name, depth in meters, related wet or dry samples, collection method, and substrate (Fig. 24 and 25).
4. Geoposition should be
  - a. Degree/Minutes/Seconds (DMS) e.g. 32 15' 43"N, 80 25' 18"W *or*
  - b. Degree/ decimal minutes (DDM) eg 32 15.23 N
  - c. Decimal Degrees (DD) **to three decimal places** e.g. 32.262 N, -80.421 E
  - d. 1 degree = 111km, so 2 decimal places is only accurate to within 1km
  - e. To convert DMS to DD,  $DD = D + M/60 + S/3600$
  - f. To convert DDM to DD,  $DD = D + DM/60$
5. Depth should be clearly defined in meters (vs fathoms, feet, km, nmi)
6. Depth may be a range of values due to gear type



**Fig 27.** This label shows collection information in pencil, not ink. The paper is durable and archival. Rubber gloves would help avoid DNA/ RNA contamination.



**Fig. 28.** 50ml vials hold small branches in ethanol, but legally require extra paperwork to ship overnight. You may drain these to ship them, and refill them later at the lab.

## Shipping

Packages shipping biological samples preserved in formalin and ethanol are subject to stringent rules and regulations. You must be certified to ship hazardous materials. **It is illegal to ship hazardous materials without proper certification.** The techniques and procedures described below are excerpted from a 49CFR/ICAO/IATA Hazardous Materials Transportation Certification class.

To summarize, preserved samples must be triple-packed in specific container types with limited quantities of fluid in order to meet the “Dangerous Goods in Excepted Quantities” provision of International Air Traffic Association (IATA) and US Department of Transportation (DOT). This protects the safety of the package and the individuals handling it.

Tissue samples preserved in ethanol must be packed in a sealed primary package (like a vial or jar) with <30ml of fluid. These must be contained in a sealed secondary package (like a plastic bag) with <500ml total fluid. A bag with 10 50ml vials (50x 100 = 500ml) will qualify as “Dangerous Goods in Excepted Quantities” when it is packed and sealed in sturdy cardboard box. The box must be no smaller than 100mm by 100mm on two sides. You can reduce the volume of ethanol in the primary package by wrapping a sample in cheesecloth or paper towel, allowing this to draw the preservative.

“Dangerous Goods in Excepted Quantities” packages must be labeled on the waybill and on the box. A “Dangerous Goods” label must be filled out and attached to the side. Check the box indicating Ethanol is Class 3 material. The UN number is 1170. The “Dangerous Goods” labels found at the websites below can be used if printed in color, no smaller than 100mm x 100mm.

If you need to ship large specimens or large numbers of specimens, be sure to check with the person at your institution responsible for shipping dangerous goods. One can ship ethanol-preserved material under the “limited quantities” designation if the inner containers hold 0.5 L or less ethanol and the total package contents are 1 L or less. There are different labeling requirements, etc., but otherwise the regulations are not much more onerous than for the “excepted quantities” designation.

These websites describe shipping regulations for biological specimens in great detail.

**Natural History Museum of LA County**      <http://collections.nhm.org/library/hazmat:hazmat>

This comprehensive and user-friendly website compiled by the NHM Marine Biodiversity Processing Center addresses invertebrate specimens directly, with links to printable labels for shipping packages.

**University of New Hampshire**      <http://www.unh.edu/ehs/shipping/Excepted-Quantities-Guidelines.pdf>

This brief .pdf document by Andy Glode at the University of New Hampshire - Office of Environmental Health and Safety (UNH-OEHS) provides a clear explanation of primary, secondary, and outer packaging. It also includes a printable Dangerous Goods label of appropriate size (100mm x 100mm).

**Stanford University**      [http://www.stanford.edu/dept/EHS/prod/researchlab/bio/Shipping\\_Guide.pdf](http://www.stanford.edu/dept/EHS/prod/researchlab/bio/Shipping_Guide.pdf)

Stanford University’s “Shipment of Biological Materials & Dry Ice Reference” is an extensive and detailed .pdf document created by trained authorities Andy Glode & David R. Gillum, UNH-OEHS.

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## Appendix 1. Classification of the Deep-sea Corals

A list of 48 families of hexacorals, octacorals, and calcified hydroids with species known to occur deeper than 200m. Common names are in parentheses.

Phylum Cnidaria (=Coelenterata)

Class Hydrozoa

Subclass Athecatae

Order Filifera

Family Stylasteridae (hydrocorals, lace corals)

Class Anthozoa

Subclass Alcyonaria (=Octocorallia) (octacorals)

Order Alcyonacea

Family Clavulariidae

Family Alcyoniidae

Family Nephtheidae

Order Gorgonacea \* (gorgonians)

Family Anthothelidae

Family Paragorgiidae (bubblegum corals)

Family Coralliidae (precious corals)

Family Keroeididae

Family Acanthogorgiidae

Family Plexauridae

Family Gorgoniidae

Family Ellisellidae

Family Dendrobrachiidae

Family Chrysogorgiidae (golden corals)

Family Primnoidae

Family Isididae (bamboo corals)

Order Pennatulacea (sea pens)

Family Veretillidae

Family Echinoptilidae

Family Kophobelemnidae

Family Anthoptilidae

Family Funiculinidae

Family Protoptilidae

Family Stachyptilidae

Family Scleroptilidae

Family Chunellidae

Family Umbellulidae

Family Halipteridae

Family Virgulariidae

Family Pennatulidae

## Appendix 1. Classification of the Deep-sea Corals (cont.)

- Subclass Zoantharia (=Hexacorallia)
  - Order Zoanthidea (zoanthids)
    - Family Parazoanthidae e.g., *Gerardia* (gold corals)
  - Order Antipatharia (black corals, wire corals)
    - Family Antipathidae
    - Family Cladopathidae
    - Family Schizopathidae
    - Family Leiopathidae
    - Family Myriopathidae
    - Family Aphanopathidae
  - Order Scleractinia (stony corals)
    - Family Caryophylliidae e.g., *Lophelia*
    - Family Dendrophylliidae e.g., *Enallopsammia*
    - Family Flabellidae e.g., *Flabellum*
    - Family Turbinoliidae e.g., *Sphenotrochus*
    - Family Rhizangiidae e.g., *Astrangia*
    - Family Fungiacyathidae e.g., *Fungiacyathus*
    - Family Oculinidae e.g., *Madrepora*
    - Family Micrabaciidae e.g., *Stephanophyllia*
    - Family Pocilloporidae e.g., *Madracis*
    - Family Guyniidae e.g., *Guynia*
    - Family Anthemiphylliidae e.g., *Anthemiphyllia*
    - Family Gardineriidae e.g., *Gardineria*

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\*Not all Taxonomists recognize the Order Gorgonacea, some believe there is a continuous variation from the Alcyonacea through the Gorgonacea

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## Appendix 3. Deep-sea corals online

### NOAA Coral Reef Information System

<http://www.coris.noaa.gov/about/deep/deep.html>

This portal favors Atlantic scleractinia, with links to myriad other online documents describing deep ahermatypic reefs. It includes lovely images, and a dense bibliography on *Lophelia sp.* and *Oculina sp.*

### Octocoral Research Center at the California Academy of Sciences

[http://www.calacademy.org/research/izg/orc\\_home.html](http://www.calacademy.org/research/izg/orc_home.html)

This website created and maintained by Dr. Gary C. Williams at the California Academy of Sciences. The bibliography from Bayer is exhaustive, at ~50 pages in its original form. The list of genera is extensive, and includes many photographs.

### Report of the International Council for the Exploration of the Sea (ICES) Study Group on Deep-sea Corals. 2003.

<http://www.ices.dk/reports/ACE/2003/SGCOR03.pdf>

### UN Status Report on Cold-water Coral Reefs. 2004.

<http://www.unep-wcmc.org/press/cold-water-coral-reefs/>

### NOAA Office of Ocean Exploration Website

#### **Gulf of Alaska**

<http://www.oceanexplorer.noaa.gov/explorations/02alaska/welcome.html>

<http://www.oceanexplorer.noaa.gov/explorations/04alaska/welcome.html>

The 2002 and 2004 Gulf of Alaska Seamount Expeditions (GOASEX) visited a dozen seamounts in the Northeast Pacific Ocean with the Alvin submersible, collecting several excellent specimens. An 2002 article in the mission background and the July 7<sup>th</sup> log describes dispersal studies. The July 14<sup>th</sup> log details some problems with morphological classification in the Family Isididae. The 2004 August 16<sup>th</sup> log discusses octocoral sample processing, while the August 19<sup>th</sup> log discusses and exhibits living polyp photography. August 17<sup>th</sup> describes the “paradox of seamounts.”

#### **North Atlantic**

<http://www.oceanexplorer.noaa.gov/explorations/03mountains/welcome.html>

<http://www.oceanexplorer.noaa.gov/explorations/04mountains/welcome.html>

The 2003 and 2004 Mountains in the Sea Expeditions also made some fascinating finds, and they detail sophisticated methods in the 2003 July 15 Daily Log by Scott France. Visiting a seamount is a special event, but returning to a previously visited seamount is truly rare. Three seamounts, Manning, Kelvin, and Bear, had spectacular gardens of octocorals.

#### **Gulf of Mexico**

<http://www.oceanexplorer.noaa.gov/explorations/03mex/welcome.html>

An interdisciplinary scientific team spends 12 days exploring deep-sea coral habitats in the Northern Gulf of Mexico. Deep corals are widely distributed in a region heavily utilized by the oil and gas industry.

#### **Hawaiian Islands**

<http://oceanexplorer.noaa.gov/explorations/03nwhi/welcome.html>

The 2003 Expedition to the Northwestern Hawaiian Islands studied the reproductive biology and population genetics of precious corals in the Hawaiian archipelago. This site contains first ever footage of an endangered monk seal foraging in precious coral beds.





