Insights into the BIODIVERSITY, BEHAVIOR, AND BIOLUMINESCENCE OF DEEP-SEA ORGANISMS Using Molecular and Maritime Technology

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ABSTRACT. Since its founding, the Monterey Bay Aquarium Research Institute (MBARI) has pioneered unique capabilities for accessing the deep ocean and its inhabitants through focused peer relationships between scientists and engineers. This focus has enabled breakthroughs in our understanding of life in the sea, leading to fundamental advances in describing the biology and the ecology of open-ocean and deep-sea animals. David Packard's founding principle was the application of technological advances to studying the deep ocean, in part because he recognized the critical importance of this habitat in a global context. Among other fields, MBARI's science has benefited from applying novel methodologies in molecular biology and genetics, imaging systems, and in situ observations. These technologies have allowed MBARI's bioluminescence and biodiversity laboratory and worldwide collaborators to address centuries-old questions related to the biodiversity, behavior, and bio-optical properties of organisms living in the water column, from the surface into the deep sea. Many of the most interesting of these phenomena are in the midwater domain—the vast region of ocean between the sunlit surface waters and the deep seafloor.

INTRODUCTION

When the Monterey Bay Aquarium Research Institute (MBARI) was founded in 1987, the biodiversity of gelatinous deep-sea organisms was mainly known from trawl-based studies and a few pioneering dives with Woods Hole Oceanographic Institution's *Alvin* submersible and Harbor Branch Oceanographic Institution's *Johnson-Sea-Link* submersibles. International teams of taxonomists and ecologists were beginning to appreciate the undocumented species of comb jellies (ctenophores), siphonophores (a clade of hydrozoans), doliolids (sea squirt relatives), and other key players in water-column ecosystems that had otherwise not been known, despite centuries of sampling using more destructive methods. Specialists focused on these poorly known invertebrates, studying everything from basic taxonomy (e.g., Pugh, 1992; Pugh and Harbison, 1986; Madin and Harbison, 1978) to metabolic rates (Bailey et al., 1995) and bioluminescence (Herring et al., 1992; Widder et al., 1992; Haddock and Case, 1999). The number of dives and sampling opportunities that were devoted to water column research, though, were numbered in the dozens to low hundreds—sufficient for making opportunistic discoveries, but not ideal for experiments or long-term studies. Even today with MBARI's marine operations and the remotely operated vehicle (ROV) dives conducted by other nongovernmental organizations and governmental ocean exploration programs around the world (e.g., NOAA Ship *Okeanos Explorer*, Ocean Exploration Trust's E/V *Nautilus*, and Schmidt Ocean Institute's R/V *Falkor*; see *Oceanography* ocean exploration supplements at https://tos.org/ocean-exploration), the combined effort covers a minute fraction of the massive volume of deep-sea habitat (Figure 1).

At the time of MBARI's founding, molecular methods were in their relative infancy, with DNA amplification via polymerase chain reaction (PCR) not widely used, and DNA sequencing achieved only through time-consuming and labor-intensive methods. As an example, the gene for the photoprotein aequorin, used in the bioluminescence systems of some jellies, had just been cloned (Prasher et al., 1986), and the gene for green-fluorescent

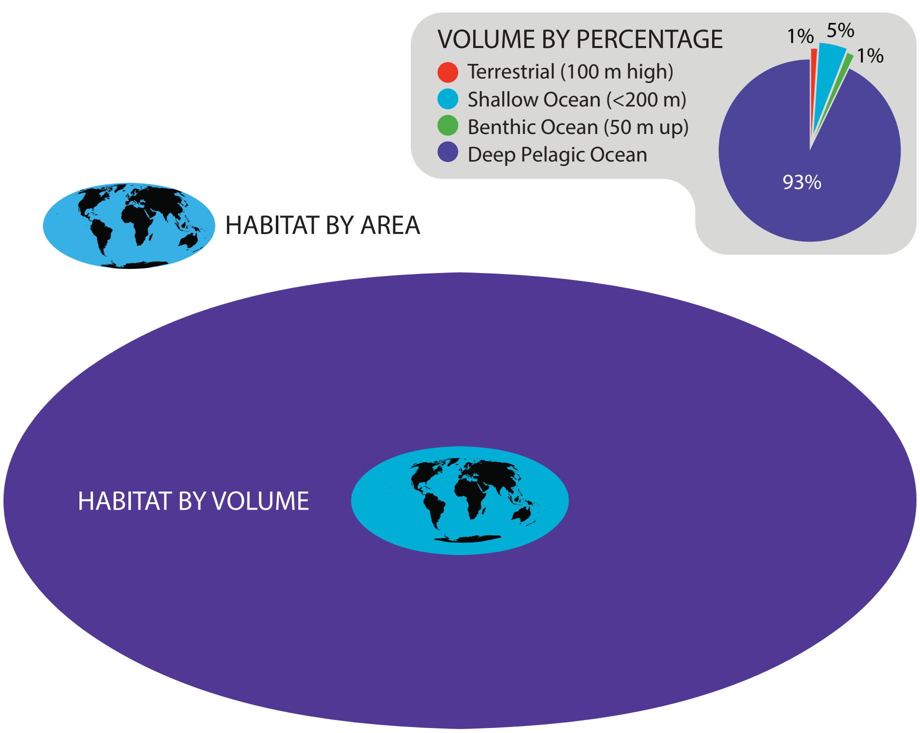


FIGURE 1. Perspective of the habitable space on the planet differs substantially whether *area* or *volume* is considered. By area, the ocean indeed covers about 70% of Earth's surface. Factoring in that the average depth of the ocean is 4,000 m, and considering that the living space over land is perhaps 100 m high, we find that 98% of the volume of the biosphere is seawater. Of that, the vast majority is the deep, dark ocean below 200 m. The field of deep-sea research has historically focused on organisms living in and on the seafloor—ignoring the kilometers of water that lie above. While unfamiliar to most, this deep water column is a source of nutrients and food for shallow-living species, and home to spectacular biodiversity, massive daily migrations, and processes controlling the cycling and sequestration of carbon. The study of midwater animals can provide unique insights into the evolutionary history of many animals, because most animals there have relatives in shallow water or the benthos. Despite its size and global importance, few institutions support research on the midwater environment.

BOX 1. EDUCATION AND OUTREACH IMPACTS

The public is often intrigued by observations and research related to the remote midwater and open-ocean environments. Anything we can do to make these habitats and organisms more approachable and part of the public's everyday life will have broad societal impacts. By shifting public perception of the deep sea from a frightening and desolate environment to one rich with fascinating diversity, we can instill an appreciation of how our fates are interlinked and inspire conservation of the ocean and life within.

One of the most direct and powerful ways to educate the public is by producing videos that combine our ROV footage with animations and scientific information. These videos reach hundreds of thousands or millions of viewers, and are widely used as content in classrooms spanning elementary schools to universities. Because many of the organisms and interactions are new to scientists, they are also novel to the general public, and appeal to our universal fascination with all things strange and wonderful.

MBARI also offers in-depth engagement with students, researchers, and teachers through many participatory programs and fellowships. Educational efforts include the summer internship program, the EARTH teacher professional development program, and the Monterey Bay Aquarium WATCH program at local high schools.

MBARI's sister institution, the Monterey Bay Aquarium, hosts nearly two million visitors per year, and MBARI research, knowledge, and images have been instrumental in the creation of several popular exhibits, including "Jellies: Living Art," "Mysteries of the Deep," "The Jellies Experience," and their LiveLink auditorium program. MBARI staff have also participated in the creation of exhibits at the Smithsonian Institution National Museum of Natural History, the American Museum of Natural History, the Exploratorium, and other international venues. The Monterey Bay Aquarium also has been involved in research collaborations that have resulted in unique studies of the origin of luciferin in jellies (Haddock et al., 2001) and the use of natural fluorescence as a lure for prey (Haddock and Dunn, 2015). Other research collaborations are with the staff of the Monterey Bay National Marine Sanctuary.

Another mechanism for dissemination of our ever-expanding knowledge of deep-ocean biodiversity is MBARI's Deep-Sea Guide (http://dsg.mbari.org; https://youtu.be/6-vpezVgD9o). This guide provides public access to the species, data, and concepts that have been identified, observed, and collected since MBARI was founded. More formally, the software to operate the Video Annotation and Reference System (VARS; http://mbari.org/vars) is also open source, so that others can use it.

Monterey Bay Aquarium Exhibits

MBARI VIDEO. There's No Such Thing as a Jellyfish » More than 300,000 views on YouTube

SpectorDance – Bioluminescence. *Photo credit: W. Roden/New Dawn Studios*

MBARI VIDEO. The Secret Life of Velella: Adrift with the By-the-Wind Sailor » More than 80,000 views on YouTube protein (GFP), which would ultimately revolutionize biotechnology and lead to the Nobel Prize in chemistry for its developers, had not yet been isolated (Prasher et al., 1992) from the jellyfish *Aequorea*. We now screen thousands of such genes for bio-optical properties, leading to leaps in biotech and evolutionary understanding, as described below.

MBARI's mission of applying technology to understand the deep sea led to the widespread use and development of ROVs that were customized and optimized for water-column exploration (detailed elsewhere in this issue by Robison et al., 2017). MBARI ranks among the preeminent institutions for deep pelagic and plankton research, and our efforts have inspired renewed interest in water-column biology. Using video cameras connected from the ROV to the surface ship by fiber optics, MBARI scientists and pilots are able to immerse themselves in the lives of organisms dwelling thousands of meters below the surface. With samplers originally designed for the *Johnson-Sea-Link* submersibles, we could bring organisms to the surface in pristine condition, suitable for morphological, molecular, physiological, biochemical, and even behavioral studies that had been previously unthinkable. Thus began three decades of research and discovery about the hidden lives and underappreciated diversity and ecological importance of deep water-column inhabitants.

ANNOTATION DATABASES

During early collection and documentation of midwater organisms from submersibles, there was no standardized procedure for quantifying the observations made during the dives, nor was there a central database for archiving the observations. As a result, most of these early records are essentially unavailable for further study. In contrast, and from the outset, MBARI established and has maintained protocols for annotating organisms seen from the ROV and entering them, along with associated oceanographic data, into a central database. This procedure and database have become the Video Annotation and Reference System (VARS; Schlining and Jacobsen Stout, 2006). The database has been a source of detailed data for hundreds of publications (Figure 2). VARS is built upon a foundation of relational databases (ROV dives, animal concepts like species within a taxonomic hierarchy, discrete observations, and oceanographic parameters such as temperature, salinity, and oxygen concentration). To facilitate the use of these databases, the bioluminescence and biodiversity research group has created Python and R scripts to retrieve and collate records spanning the history of MBARI undersea observations. For example, with a single command, we can retrieve all the instances of cephalopods consuming cnidarians (three instances), or make a histogram of the collection depths of all specimens of the ctenophore *Bathyctena chuni* (37), or retrieve the water-property data for the last 10 dives of a particular chief scientist. These computing tools are being used to rapidly generate the core data sets for publications spanning large temporal and spatial scales, including a summary of all the predator-prey interactions seen (Choy et al., 2017), and the spatial and phylogenetic distributions of the ability to emit light among water column animals (Martini and Haddock, 2017; see below.)

The up-front planning during the development of the databases does not mean that annotations have not changed over the years. For many species, as we learn more about them, we have become better able to identify them, leading to increased recognition as we continue to encounter them. To derive the full benefit of our thousands of hours underwater, we would have to re-annotate previous dives in light of current knowledge. Similarly, it is only within the last 15 years that we have paused during dives to collect and identify certain species, as different researchers with different interests have begun working with us (Figure 3). Improved video resolution has allowed better identification of taxa that we were not able to see as clearly before, and this will continue to improve as we transition to 4k video. The evolution of our annotation capabilities has resulted in a spurious increase in apparent abundance of previously unknown or unrecognized species over time in the database records, reflecting our ever-improving knowledge of the species we encounter. Some of the species we now see routinely, particularly among the ctenophores, have not been seen or collected since their original descriptions in the late 1800s or early 1900s (Figure 4).

Future work therefore includes improving our imaging capabilities, digitizing and selectively reevaluating portions of past dives that preceded our current taxonomic knowledge, and continuing to

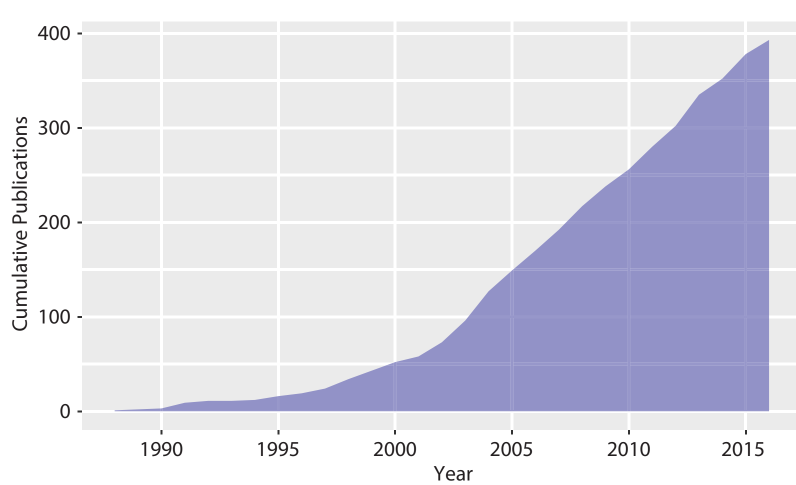


FIGURE 2. Cumulative number of publications employing the Video Annotation and Reference System (VARS) database over time.

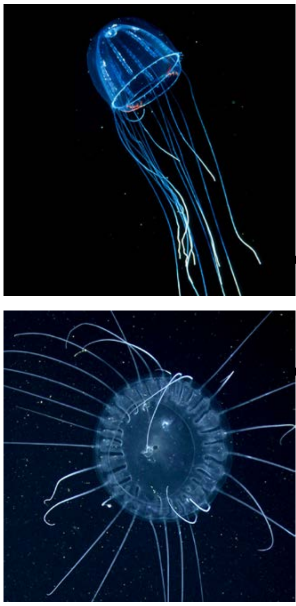
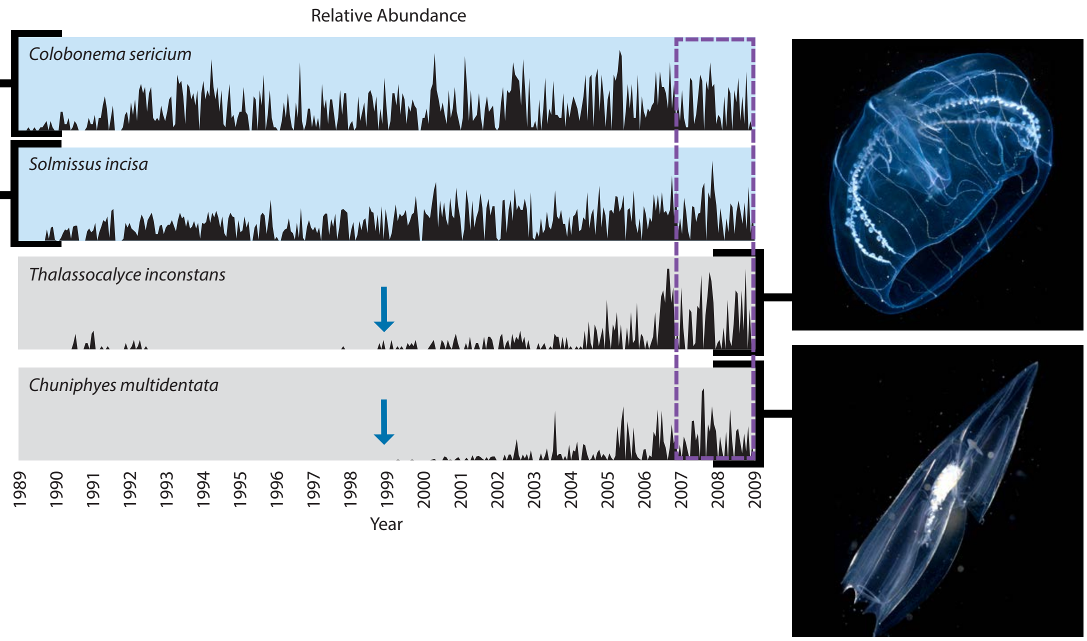


FIGURE 3. Some large and identifiable gelatinous species (top two rows, in blue) have been relatively well annotated since the very first Monterey Bay Aquarium Research Institute (MBARI) remotely operated vehicle (ROV) dives. Other species (gray panels) are more cryptic and were not collected and identified to any extent during MBARI's first decade. Once more-extensive collections (blue arrows) were initiated, they could be recognized in videos, and those species began to be quantified more accurately. In recent times (purple dashed box), we find that they are actually more numerous than their more conspicuous counterparts.



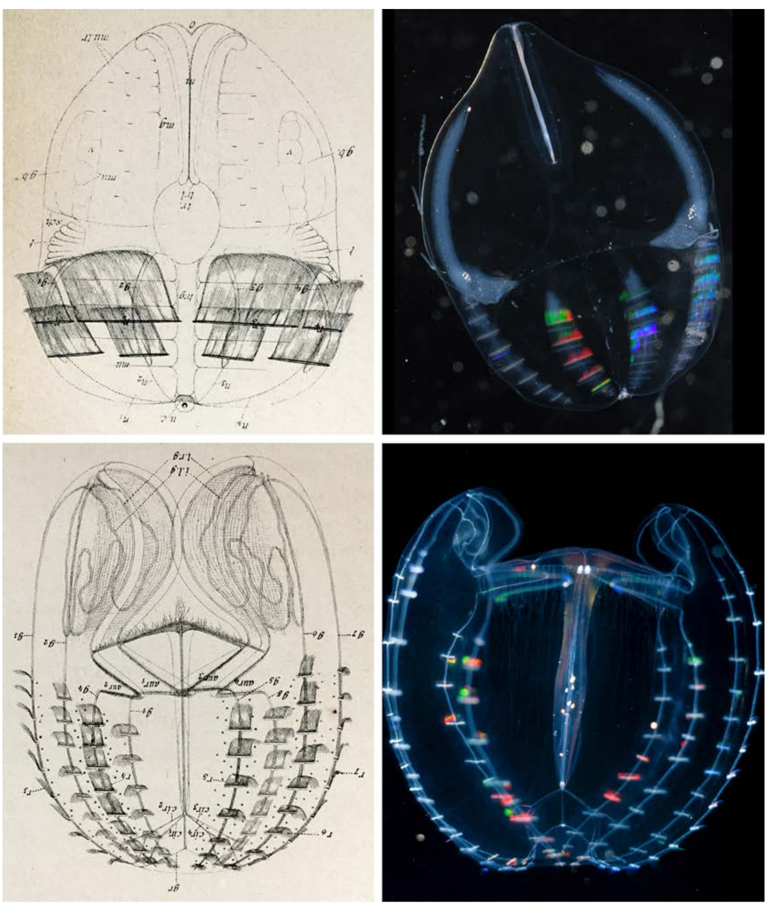


FIGURE 4. Species depicted by Chun (1880; left column) that are essentially absent in the literature, until our recent collections by ROV and blue-water scuba diving (photos at right). The nineteenth-century collections were often done by hand using a bucket and dip net, rather than large trawls towed behind a ship at relatively high speed. Top row: *Charistephane fugiens*; Bottom row: *Deiopea kaloktenota*.

perform integrative analyses of these observations. These meta-analyses can reveal patterns (temporal, vertical, geographic, carbon flow, and biomass) and correlations (predator-prey dynamics, niches, and reproductive patterns) within the diversity of midwater organisms.

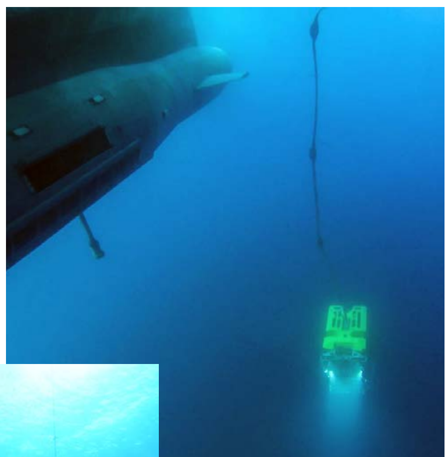
TAKING A MEASURE OF BIODIVERSITY

Many common organisms encountered in the water column are still new to science. This applies to both the deep ROV collections and shallow scuba-accessible depths (Figure 5). With the ROVs and the specialized use of blue-water scuba diving for scientific purposes (Haddock and Heine, 2005; Madin et al., 2013), we have been able to collect specimens and witness interactions that have long escaped scientific attention (Haddock, 2004). For example, about 40% of the species of ctenophores (comb jellies) that we routinely study have not yet been given scientific names, and much of this diversity is novel at the family level or even higher. Undescribed species also abound (some until just recently) among the deep-dwelling worms, amphipods, hydromedusae, and siphonophores (Dunn et al., 2005a; Haddock et al., 2005a; Osborn et al., 2009; Pugh and Haddock, 2010, 2016; Thuesen and Haddock, 2013; Gasca and Haddock, 2016). Even in the cases where we assign a provisional name to an observed organism, we often find that there are in fact several cryptic species involved (e.g., Osborn et al., 2011; Siebert et al., 2013). Application of molecular sequencing has allowed us to make progress in understanding this fine-scale diversity, the biogeographic distribution of deep-sea species, and the broad relationships between animal groups (Podar et al., 2001; Dunn et al., 2005b; Osborn, 2009; Hurt et al., 2013). The resultant "trees" depicting the relationships between organisms even include evolutionary hypotheses that challenge long-held notions of the origin of animal life (Dunn et al., 2008; Ryan et al., 2013), transforming our understanding of basic biology. Critically, these molecular data sets are grounded by morphological observations so that they can contribute to a reference database that will allow accurate assignment of community diversity through such methods as bulk sequencing of environmental DNA and metagenomics. Having genetic studies conducted in concert with taxonomic expertise is critical to the success of these fields. However, because so much of the genetic diversity is uncategorized, there are no shortcuts yet that allow a nonspecialist to "barcode" their way to an in-depth understanding of this ecosystem.

Midwater animals form a complex food web (Choy et al., 2017), and as with all networks, diversity is important for stability and ability to buffer changes. Without knowing the components and connections within this network, it is not possible to predict the outcome of any particular change. The biomass of the midwater is massive, and the connections are numerous, so a slight change in a community could lead to a large ripple effect with unanticipated consequences. This has been documented in enclosed and local systems, but it could be happening across far greater scales, and we would not notice because there is so little study of the biodiversity of the open ocean. Biodiversity is also important for all the lessons the animals present—from unique survival adaptations, to bio-inspired designs and materials, to natural products and biotechnological tools.

LIGHTS IN THE DEEP

A recent compilation of MBARI video and behavioral observations (Martini and Haddock, 2017) shows that 76% of the water-column organisms seen from the ROV, from the surface to 4,000 m depth, have the ability to make their own light—that is, to bioluminesce (Figure 6). For many deep-sea organisms, this remarkable capability was discovered for the first time by our labs when examining the live-caught specimens. For example, we have documented bioluminescence for the first time in polychaetes (*Swima,* Osborn et al., 2009; *Chaetopterus pugaporcinus,* Osborn et al., 2007; *Poeobius*, Haddock et al., 2010; Francis et al., 2016b), doliolids (*Paradoliopsis*,



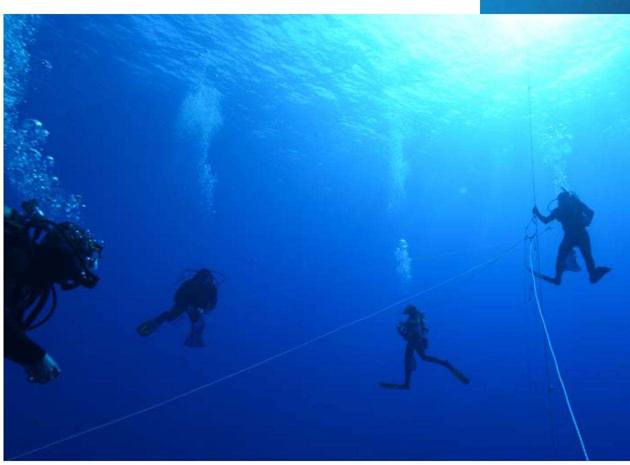


FIGURE 5. There are two main methods for observing and collecting from the open-ocean water column. In the upper 25 m, blue-water scuba diving (lower left photo) is used to observe organisms and collect them in jars by hand. Down to 4,000 m, ROVs maneuver and explore while connected to the surface for power, data, and control. The photo at upper right, by Randy Prickett and Andrew McKee, shows the ROV receding into the depths between the hulls of R/V *Western Flyer*.

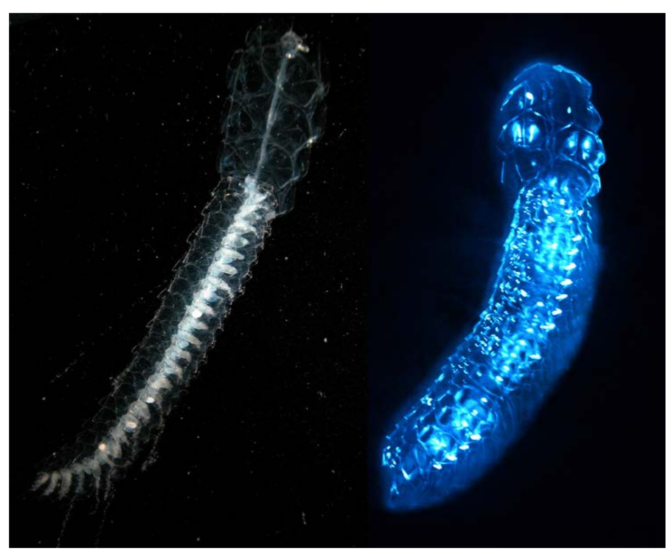


FIGURE 6. Many marine organisms, including the siphonophore *Frillagalma vityiazi* shown here, produce bright whole-body displays of bioluminescence that are thought to deter predators.

Pseudusa, *Doliolula*; Haddock et al., 2010), chaetognaths (*Caecosagitta macrocephala* and *Eukrohnia fowleri*, Haddock and Case, 1994; Thuesen et al., 2010), gastropods (two yet undescribed species), and sea anemones (Hormathiidae, Haddock et al., 2010; Johnsen et al., 2012). Bioluminescence was not known to occur in four of these taxonomic lineages before our observations. Among siphonophores, we have found five species of the genus *Erenna* (Figure 7) that use bioluminescent lures on their tentacles to attract fish (Haddock, et al., 2005b; Pugh and Haddock, 2016), and we have observed fluorescent lures in the siphonophores *Resomia ornicephala* and *Rhizophysa* and the hydromedusa *Olindias formosus* (Haddock and Dunn, 2015).

In the past, cameras with sufficient sensitivity produced only grainy black-and-white images, but now they can shoot "ultra-high-definition" 4k video and are small enough to be placed in a housing and taken to 4,000 m depth. Using customized systems mounted on the ROV, we have documented bioluminescent behaviors like "smoke screens" from fleeing fish and chaetognaths, along with other startling phenomena that are still being analyzed. This technology holds the promise of great advances in understanding the functions of luminescence, which remain one of the more challenging topics in deep-sea ecology. The emission of light is tailored to serve subtle functions, like finding prey, signaling to mates, or matching the exact color and intensity of downwelling light to provide an invisibility cloak (summarized in Haddock et al., 2010). For many species in the deep sea, interactions are rare and often sudden—a fish may eat only a few times per year, or encounter a potential mate once in a lifetime. Furthermore, the act of observing an organism almost always perturbs the environment. As imaging systems continue to improve and we become even more adept at using them on ROVs, we will be better able to use red light, for example, to lurk unseen while documenting the sporadic behavioral interactions that link members of the deep-sea community.

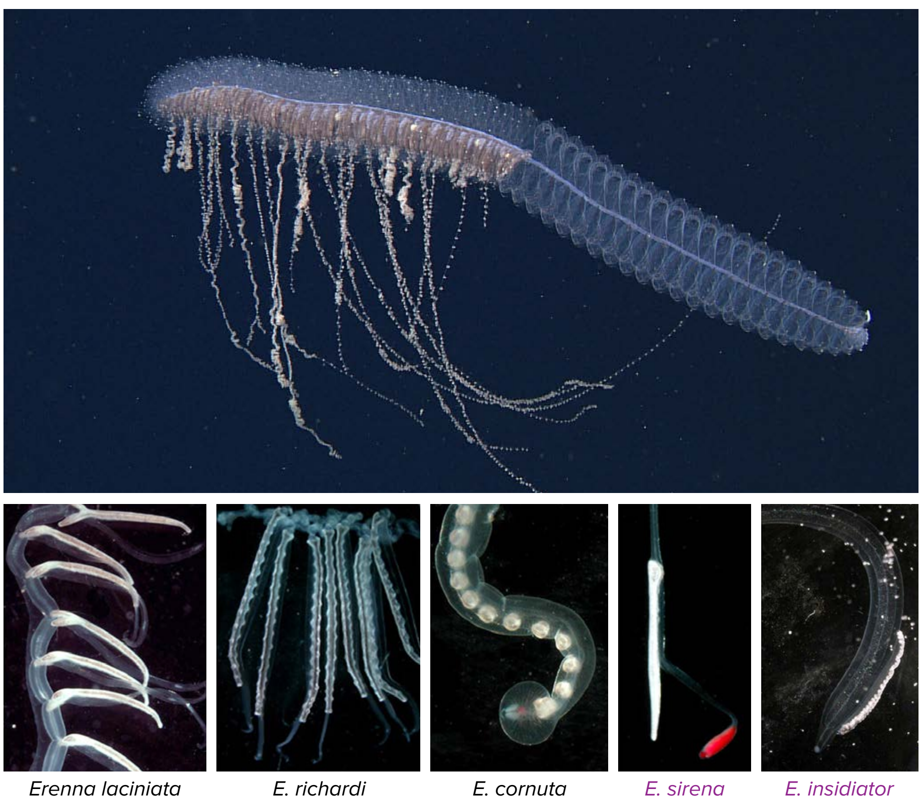


FIGURE 7. Deep-living siphonophores in the genus *Erenna* specialize in capturing fish. The top panel shows the whole animal of *Erenna laciniata*. The three known species (black labels) and two species recently described (purple labels at right) all use wriggling bioluminescent lures associated with the side branches of their tentacles. In fact, the morphology of these tentilla appears to have become specialized, perhaps for different prey, and it is the easiest way to distinguish the species (Haddock et al., 2005b; Pugh and Haddock, 2016).

AUTONOMOUS OCEANOGRAPHIC SENSORS

Instruments intended to measure the biology of the ocean automatically and at broad scales are somewhat limited. Fluorometers can detect the red fluorescence of chlorophyll, giving an idea of the amount of phytoplankton in surface waters, but they do not reveal the animals present. Towed imaging systems can photograph a range of plankton, but they require large ships, dedicated cruises, and laborious post-processing to classify the images obtained. Because bioluminescence occurs widely across diverse species, there is the potential to use luminescence as a proxy for zooplankton and dinoflagellates—one or two steps above the photosynthetic base of the food web that fluorometers sample. Since 2000, we have deployed bioluminescence sensors on autonomous underwater vehicles (AUVs) across ever-increasing ranges. Initially, a survey would be conducted on a dedicated cruise designed to study a particular process, with a surface ship tending the vehicle as it worked across a few kilometers of Monterey Bay. Now, the vehicles can be programmed to perform their missions routinely and return with a broad suite of data (Figure 8). Upcoming integration of bioluminescence sensors on long-range AUVs will allow continuous sampling of oceanographic bioluminescence for two weeks at a time, and longer-term observations are being conducted with light sensors deployed on the Monterey Accelerated Research System (MARS) underwater cabled observatory. These measurements provide a unique perspective on the composition of life in the sea, which is very different from the picture painted by chlorophyll fluorescence.

SURPRISING BEHAVIORS

Direct observations of organisms in their natural habitats, albeit illuminated by bright lights, has enabled many discoveries about the behaviors and interactions of midwater organisms, highlighting the diverse ways their lives are linked. For example, several animals, including squids, ctenophores, polychaetes, and nemertean worms, have been found to brood their young or care for egg masses during early development (Seibel et al., 2005)—a key life-history strategy in an environment where predation pressure on the vulnerable brooding mother is relatively low and where producing advanced offspring capable of hunting in a relatively food-poor environment is critical. On a particularly memorable blue-water scuba dive, we encountered the giant egg mass of a Humboldt squid (*Dosidicus gigas*) and were able to swim inside it to sample the density of eggs within the truck-sized gelatinous matrix (Staaf et al., 2008). Blue-water diving has since resulted in sightings and descriptions of six more *Dosidicus* egg masses and the parasites that live on them (Birk et al., 2016).

We have also seen and catalogued a wide array of feeding interactions (Choy et al., 2017) and parasite-host interactions (Gasca and Haddock, 2004; Gasca et al., 2015a,b), many of which cannot be observed using other technologies or approaches. Analyses of these observations reveal a complex network of predator-prey interactions, including cannibalism, detritivory, and unexpected specializations: an undescribed species of comb jelly that preys specifically on one species of worm; a giant deep-sea octopus that attains its large size by eating jellyfish (Hoving and Haddock, 2017); and a group of fish-eating siphonophores that attract their rare prey by dangling glowing lures (Haddock et al., 2005b; Pugh and Haddock, 2016). These new insights help us better understand the flow and retention of carbon and which animals are keystones in the system.

MOLECULAR BIOLOGY AND BIOCHEMISTRY

In the 1970s, researchers studying the biochemical origins of bioluminescence would collect 50,000 jellyfish each summer just to purify 500 mg of a single protein (Shimomura, 1995). Today, ultra-high-performance liquid chromatography (UHPLC) allows isolation of proteins and other substances from small amounts of starting material; this led to the isolation and purification of the yellow fluorescent compound that may give the worm *Tomopteris* its unique bioluminescence spectrum (Figure 9; Francis et al., 2014). These isolates can be used to locate the associated gene, which can then be inserted into bacteria to fabricate large amounts of protein for characterization. We can even mutate the gene to establish causal relationships between protein structure and function. Thanks to such molecular techniques, in-depth analysis of thousands of proteins of interest is now possible using a single specimen collected from the deep sea.

Advances in gene sequencing now make it possible to rapidly sequence the entire complement of mRNA—the transcriptome that encodes the proteins an organism is using at a given time. So far, our deep-sea transcriptomes have been put to a variety of uses: we have built "supertrees" encompassing hundreds or thousands of genes, instead of just one, to clarify the relationships of organisms (Dunn et al., 2008; Ryan et al., 2013). We have mined them to find and express the genes associated with bioluminescence in squids (Francis et al., 2017) and

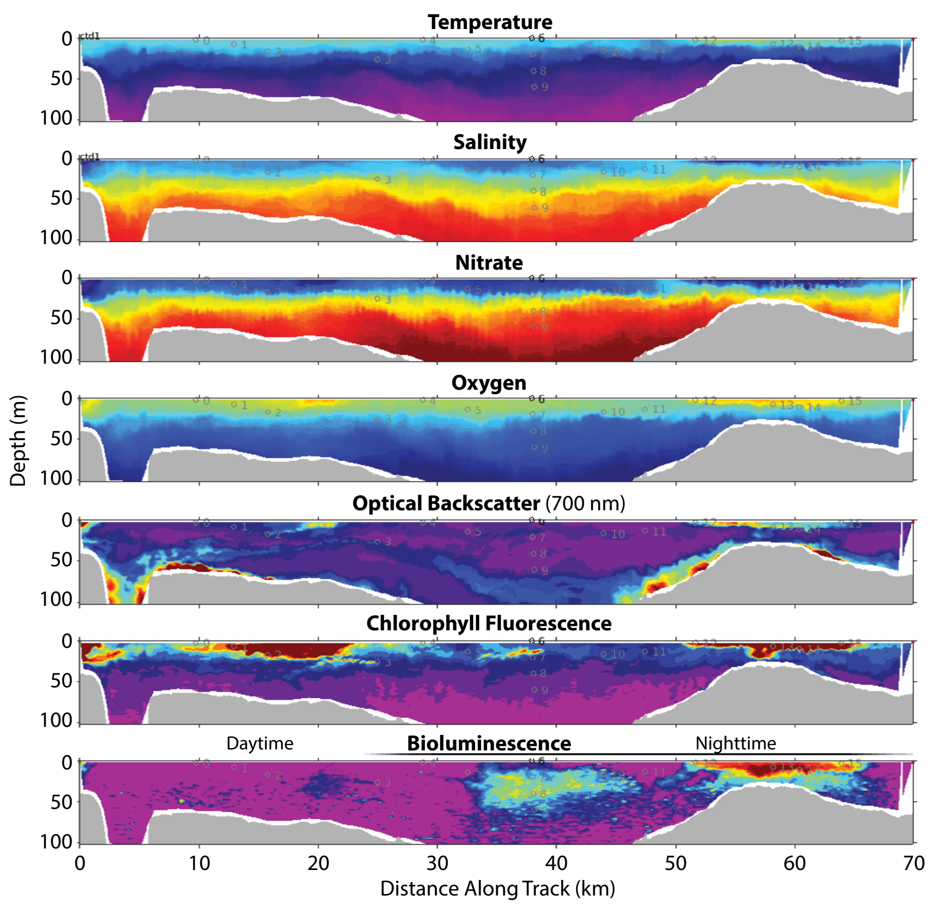


FIGURE 8. Autonomous underwater vehicles fitted with a sensor suite that includes bioluminescence (bottom panel) can capture a thorough picture of ocean chemistry and biological indicators. In this 70 km transect, the pattern of fluorescence (second pane up) correlates with bioluminescence along part of the track (right side), indicating abundant photosynthetic dinoflagellates. In contrast, the two signals diverge at the middle of the transect, with bioluminescent zooplankton forming a layer below the surface layer of phytoplankton.

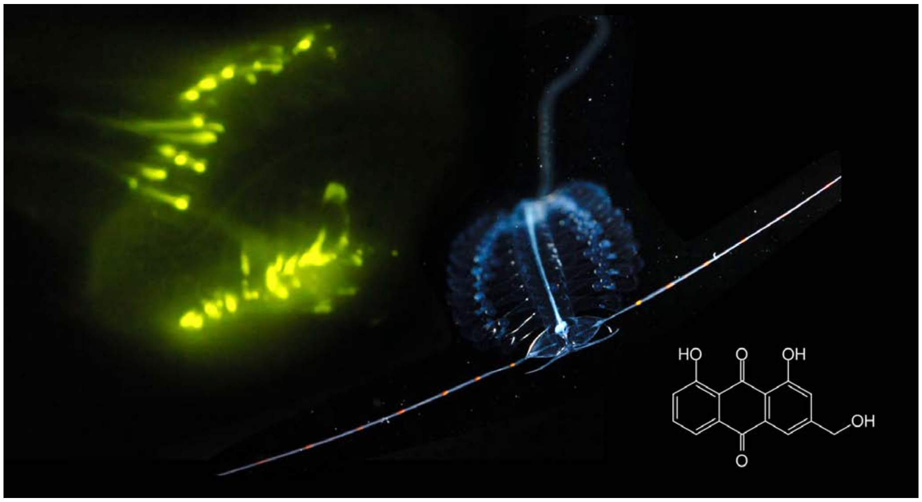


FIGURE 9. Free-swimming polychaetes of the genus *Tomopteris* are some of the very few marine organisms that produce yellow light (Haddock et al., 2010; Francis et al., 2016b). The chemistry of the light-emitter (luciferin) in this system is as yet unknown, but the chemical composition of the yellow fluorescent compound (not necessarily luminescent) has been determined in the worms (Francis et al., 2014). This molecule is likely involved in the luminescence reaction and could ultimately provide a novel biotechnological tool.

ctenophores (Powers et al., 2013; Francis et al., 2015, 2016a). They are now being queried to identify and isolate the genes involved in metabolism for deep-sea species in order to study how they are adapted to extreme pressures (DEEPC project; http://deepc.org). These data sets are a trove of valuable information that will continue to provide a rich resource for study far into the future.

SUMMARY

For the scientists in the bioluminescence and biodiversity research laboratory and their collaborators, MBARI has been a source of unparalleled opportunity. The application of the latest in molecular, maritime, imaging, biochemical, and computational technology allows new insights into outstanding questions of life in the deep sea. We have made great strides in understanding the diversity, relationships, ecology, genetics, and bio-optics of numerous fascinating and ecologically important organisms. Perhaps more importantly, these results are all woven into the same tapestry, which is beginning to provide a synthesized view of life in the deep. The insights and understanding we are gaining and opportunities we are sharing bring the distant, vast midwater into an ever-increasing public and scientific consciousness, building appreciation and fostering engagement in its protection. Many more mysteries remain to be explored as we continue to follow our founding principles by developing and applying new methods to life in Earth's most vast and perplexing habitat.

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