2 Microbial Ecology at Sea: Sampling, Subsampling and Incubation Considerations

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GENERAL INTRODUCTION

The marine environment is the largest contiguous habitat on Earth; however, it is far from being homogeneous. Relevant ecological time and space scales span more than nine orders of magnitude (Figure 2.1; Dickey, 1991), thereby contributing to the challenge of adequate and representative sampling of the marine environment.

Many distinct marine ecosystems and their microbial assemblages have been identified and studied, ranging from ice-swept polar seas to deep-sea hydrothermal vents. The diversity of microbial habitats is even greater than already implied, especially considering the fact that microorganisms live in microenvironments that are defined on space scales of millimeters or less. As a result, the environments sensed by individual microbes may be quite different from the surrounding bulk fluid. Furthermore, many microbes live in truly protected habitats such as the enteric tracks of larger metazoan organisms; sampling these microbes will require fundamentally different methods than those used to target 'exposed' microbial assemblages. In fact, the spectrum of oceanic habitats and the diversity of the associated microbial assemblages is so extreme that any broad generalizations regarding sampling, subsampling and

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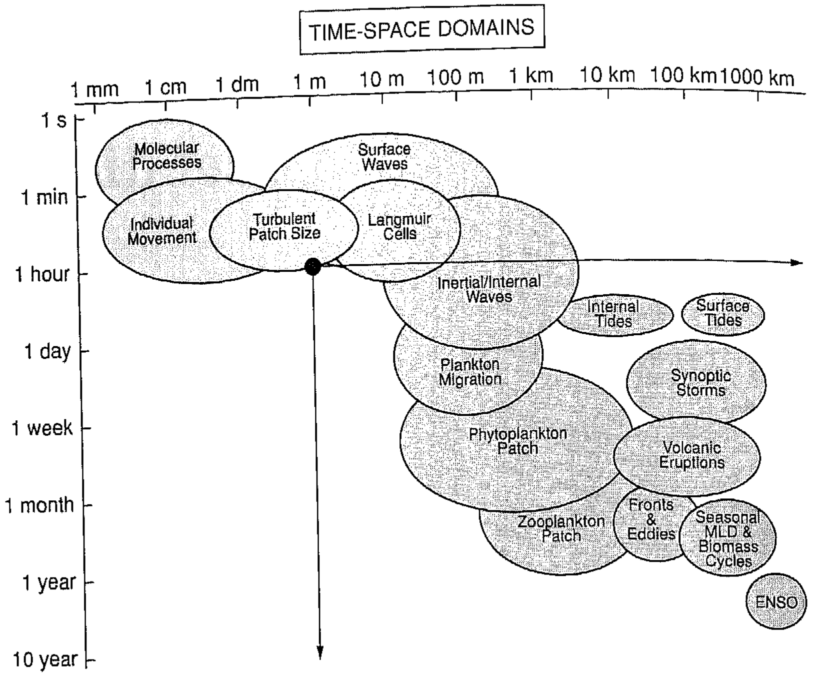
TIME-SPACE DOMAINS
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1 week
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1 year
10 year
Molecular Processes
Surface Waves
Individual Movement
Turbulent Patch Size
Langmuir Cells
Inertial/Internal Waves
Internal Tides
Surface Tides
Plankton Migration
Synoptic Storms
Phytoplankton Patch
Volcanic Eruptions
Zooplankton Patch
Fronts & Eddies
Seasonal MLD & Biomass Cycles
ENSO

Figure 2.1. Time and space scales of variability for physical and physiological processes that are relevant to marine microorganisms. The region beneath and to the right of the two arrows defines the time-space domain typically investigated by ship-based sampling programs. (From Dickey, 1991.)

measurement protocols must be carefully reviewed before application to a selected study site.

Biogeochemical cycles of carbon (C), nitrogen (N) and phosphorus (P) in the sea are ultimately driven by solar energy and a continuous supply of growth nutrients. This results in a steep gradient in potential energy in the upper 0–100 m of the water column (the so-called euphotic zone, where net photoautotrophic fixation of carbon dioxide occurs), and a vertical segregation between net autotrophic and net heterotrophic microbial processes. Significant latitudinal variations in solar radiation, as well as vertical and coastal to open ocean horizontal gradients in nutrient concentrations are also present. Furthermore, within a selected habitat there are potentially significant diel, intra- and inter-seasonal and interannual variations in microbial processes, and it now appears almost certain that decade-scale and longer climate forcing of the marine environment impacts the resident microbial communities (Tont, 1976; Venrick *et al.*, 1987; Karl, 1999).

Microbiologists now recognize three major lines of evolution: *Bacteria*, *Archaea* and *Eucarya* (Woese, 1994). In the sea, these three domains have overlapping size spectra, physiological characteristics, metabolic strategies and ecological function. Consequently it is difficult to separate these groups except by use of novel molecular biological techniques that are only now being introduced into the field of microbiological oceanography.

Microorganisms, especially *Bacteria* and *Archaea* are ubiquitous in the marine environment and are truly the 'unseen majority': it has recently been estimated that there are more than 10^{29} microbes in the world ocean (Whitman *et al.*, 1998). In addition to this sheer number, global ocean microbial biomass is also substantial and accounts for $0.6–1.9 \times 10^{15}$ g C (Karl and Dobbs, 1998). Approximately 75% of the total microbial biomass occurs in open ocean habitats with roughly half of that biomass distributed in the upper 0–100 m of the water column and the remainder in the deeper portions (>100 m) of the sea. With an average ocean depth of about 4000 m, this means that the concentration of microorganisms decreases substantially with increasing water depth.

Although microbiologists have applied laboratory-based pure culture techniques to marine isolates for over 100 years, we are still lacking a comprehensive view of the ecology of microorganisms in the sea. The subliminal fear that the laboratory-based models were fundamentally different from the native populations now seems likely (Giovannoni *et al.*, 1990). It was not until 1988 that the most abundant photoautotroph in the sea, *Prochlorococcus marinus*, was discovered and isolated (Chisholm *et al.*, 1988, 1992). Even more recently, abundant marine planktonic *Archaea* have been observed (Fuhrman *et al.*, 1992; DeLong *et al.*, 1994), but not yet cultured. DeLong *et al.* (1999) and Karner *et al.* (2000) have reported that the *Archaea*:*Bacteria* ratio approaches unity in deep waters (>500 m) of the north Pacific Ocean thereby documenting a large biomass of *Archaea* that until a few years ago were not even suspected to be present in 'normal' marine habitats. The most abundant planktonic bacterial and archaeal species in the sea have not yet been isolated, so their physiological characteristics and, therefore, ecological niches remain largely unknown.

Why sample at all? There are at least two basic objectives in marine microbiology: (1) to isolate specific microorganisms or genotypes for subsequent study; and (2) to provide quantitative information on the distribution, abundance or metabolic activities of the resident microbial assemblages. The three most fundamental microbiological properties of a given ecosystem, community structure, total standing stock of living microorganisms (also known as total microbial biomass) and rates of metabolism or growth, are still far from routine measurements. These are the master variables in the sea of microbes. Consequently if one is interested in 'marine microorganisms' it is clear that the research question or hypothesis under investigation will dictate the types of samples that are collected, the sampling frequency in time and in space, and the selection of methodologies that are to be employed. This chapter will provide a few general guidelines on sampling, subsampling and other relevant field experimental design criteria.

HOW TO SAMPLE, AND WHERE

Principle

Sampling is one of the most important, but often overlooked, aspects of oceanography. Because of the ease with which seawater or sediment is obtained, it is tacitly assumed that sampling is a straightforward and simple procedure. However, in our view, the task of obtaining an intact, representative sample of the marine habitat under investigation is the most significant challenge in microbiological oceanography.

It is now well established that the pelagic realm of the world's ocean consists of readily identifiable habitats or biogeographic provinces (McGowan, 1974). These regions coincide with major hydrographical features, and can even be surveyed from space using color-sensing satellites (Platt and Sathyendranath, 1999). Accurate and precise descriptions of spatial and temporal patterns of microorganisms in the sea are fundamental parameters for marine microbial ecology. These objectives demand a rigorous and well-designed sampling program and appropriate methods of sample collection. A sample is intended to be just that, a representative subset of the population under investigation. Questions of time and space scale of variability of the population or habitat 'unit', sampling and measurement accuracy and precision and other relevant issues need to be considered before the experiment begins, perhaps using a 'pilot study' approach (Andrew and Mapstone, 1987).

Some microbes of interest are large enough to be captured in nets (e.g. colonial or aggregated microorganisms like *Trichodesmium* or *Rhizosolenia*, and microorganisms associated with zooplankton) or in particle interceptor traps (e.g. microbes associated with rapidly sinking particulate matter). If plankton nets are used, it is up to the investigator to decide whether to tow the net at a single reference depth or over a specified depth range (i.e. a horizontal tow sampling design) or obliquely through a pre-selected depth stratum. For quantitative estimates, it is also crucial to measure the volume of water passed through the net. The larger the target organisms, in general, the fewer the number per unit water volume, and the more heterogeneous the distribution. Nets that can be opened and closed on command are preferred, and multiple opening and closing plankton samplers are ideal (Bé, 1962). However, most microorganisms are unevenly dispersed in the bulk fluid habitat, and are collected using any one of a variety of water sampling devices.

When designing an ecologically-based field program, care must also be given to the uncompromised collections of complementary data including dissolved substrates, dissolved gases, particulate matter and other parameters. In addition to the availability of numerous sampling devices, some of which will be described below, there is also a variety of potential sampling platforms, including boats and research vessels, towed vehicles and towed undulating vehicles, submersibles, remotely operated vehicles, autonomous underwater vehicles, moorings, drifters and Earth-orbiting satellites. Each platform has its own unique capabilities and limitations. Integrated measurement systems including multiple sampling platforms and fast response chemical and microbiological sensors are likely to emerge as the method of choice in future investigations of microbial processes in the sea (Dickey, 1991).

The critical importance of adequate sampling of the ocean environment can be traced back to the early nineteenth century, when serious misconceptions about the deep-sea environment were presented. A respected naturalist, Edward Forbes (1815–1854), claimed to have proven that below approximately 600 m in the open sea there was a 'probable zero of life' zone (Schlee, 1973). From an observed decrease in the number of animal species with increasing water depth, he concluded that the ocean was 'azoic,' or devoid of all life at great depths. His azoic zone theory was not refuted until the 1860s when a deep-sea cable from 2000 m was raised for repairs and revealed the presence of encrusting organisms (Gross, 1972). Clearly there had been a serious 'sampling problem.'

As mentioned above, the ocean is not a single homogeneous ecosystem, so careful consideration must be given to sampling frequency (in time and space) and location. The scale of sampling relative to the scales of variability is important if one plans to extrapolate results to the ecosystem level (Levin, 1992). In most field studies, usually due to practical considerations, the actual number of total samples collected is regrettably small, and it is often impossible to obtain truly replicate samples. If statistical methods are employed, it must be assumed that the microbial populations follow a known probability distribution (e.g. Poisson, negative binomial or log-normal). However, microoganisms generally exist in localized patches and are rarely, if ever, found in random or uniform distributions over the spatial scales used in most ecological investigations (Karl, 1982).

The investigator should be aware of at least three separate areas where variability can be introduced into field measurements: replication at the level of sampling (i.e. multiple water samples collected from a common depth); replication at the level of subsampling (multiple subsamples from a single sample); and analytical replication (i.e. multiple analyses of a single sample extract). Because of the heterogeneous distribution of microbial communities in nature, and problems that are inherent in the collection of particulate matter from aquatic environments, variance between sampling bottles is generally the largest source of error. Therefore, replication is most meaningful when performed at the highest level, i.e. multiple samples of water from a given environment (Kirchman *et al.*, 1982). It has also been demonstrated that the overall variance and the precision with which the sample variance can be estimated are functions of the procedure used to subsample the initial sample collection (Venrick, 1971).

The need for a device that is capable of aseptically collecting a sample of seawater was recognized more than 100 years ago as the field of marine microbiology emerged as a subdiscipline of oceanography. ZoBell (1941) provides a thorough historical account of the significant events during the period 1892–1940, beginning with the pioneering work of H. Russell and W. Johnston. Over the years, a variety of aseptic water samplers have been devised, deployed and re-evaluated. There are two basic approaches used in their design: (1) a device using a capillary tube inlet that is deployed in a sealed, sterile configuration, opened at depth and recovered without closure and (2) the use of a mechanical device for the removal and subsequent replacement of a stopper or similar closure mechanism (Lewis *et al.*, 1963).

In 1941, ZoBell introduced the Johnson–ZoBell (J–Z) bacteriological sampler (which of course could also be used to sample *Archaea* and

Eucarya). It consisted of a sterile, evacuated glass bottle, fixed stopper and glass and rubber tubing leading to a terminal sealed glass tube. The entire apparatus could be autoclaved at sea, then mated to a brass frame and attached to a hydrowire. A brass weight, also called a messenger, designed to travel down the same hydrowire on command, mechanically activated a lever that broke the glass tube; a water sample was aspirated into the sterile, evacuated bottle. The glass bottle could be replaced by an evacuated, compressible rubber bulb for greater depth capability (the glass bottles began to fail at about 200 m and by 600 m all bottles were crushed by the ambient hydrostatic pressure; ZoBell, 1941). A version of this modified J–Z sampler was also designed as a piggy-back microbiological sampler for use with the metallic Nansen bottle (Sieburth *et al.*, 1963) which was the most commonly used water collection device for many years (pre-1965). Most of the early water samplers had integral components that were constructed from alloys of copper, nickel, tin, zinc or lead, even though the bactericidal effects of certain metals was well known (Drew, 1914). The rubber bulbs used in the modified J–Z and piggy-back samplers were also shown to be toxic to certain microbes.

A substantially different sampler designed for the aseptic collection of seawater was introduced by S. Niskin and was termed the butterfly baggie sampler (Niskin, 1962). This bellows-type water sampler consisted of a spring-activated metal frame and a detachable 2 l sealed, sterile, disposable polyethylene baggie. On command, the messenger activated a non-sterile knife blade which cut the end seal of the inlet and released the torsion springs opening the bellows. This action created a suction and collected a water sample from any target depth in the ocean. A mechanical, spring-loaded system then resealed the inlet tube at the completion of the sampling routine to prevent water exchange during sample recovery. One reported problem with this sampler was the leakage of dissolved organic matter from the plastic bags (Sieburth, 1979), so even though the sample is collected aseptically it may not be uncompromised for certain ecological measurements.

Despite these developments, there was still concern expressed that these 'sterile' samplers might be compromised because of the requirement for *in situ* inlet tube activation by non-sterile procedures. Furthermore, the proximity of the intake of the sterile sampler to potentially contaminating non-sterile and metallic surfaces, including the hydrowire itself, raised suspicion regarding the reliability of these collections. For these reasons, Jannasch and Maddux (1967) developed a novel device consisting of a sterile syringe and glass sampling tube; the latter enclosed in a dialysis bag filled with sterile water. The apparatus is mounted on a movable arm that is attached to a frame and secured to the hydrowire (see Figures 2 and 3 in Jannasch and Maddux, 1967). A vane keeps the syringe oriented upcurrent to minimize potential contamination. When the messenger activates the sampler, the movable arm begins to fall away from the frame and its motion strips the protective dialysis bag away from the sterile glass sampling tube. At the same time, a cable attached to the syringe plunger begins to tighten and precisely when the arm is at its maximum distance from the frame assembly (approximately 75 cm) the seawater sample is aspirated. Field tests of this sampler showed a greater reduction in the recovery of contaminating bacteria (identifiable bacteria that were deliberately 'painted' onto the hydrowire for these tests) than was observed with the paired deployment of either a modified J–Z sampler or a sterile Niskin baggie sampler (Jannasch and Maddux, 1967). Ironically, this relatively simple and effective aseptic water sampler was never used extensively in subsequent field programs. This might have been due to the emergent views of that time that the need for aseptic samples, when a relatively clean and uncontaminated one would suffice, was not necessary (Sieburth, 1979).

Most of our conceptual views of the marine environment and, therefore, the basis for our sampling protocols focus on vertical profiles of oceanic parameters despite the fact that the marine environment is decidedly a 'horizontal' habitat (e.g. the horizontal-to-vertical scale of the North Pacific Ocean is >1000:1). In the open sea, this experimental protocol bisects a density-segregated water column with specific, readily identifiable layers called water masses. These components of the vertical profile vary considerably in their source and, most likely, in their chemical and microbiological properties. For example, a relative peak or minimum value for some selected parameter in a given vertical depth profile could be either an *in situ* or an advective feature. Clearly, an accurate resolution of these opposing mechanisms is desirable if not mandatory.

In the North Pacific subtropical gyre at Sta. ALOHA (A Long-term Oligotrophic Habitat Assessment), the subeuphotic zone water mass at approximately 500 m (North Pacific Intermediate Water) is formed in the NW Pacific near Japan, but the deep water mass (>3000 m) has its origin in the Southern Ocean. Mixing between water masses occurs primarily at their boundaries so it is crucial to understand and consider this vertical structure when designing a sampling program. If horizontal sampling is desired, one must be cognizant of the orientation of the plannned transect (zonal vs. meridional) and should anticipate changes in the water mass structure and positions as the source regions are approached. Because these water masses can be dated using transient chemical and radiochemical tracers, a well-designed transect can provide information on rates of change (e.g. O_2 consumption, net nutrient uptake or regeneration, net bacterial production) in this 'upstream–downstream' sample design. The use of rosette-mounted water bottles and a CTD-based environmental sensing system provides for the real-time detection of water mass structure.

In the open sea, there is a predictable vertical zonation of microorganisms including the following well-defined macro-habitats: (1) air–sea interface; (2) euphotic zone; (3) mesopelagic zone; (4) abyss; (5) water–sediment interface; and (6) sediment column. The latter topics, including both the water–sediment interface and deeper subsurface sediments will not be discussed in this chapter. While there is ample evidence to conclude that marine sediments, in both coastal and abyssal habitats, support an elevated concentration of microorganisms relative to the overlying seawaters, detailed ecological studies of microbial processes in these important habitats are severely methods- (both sampling and analysis) limited. For these reasons we will focus on the water column.

The air–sea interface, defined as the upper 150–1000 μm of the seawater, is a unique habitat characterized by high surface tension, high light (especially UV-B radiation), variable temperature, salinity, and turbulence. This specialized habitat also has generally elevated concentrations of dissolved organic matter, trace elements and microorganisms (Dietz *et al.*, 1976; Sieburth *et al.*, 1976; Carlson, 1982b; Williams *et al.*, 1986). Depending on the assumptions used for the thickness of the sea surface microlayer, the enrichment factors (i.e. the concentration in the microlayer compared to submicrolayer surface water) can be 10^2–10^3, or greater. Bubble scavenging of surface-active organic matter and microorganisms is probably one important mechanism for sustaining these enrichments (Bezdek and Carlucci, 1974; Blanchard and Syzdek, 1982). There is no doubt that the surface microlayer habitat and, presumably, its microbial inhabitants, are fundamentally different from the underlying euphotic zone. Because the skin of the ocean is so important for heat, momentum and mass exchange, including gas fluxes, this under-studied habitat may be very important in issues related to global environmental change (GESAMP #59, 1995).

The sea surface microlayer habitat is most likely composed of a series of overlapping zones that are difficult to sample quantitatively. Over the years a variety of instruments have been used, including: (1) the prism dip (Baier, 1972); (2) screen sampler (Garrett, 1965); (3) rotating ceramic drum (Harvey, 1966); (4) stainless steel tray (Hatcher and Parker, 1974); and (5) glass plate sampler (Harvey and Burzell, 1972). The efficacy of these methods has been evaluated in the laboratory (Hatcher and Parker, 1974; Van Vleet and Williams, 1980) as well as under field conditions (Carlson, 1982a). A mobile platform for studying the sea-surface film has also been described (Williams *et al.*, 1982).

The euphotic zone of the ocean is probably the most well-studied region with regard to microorganisms. Although vertically stratified, the seawater can be easily sampled and subsampled in time, using any one of a number of commercially available or homemade water collection devices. The water sampled with these devices includes dissolved constituents, viable planktonic microorganisms and some fraction of the total non-living particulate matter pool. There are at least two fundamentally distinct classes of particles in the sea: (1) particles that sink or rise and (2) particles that are approximately neutrally buoyant in the water column. Only those that are nearly neutrally buoyant can be sampled effectively with water bottles. The remainder of the particulate matter inventory must be collected using specialized devices such as sediment traps or large volume *in situ* pumps (Gardner, 1997).

Depending upon the experimental objectives, the sample volume can range from <1 ml to >30 l; the former to sample discrete microenvironments (DiMeo *et al.*, 1999), and the latter for routine sampling of dissolved and particulate matter. The most commonly deployed water sampler in contemporary microbiological oceanography is the Niskin® bottle (or its equivalent) which in its simplest configuration is a polyvinyl chloride (PVC) cylinder with end caps secured by an internal elastic cord or spring. This non-sterile sampler is usually deployed open, by attachment to the hydrowire, lowered to the target depth, then mechanically triggered to close by a messenger. Variations on this theme include more elaborate bottle designs that can pass through the sea surface microlayer prior to opening at depth by a pressure-activated switch, to external closure mechanism bottles designed to reduce the potential for chemical contamination. Of course, like any other sampling device, the 'devil is in the details,' and so it is with water sampling bottles, especially with regard to the aspect ratio and inherent flushing characteristics (Weiss, 1971). Concerns have also been expressed about the potential incomplete recovery of large particles due to the positioning of the drainage spigots (Gardner, 1977). To the extent that microorganisms are unevenly distributed between free-living and attached forms, this mechanical sorting and subsampling selection against large particles may be a significant source of sampling error.

Most modern oceanographic investigations deploy a carousel of bottles attached to a large 1–2 m diameter circular frame referred to as a rosette. Typically, these 12–24 bottle packages are activated from the surface vessel using an electrically-controlled device on the rosette called the pylon. An essential requirement for this type of water sample collection is a hydrowire with electrical conductors and usually an environmental sensing device, termed a conductivity–temperature–depth (CTD) instrument, to provide real-time information on water depth and other habitat characteristics. Modern CTD devices provide the option for including additional underwater environmental sensors for relevant ecological parameters such as sunlight, light absorption and scattering, fluorescence and dissolved oxygen. These real-time data are invaluable for positioning the water bottles in zones of greatest potential interest (e.g. fluorescence maximum, particle maximum, O_2 minimum). Rosette-assisted sampling of the water column also provides for multiple water bottle sampling at a single depth, as needed for statistical evaluation or for large volume demands. Both hydrowire and rosette-mounted PVC water bottles can be thoroughly cleaned with 1 M hydrochloric acid and rinsed with distilled water prior to use. To our knowledge, there are no readily available procedures for the truly aseptic collection of large volume (>5 l) samples using rosette-assisted protocols. Other ingenious devices such as the tidal-powered (Hayes *et al.*, 1980) and osmotic pressure-driven (Jannasch *et al.*, 1994) water samplers can be used for unattended time-series collections.

Sampling the marine environment at depths greater than approximately 2000 m (the abyssopelagic and bathypelagic zones) requires special considerations and, depending upon the expedition objectives, sophisticated sampling gear. The successful isolation of deep-sea bacterial isolates that are obligately barophilic (Yayanos *et al.*, 1979, 1981) has emphasized that pressure is an important determinant of microbiological zonation in the sea. Although some obligately barophilic bacteria may survive decompression, others may not. This implies that we may still have an incomplete understanding of microbial processes in the abyss. As Yayanos (1995) remarked, 'An ideal microbiological sample would remain in the dark, at the temperature and pressure of the deep sea, and mechanically and chemically undisturbed.' This is, unfortunately, generally not practical. While specialized pressure-retaining devices, including both water samplers and macroorganism traps, have been devised and deployed, the sample eventually needs to be decompressed for subsequent processing. To circumvent this problem, Jannasch *et al.* (1973, 1976) have developed a 1 l pressure- and temperature-retaining deep-sea water sampler that, in connection with a transfer unit for the addition and withdrawal of 13 ml subsamples, can be used as an incubation vessel aboard ship or in the laboratory. A modified version of the deep-sea sampler can concentrate the water sample several hundred-fold, by *in situ* filtration (Jannasch and Wirsen, 1977). A high-pressure chemostat for field and laboratory-based studies has also been described (Wirsen and Molyneaux, 1999). However, these prototype devices are not commercially available and therefore are not generally employed. Recently, a sterile, rosette-mounted high-pressure sampler capable of serial subsampling without decompression has been described (Bianchi *et al.*, 1999) and employed to evaluate the pressure effects of microbial assemblages from the NW Mediterranean Sea (Tholosan *et al.*, 1999).

◆◆◆◆◆◆ WHEN TO SAMPLE, AND HOW OFTEN

Principle

The ocean is an ephemeral ecosystem, both in terms of the physical habitat and the microbial assemblages. This is especially true when one considers the *in situ* generation times of most bacteria (<1 day) and their potentially rapid response to perturbation. Even the duration and frequency of the El Niño Southern Oscillation (ENSO) system, which itself exhibits a 3–5 year frequency of occurrence with well-documented effects on biogeochemical processes (Chavez *et al.*, 1999), varies substantially on the decadal scale (Trenberth and Hoar, 1997). The impact of these lower-frequency changes on ocean biogeochemistry remains largely unknown. The ENSO observing system in the equatorial Pacific Ocean, which includes moored and drifting buoys, a network of voluntary observing ship lines, and satellite surveys, is in place to observe the coupled physical–biogeochemical impacts of ENSO cycles.

Furthermore, variation in time can produce coherent variation in space (Bennett and Denman, 1989); consequently separation of temporal and spatial variability of a given study region may be artificial (Jumars, 1993). Persistent coastal or equatorial upwelling, and the presence of quasi-stationary open ocean frontal regions such as the N Pacific Subtropical Front and the Polar Front, are fairly well studied. Less well documented are the sources of mesoscale (10–100 km) variability in the pelagic habitat. Direct evidence for the role of mesoscale eddies as an important control on nutrient delivery and sustained microbial primary and secondary productivity, especially in chronically starved, low nutrient open ocean ecosystems, has recently appeared (Falkowski *et al.*, 1991; McGillicuddy *et al.*, 1998; Oschlies and Garçon, 1998). These stochastic, pulsed processes disrupt the ecosystem steady-state and can lead to the selection of fundamentally distinct microbial assemblages. Detection of these short-lived processes and integration of their effects into our conceptual models is only now starting to be achieved.

There are at least two aspects of oceanic habitat variability that are relevant here. First, in order to ensure that a given sample is representative of the ecosystem under investigation, it may be necessary to obtain multiple samples in time. In the open ocean there may be significant changes in heterotrophic bacterial processes that are coupled to the diel production of utilizable organic substrates by photoautotrophs. Likewise, in coastal habitats there may be a significant tidal influence on microbial processes that may be out of phase with diel periodicity. Probably all marine habitats, including the deep sea, experience intra- and inter-annual variations in the distribution, abundance and metabolic activities of the resident microbial assemblages. How many samples do we need to define this well-documented and fully expected temporal variability? There is, unfortunately, no simple answer to this question.

Because seasonal changes in many marine ecosystems are substantial, the annual changes that integrate them from year to year are best evaluated over periods of decades to centuries. However, comprehensive long-term (>10 year) time-series observations of marine ecological or biogeochemical processes are rare. The >50 year Hardy continuous plankton record from the North Sea and North Atlantic and the >50 year plankton observations collected off Mexico and the southwest US coast as part of the California Cooperative Ocean Fisheries Investigation (CalCOFI) program are notable exceptions. Both of these studies focused, ultimately, on pelagic fisheries and neither contained a comprehensive study of the microbial assemblages or their controlling growth substrates. Relationships between upwelling intensity and plankton production was one physical link that emerged.

In 1988, we began a systematic examination of microbial and biogeochemical processes in what was, at that time, considered to be a temporally stable habitat — the North Pacific Subtropical Gyre. After the first decade of approximately monthly research cruises it was concluded that this sampling frequency was too coarse in time to fully resolve even the most important physical–biological interactions (Karl, 1999). The greater the number of time periods and space scales that are involved, the greater the measurement intensity to achieve even a basic understanding. As Stommel (1963) cautioned, 'Where so much is known, we dare not proceed blindly — the risk of obtaining insignificant results is too great.' Undersampling is, unfortunately, a sobering fact of life in microbiological oceanography.

INCUBATION EXPERIMENTS AND RATE DETERMINATIONS

Principle

Many techniques currently employed in microbiological oceanography require incubation of a seawater sample for various periods of time. The underlying assumption of these methods is that the subsequent incubation conditions do not alter the *in situ* rates of metabolism or biosynthesis. This assumption is usually impossible to verify. Consequently it is imperative that the water sample is collected without chemical or environmental perturbation, and is subsequently incubated under conditions that duplicate those of the native habitat.

Most microbiologists have a greater appreciation and concern for an aseptic sampling technique than they do for a chemically-clean sampling technique. Both are equally important but rarely enforced in field studies. For example, the most commonly used water sampling devices such as PVC water bottles are neither sterile nor necessarily free from contaminating materials. Furthermore, even if all viable microorganisms associated with a particular sampling device are killed, there is no assurance that specific cell biomarkers (e.g. lipopolysaccharide, nucleic acids) have been eliminated. Likewise, although an ethanol rinse might be recommended as a method to sterilize a sampling device or a subsampling instrument, it could grossly contaminate the sample with dissolved organic matter and otherwise preclude any reliable post-sampling incubation procedures. Although an aseptic technique is probably not required for most routine field studies, to determine the presence or absence of a specific microorganism such a technique is imperative.

An equally important concern, especially for post-collection incubation measurements, is attention to a clean sampling technique. Metal samplers, toxic closure components or other potentially detrimental materials should be avoided. Carpenter and Lively (1980) and Fitzwater *et al.* (1982) have warned of point source contamination by toxic trace metals during water sampling and subsampling procedures. This potential problem is especially acute when sampling surface waters of the open ocean where trace element concentrations are very low. Likewise, butyl rubber, latex rubber and neoprene tubing and o-rings should also be avoided because they have been shown to be very toxic to marine microorganisms (Price *et al.*, 1986; Williams and Robertson, 1989). Only silicone materials appear to be acceptable and most chemically-clean samplers are not sterile. Depending upon the precise objectives of the particular study, one or both of these requirements can be ignored. However, while one should let common sense dictate, it is equally critical to know when and where point sources of microbial or chemical contamination are likely to occur.

In order to ensure that the rates measured during the post-collection incubation procedure are representative of those occurring in nature, several precautions must be taken. First and foremost, the initial sample must be collected with great care so as to minimize chemical and microbiological contamination. Furthermore, exposure of viable microorganisms to environmental conditions that are substantially different from those at the collection site should be avoided so as to minimize any deleterious effects ranging from short-term transitions in metabolism to death. For selected habitats this will be virtually impossible, as described above for samples derived from deep-sea habitats.

A major limitation in microbial ecology is the lack of absolute standards or certified reference materials that can be measured along with the incubated samples. This places the entire burden for accuracy on the investigator, so the sampling and incubation design then become even more critical. In this regard, multiple complementary and even redundant assays should be performed, preferably using independent sample collections and incubation protocols, so as to constrain the specific ecological processes under investigation.

The use of exogenous isotopic tracers has become a routine procedure for most field studies in marine microbiology. Often this is the only approach that is sensitive and specific enough to measure the sometimes low fluxes of carbon and other bioelements that occur in natural ecosystems. For example, the use of ^{14}C-bicarbonate as a tracer for carbon in marine photosynthesis (the so-called ^{14}C method; Steemann Nielsen (1956)) is probably the most widely used method in biological oceanography. Other exogenous isotope-based methods also exist for tracking heterotrophic microbial processes.

The details of selected individual methods are discussed elsewhere, however there are several general considerations regarding the use of stable and radioactive isotope tracers in studies of microbial ecology that merit attention. These include: (1) the overall reliability of the added element (or compound) as a tracer, including an evaluation of the site of labeling, its uniqueness and stability during cellular metabolism and biosynthesis; (2) isotope discrimination factors; (3) the partitioning of the added tracer with existing exogenous and internal pools of identical atoms, molecules, or compounds and the importance of measuring the specific activity of the incorporated tracer; and (4) the design and implementation of experimental procedures and proper kinetic analysis of the resulting data.

The use of isotopic tracers, especially radioisotopes, in ecological studies is often perceived as being straightforward and well documented. Consequently, a detailed working knowledge of the basic chemical, physical, statistical, and analytical principles on which these methods are founded is generally considered unnecessary. However, without such basic background information, it is possible to commit inadvertent errors in design or sample analysis that could result in gross misinterpretation of experimental data. In using commercially available isotopes, one relies to a large extent on the manufacturer's claims regarding the radiochemical, radioisotopic, and chemical purity of the product, the position and pattern of labeling, and the measured specific radioactivity. Foreign chemicals are sometimes added deliberately to labeled compounds in order to improve the chemical stability (e.g. antioxidants) or radiochemical stability (e.g. radical scavengers) or as bactericidal agents. Impurities may also arise during the chemical, enzymatic or *in vivo* microbial synthesis of the specific compound or as a result of chemical hydrolysis (especially during long-term storage) and radiolysis. For example, both organic ^{14}C (Williams *et al.*, 1972; Smith and Horner, 1981) and trace metals, including Mn, Zn, Cu, Ni, Pb and Fe (Fitzwater *et al.*, 1982), have been detected as contaminants in commercial preparations of [^{14}C]sodium bicarbonate used routinely for primary production estimation. The presence of these contaminants may grossly affect the reliability of the tracer for measuring the rate of photosynthesis in aquatic environments.

Another potential problem in tracer experiments results from the common use of ^{3}H-labeled organic molecules as auxiliary labels for carbon. The primary advantage of the ^{3}H-labeled compounds is their extremely high specific radioactivities, general availability and relatively low cost (per Bq). The former consideration is especially important for many ecological applications to avoid problems arising from organic nutrient perturbation resulting from the addition of the tracer (Azam and Holm-Hansen, 1973). The major disadvantage is the tendency of certain C–^{3}H bonds to exhibit exchange reactions with H in the solvent (generally H_2O). This exchange reaction may occur without any chemical change in the organic compound. Such labilization of tritium can occur due to chemical or enzymatic release during the intermediary metabolism of the tracer, during sample storage or during the extraction, purification and isolation of intermediate precursors or products.

A very important but often overlooked principle in the use of isotope tracers in marine ecological studies is the evaluation of the specific activity (or atom % enrichment) of the added, incorporated or metabolized element, molecule or compound. The ideal tracer is one that can be added without perturbing the steady-state concentration of the ecosystem as a whole. The supplier is generally the source for specific labeling information; however, errors of up to 500% in the quoted specific activities of tritiated nucleosides from one supplier have been reported (Prescott, 1970). A detailed discussion of numerous potential sources of error in the calculation of specific activity has been presented by Monks *et al.* (1971). In ecological studies, an accurate assessment of the specific activity is further complicated by the dilution of the added tracer with exogenous pools present in the environment and by endogenous pools present in living microbial cells. Without a reliable measurement of the extent of dilution prior to incorporation, tracer uptake data by themselves are of limited use in quantitative microbial ecology. Furthermore, isotope specific activities may change over the course of the labeling period due to the combined effects of depletion (uptake) of the added tracer or isotope dilution by a constant regeneration of the exogenous pools (assuming steady-state conditions). In fact, NH_4^+ (Blackburn, 1979; Caperon *et al.*, 1979) and HPO_4^{2-} (Harrison, 1983) regeneration rates have been estimated in environmental samples by measuring the extent of isotope dilution during short-term sample incubation periods.

The final point of concern regards the theoretical bases and mathematical formulations required for the proper interpretation of data arising from the use of isotopes. This topic has been summarized and explicitly discussed by Smith and Horner (1981), who are of the opinion that ecologists in general and marine biologists in particular are largely ignorant of the vast body of literature available regarding the proper use of stable and radioactive isotopes. Smith and Horner (1981) present several multicompartment models and discuss the assumptions, restrictions and advantages of each approach. This type of rigorous kinetic treatment of tracer data is only now becoming recognized as an essential component of the study of microbial processes in nature.

A final consideration is the incubation itself; what is the best method for obtaining reliable rate estimates? Unfortunately there is no simple answer, but there are some recommendations to consider. First and foremost, the incubation conditions should match, to the extent possible, the sampled habitat. This simulation should mimic *in situ* light, temperature, pressure and all related chemical conditions. If a sample is collected from a lighted habitat, the incubation should also provide a similar flux of photons, even if the desired measurement is not directly coupled to sunlight. This is especially critical for measurements in ecosystems with a rapid turnover and, therefore, tight coupling between photoautotrophic and heterotrophic processes. If heterotrophic bacterial production is measured in the dark, it may underestimate the true *in situ* rate by depriving the heterotrophs of contemporaneously produced organic substrates. When in doubt, light vs. dark replicate treatments should be performed to assess the potential impact of this effect.

In general, *in situ* incubation of samples is preferred to shipboard deck incubations. The *in situ* protocol ensures a match for temperature as well as light quality (wavelength) and quantity (Lohrenz *et al.*, 1992). Even when water samples are incubated under *in situ* or simulated (shipboard) *in situ* conditions there is a potential for metabolic perturbation during the initial sample collection process and subsequent pre-incubation handling. In order to circumvent the effects of light, temperature and pressure shock and in an attempt to obtain the most reliable estimation of the true *in situ* rate of primary production, Gundersen (1973) designed a clever device that he termed the *in situ* incubation sampler or ISIS (ISIS is also the Egyptian goddess of fertility!). The chemically-clean, non-metallic (but non-sterile) sampler is deployed on a hydrowire or synthetic rope to the depth of interest; multiple samplers can be deployed on a single wire. Each sampler has one opaque (PVC) and one transparent (polycarbonate) chamber. A sealed glass ampoule containing the ^{14}C-bicarbonate radioisotopic tracer is positioned in a special holder. When the sampler is activated by a messenger, the spring-loaded motion of the end-caps breaks the glass ampoule, thus inoculating the sample with the radioisotopic tracer and initiating the *in situ* incubation. The device remains in position for the duration of the pre-determined incubation period before it is recovered and processed. Dandonneau and Le Bouteiller (1992) have devised a 'clean,' *in situ* sample collection-incubation device which they termed 'let-go' because of its relatively simple deployment requirements.

When natural populations of aquatic microorganisms are contained in glass bottles for periods of approximately 24 h, the composition of the population and rates of metabolism can change drastically and elicit the so-called 'bottle effect' (ZoBell and Anderson, 1936; Venrick *et al.*, 1977). Bottle effects are totally unpredictable and vary considerably among taxa, location of sample and incubation conditions. In this regard, it would seem advantageous to keep incubations to a minimum duration that would still satisfy the prerequisites of the individual method (e.g. sensitivity in uptake, production or release of substance being measured, intracellular radiotracer precursor equilibration). Even during relatively short-term incubations (1–3 h), there is the possibility of a nonlinear time-course of metabolism as a result of confinement. One potential drawback with *in situ* experiments is the fact that they are generally end-point determinations and, therefore, lack information on changes that might occur during the incubation period. A recently devised sampler incubation device (SID) now provides the opportunity for an *in situ*, time-course incubation (Taylor and Doherty, 1990). Comparison of data collected with this method to standard end-point measurements have documented a serious, potential problem with prolonged end-point incubation experiments (Taylor and Doherty, 1990).

◆◆◆◆◆◆ ECOSYSTEM LEVEL EXPERIMENTS

It is often desirable to deliberately manipulate the nutrient, trace element or other metabolic status of natural microbial communities in order to study their physiological and ecological responses over both the short (minutes to hours) and long term (days to weeks). However, as Strickland (1967) lamented, 'the open sea is too big; the laboratory beaker is too small.' Research using large plastic enclosures in the open sea has demonstrated the merits of this experimental design especially for the study of nutrient dynamics, organic matter production and recycling and the response of the microbial assemblages to short-term perturbations (Kuiper, 1977; Menzel and Case, 1977; Steele, 1979; Davies, 1984). The enclosures, though not meant to duplicate the habitat under investigation, are analogous to controlled experimental plots used in agricultural research (Menzel and Steele, 1978). In selecting a specific experimental design it is important that the volume selected is large enough to contain all components of the microbial food web including predators up to a few millimeters in diameter. Trade-offs between size of the experimental unit and replication is an important design consideration. Carpenter *et al.* (1998) conclude that it may be more informative to increase the number of treatments rather than to replicate separate treatments. The key question of fundamental concern in the design of these experiments is: How do ecosystems respond non-randomly to physical, chemical or biological manipulation?

In a series of very influential papers, Schindler (1988, 1990) and Levine and Schindler (1992) compared the results from long-term ecological experiments performed in whole lakes with results from lake subsamples contained in smaller enclosures, ranging from liter-scale bottles to cubic-meter-scale mesocosms. Unfortunately, the results from these two sets of protocols oftentimes do not track each other. Schindler (1998) has recently reviewed the probable reasons for this ecological mismatch, including physical and biological shortcomings of the subsampled scales. The principal conclusion from these observations is that the whole ecosystem results must be correct, so this is the standard for subsample comparison.

While the major limitation in these whole lake experiments is the identification of an acceptable 'twin' for replication treatment, the ocean, because of its large scale, provides an opportunity for replication of a defined ecological unit. Nevertheless, 'whole ecosystem' studies, including manipulation experiments, are rare in the marine environment. The Controlled Ecosystem Pollution Experiment (CEPEX) employed 10 m × 3 m plastic enclosures as experimental marine 'plots' to study the response of marine communities, from bacteria to fish, to the addition of nutrients, trace metals and petroleum hydrocarbons (Menzel and Case, 1977). Recently, oceanographers have used unenclosed, ecosystem-level fertilization to test the hypothesis that primary productivity in selected high-nutrient, low-chlorophyll regions of the world ocean is Fe-limited (Martin *et al.*, 1994; Coale *et al.*, 1996). These perturbations typically lasted for a period of several weeks, at most, so long-lived changes including succession and negative/positive feedbacks were probably not well documented. Nevertheless this 'whole ecosystem' experimental approach is probably the most direct and most relevant for understanding ecosystem dynamics and for improving our prediction of the response of the ocean to natural and anthropogenic change (Carpenter *et al.*, 1995).

THE HOT PROGRAM PROTOCOLS: A CASE STUDY

Background

Since October 1988, scientists with the Hawaii Ocean Time-series program (HOT) have been evaluating the temporal variability of physical and biogeochemical processes in the subtropical North Pacific Ocean. Our primary sampling site is the deep-water Station ALOHA (A Long-term Oligotrophic Habitat Assessment; 22°45′ N, 155° W). This open ocean site lies approximately 100 km north of the island of Oahu, upwind of the Hawaiian island chain and over flat topography, with a water depth of 4750 m (Karl and Lukas, 1996). The waters around Station ALOHA are considered to be representative of the North Pacific subtropical gyre (NPSG), which is the largest circulation feature on Earth and our planet's largest contiguous biome (Karl, 1999). The water column of the NPSG hosts a vast (in area and depth), low-biomass, microbially-dominated ecosystem that exhibits changes on a variety of timescales. Meaningful sampling of this habitat requires a robust, interdisciplinary field program and modern oceanographic sampling methods.

Site selection

As discussed above, effective sampling of marine ecosystems requires careful selection of sampling locations. While spatial studies of microbiological oceanographic processes may rely on sampling transects or grids for adequate areal coverage, long-term time-series programs typically visit a specific site at regular intervals over an extended period of time. In this model the sampling is Eulerian rather than Lagrangian and this is clearly a critical and debatable sampling strategy. No single site is perfectly representative of the entire oceanic province under study, but the time-series researcher must select a site that is as representative as possible, while keeping in mind the considerable logistic constraints associated with field oceanography. In selecting Station ALOHA for our research, we maintained the absolute criteria that the station must be in deep water (>4000 m), upwind (NNE) of the Hawaiian island chain, and sufficiently far from land to be free from coastal ocean dynamics and terrestrial biogeochemical influences. Within these absolute requirements, we chose a site that would be close enough to the port of Honolulu to make regular (approximately monthly) visitation logistically and financially feasible. At the beginning of each cruise, we also visit the near-shore Station Kahe (21°20.6′ N, 158°16.4′ W) to collect comparative data and to test equipment before proceeding to the open-ocean site.

High-resolution depth profiles

HOT cruises are conducted approximately monthly and are typically 4 days in duration, with 60–72 h spent at Station ALOHA. This frequency of station occupation allows for some resolution of seasonal and interannual variability in ecosystem parameters. Data collected from moored instruments (see below) helps to fill in the gaps. During each occupation of Station ALOHA, repeated CTD hydrocasts to 1000 m are performed at approximately 3 h intervals for at least 36 h, in order to assess the physical characteristics of the water column. These data are used to produce a cruise-average density profile, which becomes useful for filtering out high-frequency fluctuations in the depth–density structure of the water column caused by tides and internal waves. The relative depths of biogeochemical features such as particle maxima and nutrient gradients can then be directly compared between cruises. At least one deep hydrocast per cruise is also carried out, to within a few meters of the bottom.

Discrete depth measurements

During each monthly station occupation, whole water samples are collected at discrete depths using a 24-place rosette with 12 l PVC bottles (see above). These bottles utilize teflon-coated stainless steel springs and silicone o-rings for closure to minimize sample contamination with leached chemicals. From these primary samples, subsamples are drawn for a suite of chemical and microbiological measurements (Table 2.1). The detailed protocols for processing, storage, and analysis of these subsamples are beyond the scope of this chapter, but are available online at the HOT program website (http://hahana.soest.hawaii.edu /hot/hot_jgofs.html). For most discrete water column measurements, we collect samples at fixed depths from surface to bottom, but the sample spacing is wider in the deep ocean and compacted in the euphotic and shallow aphotic zones (0–250 m). This uneven spacing reflects the decreasing distribution of microbial biomass and biogeochemical variability with depth. We collect

Table 2.1 Core parameters measured in the Hawaii Ocean Time-series program

Parameter	Depth or depth range (m)	Sensor or analytical procedure
Continuous profiles		
Depth (pressure)	0–4800	Pressure transducer on SeaBird CTD
Temperature (*in situ*)	0–4800	Thermistor on SeaBird CTD
Salinity (conductivity)	0–4800	Conductivity sensor on SeaBird CTD
Dissolved oxygen	0–4800	YSI polarographic sensor on SeaBird CTD
Fluorescence	0–1000	Sea-Tech fluorometer on SeaBird CTD
PAR and spectral irradiance	0–150	Biospherical Instruments, PRR-600
Natural fluorescence	0–150	Biospherical Instruments, PRR-600
Discrete water bottle samples		
Salinity	0–4800	Conductivity
Dissolved oxygen	0–4800	Automated Winkler titration
Dissolved inorganic carbon	0–4800	Coulometry
Alkalinity	0–4800	Automated Gran titration
Dissolved nitrate and nitrite	0–4800	Chemiluminescence and autoanalyzer
Soluble reactive P	0–4800	MAGIC, spectrophotometry and autoanalyzer
Soluble reactive Si	0–4800	Autoanalyzer
Dissolved organic C	0–1000	HTCO, IR detection
Dissolved organic N and P	0–1000	UV digestion, autoanalyzer
Particulate C and N	0–1000	High-temperature combustion, gas chromatography
Particulate P	0–1000	High-temperature ashing, spectrophotometry
Pigments, chlorophyll *a*	0–200	High-pressure liquid chromatography and fluorometry
Primary production	0–200	'Clean' ^{14}C *in situ* incubations
Bacteria and cyanobacteria	0–200	Flow cytometry
Respiration	0–200	Incubation, Winkler O$_2$ determination
Bacterial production	0–200	Incubation, ^{3}H-leucine uptake
Free-drifting sediment traps		
Particulate C, N and P	150	As above
Particulate calcium carbonate	150	Acidification, IR analysis of CO$_2$
Particulate biogenic silica	150	Alkaline digestion, spectrophotometry
Bottom-moored sequencing sediment traps		
Particulate C, N and P	1500,2800,4000	As above
Particulate calcium carbonate	1500,2800,4000	As above
Particulate biogenic silica	1500,2800,4000	As above
Net tows		
Meso- and macrozooplankton	0–150	C, N, mass, identification, gut pigments
HALE ALOHA mooring		
Meteorological measurements	Surface	Thermistor, anemeter, pyranometer, rain gauge
Dissolved gases	50	GTD (Gas Tension Device)
Nutrients	120,180	Osmoanalyzer
Optics	25	Spectral radiometer
Temperature	0–200	Thermistor

additional samples at the depths of specific hydrographic features, such as the shallow salinity maximum and the deep oxygen minimum. Our sampling depths below the euphotic zone typically coincide with discrete, physically and chemically defined water masses. Also, we collect 'samples of opportunity' from depth strata in which our real-time instruments reveal interesting or unusual phenomena.

Flux and rate measurements

The rate of primary photosynthetic production is a critical parameter for the determination of energy and carbon flows through oceanic ecosystems. We measure the depth profile of planktonic carbon assimilation on each HOT cruise, using ^{14}C tracer methodology. Because of the potential depression of production rates in incubated water samples due to the toxicity of contaminating trace metals, ultra-clean techniques are utilized for these measurements (Fitzwater *et al.*, 1982). A dedicated winch with a kevlar line is used to collect water samples at eight depths from 5 to 175 m, using special Go-Flo® sampling bottles. These bottles are teflon-coated, and are deployed in a closed position so as to prevent contamination from compounds concentrated within the surface microlayer. Subsamples are drawn into acid-cleaned polycarbonate bottles and spiked with ^{14}C-labeled bicarbonate, then attached to a free-floating array at the depths from which they were originally sampled. The Go-Flo® cast is conducted at night to avoid light shock to the organisms, and the array is deployed from the ship at dawn and recovered again at dusk. The incorporation of ^{14}C label into particulate matter during this *in situ* incubation serves as an estimate of the net photosynthetic fixation of carbon during the daylight hours.

The gravitational flux of particles from the euphotic zone is another key rate measurement carried out regularly at Station ALOHA. This sinking material is not only the primary export term for organic carbon and other bioelements, it is also the primary source of energy for the metabolism of mid-water and deep-sea communities. In order to quantitatively estimate this downward flux, we collect particles using a free-drifting sediment trap array, following the Multitrap design (Knauer *et al.*, 1979). The particle interceptor traps (PITs) are cylindrical polycarbonate tubes containing a high-density formalin-amended salt solution, which serves both to prevent wash-out and to preserve the collected particles. Twelve PITs at each depth are attached to the array line with a PVC cross-bar, and the array is left to float freely for 60–72 h. We routinely deploy these sediment traps near the base of the euphotic zone (150 m) to catch the euphotic zone export flux, and at other depths as desired for additional information about particle decomposition dynamics.

Plankton net tows

If we are to acquire a holistic understanding of microbial dynamics in the sea, we must include studies of microbial mortality and the passing of carbon and energy to higher trophic levels. In the HOT program, the community structure of meso- and macrozooplankton is examined through the routine collection of these larger organisms using towed plankton nets. This method of sampling is not only useful for collecting zooplankton grazers, but it is also effective for collecting the rare large (>64 μm) microalgal and cyanobacterial cells, and in particular the colonies and aggregates thereof.

Light and meteorology

Biological processes in the upper ocean are strongly affected by weather, for example, through the influence of wind on water column mixing, and through the influence of light on photosynthesis. Accordingly, we routinely collect data on incident solar irradiation and spectral radiance with depth in the upper water column (0–175 m), as well as a suite of shipboard meteorological measurements (Table 2.1).

Moored instruments

Because monthly sampling cannot effectively resolve short-term (minutes–weeks) variability of ocean biogeochemistry, we utilize electronic data-collecting instruments attached to a moored buoy system as much as possible. The HALE ALOHA mooring (Hawaii Air-sea Learning Experiment at Station ALOHA) is deployed at a fixed position near the station for several months at a time. The mooring is outfitted with a variety of devices, including CTDs, optical sensors, dissolved gas sensors, nutrient analyzers and meteorological instruments. Although not a substitute for hands-on microbiological sampling and experimentation, the high-frequency data sets obtained from the moored instruments provide an unprecedented window on the environmental variability in the NPSG, and complement the monthly sampling scheme. Details of the instrumentation used on HALE ALOHA can be found on the HOT program website (http://hahana.soest.hawaii.edu/hot/hale-aloha/ha.html). In addition to HALE ALOHA, we have repeatedly deployed an array of time-series sequencing sediment traps. These bottom-moored instruments collect the flux of sinking particles reaching the deep ocean (4000 m) over weekly intervals, and thus provide a detailed export data set complementary to that obtained from the monthly free-floating sediment trap deployments.

Ancillary measurements and experiments

The core physical and biogeochemical data collected by HOT program scientists represent more than just an exercise in long-term microbial habitat monitoring — they represent a framework upon which a conceptual and mechanistic understanding of ocean ecosystem dynamics may be built. To that end, the HOT program has hosted a wide array of collaborating researchers, whose measurements and experiments complement our own efforts. These ancilliary projects have included investigations of phytoplankton population dynamics, microbial production of trace gases, isotopic constraints on the ocean's role in the global carbon cycle, and molecular biological evaluations of nitrogen fixing genes, to name but a few. A list of these collaborations may be found on the HOT program website (http://hahana.soest.hawaii.edu/hot/ancillary.html). The success of each of these endeavors relies, ultimately, upon the proper collection of appropriate samples.

◆◆◆◆◆◆ CONCLUSION AND PROSPECTUS

Seagoing microbiological oceanographers labor under several disadvantages compared to their land-based counterparts. First and foremost is the immense scale of the habitat, relative to the size of the sample that is generally removed for quantitative analysis. In addition, it is no longer reasonable or acceptable for marine microbiologists to focus exclusively on the microbial inhabitants of the sea, especially if *in situ* ecological process understanding is a primary objective of the investigation so great care must be given to collections and analyses of complementary physical and biogeochemical data sets. Second is the difficulty in conducting controlled, replicated field experiments. Finally, the complexity and ephemeral nature of most marine ecosystems precludes straightforward scaling from the experimental measurements to regional or basin-wide scales with any degree of certainty. Empirical modeling approaches, that have been used extensively in ecological studies, must be supplemented with process-oriented studies of the physical, chemical and biological interactions before a comprehensive understanding can be achieved. High on the relatively long list of research priorities is the design and implementation of a rigorous sampling program.

It is dangerous to predict the future progress of any scientific discipline. In the case of microbiological oceanography, however, there is ample evidence to suggest that the field will experience a rapid increase in technology and instrumentation, including sampling, over the next few decades. Field researchers should anticipate increased automation both in sample collection and in the remote, autonomous detection of microorganisms (including specific microbes) and their *in situ* metabolic activities. Futhermore, advanced statistical treatments of ecological data and the development of comprehensive models should provide better understanding of the complex interactions between microorganisms and their environment. It is very likely going to be an exciting few decades.

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