MOLECULAR APPROACHES TO MICROBIAL BIOMASS ESTIMATION IN THE SEA

2

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2.1 INTRODUCTION

The marine environment is the largest contiguous habitat on Earth. It is composed of several distinct ecosystems including high-latitude (polar) zones, mid-latitude open-ocean gyres, equatorial upwelling systems and a diverse assemblage of coastal habitats (e.g. continental shelves, bays, coral reefs, mudflats and estuaries). These individual ecosystems have characteristics that collectively influence both their populations (e.g. types of organisms present, trophic structures) and their *in situ* rates of biogeochemical processes (e.g. primary production, respiration and nutrient cycling). This diversity makes broad generalizations about microbial processes in the sea impossible. It is also important to remember that our current understanding of ecological processes in the largest marine habitat (by volume), the deep sea, is rudimentary. An excellent example of our lack of general understanding of deep-sea processes was the unexpected discovery, in 1977, of luxuriant communities of previously unknown organisms surrounding regions of hydrothermal fluid discharge (Corliss *et al.*, 1979). This discovery challenged our basic views on the obligate role of sunlight in global ecology, and presented a new paradigm for the existence of life in the biosphere (Karl, 1995a).

The biogeochemical cycles of carbon (C), nitrogen (N) and phosphorus (P) in the sea are driven primarily by energy supplied to the surface ocean by sunlight. Photosynthetic production of organic matter fuels a complex series of feeding interactions (i.e. food-web trophic interactions) and organic matter transport processes that ultimately sustains life throughout most of the world ocean (Fig. 2.1). During cell metabolism and growth, nutrients are assimilated and incorporated into living organic tissue, i.e. biomass, a portion of which is transported by the combined active and passive movements of living and nonliving particulate matter and, eventually, recycled back to inorganic forms to start the cycle again. These C, N and P nutrient cycles may have both regional and global components with regeneration time scales ranging from hours to days for local processes, to geological time scales for materials buried in deep-sea sediments. The cycles provide a constant resupply of nutrients necessary to sustain trophic interactions.

By contrast, a portion of the energy originally supplied to the system in the form of sunlight is converted first into chemical energy by the reduction of inorganic carbon to organic carbon and, eventually, into heat as the organic matter is transported, transformed and finally recycled within the

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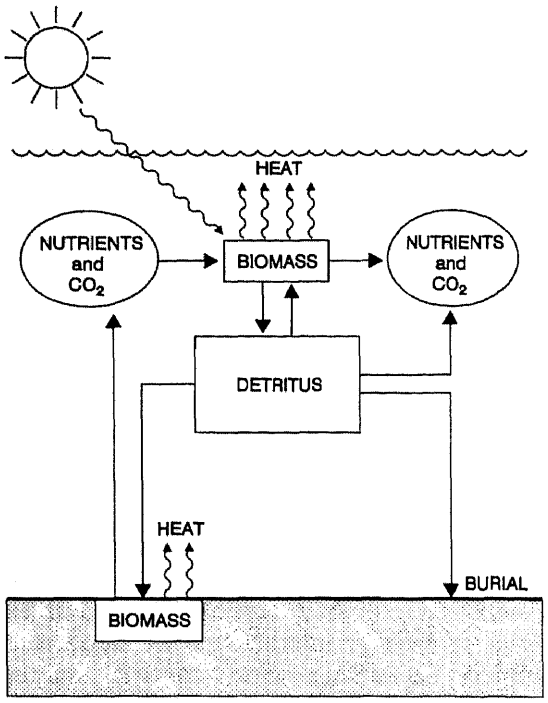


Fig. 2.1 Schematic representation of the role of microbial biomass in oceanic carbon and nutrient cycles. Photoautotrophic microbes convert radiant energy and inorganic carbon into cell biomass, a portion of which is converted back into inorganic carbon and the remainder into detritus. During the flow of energy into the biosphere, heat is produced as a waste product as predicted by the second law of thermodynamics. The nutrients (N, P, S), on the other hand, are largely recycled during this process, but small amounts are buried in deep-sea sediments, where they are mobilized over much longer time scales than are upper ocean nutrients. In the ocean, the amount of organic matter that is present as detritus is much larger (by several orders of magnitude) than that present as microbial biomass.

marine environment (Fig. 2.1). This fundamental difference in the cyclic flow of mass versus the unidirectional flow of energy is common to all ecosystems (Kormondy, 1969). Fluctuations in the biomass of living organisms are a reflection, in part, of the rate of supply of nutrients and energy to the system (biomass would change if either the nutrient or energy flux were altered) and partly a result of the structure and efficiency of the food web.

Two fundamental properties of all ecosystems are: (1) biomass, the standing stock of living organisms and (2) metabolism, the driving force behind biogeochemical cycles. Microorganisms comprise a dominant portion of the total biomass in the sea and their metabolic activities and specific growth rates are responsible for most of the carbon and energy flow in the biosphere. Consequently, there has been a logical focus on the study of microbial processes in the sea.

Biologists now recognize three major lines of evolution: Bacteria, Eucarya and Archaea (Woese, 1994; also see Chapter 1). Photoautotrophic marine microorganisms include representatives of the Domains Bacteria and Eucarya; however, the dominant photoautotrophic microorganisms in any given ecosystem can vary depending upon geographic location. For example, in most open-ocean, mid-latitude habitats (e.g. low-nutrient gyres in all major ocean basins), photosynthetic biomass is dominated by Bacteria (Fig. 2.2). Although now well-documented, this phenomenon was not recognized until the late 1970s and even today is not always considered in the design of field studies. Furthermore, the terms 'bacterial biomass' and 'bacterial productivity' are generally reserved for the heterotrophic bacterial populations and not for photoautotrophic Bacteria, so the scientific literature may be a source of confusion on this point. By comparison to the open-ocean habitats, most coastal and shelf regions and all high-latitude zones are dominated by Eucarya, typically diatoms, coccolithophorids and photosynthetic flagellates (Fig. 2.2).

As will become apparent in subsequent sections of this chapter, biomass is one of the most important master variables in micro-bial ecology. Biomass estimations help to provide an understanding of the

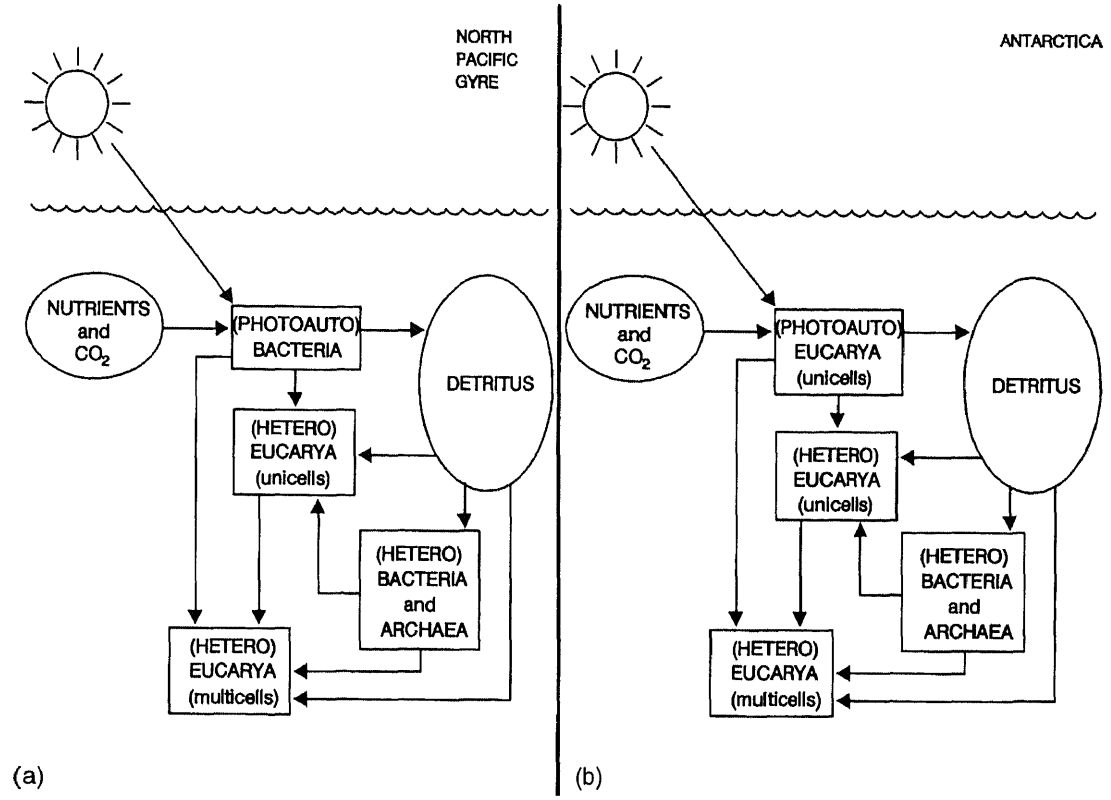


Fig. 2.2 Comparison of microbial food webs from two different marine ecosystems. (a) The typical subtropical, oligotrophic ocean food web that has been described for the North Pacific Ocean. Photoautotrophic Bacteria dominate the radiant-energy capture processes, with Eucarya playing a relatively minor role. These small photoautotrophic Bacteria (0.8–1.5 μm) are consumed by small unicellular Eucarya (primarily heterotrophic nanoflagellates), which also prey on Bacteria and, perhaps, Archaea. The protozoans are in turn consumed by small multicellular organisms and eventually by higher trophic level predators. The large pool of detritus fuels a heterotrophic bacterial-based food web which recovers some of the energy released at the primary producer level. (b) By comparison, primary production in the coastal ecosystem of Antarctica is by relatively large (>20 μm) Eucarya which are consumed directly by larger multicellular organisms. Although the microbial food web is still present, photoautotrophic bacteria are absent. Archaea are present in relatively large concentrations in coastal Antarctic environments, especially when compared to the subtropical ocean ecosystems.

trophic structure and bioenergetic relationships within an ecosystem and set limits on the fluxes of mass and energy. Along with quantitative determinations of growth rate, bio-mass estimations can be used to quantify the productivity of ecosystems and to predict and model carbon-pool dynamics. However, there are also limitations. For example, there is no a priori relationship between biomass and growth among diverse microorganisms. Regions having high biomass do not always coincide with areas of high productivity, so concurrent data on *in situ* metabolic activity are also required to describe the role of microbes in the sea (see Chapter 3).

Despite the differences among the various marine ecosystems, there are certain shared characteristics that favor the formation of organic matter in both living and nonliving forms. In this chapter, we discuss those basic properties of the oceanic carbon cycle that lead to the production and accumulation of living microorganisms, their removal with time and the remineralization of reduced organic matter to its oxidized form (i.e. carbon dioxide). These reversible, largely microbiologically mediated interconversions among the dissolved inorganic and the dissolved and particulate organic pools are fundamental processes in biological oceano-graphy. Additionally, the oceanic carbon cycle affects the atmospheric accumulation of greenhouse gases and the potential for global environmental change. We shall focus on the rationale for measuring total standing stocks of biogenic matter (usually expressed in terms of carbon per unit ocean surface area or volume of seawater), and will present a comprehensive summary of the analytical methods used to estimate the biomass of marine microorganisms, with an emphasis on molecular techniques. Our primary objective is to assess what these methods have revealed about the distribution and abundance of microbial biomass in the sea and how that information has improved our overall understanding of the global carbon cycle. This chapter focuses on the biomass in the ocean *per se*, rather than the subseabed; the latter may be an equally important habitat though we have less information about sediment microbial processes.

In preparing this chapter we have tried to avoid duplication of the excellent presentations by Brock (1971), van Es and Meyer-Reil (1982), White (1983), Leftley *et al.* (1983) and Karl (1986) concerning related issues of microbial biomass and metabolic activity determinations in nature, and the comprehensive treatise, *Handbook of Methods in Aquatic Microbial Ecology* (Kemp *et al.*, 1993).

2.2 OCEANIC CARBON CYCLE

The global inventory of carbon, approximately 4–5 $\times$ 10^4 gigatonnes (Gt = 10^{15} g), is distributed unequally among four active reservoirs (Post *et al.*, 1990): atmospheric (1.7%), terrestrial (4.5%), geological (8.9%) and oceanic (85%). The comparative sizes of the atmospheric and ocean reservoirs are of great importance in scientific issues related to global climate and environmental variability. However, at present we have only a rudimentary understanding of the mechanisms and rates of transfer of carbon among these major pools.

The large and dynamic oceanic reservoir of carbon, approximately 4 $\times$ 10^4 Gt distributed among dissolved and particulate constituents with various redox states, plays an important role in biogeochemical cycles. The two largest pools are dissolved inorganic carbon (DIC = $[H_2CO_3]$ + $[HCO_3^-)$ + $[CO_3^{2-}]$) and the less oxidized pool of mostly uncharacterized dissolved organic carbon (DOC). A chemical disequilibrium between DIC and organic matter is produced and maintained by microbiological processes. These reversible interconversions between dissolved and particulate carbon pools in the sea define the oceanic carbon cycle.

Primary conversion of oxidized DIC to reduced organic matter (dissolved and particulate pools) is generally restricted to the euphotic zone (0–200 m) of the world ocean where photosynthesis occurs. A notable exception may be the flow of energy at deep-sea hydrothermal vents; however, the thermodynamic basis for production in these 'dark' habitats is not firmly established (Karl, 1995b). The supply of reduced carbon and energy required to support subeuphotic zone (>200 m) metabolic processes is derived from upper ocean photosynthesis and is transported downward by advection and diffusion of dissolved organic matter (Toggweiler, 1989), gravitational settling of particulate matter (McCave, 1975) and the vertical

migrations of pelagic animals (Longhurst and Harrison, 1989). Each of these processes, collectively termed the 'biological pump' (Volk and Hoffert, 1985), is controlled by distinct environmental factors and, therefore, the relative contribution of each process varies with changes in habitat or with water depth.

Each year, the biological pump removes an estimated 5 Gt of carbon from the surface waters of the world ocean, a value equivalent to approximately 10% of the annual global ocean primary production (Karl *et al.*, 1996). It is important to emphasize that this relatively small percentage of the total carbon and energy captured in the euphotic zone is all that is available to fuel life processes in the remaining 90–95% of the volume of the world's oceans. This understanding predicts that both microbial biomass and total metabolic activity are concentrated near the sea surface and decrease with increasing water depth. These expectations are generally supported by extensive field data, some of which are summarized in subsequent portions of this chapter.

2.3 SIZE SPECTRUM OF ORGANIC MATTER

In the sea, the pool of total organic carbon (TOC) is distributed among three major, operationally defined pools: dissolved organic carbon (DOC), nonliving particulate organic carbon and living, or biomass, carbon (Table 2.1). The arbitrary boundary between dissolved and particulate is defined on the basis of procedural and analytical considerations. Typically, a membrane filter with a 0.2–1 μm diameter porosity is used to separate dissolved from particulate organic matter. By this operational definition, colloids and small particles, including some bacteria and most viruses, are 'dissolved'.

In most marine habitats, DOC dominates the TOC inventory (Table 2.1). Consequently, one cannot ignore the presence of the DOC and colloid pools when discussing biomass distributions in the sea. DOC in the sea is thought to originate from living organisms, although abiotic formation of organic matter is possible, for example, at mid-ocean spreading centers (Karl, 1995b). On the global scale, however, abiotic production is of minor importance compared to biogenic processes.

The oceanic DOC pool is a diverse and complex mixture of largely uncharacterized materials. Even with modern analytical procedures, generally less than 20% of the total DOC in seawater is identified (Druffel *et al.*, 1989). Both low molecular weight (e.g. methane, glucose, amino acids) and high molecular weight (e.g. proteins, nucleic acids) compounds are routinely detected at all geographic locations. It is likely that hetero-trophic bacteria from different marine

Table 2.1 Approximate sizes of the major organic carbon pools in the sea

Pool[a]	*Inventory (Gt)*	*Percentage of total*
Dissolved organic carbon	100	45
Colloidal organic carbon	100	45
Nonliving particulate organic carbon	20	9
Living carbon	1.6	0.7
Bacteria	0.4	0.2
Protozoa	<0.1	<0.1
Plankton (phyto- and zoo-)	1.2	0.5
Total organic carbon	222	100

[a]Data from Cauwet (1978) and Ajtay *et al.* (1979).

ecosystems utilize different DOC components and may even have unique substrate preferences (Christian and Karl, 1995).

Despite the fact that biogenic processes produce DOC, not all organic compounds are readily available as substrates for chemoheterotrophic bacterial assimilation. At least half of this large reservoir (200 Gt of carbon) may be refractory (mean age ≥6000 years; Williams and Druffel, 1987). It is possible, and indeed probable, that these compounds represent relatively rare or unusual metabolic by-products that accumulate over long periods of time, relative to the generation times of the organisms that produce them. If this is the case, then the more relevant DOC measurement for studies of microbiological oceanography might be the 'bioavailable' DOC pool rather than the TOC or DOC pools. Unfortunately, even if bioavailable DOC could be defined and measured, it is not straightforward to relate steady-state concentrations to mass flux for the oceanic DOC pool.

The concentration of the particulate organic carbon (POC) pool in seawater (nonliving plus living) is small relative to DOC, and ranges from <1% of TOC in subeuphotic zone waters to 20–40% of the TOC pool at the height of a coastal plankton bloom. Dissolved organic compounds can be transformed into POC by microbial uptake and assimilation, or by other physical processes including coagulation, adsorption, flocculation or precipitation (Karl *et al.*, 1991). One well-studied process is the transformation of DOC to POC by bubble bursting (Johnson and Cooke, 1980; Kepkay and Johnson, 1988, 1989), a common phenomenon at the air–sea interface.

In the surface waters of most open-ocean habitats and throughout the intermediate and abyssal ocean worldwide, the concentration of nonliving POC exceeds that of the biomass pool, in some cases (e.g. deep-sea habitats) by two orders of magnitude (Table 2.1). This dominance of nonliving organic matter makes direct estimates of microbial biomass from measurements of total particulate carbon, nitrogen or protein or dry-weight determinations impossible. Even though microbial biomass is a relatively small pool of carbon, it is the catalyst that controls the fluxes of all other pools, including dissolved inorganic carbon. It will be important to consider the low concentration of 'living carbon' in a soup of 'nonliving' organic matter as we later discuss the analytical methods for biomass estimation and their implications.

The large and regionally variable pool of nonliving organic matter (both dissolved and particulate) is sometimes called 'organic detritus'. We shall adopt the definition proposed by Wetzel *et al.* (1972): 'detritus – any nonpredatory losses of organic carbon from any trophic level (including egestion, excretion, secretion, etc.) or inputs from sources external to the ecosystem that enter and cycle in the system (allochthonous organic carbon)'. This definition eliminates the arbitrary distinction between dissolved and particulate phases and is more consistent with the recognized common fates of these two classes of organic matter in the ocean. Although not explicitly discussed by Wetzel *et al.* (1972), we will classify virus particles together with living carbon, rather than detritus (see following section), and we will also leave open the possibility that certain resting, arrested or otherwise inactive or debilitated but potentially viable cells may also be part of the detrital pool in seawater. This distinction becomes important in the intercomparison of independent methods for biomass estimation in the sea and in the overall interpretation of our field data.

2.4 MICROBIAL INHABITANTS OF THE SEA

Studies on the distribution and abundance of marine microorganisms date back to the pioneering nineteenth-century research efforts of Certes, Fischer and Russell (discussed by Gee, 1932; Benecke, 1933). During this embryonic phase of marine microbial ecology, it was independently established that heterotrophic

bacteria and other microorganisms are ubiquitous in the world's oceans regardless of latitude, water depth or distance from continental land mass. Quantitative methods for determination of cell abundance or biomass were first developed in the 1960s. However, the complexities of microbial communities and their environments still challenge the capabilities of existing analytical methods. Although considerable progress has been made over the past three decades, an accurate and precise estimation of microbial biomass in the marine environment will probably require improved or novel methodologies (Karl, 1994).

Microorganisms are generally classified on the basis of size, nutritional characteristics or phylogeny. Regardless of the criterion used, a broad diversity in function and form is revealed. Living organisms are often classified as unicellular or multicellular. One major group that does not fit easily into this classification scheme comprises virus particles. Viruses contain nucleic acid (either RNA or DNA) and protein, but are otherwise acellular. They are obligate parasites and cannot grow or reproduce outside of their host. With regard to physical dimensions, marine microbes range from a few nanometers (1 nm = 10^{-9} m) for the smallest virus particles to >100 μm (1 μm = 10^{-6} m) for certain unicellular protozoans and large phytoplankton cells (e.g. diatoms). For the purposes of this chapter we will define 'microorganism' broadly, so as to include all small (≤200 μm), acellular and unicellular and even some small multicellular organisms that inhabit the sea.

A differential staining technique of great practical value, which subsequently became known as the Gram stain, was discovered empirically by Christian Gram in 1884 (Stanier *et al.*, 1986). This procedure divides all Bacteria into either Gram-negative or Gram-positive species depending upon whether they retain the dye crystal violet following alcohol treatment. The outcome reflects major differences in the chemical composition and structure of the cell wall (see Section 2.6.7).

The phylogeny of marine microorganisms is thoroughly reviewed in Chapter 1, but several points are re-emphasized here. First, by the above definition based on particle size spectrum, marine microorganisms represent all three major lines of evolution: Bacteria, Archaea and Eucarya (Woese, 1994). Although the terms 'bacteria' and 'algae' have been widely used in the marine microbiology literature to refer to the 'heterotrophic bacteria' (bacterioplankton) and the 'photosynthetic eukaryotic algae' (phytoplankton), it is now well known that most of the algae in the open sea are actually Bacteria (i.e. cyanobacteria, formerly known as blue-green algae, especially of the genera *Synechococcus* and *Prochlorococcus*; see Waterbury *et al.*, 1979; Chisholm *et al.*, 1988). Furthermore, members of the Domain Archaea are now known to be widely distributed in the marine environment and may be a major constituent of microbial biomass in selected habitats (DeLong *et al.*, 1994). Most previous analyses of 'bacteria', for example by direct microscopic techniques, inadvertently included Archaea. These previous misrepresentations of fundamentally different phylogenetic groups underscore the diversity and complexity of natural microbial communities and the current limitations of our analytical methods. One might legitimately ask, 'What else don't we know?'

With regard to nutrition and metabolism, there also is a large range of characteristics. Furthermore, the recent discovery of novel genomes (Bult *et al.*, 1996) suggests that additional diversity of cell function exists, and this may have important implications for our current models of global carbon-pool dynamics. Consequently, we must view our present knowledge of bacterial metabolism as a flexible outline, rather than as rigid dogma (Karl, 1995b).

This broad diversity in microbial form, function and phylogeny is an important

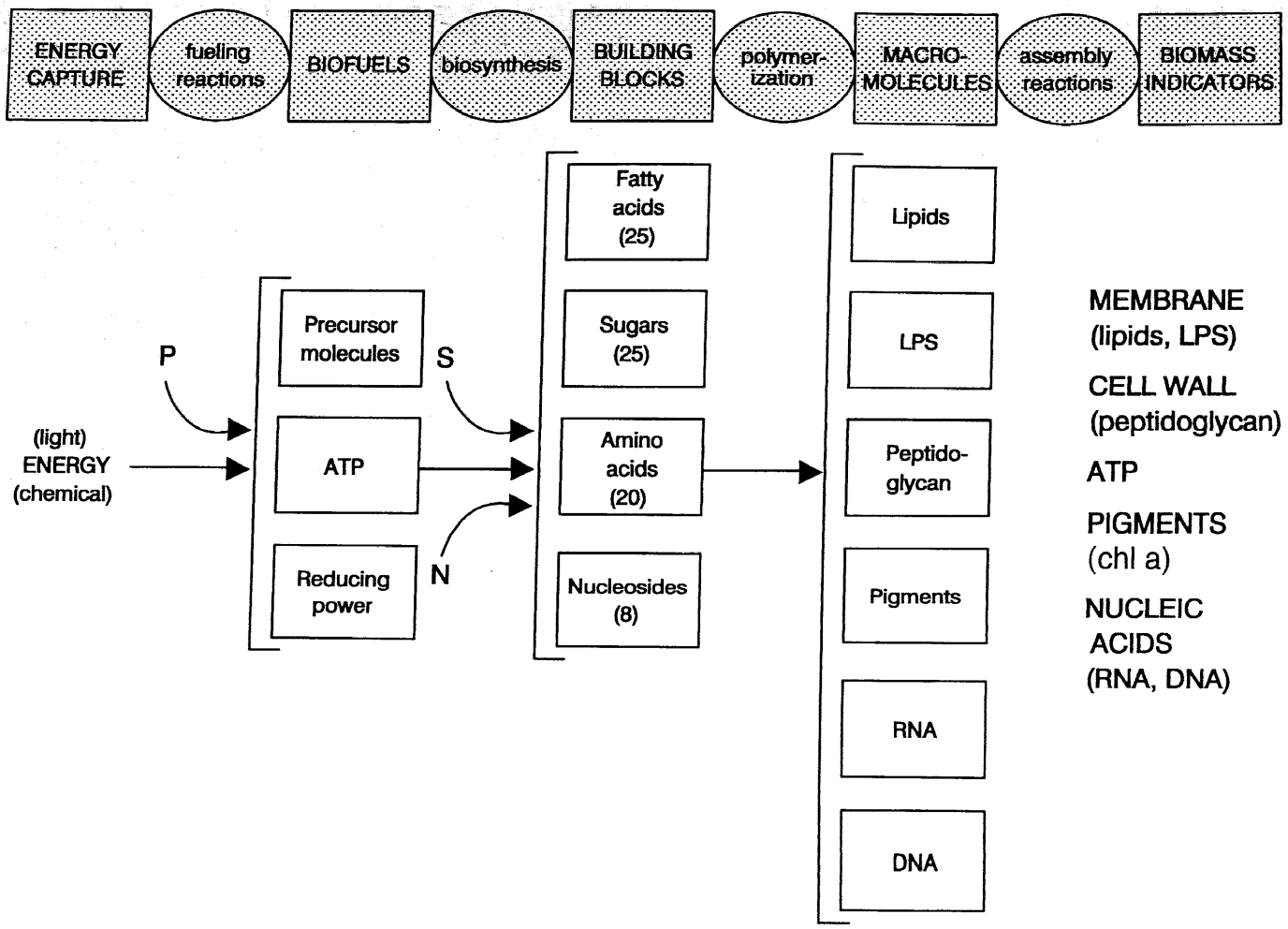


Fig. 2.3 Schematic representation of the sequential steps involved in the synthesis of the biofuels, building blocks and macromolecules that comprise living cells. Shown at right are biomarker compounds or compound classes used to estimate microbial biomass in the sea.

consideration when designing analytical methods to estimate microbial biomass. As discussed in subsequent sections of this chapter, biomass estimation techniques target either the entire microbial community or a specific portion thereof (Fig. 2.3 and Table 2.2). The concentrations of specific biofuels, building blocks and macromolecules have all been used as biomarkers to estimate microbial biomass. The overall reliability of these methods is determined by the presence of either universal or unique biomolecular characteristics. Given the broad diversity of the microbial world, the task of biomass estimation is a substantial intellectual and analytical challenge with great scientific reward if done properly.

2.5 SAMPLING

The reliability of any field-collected data set is determined by the methods used for sampling and analysis. Although this chapter focuses attention on methods of analysis, it is important to keep in mind that a precise and accurate measurement is compromised by improper collection of the sample. Both the method used to collect the sample from nature and the challenge of obtaining a truly representative sample are primary concerns in studies of marine microbial ecology.

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 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'.

A number of specialized sampling devices have been developed over the years to collect microorganisms from ocean depths (Jannasch and Wirsen, 1977; Atlas and Bartha, 1993). These devices range from sterile and trace metal-free collectors to pressure-retaining samplers designed to recover and manipulate deep-sea microorganisms in the absence of sample decompression. Although aseptic technique is not required for most routine field studies, to determine the presence or absence of a specific microorganism such technique is imperative.

The scale of sampling is an important factor to consider when extrapolating results to the ecosystem level (Levin, 1992). In most field studies, usually due to practical considerations, the number of samples collected is regrettably small, and it is often impossible to obtain replicate samples. If statistical methods are employed, it is assumed that the microbial populations follow a known probability distribution (e.g. Poisson, negative binomial or log-normal). However, microorganisms 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 vessels 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).

Table 2.2 Summary of methods used for estimating microbial biomass in seawater samples

Method	*Target microorganisms*	*Assay assumptions*	*Limitations*	*Comments*	*References*
Enumeration					
Culture methods (spread/pour plate, dilution MPN, selective media, roll tube)	Selected groups of Bacteria, Archaea, Eucarya	Viable cells grow to form colonies (solid medium) or turbid solutions; one cell – one colony	Diverse metabolic characteristics preclude existence of a universally acceptable medium or set of growth conditions; cells often found in clumps or attached to particulate matter; numbers based on plate counts typically a small percentage (1–10%) of that estimated by direct microscopy; culture methods set a lower limit on the number of viable bacterial cells in the sea	Cannot be used for reliable quantitative biomass determination, but does have other specialized applications for autecological studies and enumeration of certain target organisms	Lewin, 1974; Hattori, 1988
Microscopic methods (epifluorescence)	Bacteria, Archaea, Eucarya and virus-like particles (VLP)	Cell biovolume can be derived from estimates of cell size and number, both determined by direct microscopy following the addition of a nucleic acid stain; biomass is estimated by extrapolating biovolume to mass, using an empirical relationship	Small cells are difficult to see and impossible to size accurately; heterotrophic bacteria cannot be easily distinguished; not all stained particles are viable cells; volume estimation is difficult from 2-D image; there may be postcollection changes in biovolume; large range in reported biovolume-to-C extrapolation factors	Most widely used method for biomass estimation despite uncertain accuracy	Hobbie *et al.*, 1977; Porter and Feig, 1980; Hennes and Suttle, 1995
Molecular biomarkers					
ATP	Bacteria, Archaea, Eucarya	All living microorganisms contain ATP; ATP is not associated with organic detritus; constant C:ATP ratio in all target organisms	'Nonspecific' measure of microbial biomass; cell quota of ATP varies with nutrient limitation, especially P; dissolved ATP is present in most marine environments	Most reliable measure of total microbial biomass	Holm-Hansen and Booth, 1966; Karl and Holm-Hansen, 1978

Chlorophyll *a* (chl *a*)	Photoautotrophic Bacteria and Eucarya	Chl *a* is a major light-harvesting pigment in all photoautotrophic microbes; chl *a* is not associated with organic detritus; constant C:chl *a* ratio in all target organisms	Cell quota of chl *a* varies with nutrient limitation and, especially, light levels; dissolved chl *a* is present in the marine environment	One of the most commonly measured molecular biomarkers; methods are available for autonomous, continuous chl *a* measurements including satellite-based sensors	Holm-Hansen *et al.*, 1965 Holm-Hansen and Riemann, 1978
Membrane phospholipid phosphate (PL-P)	Bacteria, Archaea, Eucarya	All living microorganisms contain membrane phospholipids; rapid turnover rate of PL-P following cell death; C:PL-P ratio is constant	'Nonspecific' measure of microbial biomass; C:PL-P ratios are not well constrained	Phospholipid assay based on measurement of PL-P is reliable for detection of total microbial biomass; alternative detection system requires specialized instrumentation	White *et al.*, 1979b
Membrane phospholipid fatty acids (PLFA)	Bacteria and Eucarya	All Bacteria and Eucarya contain PLFAs (Archaea do not); rapid turnover of PLFAs following cell death; C:PLFA ratio is constant	'Nonspecific' measure of microbial biomass; C:PLFA ratios are not well constrained		Baird and White, 1985
Ergosterol	Selected groups of Eucarya (fungi)	Ergosterol is present in the membranes of all fungi; ergosterol is not present in organic detritus; C:ergosterol ratio is constant	C-ergosterol extrapolation factor not well constrained; fungi rarely dominate microbial biomass; ergosterol is present in selected algae and protozoans		Newell *et al.*, 1988 Newell, 1993
Lipopolysaccharide (LPS)	Selected groups of Bacteria (gram-negative Bacteria)	LPS is a component of the outer membrane of all gram-negative Bacteria, including photoautotrophs and heterotrophs; LPS is not present in organic detritus; C:LPS ratio is constant	Not all cyanobacteria contain LPS; dissolved LPS is present in most marine environments	Most reliable biomarker for gram-negative Bacteria in the sea	Watson *et al.*, 1977
Muramic acid (MA)	Bacteria (gram-negative, gram-positive, including cyanobacteria)	Rigid peptidoglycan layer of all Bacteria contains MA; amount of MA per cell varies systematically among gram-positive and gram-negative Bacteria; MA is not present in organic detritus	Assay is not straightforward and requirement for independent determination of gram-positive and gram-negative Bacteria populations limits accuracy; MA is present in 'empty' cells		Millar and Casida, 1970; Moriarty, 1978; White *et al.*, 1979a
DNA	Bacteria, Archaea and Eucarya	DNA is present in all living organisms; DNA is not present in organic detritus; C:DNA ratio is constant	'Nonspecific' measure of microbial biomass; dissolved and nonliving particulate DNA is present in most marine environments	Can be used in phylogenetic studies with appropriate oligonucleotide probes	Holm-Hansen *et al.*, 1968; Holm-Hansen, 1969a

Unfortunately, in many oceanographic studies, a single sample often represents an area several hundred meters vertically and several to tens of kilometers horizontally. Finally, the importance of repeated measurements over time to ascertain diel, seasonal, interannual and decadal-scale variability has not been taken account of in most field studies.

Exposure of viable microorganisms to environmental conditions substantially different from the *in situ* conditions should be avoided to minimize changes in growth, metabolism or viability. However, keeping conditions constant is impossible during the collection of many samples, for example abyssal water samples from the equatorial ocean. Decrease in pressure and increase in temperature during the time required for the sample to reach the ocean's surface alters certain biomass-related cellular constituents or affects cell size and viability. However, at the present time these considerations have not been systematically evaluated.

2.6 METHODS FOR BIOMASS ESTIMATION

No single approach to the study of microbial ecology is universally accepted or acceptable (Karl, 1986). One option is to investigate the occurrence of a single taxon or metabolic process. However, the presence of a particular microorganism or phylogenetic group does not necessarily imply an important ecological role. An alternative approach is to study the ecosystem or the microbial assemblage *in toto*. Because most microbial communities cannot be separated easily into discrete metabolic categories, much less into defined taxonomic groups, prior to *in situ* measurements, the resultant synecological data are generally more difficult to interpret.

A variety of methods is currently available for the estimation of the diversity, biomass, metabolic activity, and growth of microorganisms (Brock, 1971; van Es and Meyer-Reil, 1982; White, 1983; Karl, 1986; Kemp *et al.*, 1993). The interested reader should refer to these articles and to their primary references. In this chapter, we focus on estimation of microbial biomass, the standing stock of living microorganisms. We discuss only those selected biochemical/molecular methods used extensively in oceanographic field studies to estimate the biomass of a specific group of microorganisms or total microbial biomass (Table 2.2). Direct microscopic methods and laser-based flow cytometric techniques that rely upon the use of molecular discriminators (e.g. immunofluorescence, nucleic acid fluorochromes and pigments) are briefly discussed (Table 2.2).

2.6.1 CARBON-POOL MEASUREMENTS

Carbon is traditionally used in ecological studies as the basic unit of living biomass, production and fluxes among the various trophic levels (Karl, 1986). As discussed previously (Sections 2.2 and 2.3), biomass (living) carbon is generally a small percentage of TOC, which itself is dwarfed by the magnitude of the DIC pool. Nevertheless, direct measurements of DIC, DOC and POC pools are extremely important in studies on the distribution and control of microbial biomass. For example, DIC is the carbon source for the growth of photo- and chemolithoautotrophic microbial populations and is a metabolic by-product of all living organisms. Furthermore, chemoheterotrophic bacterial populations rely upon DOC as a source of carbon and energy. DOC has numerous sources in the marine environment, and so the chemical composition of the DOC pool is generally fairly complex.

The DIC pool in seawater can be measured indirectly by alkalinity and pH measurements (Strickland and Parsons, 1972) or directly by acidification, gas CO_2 stripping and analysis by infrared light absorption. More recently, a coulometric-based DIC detection system has been introduced to oceanography (Johnson *et al.*, 1985, 1987). In this method, the CO_2 gas is stripped from an acidified sample of seawater

and is subsequently absorbed into an alkaline solution containing ethanolamine. The weak acid generated by the absorbed CO_2 is titrated by a strong base that is produced electrolytically. The equivalence point is detected photometrically with an indicator dye. This method is extremely precise and accurate and now can be used to routinely achieve a precision of 0.05% (approximately 1 µmol carbon per liter) and an accuracy of 1 µmol/l. This high level of accuracy and precision provides a detection system that can be used for direct determination of respiration rate during timed incubations.

The measurement of DOC relies upon chemical oxidation to CO_2 followed by detection of the CO_2, generally by infrared light absorption. Prior to 1990, an acid persulfate wet chemical oxidation method was commonly employed to convert DOC to CO_2 (Menzel and Vacaro, 1964). However, recently it has become evident that high-temperature combustion (HTC) methods may be more efficient than wet chemical oxidation methods and a variety of instruments are now available for HTC-DOC analyses (Sharp *et al.*, 1995). At the present time, the accuracy of routine HTC-DOC determinations is approximately 1% (0.8–1 µmol/l). This level of precision now makes it possible to measure DOC changes during timed seawater incubations (Kirchman *et al.*, 1991). When combined with coulometric DIC determinations, direct measurement of the net efficiency of DOC utilization by chemoheterotrophic bacteria (DIC produced/DOC utilized) is now possible.

Finally, POC measurements also rely upon high-temperature combustion of particulate matter collected usually on a combusted glass fiber, silver or aluminum oxide filter. Most commercially available instruments oxidize the organic matter in a pure O_2 environment, followed by CO_2 measurement using a gas chromatograph equipped with a thermal conductivity detector. The precision of POC determinations, approximately 10–15%, is not nearly as good as the precision of DIC or DOC measurements.

2.6.2 OBSERVATIONAL METHODS AND ENUMERATION OF MICROORGANISMS

Enumeration of microorganisms by spread or pour plate, most probable number (MPN) techniques, enrichment cultures or direct microscopy was traditionally used to estimate total biomass (Table 2.2). There are numerous limitations and shortcomings to these techniques, not the least of which is the difficulty of converting cell numbers to biomass. Cell numbers by themselves are of limited ecological value because of the wide range in the dimensions of individual microorganisms. For most field studies, a more relevant expression of viable microorganisms is the total amount of living organic matter.

Culture techniques are further complicated by the inability of certain viable cells to proliferate under laboratory incubation conditions. Consequently, the accuracy of these methods depends upon the capacity of the selected medium and conditions to satisfy the growth requirements of a significant proportion of the organisms present. Concerns over the reliability of culture methods are compounded by the problems arising from clumps, aggregates and the association of cells with nonliving particulate matter. Given the physiological diversity of microbial groups and the physical and chemical diversity of the microenvironments, it is not surprising that only a small percentage of the total microorganisms from most natural habitats can be cultured.

The microscope is an important research tool in microbial ecology and microscopic observations and enumeration of bacteria and other unicells date back to the seventeenth century (van Leeuwenhoek, 1677). In principle, microscopic observations of cell numbers, in conjunction with estimates of cell size, should provide the absolute standard with which to compare the results derived from

less direct methods. The major criticisms of direct microscopy are that the method is (1) highly subjective, (2) labor intensive and (3) relatively insensitive (Karl, 1986).

Optical microscopy is limited by resolution, i.e. 0.25 μm for visible light (Barer, 1974). The twentieth-century development and refinement of electron-beam technology (e.g. scanning electron microscopy, transmission electron microscopy, scanning transmission electron microscopy, high-voltage electron microscopy and analytical electron microscopy) have provided instruments with resolving power orders of magnitude greater than that of conventional optical microscopes.

When microscopic cell counts are compared with plate counts for a given water sample, the former method generally yields values that are two to four orders of magnitude greater than the latter (Jannasch and Jones, 1959; Jensen, 1968). However, controversy has resulted over the interpretation of these data. Certain investigators conclude that plating methods underestimate bacterial population densities. Others believe that direct microscopic methods overestimate the true population size because of the difficulty in distinguishing bacterial cells from nonliving particles and the assumption that all recognizable bacteria are viable. For example, Koike *et al.* (1990) have reported that >95% of the suspended submicrometer-sized (0.38–1 μm) particles in the upper ocean, up to 10^7 per ml, are nonliving particulate organic matter. Distinguishing between living and nonliving particles in the sea is important for accurate determinations of cell numbers and biomass.

The various technical problems and limitations inherent in the classic, visible-light microscopic methods were alleviated in part by recent developments in fluorescence microscopy, computer-assisted selective microscopic counting and laser-induced flow cytometry. The use of transmitted and UV epifluorescence microscopy greatly facilitates the detection, recognition and enumeration of certain microbial cells in their natural habitats. Partially obscured organisms are more easily discerned with fluorescence microscopy and, in principle, cells below the theoretical resolution of the optical microscope might also be detected because cells stained with fluorescent dyes emit light.

Autofluorescence is used to detect and enumerate phototrophic Bacteria, Eucarya and certain Archaea (e.g. methanogens). Because autofluorescence decays following cell death, it may be possible to enumerate active cells selectively by this technique. However, the primary fluorescence in photoautotrophs is determined by the cell content of chlorophyll, which is known to vary considerably in response to environmental conditions (especially light history). Consequently, the use of fluorescence intensity to distinguish viable microorganisms can yield a lower bound on true population density.

The use of exogenous fluorochromes has evolved from the initial investigations by Strugger (1948) to become the method of choice for direct counting of microorganisms from natural environments (Hobbie *et al.*, 1977; Porter and Feig, 1980). Routine methods now exist for the staining, observation and enumeration of Bacteria, Eucarya and Archaea. The most frequently employed techniques use acridine orange (AO), and the DNA-specific dyes 4,6-diamidino-2-phenylindole (DAPI) and Hoechst dye no. 33258. Many refinements, modifications and methodological improvements have been described over the past few years to optimize counting procedures (Turley, 1993). Recently, it has been suggested that many (and in some cases the majority of) stained cells actually lack nucleoids and, hence, are either inactive or nonviable (Zweifel and Hagström, 1995). If this turns out to be a general phenomenon then it may be necessary to revise, downwards, our microscopically derived estimates of bacterial cell numbers.

Immunofluorescence (fluorescence of an antibody) is a specific application of fluorescence microscopy that has been adapted

successfully for autecological studies. Methods now exist for the detection of numerous microbial genera (reviewed by Stanley *et al.*, 1979; Bohlool and Schmidt, 1980). Application of the fluorescent antibody technique is limited by (1) specificity of the antigenic reaction, (2) interference from background autofluorescence, (3) nonspecific adsorption of the antibody, and (4) the lack of distinction between viable and nonviable cells. The problem of counting a specific microbial population within a mixed assemblage may seem formidable but monospecific enumeration is, in many respects, easier than estimating total microbial biomass.

Total cell numbers do not always correlate with biomass because of the variation in the size of individual microorganisms. In principle, electronic particle counters such as the Coulter counter could be used to estimate the biovolume in a particular sample (Cushing and Nicholson, 1966; Kubitschek, 1969). However, this method can only be applied when the source habitat is devoid of nonliving particles and the biomass is dominated by relatively large cells. Most often, biomass is extrapolated from estimates of biovolume, density (mass per unit volume) and percentage dry weight. The first estimate is dependent on microscopic cell sizing, which is tedious and labor intensive. Furthermore, the lower limit of practical particle-size measurement with the optical microscope is approximately 0.8 μm; even in the 2–3 μm range, the errors are appreciable (Humphries, 1969). Developments in computer-assisted image microanalysis now provide an alternative to the laborious task of bacterial cell counting and sizing (Sieracki *et al.*, 1985; Bjørnsen, 1986). Nevertheless, the accuracy of cell sizing with optical microscopes decreases as the resolution limit is approached, and the halo effect (Watson *et al.*, 1977) observed with fluorochrome staining and epifluorescence microscopy can also affect the accuracy of all microscopic biovolume extrapolations. Furthermore, it may be difficult to resolve the dimensions of large cells if only their nucleic acids are stained, e.g. with DAPI, and there still appears to be uncertainty in our current methods of biovolume estimation. For example, Suzuki *et al.* (1993) have recently reported that bacterial biovolume (extrapolated from cell counts and size determinations) derived from the commonly used DAPI staining technique ranged from 25% to 61% of the biovolume derived from AO staining. They attributed these results to the more specific target of the DAPI stain (double-stranded DNA) compared to AO. On the other hand, AO staining is less selective and could overestimate biomass by interacting with particulate detritus. Consequently, it is not obvious from their data which of these two independent methods is more accurate.

Once determined, biovolume can be extrapolated to biomass C if the density, percentage dry weight and dry weight-to-carbon relationships are known. Bakken and Olsen (1983) measured the buoyant densities and dry matter contents of selected bacteria and fungi. Their results indicated densities of 1.035–1.093 g/cm^3 for bacteria and 1.08–1.11 g/cm^3 for fungal hyphae. However, their results for dry matter content were more variable, ranging from 12% to 33% and 18% to 25% (wt/wt) for bacteria and fungi, respectively. Bratbak and Dundas (1984) reported that the dry matter content of three different bacterial strains ranged from 31% to 57%, suggesting that the 'standard' value of 20% may be inaccurate. Furthermore, Romenanko and Dobrynin (1978) report dry-weight:wet-weight ratios of 0.082–0.291 (mean = 0.176) for a single species of *Pseudomonas*.

Finally, if biomass is expressed in carbon units, it is also necessary to apply a third correction to the biovolume estimates. Luria (1960) reported a carbon:dry-weight ratio of 0.50 ± 0.05 for bacteria, while Ferguson and Rublee (1976) used 0.344 as their conversion factor for marine bacteria. Newell and Statzell-Tallman (1982) gave a range of 0.255–0.470 for marine fungi. For photosynthetic Eucarya, the

cell carbon-to-biovolume conversion varies inversely with cell volume from 0.2×10^{-12} g/μm³ for small (10–20 μm³) cells to 0.02×10^{-12} g/μm³ for cell volumes >120 000 μm³ (Mullin *et al.*, 1966). Norland (1993) presents a comprehensive review of the relationship between bacterial biomass and biovolume with special reference to calibration and size scaling. He rejects the published values that are at the upper end of the range (i.e. $>5 \times 10^{-13}$ g/μm³) as being 'physiologically dubious'. The carbon content of single cells can be measured directly by energy dispersive X-ray microanalysis with transmission electron microscopy (Heldal *et al.*, 1985). This method obviates the need for biovolume estimation and provides for a more reliable discrimination between small bacteria and nonliving particulate matter. One must conclude that the accuracy of a biomass C estimate based solely on direct microscopic analyses cannot be greater than ±100%, considering the extrapolation factors available and the errors in enumeration and cell sizing.

With the recent development and refinement of laser-based flow cytometry, a new approach to cell enumeration and sizing is available for aquatic microbial ecologists (Monger and Landry, 1993). This method has the advantages of (1) high statistical precision due to the large number of cells enumerated, (2) elimination of subjectivity and fluorescence fading inherent in epifluorescence microscopy, (3) the ability to distinguish between photoautotrophic and heterotrophic Bacteria, (4) detection of ultramicrobacteria and viruses that are below the size resolution of optical microscopes, (5) multiparameter analysis capability (for enumeration, size estimation, DNA content, etc.), and (6) cell-sorting capabilities (Muldrow *et al.*, 1982; Yentsch *et al.*, 1983). Flow cytometry can also be used to enumerate specific groups of Bacteria, Eucarya and Archaea using rRNA-targeted universal or group-specific oligonucleotide probes (DeLong *et al.*, 1989; Wallner *et al.*, 1993). These procedures have been employed in combination with specific fluorochromes for the detection and estimation of cellular macromolecules (e.g. protein, RNA, DNA), with fluorescent-labeled antigens or antibodies for specific autecological applications.

2.6.3 MOLECULAR BIOMARKERS

Selectivity of culturing methods, subjectivity of microscopic methods and uncertainties in the extrapolation factors used to estimate microbial biomass from enumeration and cell sizing have encouraged the development of alternative approaches. As mentioned already, the direct determination of total organic C, N, P or protein is unacceptable as a surrogate measure of biomass due to the long residence times of these compounds in the environment following cell death and lysis. These bulk chemical measurements can, however, be used to set an upper bound on total living carbon, and in this regard they are extremely useful parameters in marine microbial ecology.

An effective biochemical or molecular biomarker of microbial biomass should satisfy the following criteria (Karl, 1986): (1) the biomarker selected must be present in all living cells or in all organisms of a specific population, (2) it must be readily metabolized, hydrolyzed or otherwise decomposed following cell death, (3) it must exist as a uniform and constant percentage of total biomass regardless of environmental or physiological conditions, (4) there must exist a convenient method to extract and purify (if necessary) the compound from environmental samples and (5) a sensitive quantitative assay procedure must be available. Microbial biomass in the ocean is low, so these techniques usually require sample concentration; assay sensitivity is an important criterion.

Because culture methods are ineffective with most marine microorganisms from nature, one must question whether the pure cultures that we use in laboratory experiments are representative of the assemblages

in nature. This question is important for the application of molecular biomarkers because the factors used for extrapolation to cell C must be calibrated with laboratory cultures. Our suspicion regarding the potential phylogenetic mismatch between laboratory isolates and *in situ* bacterial assemblages was supported by recent 16S rRNA sequence analyses of open-ocean populations (Giovannoni *et al.*, 1990). It is still not known to what extent the accuracy of our current molecular biomarker protocols may be affected by this general lack of representative laboratory strains.

There are a number of potential biomarker compounds to estimate microbial biomass in environmental samples (Karl, 1986; Kemp *et al.*, 1993). However, only a few have withstood the tests of time and peer review (Tables 2.2 and 2.3). We shall focus on the theory and practice of these selected procedures. In developing the rationale for the application of each method, we present only the conceptual justifications supporting each method and the limitations. Detailed protocols are not reproduced here but can be found in the primary literature.

2.6.4 ATP

Levin *et al.* (1964) first used ATP measurement as a sensitive method for the detection of living microorganisms in natural samples. Two years later, Holm-Hansen and Booth (1966) suggested that ATP measurements could be used to estimate total microbial biomass in marine ecosystems. These pioneering efforts evolved into a simple and extremely sensitive assay that has widescale use in oceanography. Although the ATP method does not differentiate among individual groups of microorganisms, the value of the procedure lies in the determination of total microbial community biomass. Without exception, all living organisms contain ATP, so the basis for the use of this molecular biomarker is well-founded. Virus particles have no endogenous metabolism and contain no ATP, so their biomass is not detected by this method.

ATP functions as an essential link between catabolism and biosynthesis, as a precursor for RNA and DNA synthesis, and, in concert with ADP and AMP, for regulation of cell metabolism. In general, the steady-state intracellular concentration of ATP is maintained at

Table 2.3 Summary of biomarkers, assay target molecules and detection systems used for estimation of microbial biomass in the sea

Reference biomarker(s)	*Assay target molecule(s)*	*Detection system*	*Selected references*
ATP	ATP	Firefly bioluminescence	Holm-Hansen and Booth, 1966
		HPLC-fluorometry	Davis and White, 1980
chl *a*	chl *a*	Fluorometry	Holm-Hansen *et al.*, 1965
		Spectrophotometry	Lorenzen, 1967
		HPLC-fluorometry	Latasa *et al.*, 1996
Phospholipids	P	Colorimetry	White *et al.*, 1979b
	Fatty acids	GC-MS	Baird and White, 1985
Lipopolysaccharide	LPS	Spectrophotometry	Watson *et al.*, 1977
	LPS	Colorimetry	Nakamura *et al.*, 1977
	Lipid A fatty acids	GLC	Parker *et al.*, 1982
Ergosterol	Ergosterol	HPLC	Newell *et al.*, 1988
Muramic acid	Lactate	Enzymatic	Moriarty, 1975
	Lactate	TLC/colorimetry	King and White, 1977
	Muramic acid	TLC/GLC	Fazio *et al.*, 1979
	Muramic acid	HPLC	Moriarty, 1983
DNA	*d*-ribose	DABA fluorescence	Holm-Hansen *et al.*, 1968
	DNA	DAPI/Hoechst fluorescence	DeFlaun *et al.*, 1986

a basal level proportional to biovolume (i.e. at a constant concentration, approximately 1–2 mM). Thus, ATP measurements are more likely to track total cell biovolume than total cell biomass despite the fact that most calibrations have focused on the relationships between ATP and total cell carbon.

ATP is biologically labile but relatively stable chemically, as evidenced by the variety of methods successfully employed to extract ATP from living cells (Karl, 1980). Significant concentrations of dissolved ATP have been detected in marine (Azam and Hodson, 1977; Nawrocki and Karl, 1989) and freshwater (Riemann, 1979; Maki *et al.*, 1983) environments. It is unknown whether this dissolved ATP interferes with the conventional measurement of particulate ATP in aquatic environments (Holm-Hansen and Booth, 1966). However, because most water samples are concentrated onto filters before extraction, the relative contribution from dissolved ATP should be minimal. Once extracted from living cells, ATP can be measured at extremely low concentrations (picomolar, or less) using either the firefly bioluminescence assay (Holm-Hansen and Booth, 1966) or high-performance liquid chromatographic (HPLC) procedures (Davis and White, 1980).

Of the criteria for an effective biomarker, most critical is that it is a constant percentage of the total cell mass, or cell C. There is no question that the C:ATP ratio varies considerably, and somewhat predictably, among microbial taxa and even within a given species as a function of culture conditions (data summarized in Karl, 1980). Among the most conspicuous differences in the C:ATP ratio are those observed between unicells (C:ATP = 220–350) and multicellular Eucarya (C:ATP = 50–150), and the large increases in C:ATP observed during P starvation (Karl, 1980). It has been suggested that the total adenine nucleotide concentration (i.e. A_T = [ATP] + [ADP] + [AMP]) might be a better measure of microbial biomass than ATP (Davis and White, 1980); little is known, however, about the variability or calibration of C:A_T in microorganisms.

In the past decade, the basic method of ATP measurement as an indicator of total microbial biomass has given rise to the more comprehensive protocol of environmental nucleotide fingerprinting (Karl, 1980). The development of specific and sensitive techniques for quantitative measurements of (non-ATP) intracellular nucleotides (e.g. GTP, UTP) has enabled researchers to estimate the rates of protein and nucleic acid biosynthesis, nucleotide metabolism, metabolic activity, and growth. More recently, Karl and Bossard (1985) developed a method to estimate the turnover rates of ATP to quantify energy flux in natural populations of marine microorganisms. This expanded approach of nucleotide fingerprinting is strongly recommended for field investigations to obtain corroborative data relating to the *in situ* physiological states of microbial assemblages.

Regional, seasonal and depth variations in ATP

ATP concentrations in the marine environment are variable on both time and space scales. Systematic spatial variations are evident in nearshore-to-offshore transects off the continental shelf (Fig. 2.4) and in vertical profiles (Fig. 2.5). ATP has been detected throughout the world's oceans, from the Arctic Basin to Antarctica, including deep-sea trenches (Fig. 2.6). ATP measurements have substantially advanced our understanding of basic microbiological oceanographic processes, especially those regarding food-web dynamics and spatial and temporal distributions of living organisms. Since the first vertical profile appeared (Holm-Hansen and Booth, 1966), more than 200 papers have been published concerning specific applications of ATP measurements in the ocean, and more can be expected.

Although ATP concentrations vary with geographic location and depth (Figs 2.4–2.6),

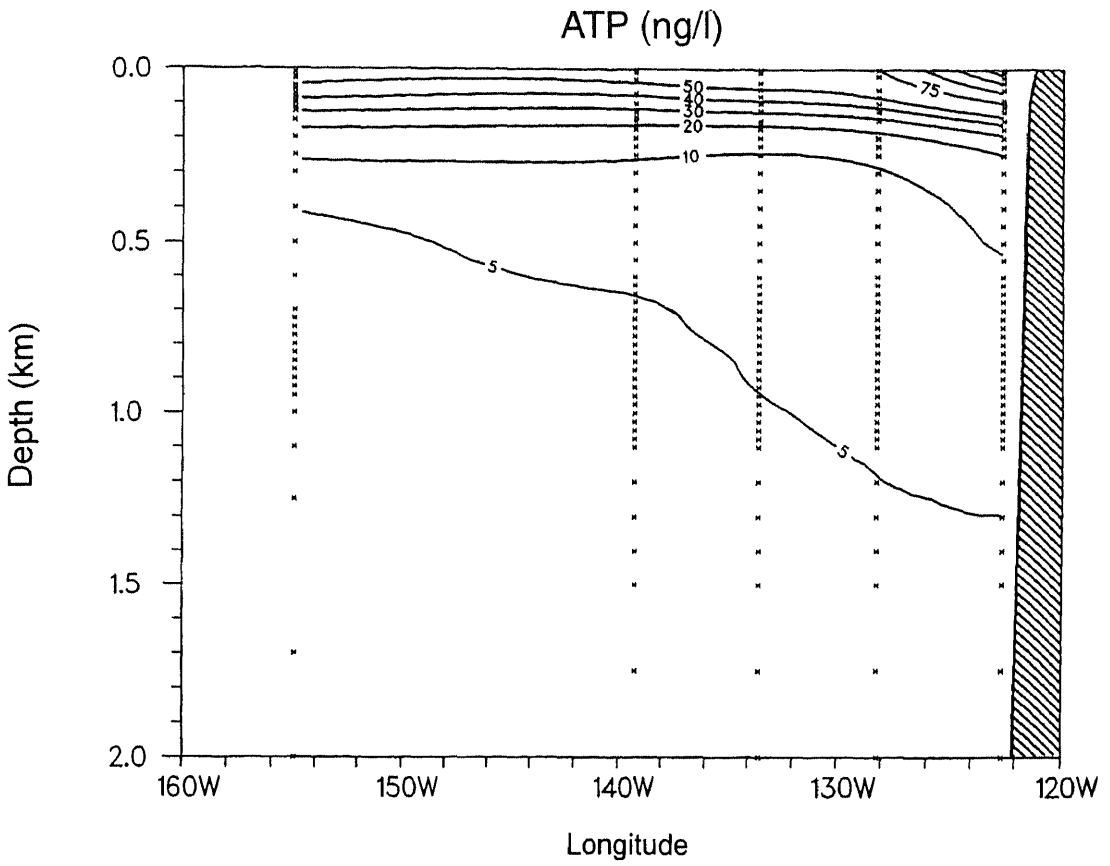


Fig. 2.4 Nearshore-to-offshore gradients in ATP concentrations off central California. Microbial biomass in nearshore waters is enriched by the flux of inorganic nutrients transported into the surface waters by coastal upwelling processes. The biomass enrichment is not restricted to the surface waters but is reflected in the mesopelagic zone (200–1000 m) as well. This enrichment in the subeuphotic zone microbial biomass is probably sustained by an increased flux of reduced carbon and energy from the euphotic zone in the form of sinking particulate matter.

there are characteristic features of the distribution of ATP in the ocean including the following. (1) Elevated ATP concentrations typically are found at the sea surface microlayer (0–150 μm depth), and may be the result of physical concentrating mechanisms or microbial growth. (2) Relatively high concentrations of ATP are present between 0 and 100 m, the high-productivity zone of the ocean. Frequently, a subsurface peak at or near the chlorophyll *a* maximum layer is also apparent. The concentration of ATP in the euphotic zone varies regionally and is usually positively correlated with photoautotrophic biomass and rates of primary production (Figs 2.4 and 2.5). Typically, near-surface ATP concentrations range from >500 ng ATP per liter for eutrophic waters, to 100–500 ng ATP per liter for regions with moderate productivity, and to <50 ng ATP per liter for oligotrophic portions of the ocean (Fig. 2.5). (3) Below the euphotic zone, the concentration of ATP is more variable but usually decreases rapidly with increasing water depth to a

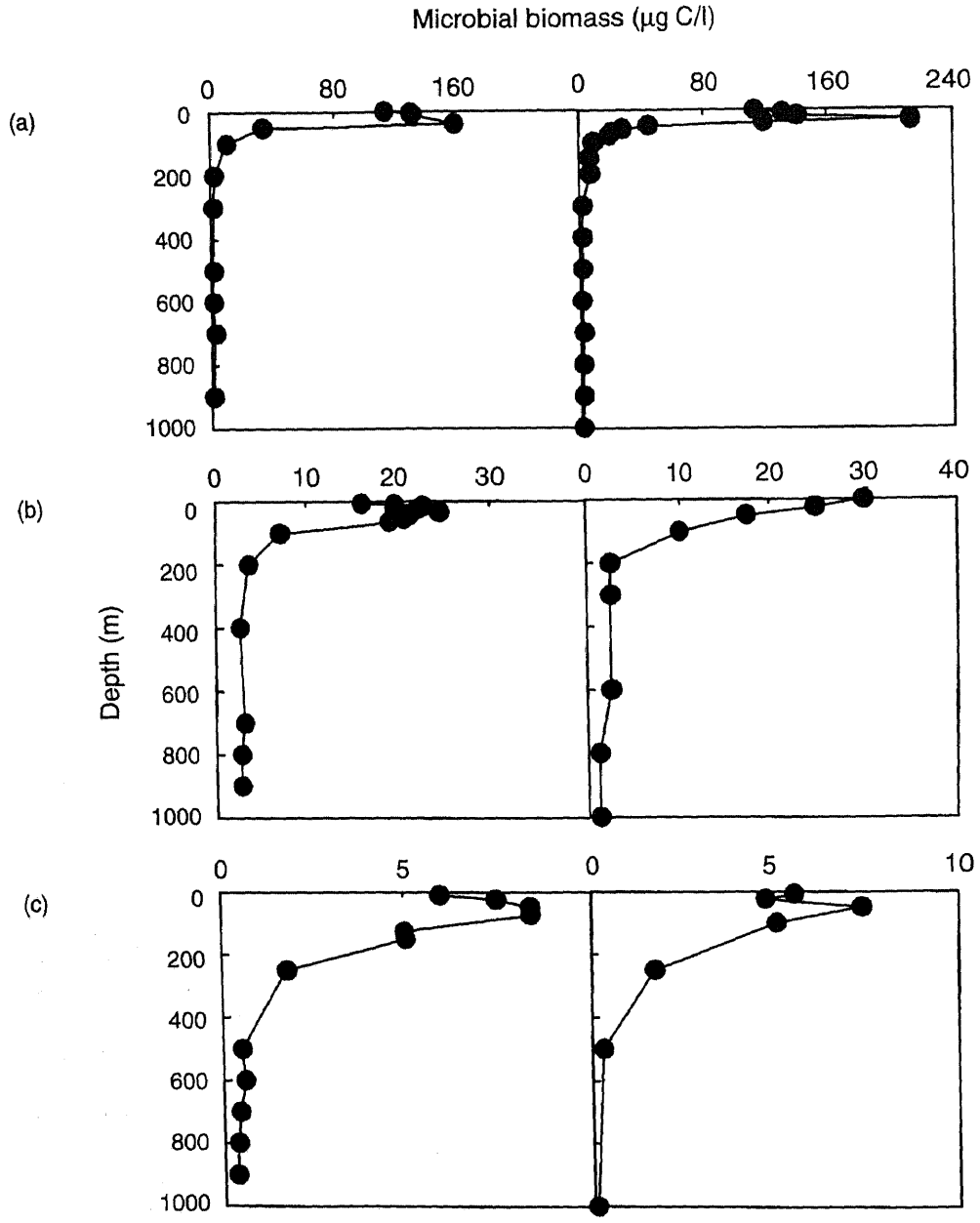


Fig. 2.5 Microbial biomass derived from ATP measurements assuming a C:ATP ratio of 250 (Karl, 1980) versus depth for several representative marine ecosystems. (a) (left) Pacific Ocean continental slope waters off central California at 123°45'N, 35°45'W (Karl and Knauer, 1984), (right) Atlantic Ocean continental slope waters off southwest Africa at 22°S, 12°W (S. Watson, unpublished). (b) (left) Eastern tropical Pacific Ocean at 18°N, 108°W (D. Karl, unpublished), (right) Mediterranean Sea at 42°12'N, 05°36'E (Laborde, 1972). (c) (left) Central North Pacific Ocean at 18°43.8'N, 156°50.4'W (Winn and Karl, 1984), (right) Central North Atlantic Ocean (Sargasso Sea) at 32°50'N, 62°30'W (Gordon *et al.*, 1979). Note scale changes in microbial biomass axes from coastal waters to the open ocean.

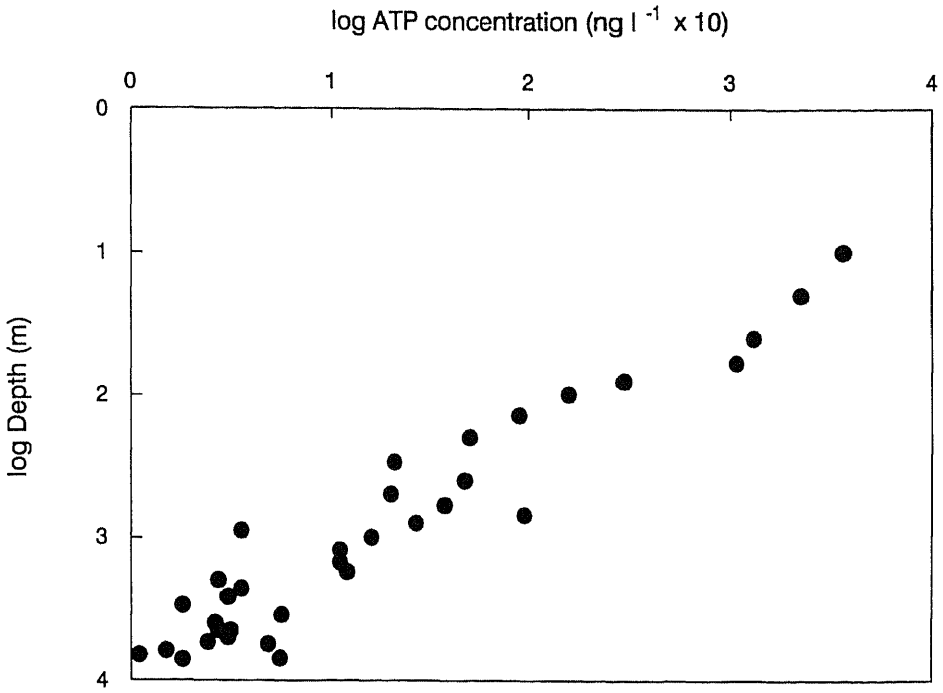


Fig. 2.6 ATP concentration versus depth for samples collected over the Aleutian Trench (50°53'N, 177°31'W) showing a systematic decrease in microbial biomass with increasing water depth. Even at great ocean depths (5000–7200 m), there is detectable ATP ranging from 28 to 140 mg C per m^3. (Redrawn from Holm-Hansen *et al.*, 1975.)

value <10% of the surface concentration by a depth of 400–600 m (Figs 2.5 and 2.6). (4) Below 400 m there is a more gradual decrease in ATP concentration to minimum values of approximately 0.5–2 ng ATP per liter regardless of the productivity of the surface waters or total water depth (Figs 2.4–2.6).

Superimposed on these geographic and depth variations in microbial ATP are seasonal changes that are especially evident in near-surface waters (0–200 m) of high-latitude regions. For example, the ocean waters surrounding the Antarctic Peninsula are characterized by a diverse and seasonally variable assemblage of microorganisms. During the spring–summer bloom of plankton, microbial biomass is equal to or greater than that found in any other nonpolluted coastal waters (Fig. 2.7). Total microbial biomass (assuming a C:ATP conversion factor of 250) of approximately 375 mg C per m^3 in surface waters is not uncommon. During winter, however, these same surface waters are among the most oligotrophic on earth with a microbial biomass of approximately 4 mg C per m^3 (Fig. 2.7). This very large seasonal change in epipelagic biomass has a major influence on the structure and function of the ecosystem and on associated biogeochemical fluxes, and emphasizes the importance of year-round investigations of the marine ecosystem.

Microbial biomass at deep-sea hydrothermal vents

The recent discovery and subsequent exploration of deep-sea hydrothermal vents revealed unexpected biomass-enriched, meta-

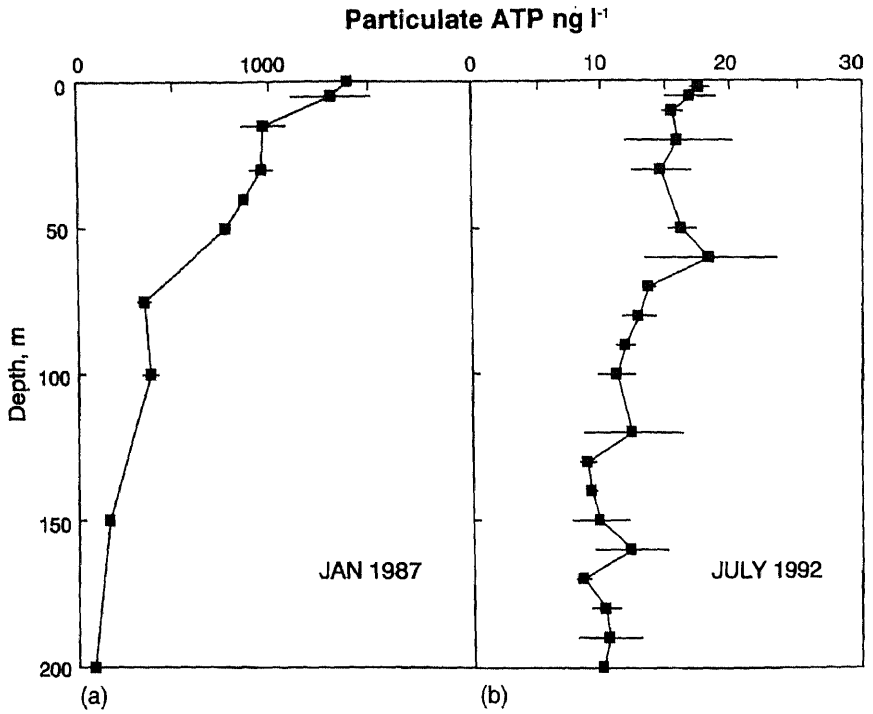


Fig. 2.7 Particulate ATP concentration profiles for a station located in Gerlache Strait, Antarctica. (a) Conditions during the spring–summer bloom in the 1986–87 austral summer showing surface ATP concentration of nearly 1500 ng/l (equivalent to a microbial biomass of approximately 375 mg C per m^3) and a steep concentration gradient with depth to a value of 15 mg C per m^3 at 200 m. These summertime ATP concentrations are among the highest measured in nonpolluted waters. (b) Conditions in winter for the same location showing a uniform depth distribution and a reduced microbial biomass of 2.5–4 mg C per m^3 throughout the upper 200 m of the water column. These wintertime ATP concentrations are among the lowest ever reported for a surface ocean ecosystem. (Redrawn from Tien *et al.*, 1992.)

bolically active microbial communities, and thus challenged dogma that life in the deep sea is limited by availability of reduced carbon and energy (Corliss *et al.*, 1979). This serendipitous discovery also casts doubt on a basic tenet of ecology that solar radiation is the sole source of energy for all biota. It was hypothesized prior to this discovery that the internal heat of our planet, produced and maintained through the radioactive decay of long-lived isotopes of uranium, thorium and potassium, provides an alternative energy source to incoming electromagnetic radiation (Kriss, 1963; Hutchinson, 1965), although the utilization pathway from radioactive decay (i.e. heat) to living organisms was not apparent. The discovery of deep-sea hydrothermal vents provides an interesting case study for testing this alternative-ecosystem hypothesis (Karl, 1995b) and ATP-based microbial biomass measurements are a key element of the test.

When the slightly turbid hydrothermal vent waters were sampled, filtered and viewed using brightfield, epifluorescence or electron microscopy, some of the turbidity was ascribed to the presence of bacterial cells (Corliss *et al.*, 1979). Initial estimates suggested that bacterial cell densities in vent fluids were approximately four orders of magnitude greater than in ambient deep-sea water.

Cell counts of bacteria in vent waters later collected from the Galapagos Rift were on the order of 5×10^5 to 1×10^6 cells/ml (Jannasch and Wirsen, 1979; Karl *et al.*, 1980), approximately two orders of magnitude lower than the first Galapagos Rift vent-water samples, but still elevated relative to deep-sea controls. Quantitative estimates based on ATP measurements from the Garden-of-Eden vent fluids (Galapagos Rift zone) exceeded those in the control deep water by a factor of >300,

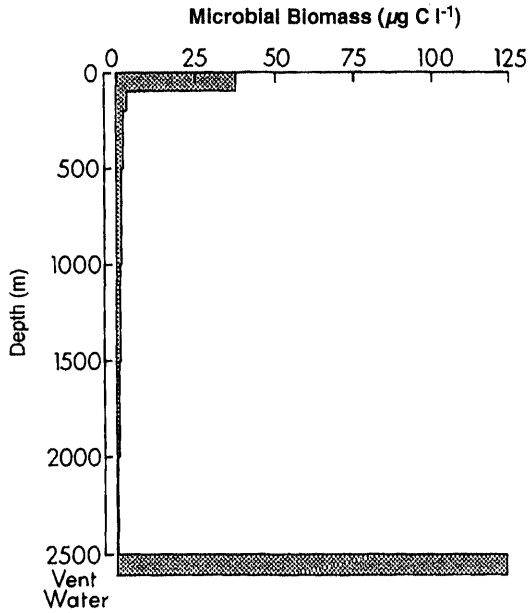


Fig. 2.8 A profile of microbial biomass, based on ATP measurements and expressed as biomass-C (ATP = 250), for the water column overlying the Galapagos Rift hydrothermal vents (Karl *et al.*, 1980). As expected, there is a large decrease in biomass with increasing water depth throughout the upper water column and mesopelagic zones. The vent waters themselves, however, are enriched nearly 300-fold relative to the deep-sea water and are enriched fourfold compared to the productive surface ocean waters. These were the first data to suggest that hydrothermal vent ecosystems sustain large, viable microbial populations that could be uncoupled from the solar energy previously thought to sustain all life on Earth. (Redrawn from Karl, 1987.)

and were approximately fourfold greater than ATP concentrations measured for photosynthetically active surface waters (Karl *et al.*, 1980; Fig. 2.8).

Subsequent microbial biomass investigations of geographically distinct hydrothermal vent ecosystems at 11° and 21°N along the East Pacific Rise, at Guaymas Basin and at the Endeavour Ridge, consistently revealed an elevated microbial biomass relative to ambient bottom seawater (data summaries presented in Karl, 1987, 1995b). Biomass enrichment factors (i.e. ATP in vent water: ATP in ambient deep-sea water) ranged from <3 to >500 for 23 vent samples. Total microbial biomass and metabolic activity were more elevated in mesophilic (20–50°C) than in thermophilic (>80°C) habitats (Karl, 1985), and high-temperature vents (>110°C) contained negligible concentrations (<5 ng/l) of ATP (Karl *et al.*, 1988b).

These cell number and biomass data confirm the presence of bacteria in hydrothermal vent fluids and support the hypothesis of localized microbial production in the deep sea. The maximum ATP concentrations of 0.5–1 μg/l (equivalent to 125–250 μg C per liter) measured at different hydrothermal vents suggest that biomass production is similar for all hydrothermal vent fields.

Deep-sea hydrothermal vents provide access to a vast subsurface habitat where elevated microbial biomass and accelerated biogeochemical transformations could occur. Although there is uncertainty over the size (volume) of this subseabed habitat, estimates suggest that the total habitable pore space in the Earth's lithosphere is 2×10^7 km^3, assuming that life exists to a maximum temperature of 110–150°C (~5 km deep) and an average porosity of 3% (Gold, 1992). If completely filled with water, this interstitial volume is 2% of the global ocean volume, or approximately equal to 10% of the volume of seawater contained in the abyssopelagic zone (i.e. ocean depths >4000 m). Although there are few measurements of microbial biomass in the

lithosphere (mainly from deep-core drilling), if the average standing stock were an order of magnitude greater than that measured in the deep abyss (as the hydrothermal vent ATP data suggest), then microbial biomass in the hot biosphere, by our estimation, could approach 10^{13} g C. This extrapolation, based on data collected at deep-sea hydrothermal vents, is four orders of magnitude lower than another estimate (Gold, 1992), which is based on the assumption that bacteria are 1% of the total mass of the lithospheric pore water habitat. In the absence of additional field data, neither extrapolation can be rejected. Regardless of the total biomass, the mere presence of microorganisms in the deep, hot biosphere deserves attention as a new habitat.

Microbial biomass as a percentage of total particulate carbon

As discussed previously, the presence of nonliving particulate organic matter precludes the direct measurement of microbial biomass from total POC determinations. One application of ATP as a total biomass indicator is to assess microbial biomass C relative to total POC. Studies revealed that between 30% and 80% of the total POC within the euphotic zone is living C, with the higher values corresponding to eutrophic ecosystems (e.g. Table 2.4). The proportion decreases to <10% at depths exceeding 250 m (Table 2.4). From the perspective of global carbon cycles, it is imperative to focus on the formation and utilization processes of these dominant particulate (and dissolved) detrital pools in the sea for a complete understanding of carbon-pool dynamics.

2.6.5 CHLOROPHYLL *a*

A common feature of photosynthetic microorganisms in the sea, both Bacteria and Eucarya, is chlorophyll *a* (chl *a*), which functions as a major light-harvesting pigment. This observation provides the primary justification for estimating photoautotrophic biomass from measurements of chl *a*. Chl *a* can be easily extracted from particulate matter using acetone or methanol and measured with a high level of precision and with adequate sensitivity to obtain quantitative determinations (Holm-Hansen *et al.*, 1965; Holm-Hansen and Riemann, 1978). Although chl *a* can be measured by fluorometry, spectrophotometry or HPLC, fluorometry is widely used in oceanographic field studies. HPLC methods can isolate and quantify the entire pigment-biomarker spectrum, including the major chlorophylls (monovinyl and divinyl chl *a*, monovinyl and divinyl chl *b*, chl *c*), carotenoids and most other accessory pigments (Mantoura and Llewellyn, 1983; Latasa *et al.*, 1996). Such a detailed analysis is essential when using pigment signatures for taxonomic characterization of the photoautotrophic microbial assemblage (Letelier *et al.*, 1993). Intercomparisons among spectrophotometric, fluorometric and HPLC methods yield comparable results, but with systematic offsets depending upon the source materials analyzed (Murray *et al.*, 1986; Pinckney *et al.*, 1994). The most time-consuming and expensive method, HPLC, is the most accurate.

A logical extension of the *in vitro* fluorometric determination of chl *a* in particulate matter concentrated from discrete seawater samples was the introduction of *in vivo* methods for continuous measurements (with depth, space or time) of chl *a* in the ocean (Lorenzen, 1966). This concept evolved into *in situ* instrumentation for the rapid profiling of fluorescence (Strickland, 1968; Kiefer, 1973) and for unattended measurements from buoys (Dickey, 1990), with results that have greatly improved our understanding of photoautotrophic processes in the sea. Although the *in vivo* measurement techniques are less precise and probably less accurate estimators of total chl *a* (and hence, photoautotrophic biomass) than the *in vitro* chl *a* extraction techniques, they do provide

Table 2.4 Distribution of particulate ATP and organic carbon (POC) in representative coastal and oceanic habitats

Station location and type	*Water depth (m)*	*ATP concentration (ng/l)*	*Microbial biomass*[a] *(mg C/m³)*	*POC concentration (mgC/m³)*	*Percentage living (biomass/POC × 100%)*
Coastal	10	126	31.5	79	39.9
California[b]	30	357	89.3	120	74.4
(33°18.5'N,	103	17.3	4.3	33	13.0
118°40'W)	183	6.1	1.5	51	2.9
	200	10.7	2.7	44	6.1
	315	6.4	1.6	40	4.0
	500	6.4	1.6	57	2.8
	722	4.0	1.0	45	2.2
	1025	0.5	0.13	20	0.7
North Atlantic,	10	22.4	5.6	15.9	35.2
open ocean[c]	25	19.2	4.8	18.3	26.2
(32°50'N,	50	29.6	7.4	16.5	44.9
62°30'W)	100	20.4	5.1	17.8	28.7
	250	6.8	1.7	22.8	7.5
	500	1.2	0.3	8.0	3.8
	1000	0.4	0.1	6.7	1.5
	2500	0.4	0.1	4.6	2.2
	3500	1.2	0.3	4.2	7.1

[a]ATP concentration × 250.
[b]Selected data from Holm-Hansen and Booth (1966).
[c]Selected data from Gordon *et al.* (1979).

continuous biomass measurements that are not possible with any other molecular biomarker procedure.

One of the most impressive achievements in modern biological oceanography has been the successful measurement of ocean color from space (Hovis *et al.*, 1980). Earth-orbiting detectors relate the remotely sensed upwelling radiance signal to chl *a* concentration and, hence, to photoautotrophic microbial biomass, thereby giving a truly synoptic view of the surface ocean on a global scale (Gordon *et al.*, 1980). Data sets obtained by the Coastal Zone Color Scanner (CZCS) have been used to study (1) both small- (>50 km) and large-scale (ocean basin) distributions of photoautotrophic microorganisms, (2) seasonal biomass fluctuations, including the timing and evolution of vernal and autumnal phytoplankton blooms, and (3) the effects of mesoscale ocean-circulation processes on the distribution and abundance of photosynthetic organisms.

The half-life of chl *a* after cell death is not well studied. It is generally believed that chl *a* is rapidly decomposed by a combination of bacterial degradation and, at least in near-surface waters, by photochemical processes. Nelson (1993) estimated a half-life of approximately 10 days for detrital pigments, assuming that the primary removal mechanism is photooxidation. The presence of cell-free chl *a* is a potential limitation to its use as a biomass-indicator molecule.

The recent study of Bianchi *et al.* (1995) documented the presence of chl *a*, chl *b* and other pigment biomarkers in the high molecular weight DOC pool (>1000 Da) of the Gulf of Mexico. Although the amount of pigment per unit C was much lower in the DOC pool compared to POC (implying either a selective retention of pigments by living photoau-

totrophs or a rapid decomposition rate of pigments following cell death or predation), the total amount of detrital pigments exceeded those in living organisms due to the high DOC:POC ratio characteristic of the marine environment. If these detrital pigments were concentrated with living microorganisms during sample processing, photoautotrophic biomass might have been overestimated. This concern is likely to be larger for *in vivo* and satellite remote-sensing applications than for discrete sample collections, which are usually filtered to concentrate the particulate signal, thereby reducing the dissolved chl *a* interference.

The primary limitation of this biomarker approach is that the chl *a*:C ratio can vary significantly among algal species and within a given organism as a function of many factors, including time, cell size, temperature, nutrient conditions, growth rate, and, especially, light history (reviewed by Falkowski, 1981). Variations of 26-fold have been observed for the chl *a*:C ratio of a single algal species as a function of environmental conditions and growth rate (Laws *et al.*, 1983). Consequently, reliable extrapolation of chl *a* concentrations to photoautotrophic biomass is dependent upon the availability of additional information on species composition, nutrient concentration and limitation, light levels and growth rate.

One approach used in aquatic field studies establishes the relationship between POC and chl *a* for a given set of samples and estimates the mean *in situ* chl *a*:C ratio from the slope of the regression line. Banse (1977) evaluated the assumptions inherent in this method and discussed its problems and limitations. Eppley *et al.* (1977) calculated *in situ* chl *a*:C ratios using a variety of methods for samples collected in the Southern California Bight. Their estimates ranged from 3.5 to 125 mg chl *a* per g C, with systematic variations in response to seasonal changes in temperature and irradiance.

Redalje and Laws (1981) described a novel method for estimating photoautotrophic biomass C in mixed assemblages of microorganisms. The method requires a 6–12 h incubation with ^{14}C–HCO_3^-, followed by chl *a* extraction, isolation and determination of the specific radioactivity (i.e. Ci/g C in chl *a*) as well as an independent measurement of total radioactivity (Ci) incorporated into the total photoautotrophic carbon pool. After an appropriate incubation period, the specific activity of the chl *a* carbon pool is equivalent to the specific activity of the total phytoplankton carbon pool. The total biomass C can then be calculated by dividing the total ^{14}C activity in the photoautotroph fraction by the specific activity of the chl *a* pool. The unique distribution of chl *a* in photoautotrophs and the obligate association of chl *a* with living cells confers a high degree of selectivity on this method. Redalje (1983) compared the ^{14}C incubation method with biomass estimates derived from microscopic enumeration and sizing of the dominant photoautotrophic cells. The direct microscopic values ranged from 89% to 193% (mean = 146%) of the ^{14}C incubation method for six samples from Southern California coastal waters.

Regional distributions of chl *a* in the North Pacific Ocean

One of the most comprehensive ship-based studies on the large-scale distribution of chl *a* and accessory pigment-biomarker compounds was conducted during two trans-Pacific cruises, one along 24°N and the other at 47°N (Ondrusek *et al.*, 1991). The cruise tracks crossed several major hydrographic features (California Coastal Current, North Pacific gyre, Kuroshio Current, Subarctic gyre and North Pacific current) and their transition zones. Although the HPLC pigment signatures obtained on these cruises provided a comprehensive data set on the regional variations in major groups of photosynthetic microorganisms, only the chl *a* (biomass) data are discussed here.

A comparison of the zonal changes in chl *a*,

temperature and nitrate concentrations for each transect revealed several consistent basin-wide patterns (Fig. 2.9). For the 24°N transect, upwelling of cold, nutrient-rich waters near the California Current (122°W longitude) resulted in a large increase in the upper water column (0–50 m) concentration of chl *a* to values in excess of 100 ng/l (Fig. 2.9). A smaller increase in surface nutrients and chl *a* concentrations was also observed in the Kuroshio Current at approximately 130°E longitude. Throughout the remainder of the transect, a horizontal distance of more than 800 km, the near-surface chl *a* concentrations were homogeneous at 20–40 ng/l (Fig. 2.9). Below 50 m, however, a different pattern was observed. First, a well-defined and relatively large chl *a* maximum layer at 100–125 m was evident throughout the transect. It should be emphasized that most of the total euphotic zone chl *a* is located below, not above, the 50 m depth horizon. This is an important point to remember because chl *a* estimates based on satellite remote-sensing techniques do not detect chl *a* at depths below 25–30 m, even in the clearest ocean waters. The horizontal variability in chl *a* concentrations in the subsurface maximum is much greater than in the

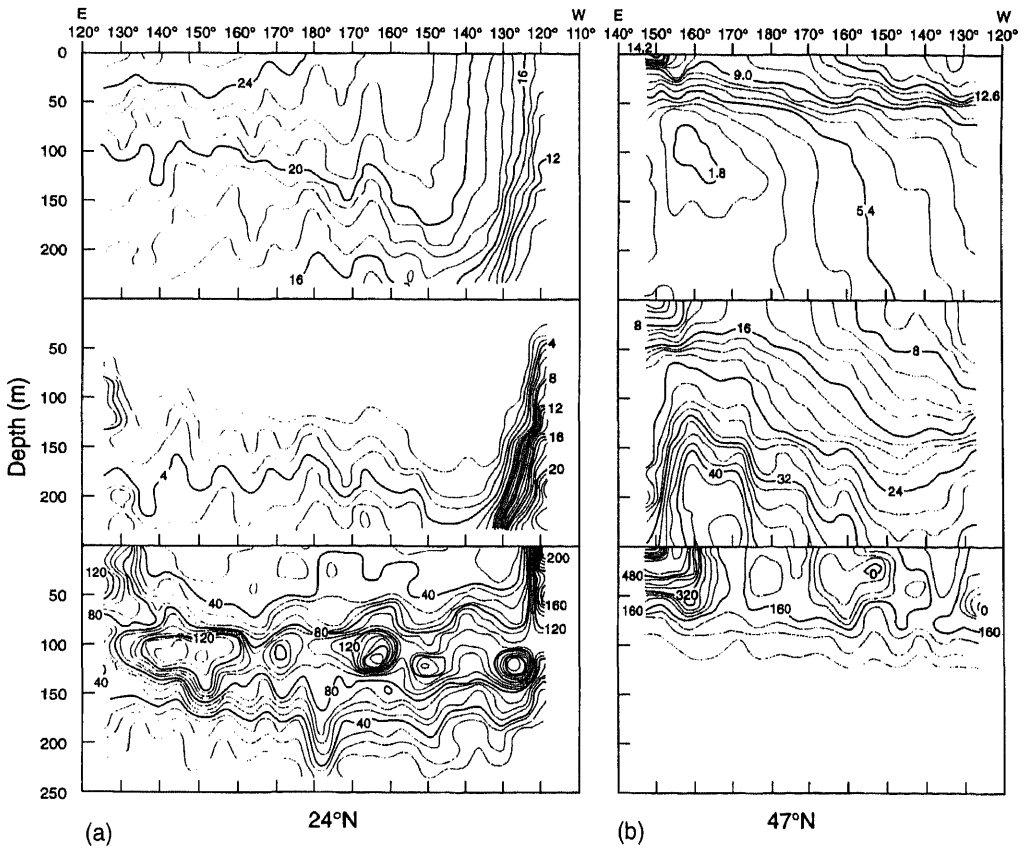


Fig. 2.9 (a) Distributions of (top) seawater temperature (°C), (middle) nitrate (μmol/l) and (bottom) chl *a* (ng/l) for a North Pacific Ocean transect along 24°N latitude. (b) As above, except for transect at 47°N latitude. (Redrawn from Ondrusek *et al.*, 1991.)

surface waters and is controlled by independent processes. Of particular interest were the elevated pigment concentrations at 165°W longitude, near the Hawaiian Ridge (Fig. 2.9). A similar pattern of chl *a* west of the Hawaiian Ridge was noted by Venrick during an independent trans-Pacific survey along 24°15'N (Venrick, 1990). She suggested that high chl *a* concentrations may be related to nutrient injections associated with cyclonic eddies.

At 47°N latitude, chl *a* depth distributions were fundamentally different from those observed along the 24°N transect (Fig. 2.9). These colder and more nutrient-enriched waters sustain a higher concentration of near-surface chl *a* (>100 ng/l) and have a shallow and less well-defined subsurface chl *a* maximum layer. The summertime (August–September) chl *a* values at this latitude are near the maximum annual values observed for seasonally phased, high-latitude ecosystems. There was also a strong zonal gradient in surface chl *a*, with greatest concentrations in the western Pacific associated with the Kuroshio extension (Fig. 2.9). A principal-component analysis of these transect results, which included data on chl *a* and accessory pigment biomarkers, defined three ecosystems that corresponded to specific hydrographic characteristics: low biomass (oligotrophic), high biomass (coastal and upwelling) and transitional regions (Ondrusek *et al.*, 1991).

Finally, Chavez *et al.* (1995) have compiled an extensive regional data set for the Pacific Ocean basin from 60°S to 50°N latitude, including samples collected from equatorial upwelling regions, nutrient-poor mid-latitude subtropical gyres and nutrient-enriched high-latitude zones. When expressed as total depth-integrated (euphotic zone) chl *a* concentrations, they observed a basin-wide variability of approximately six-fold in the total chl *a* with values ranging from <10 mg/m^2 in the subtropical gyres to >50 mg/m^2 at the equator (Fig. 2.10).

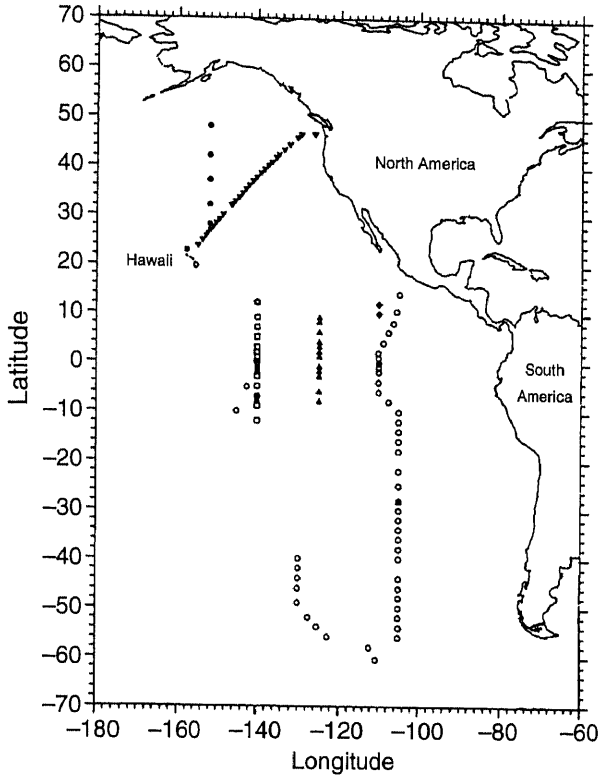
Seasonal variability of chl *a* in the subtropical North Pacific Ocean

The spring bloom of photoautotrophic microorganisms in high-latitude regions of the ocean is well characterized and understood in terms of the critical-depth model (Sverdrup, 1953). This predictable, physically driven event plays an important role in food-web dynamics and in the breeding and reproductive cycles of higher organisms dependent on plankton as a source of nutrition. Even aspects of the ecology of deep-sea abyssal communities appear to be tied into this annual maximum in organic biomass production and particle export.

The intensities of seasonal cycles in light, temperature and upper ocean turbulence, however, decrease at lower latitudes and a coherent seasonality in subtropical and equatorial regions is more difficult to demonstrate. On the basis of satellite remote-sensing observations, Yoder *et al.* (1993) reported that chl *a* concentrations in the subtropical region of the North Pacific display an annual cycle with a maximum chl *a* in winter.

Winn *et al.* (1995) re-evaluated the seasonality of chl *a* in the North Pacific gyre with a focus on the ecological control mechanisms. Using a 5-year data set collected as part of the Hawaii Ocean Time-series program at Station ALOHA (22°45'N, 158°W), which included *in vivo* and extracted chl *a* concentrations as well as estimates of chl *a* per cell measured using a dual-laser flow cytometer, they detected two opposing annual cycles (Fig. 2.11): (1) an upper ocean (0–50 m) chl *a* concentration maximum in winter and (2) a lower water column (100–175 m) chl *a* concentration increase in spring, with a minimum in winter. The winter chl *a* increase, observed previously by remote sensing of ocean color, is due to photoadaptive changes in chl *a* per cell, rather than a result of increased photo-autotrophic biomass. In the lower water column, both chl *a* and photoautotrophic biomass increase as a consequence

(a)



(b)

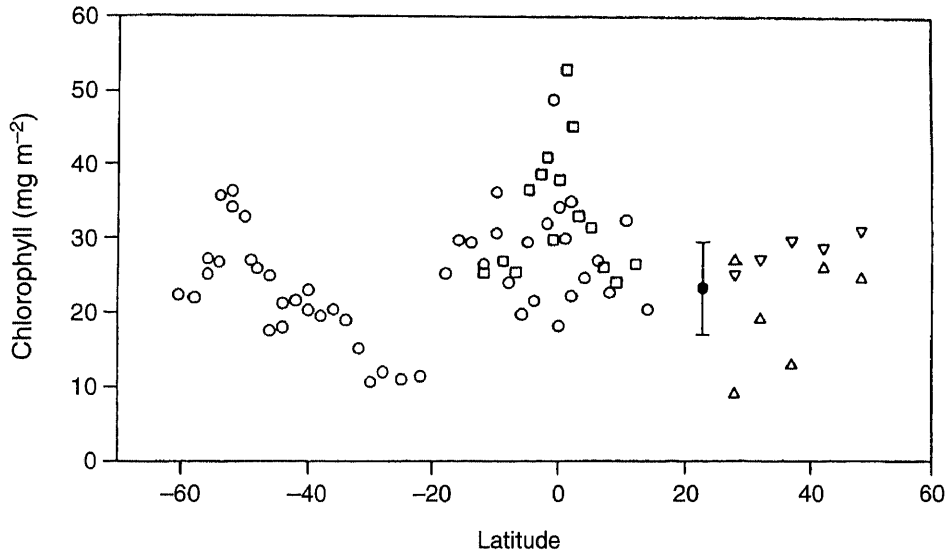


Fig. 2.10 (a) Map showing the locations of stations where chl *a* observations have recently been made. (b) Euphotic zone depth-integrated chl *a* from 50°N to 60°S in the Pacific Ocean. Different symbols refer to separate field studies. The solid circle with error estimates is the mean (±1 standard deviation) of the 4-year data set from Station ALOHA located at 22°45'N, 158°W. (Redrawn from Chavez *et al.*, 1955.)

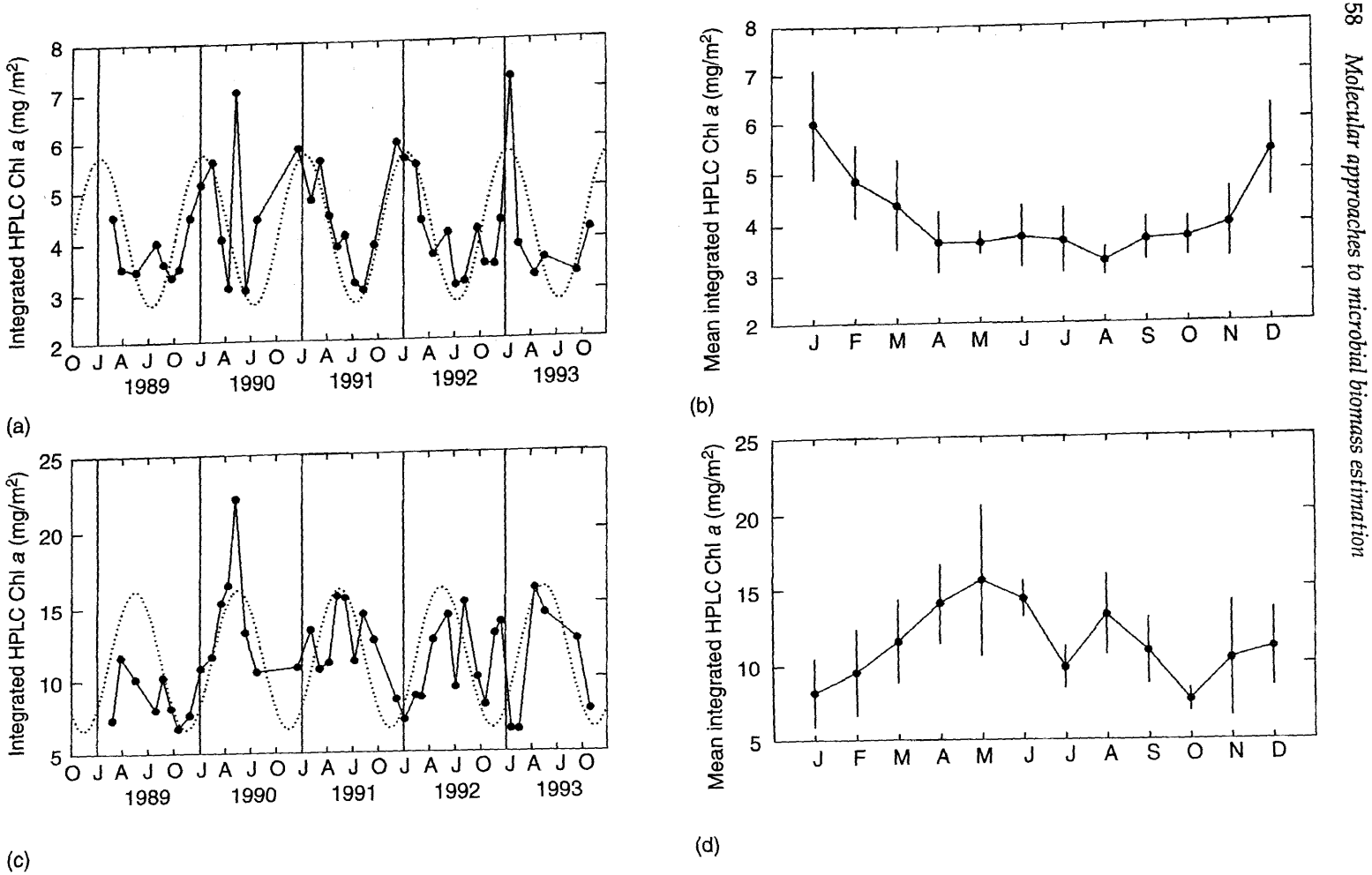


Fig. 2.11 (a) Time series of depth-integrated chl *a* (in mg/m²) in the upper euphotic zone (0–50 m), from October 1988 through October 1993 at Station ALOHA. (b) Mean values for the time series binned into monthly intervals with error bars representing the standard deviation of the binned values. (c) Corresponding time series of the depth-integrated chl *a* (in mg/m²) for the lower euphotic zone (100–175 m). (d) Mean values for monthly intervals, as in (b), above.

of increased light intensity in spring and summer (Winn *et al.*, 1995).

These results are a reminder that molecular biomass indicators are only useful when the extrapolation factors are well constrained. In addition to a comprehensive on-site evaluation of the accuracy of the extrapolation factors, one must also establish variability in the extrapolation factors with time (season) and depth.

Interannual variability of chl *a* in the subtropical North Pacific Ocean

With increased interest in patterns of global environmental variability and the potential for human-induced environmental change, researchers have begun to focus on interannual and decadal trends in ecosystem processes. Venrick *et al.* (1987) reported that the summertime (May–October) chl *a* concentrations in the central North Pacific Ocean had nearly doubled since 1968 (Fig. 2.12). An extension of these subtropical North Pacific Ocean observations using data collected during the Hawaii Ocean Time-series (HOT) research program (Karl and Lukas, 1996) confirms their observations of higher chl *a* concentrations in recent years. Venrick *et al.* (1987) hypothesized that one potential mechanism for this decade-long accumulation of photoautotrophic biomass was a change in large-scale atmospheric forcing of the upper ocean, which resulted in an increased flux of nutrients into the euphotic zone. If supported by other retrospective analyses, these changes in the carrying capacity of one of the dominant (by size) ecosystems on our planet may have important consequences for the global carbon cycle and, especially, for carbon sequestration in the ocean.

2.6.6 LIPIDS

Lipids are major constituents of all living cells. As a class of biomolecules, lipids have enormous structural diversity that, in part, reflects phylogenetic diversity. Consequently, lipid analysis offers an alternative or complement to rRNA-based taxonomic investigations (Findlay and Dobbs, 1993). Lipids are diagnosed on the basis of solubility, more so than chemical composition or function within the cell. Consequently, lipids are a chemically heterogeneous group that includes fats, phospholipids, steroids, isoprenoids and poly-β-hydroxyalkanoate (Stanier *et al.*, 1986).

All microorganisms are bounded by a cytoplasmic membrane composed of lipid and protein. However, the similarity ends there. A dichotomy exists in the chemical composition of lipids from Bacteria and Eucarya membranes compared to those isolated from Archaea. Whereas Bacteria and Eucarya contain phospholipid ester-linked fatty acids (PLFA), membrane lipids in Archaea consist primarily of two C_{20} isoprenoid-branched hydrocarbon side-chains that are ether-linked to a glycerol backbone (Tornabene and Langworthy, 1979). Other, less spectacular differences in the structure of membrane lipids can be used to distinguish Bacteria from Eucarya. The largest data set on bacterial lipids is based on their fatty-acid composition, and these biomolecules have been used extensively in taxonomic studies (Lechevalier, 1989).

At least three distinct groups of lipids, all components of the cell membrane, have been suggested as molecules useful for estimating microbial biomass: (1) total phospholipid, (2) lipopolysaccharide (LPS) and (3) ergosterol. In addition to their utility as indicators of microbial biomass, certain lipids (so-called signature lipids) have been used to detect the presence of specific groups of microorganisms (e.g. sulfate-reducing bacteria; Dowling *et al.*, 1986), to ascertain the nutritional status of microbial populations, to detect unbalanced growth and to quantify changes in microbial community structure (Guckert *et al.*, 1985). Consequently, analysis of the full lipid spectrum recovered from a one-phase solvent extraction can provide useful information regarding microbial processes in nature (White, 1994).

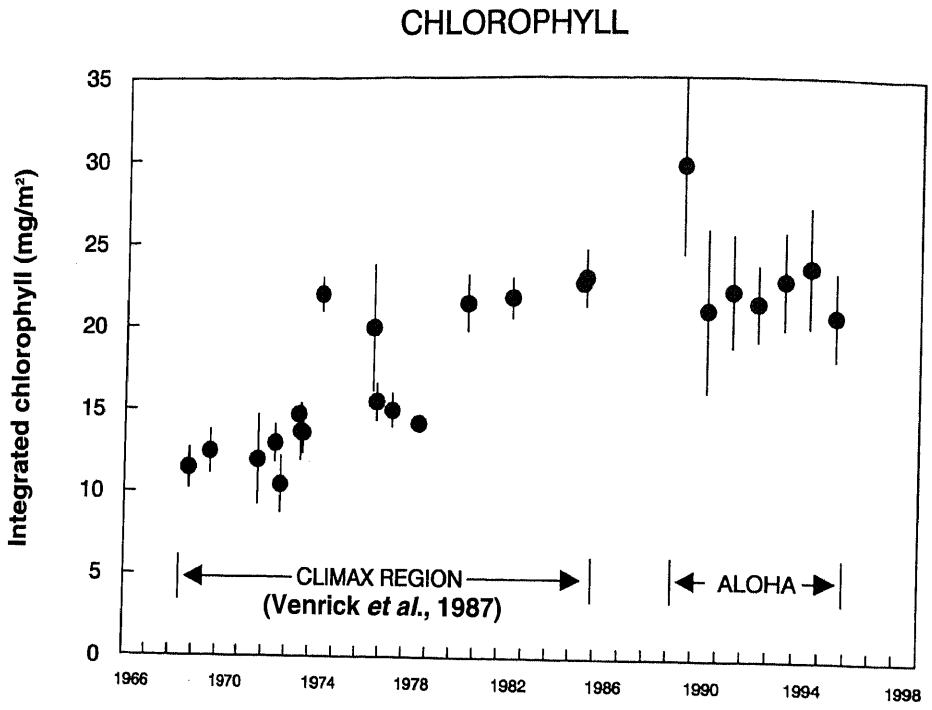


Fig. 2.12 Time series of euphotic zone depth-integrated chl *a* (mg/m²) in the subtropical North Pacific for the period 1968–present based on observations in the CLIMAX region (near 28°N, 155°W; redrawn from Venrick *et al.*, 1987) and at Station ALOHA (22°45'N, 158°W). The bars indicate the 95% confidence intervals for CLIMAX data and annual means (±1 SD) for the HOT program data set. The approximately two-fold 'change' in chl *a* content of the subtropical gyre waters described by Venrick *et al.* (1987) has continued through the 1990s.

In this section we shall discuss the application of two lipid-based, microbial biomass indicators: (1) phospholipid-phosphate (PL-P) for the determination of total microbial biomass, and (2) ergosterol for the determination of fungal biomass. The measurement of LPS as an indicator of Gram-negative bacterial biomass will be presented in the following section.

Phospholipids

Phospholipids are ubiquitous in Bacteria and Eucarya and present to a lesser extent in Archaea, so biomass estimation based on phospholipid analysis is a nonselective measure of total microbial biomass. Phospholipids function primarily as structural components of the membrane and are not involved in energy storage. Consequently, the concentration of phospholipids (relative to total cell carbon) is relatively constant regardless of environmental conditions or growth rate (White *et al.*, 1979b). The turnover time of phospholipids following cell death is rapid (2–10 days), at least in aerobic sedimentary habitats, but lipid turnover has not been investigated in the more dilute, pelagic ecosystem.

Phospholipids are easily separated from other cell components because of their polar nature; although soluble in organic solvents,

they have an associated hydrophilic group at one end. Several different types of phospholipids are present but, for the purpose of biomass estimation, the measurement of 'total phospholipid' is preferred (White *et al.*, 1979b). Phospholipids are efficiently extracted from cells using a modified Bligh and Dyer (1959) procedure (single phase chloroform:methanol:buffer extraction), and can be quantified by measuring the amount of phosphate released from the extract's polar-lipid fraction following sample hydrolysis (White *et al.*, 1979b; Findlay *et al.*, 1989). Although this technique has been applied almost exclusively to estimate microbial biomass in sedimentary habitats, samples from other habitats should be easily accommodated.

As with all other molecular biomarkers, the accuracy of the conversion from the compound of interest (in this case total phospholipid) to microbial biomass C is critical. Based on a compilation of PL-P:C ratios that have been obtained for a variety of different groups of Bacteria and Eucarya, a PL-P value of 50–150 μmol/g microbial C appears to be most appropriate for applications in marine microbial ecology (Dobbs and Findlay, 1993).

The lower limit of detection for the colorimetric determination of phosphorus is approximately 0.5 nmol P, even when using high extinction coefficient dyes such as malachite green. Consequently, for a typical subeuphotic zone seawater sample containing a microbial biomass of 1–2 μg C per liter, one would need to concentrate the particulate matter from at least 1 to 10 l for a single PL-P determination, assuming a conversion factor of 100 μmol PL-P per g cell C (Dobbs and Findlay, 1993). Difficulties encountered with large sample collection and processing may be prohibitive.

The analysis of phospholipid ester-linked fatty acids (PLFA) derived from the membrane-lipid fraction can be used as a more sensitive measure of total biomass. However, Archaea are not detected by this procedure (see below). In this analysis, the PL fraction is subjected to mild alkaline methanolysis, and purified by thin-layer chromatography, and then PLFA are detected and identified by gas chromatography–mass spectrometry or, potentially, tandem mass spectrometry (Baird and White, 1985; Tunlid *et al.*, 1989). Although this procedure is much more sensitive than the PL-P assay, it is also more time-consuming and costly. PLFA analysis has been successfully employed to estimate the biomass of microorganisms at Station ALOHA. The results, obtained from <10 l of seawater per sample, indicated a steep concentration gradient with depth with PLFA values ranging from 500–900 pmol/l in surface (0–200 m) waters to 70 pmol/l at 1000 m (F. Dobbs, unpublished data). The measurement of palmitic acid, a major fatty acid in most microorganisms, has also been suggested as a surrogate for total biomass (Federle *et al.*, 1983).

Like ATP, PL-P or PLFA values are 'nonspecific' measures of microbial biomass, which is both an advantage (universal distribution of PL in all microorganisms) and a disadvantage, depending upon the objectives of the particular field of study. When used in conjunction with signature lipids, a comprehensive description of the total microbial assemblage is possible (White, 1994). Finally, methods have also been developed to measure PL synthesis using either $^{32}PO_4$ or [1-^{14}C] acetate as precursors, so measurements of microbial activity and growth are also possible (White *et al.*, 1977).

The procedure to assess the PLFA of Bacteria and Eucarya does not effectively isolate the ether-linked lipids of Archaea. Stronger hydrolysis conditions are necessary to break the ether bonds before measurement using gas chromatography (Nichols *et al.*, 1993). A sequence of mild and strong hydrolyses, therefore, can provide a biochemical assay specific for Archaea biomass (Dickens and Van Vleet, 1992; Hedrick *et al.*, 1992).

Ergosterol

Ergosterol is a principal component of the membranes of fungi and has been used to estimate total fungal biomass in diverse environments (Lee *et al.*, 1980; Newell *et al.*, 1988; Nylund and Wallander, 1992). Originally, this procedure involved sample extraction in methanol, saponification, partial purification by column chromatography and final detection by HPLC (Seitz *et al.*, 1977). Over the years, several improvements have been introduced (see Newell *et al.*, 1988; Nylund and Wallander, 1992; Newell, 1993). Although other microorganisms also contain ergosterol (e.g. eukaryotic green algae, protozoans; Newell, 1993), the principal application of this method has focused on decomposing plant litter where fungi are known to be a large proportion of total microbial biomass. More recently, Newell and Fallon (1991) have extended the scope of this procedure to include ergosterol synthesis and, hence, specific growth rates of the fungal community.

Unfortunately, the extrapolation factors to convert ergosterol concentrations to fungal carbon are not yet well established. The majority of data available from diverse culture studies indicate ergosterol levels of approximately 1–10 mg/g fungal tissue (Newell *et al.*, 1987; Nylund and Wallander, 1992; Gessner and Chauvet, 1993). The ecological role of fungi as decomposer organisms in coastal marine habitats is well established. However, in open-ocean ecosystems, the absence of vascular plant litter may preclude fungal decomposition as a major pathway of carbon and energy flow. Under these conditions, filamentous fungi are not expected to be a large proportion of the total microbial biomass. Much less research has been conducted on the quantitative distribution and abundance of yeasts (unicellular fungi), despite the fact that they are ubiquitous in the world ocean (Kohlmeyer and Kohlmeyer, 1979).

2.6.7 LIPOPOLYSACCHARIDE

Early comparative studies of the cell walls of Gram-negative and Gram-positive bacteria revealed fundamental differences in composition, form and function (Fig. 2.13). Fore-most among these differences is the presence of an outer membrane containing lipopoly-saccharide (LPS) in gram-negative bacteria, and a much more dense cell wall (i.e. murein sacculus) in gram-positive bacteria. Peptido-glycan, an integral polymer in bacterial cell walls, can vary from 1–10% of the dry weight of the wall in gram-negative cells to approximately 50% in gram-positive bacteria (Cummins, 1989). The chemical composition of the peptidoglycan fraction and general molecular architecture of the cell wall, however, is similar in both groups.

LPS is a characteristic component of the cell wall of all gram-negative bacteria and some cyanobacteria (Mayer *et al.*, 1985). It is localized in the center portion of the outer membrane (Fig. 2.13), where it contributes to cell integrity. Injected into higher organisms, purified LPS causes fever, shock and even death (Mayer *et al.*, 1985). For this reason, LPS is termed an 'endotoxin'. The toxic effect resides with the lipid A moiety of LPS, not with the polysaccharide. Distinct chemical architectures of LPS reflect genetic differences, so measurements of the specific lipid A structures are used to reveal biodiversity or community structure.

The measurement of LPS is used as an indicator of 'bacterial' biomass in the marine environment (Watson *et al.*, 1977; Watson and Hobbie, 1979). This assay is valid when gram-negative bacteria dominate the total bacterial biomass. It is important to emphasize that the bacterial biomass in surface waters of many open-ocean ecosystems is dominated by photoautotrophic bacteria with very different ecological roles from the heterotrophic bacteria. For example, *Synechococcus* contains LPS and has endotoxin activity (Weckesser *et al.*,

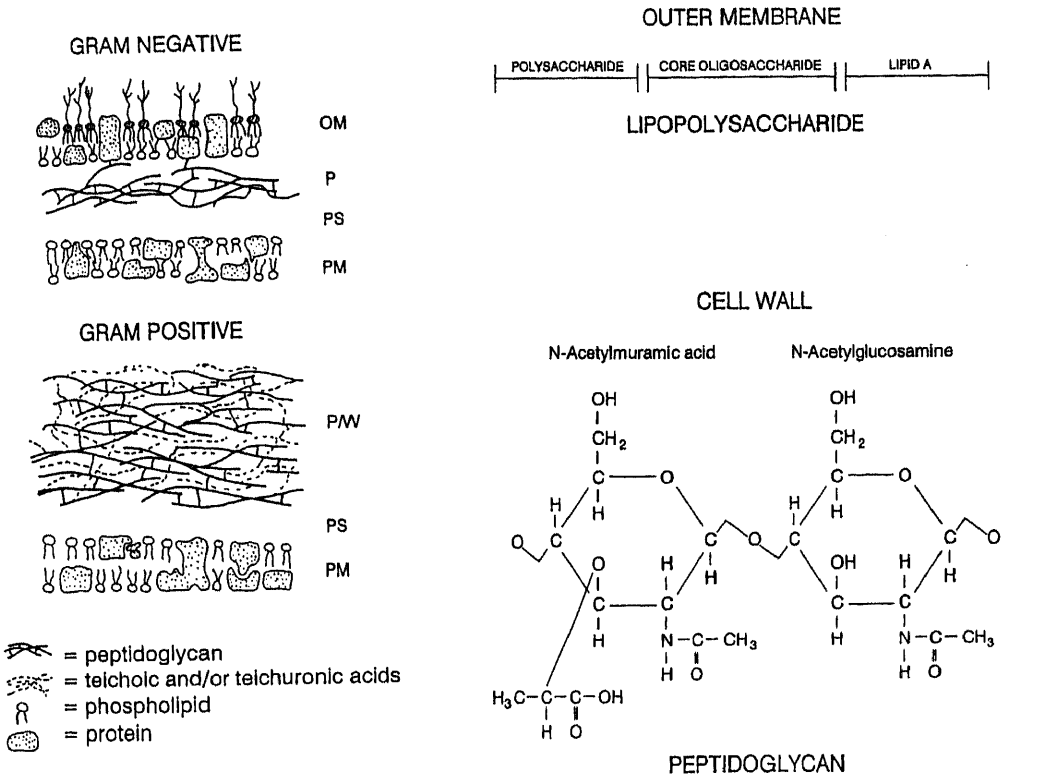


Fig. 2.13 (Left) Schematic representation of the arrangement of macromolecules in the gram-negative (top) and gram-positive (center) bacterial cell envelope. The main differences between these two groups are: (1) the organization and dimensions of the peptidoglycan (P) layer (P/W is peptidoglycan and outer cell wall in gram-positive bacteria), (2) the presence of teichoic and teichuronic acids in gram-positive cells and (3) the presence of a lipopolysaccharide outer membrane (OM) in gram-negative Bacteria. PS and PM are the periplasmic space and plasma membrane, respectively. (Redrawn from Beveridge, 1989.) (Right) Schematic composition of the lipopolysaccharide outer membrane in gram-negative Bacteria (top) and peptidoglycan layer (bottom) found in both gram-negative and gram-positive bacterial cells. (Redrawn from Doetsch and Cook, 1973.)

1979). LPS has also been found in both heterocystous and nonheterocystous subgroups of filamentous cyanobacteria as well as in the photosynthetic purple-sulfur and nonsulfur bacteria although certain groups have reduced or absent endotoxin activity. Clearly, one must carefully define 'bacterial biomass' for LPS-based ecological studies in marine ecosystems.

Watson *et al.* (1977) adapted the *Limulus* amoebocyte lysate (LAL) test for the *in vitro* detection of bacterial LPS as endotoxin. LAL contains a clotting enzyme and coagulogen. When activated by LPS, the enzyme hydrolyzes coagulogen and converts it to a gel protein (Levin and Bang, 1964). The coagulation endproduct is detected by turbidometric measurement or by colorimetry when coupled to hydrolysis of an exogenous chromogenic protein analog (Nakamura *et al.*, 1977).

The LAL assay is extremely sensitive and detects approximately 1 pg LPS per ml. This concentration of LPS is equivalent to 5–10 pg bacterial C or 10^2–10^3 bacterial cells. For most ecological applications, sample dilution rather than sample concentration is required prior to analysis. This extreme sensitivity is ideally suited for applications in oligotrophic marine habitats. Alternative methods of LPS detection involve isolation of lipid A and detection of the associated hydroxy fatty acids, as 2-keto-3-deoxyoctonate (KDO) or β-hydroxymyristic acid (Table 2.3). However, the empirical calibration experiments required to extrapolate LPS to bacterial C were made by the LAL assay procedure.

The LAL test reacts with both living and dead cells (Jay, 1977; Jorgensen *et al.*, 1973), and there is at least an order of magnitude variability in the threshold sensitivity of different gram-negative bacteria to this assay (Jay, 1977). Whether this latter observation was the result of variable cell sizes (hence variable absolute concentrations of LPS) cannot be determined from the data presented. In calibrating their method, Watson *et al.* (1977) selected *Escherichia coli* as the laboratory test organism and found that the C:LPS ratio averaged 6.35 (range 3.5–9.0). Based on a regression analysis of 188 seawater samples collected from coastal and open-ocean habitats, Watson *et al.* (1977) suggested that marine bacteria contain 2.78 ± 1.42 fg LPS per cell, compared to 50 fg/cell for *E. coli* in culture. Sledjeski and Weiner (1991) have also examined the LPS content of 19 species of marine bacteria. Their results indicated that LPS varies between 1% and 10% of the cell dry weight, compared to 3–5% for *E. coli*. While there is variability in the C:LPS ratio among bacterial species, the range is not extreme. One limitation is the uncertainty of the C:LPS ratio of phototrophic bacteria (e.g. *Prochlorococcus* and *Synechococcus*) that dominate the surface waters of most open-ocean habitats.

Although LPS is rapidly degraded following cell death and lysis, the polysaccharide is more readily attacked than the lipid (Saddler and Wardlaw, 1980). This relative stability of the lipid may have serious consequences for the estimation of LPS by analysis of the lipid A components or by endotoxin activity. Cell-free LPS has been detected in aquatic environments and may represent up to 92% of the total LPS in certain habitats (Table 2.5). In this regard, detrital LPS may dominate the total inventory in certain ecosystems. Spontaneous release of LPS is documented for many bacteria and appears to occur during normal growth (Cadieux *et al.*, 1983). Detrital LPS may also accumulate as a consequence of protozoan grazing activities or viral lysis.

LPS distribution in Antarctic coastal ecosystems

Although Antarctic waters are perennially cold and in some locations permanently ice-covered, there is a large diversity of microbial habitats and, therefore, microbial assemblages. Superimposed on these spatially diverse marine habitats is an intense temporal variability, perhaps the most extreme seasonality observed anywhere in the world ocean. Many microbiological studies in Antarctica have focused on the controls of photoautotrophic production, and autotroph–heterotroph coupling.

An extensive 4-month field experiment, RACER-I, was designed to quantify the flux of carbon from primary producers to higher trophic levels and decomposer populations, with a component focused on the microbial food web. Contrary to expectations, bacterial abundance was uncoupled from photoautotrophic production (Karl *et al.*, 1991) and appeared to respond only at the demise of the seasonal bloom. By comparison to the empirical relationships derived from temperate aquatic ecosystems (Bird and Kalff, 1984; Cole *et al.*, 1988), the heterotrophic bacterial abundances in Antarctica were an order of

Table 2.5 The distribution of dissolved and particulate LPS in selected aquatic habitats

Study area	*Bound LPS (ng/ml)*	*Free LPS (ng/ml)*	*Total LPS (ng/ml)*	*Free LPS (% total)*	*Reference*
Freshwater drainage system, Montana					
Sta EF1	0.7	9.0	9.7	93	Evans *et al.*, 1978
Sta EG4	7.5	11.5	19.0	61	
Sta EG5A	32.4	16.2	48.6	33	
Central North Pacific Ocean					
50 m	0.23	0.22	0.45	49	HOT program,
100 m	0.15	0.14	0.29	48	unpublished data
400 m	0.05	0.03	0.08	38	
1000 m	0.04	0.01	0.05	20	
Western Pacific Ocean					
0 m	1.4	1.5	2.9	52	Maeda and Taga,
25 m	1.7	1.5	3.2	47	1979
50 m	3.1	1.9	5.0	38	
75 m	4.8	1.3	6.1	21	
100 m	4.0	0.6	4.6	13	
150 m	1.8	0.3	2.1	14	
200 m	2.1	0.5	2.6	19	

magnitude lower than predicted on the basis of chl *a* concentrations. If these results are characteristic of polar habitats in general, then current biogeochemical models developed for tem-perate ocean regions need to be revised for high-latitude ecosystems.

A subsequent investigation focused on the relationship between bacterial biomass (based on LPS), phototrophic biomass (based on chl *a*) and total biomass (based on ATP) in Gerlache Strait, Antarctica, during the development of the extensive austral spring bloom. Within an approximately one-month period, ATP increased nearly 10-fold in the near-surface waters as the bloom developed (Fig. 2.14). Chl *a* concentrations generally paralleled the ATP trends. Compared to these large temporal changes in the upper portion of the water column, ATP concentrations (i.e. total microbial biomass) below 50 m were relatively constant, suggesting a very localized effect (Fig. 2.14). Particulate LPS concentrations for the same water samples also increased systematically within the upper portion of the water column, again with little change below 50 m (Fig. 2.14). Although the bacterial biomass tracked ATP as expected, it was lower overall than would be predicted from empirical models of nonpolar habitats. For example, gram-negative bacterial biomass (based on particulate LPS concentrations and assuming a C:LPS ratio of 6.35) varied from 0.5 to 5 μg/l in the upper 10 m of the water column compared to total microbial biomass values of 43–375 μg C per liter. Estimates of photoautotrophic biomass, which in this ecosystem is dominated by Eucarya (see Fig. 2.2), was extrapolated from chl *a* (assuming a C:chl *a* of 50) and confirmed that >95% of the microbial biomass in this polar ecosystem was algal (Karl and Tien, 1991). It appears that there is an uncoupling of bacterial and algal processes during the development of austral blooms. Not until later in the year, during the demise of the bloom, do microheterotrophic processes increase to appreciable levels (Bird and Karl, 1991).

2.6.8 MURAMIC ACID

The rigid peptidoglycan cell wall or murein layer is found in all Bacteria (gram-positive and gram-negative bacteria, and cyanobacteria) with the possible exception of *Planctomyces*, and is absent in all Eucarya and most Archaea. The murein helps to maintain cell shape (Schleifer, 1985). It consists of a linear chain of alternating β1–4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues containing cross-linked tetrapeptide side-chains having both D- and L-protein and nonprotein amino acids (Fig. 2.13). The cross-linking results in a mesh-like structure that varies from group to group depending upon the degree of cross-linking and the composition of the amino acids. On the basis of these variations, over 100 different chemotypes of murein can be identified (Stanier *et al.*, 1986). Representatives of the Domain Archaea have a different form of peptidoglycan called 'pseudomurein' that differs primarily in that muramic acid is replaced by *N*-acetyltalosaminuronic acid. The measurement of muramic acid (MA) as a biomarker for bacteria was originally suggested by Millar and Casida (1970) and was expanded into a method for estimating marine bacterial biomass by Moriarty (1975). To our knowledge, the measurement of *N*-acetyltalosaminuronic acid has not yet been used to estimate the biomass of Archaea but, for reasons parallel to those described here for MA, it could be a relevant biomarker.

In its simplest form, extrapolation of MA concentrations to bacterial carbon assumes a constant MA:carbon ratio for all bacteria and rapid degradation of bacterial cell walls (or at least MA) following cell death. However, the composition of bacterial cell walls is highly variable among species and can change as a function of growth rate and environmental conditions (Ellwood and Tempest, 1972). Furthermore, gram-positive bacteria have far more MA per cell than gram-negative cells, and cyanobacteria, actinomycetes and bacterial endospores all have higher concentrations of MA (on a per cell basis) than most bacteria (Millar and Casida, 1970; Moriarty, 1978; White *et al.*, 1979a; Fig. 2.13). For example, in gram-positive bacteria, peptidoglycan can be up to 50% or more of the total dry weight of the cell wall compared to 10% in gram-negative cells (Cummins, 1989). Consequently, one must know the proportion of gram-positive to gram-negative bacterial cells and must also have information regarding the presence of actinomycetes, cyanobacteria and bacterial spores in each sample. Furthermore, because the MA content of a cell is expected to be proportional to surface area rather than to biomass *per se* (Ellwood and Tempest, 1972), an estimate of the size distribution of prokaryotic cells is also required to extrapolate the surface area measurement to biovolume and ultimately to biomass.

The measurement of MA is neither simple nor rapid (Moriarty and Hayward, 1982). No standardized protocol exists, and it is possible that the reported variability in MA may be due in part to the variation in analytical procedures. For example, King and White (1977) reported that the enzymatic assay of MA (actually of D-lactate derived from alkaline hydrolysis of MA) indicated concentrations 10–20 times greater than the colorimetric lactic acid assay procedure. Direct measurement of MA, either by gas-liquid chromatography or HPLC (Fazio *et al.*, 1979), appears superior to less direct procedures (e.g. measurement of D-lactate or NADH). Although MA has been shown to be readily degraded by natural microbial communities under certain environmental conditions (King and White, 1977; Moriarty, 1977), transmission electron microscopic analysis of sediments revealed that up to 40% of the total MA may be present as empty cell walls ('ghosts'); this observation obviously affects the accuracy of this method for estimating bacterial biomass (Moriarty and Hayward, 1982).

Much like the PL-P and PLFA protocols

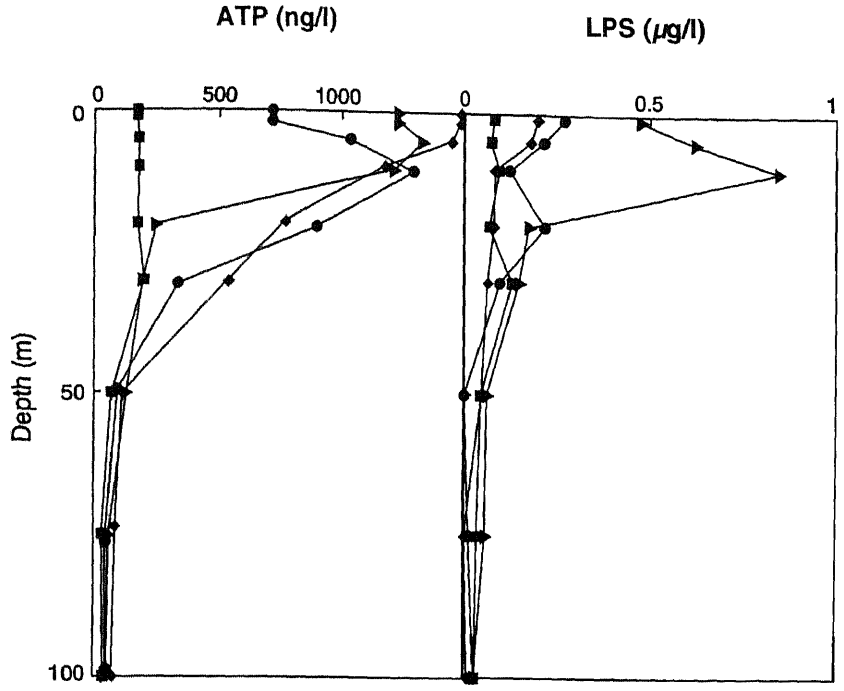


Fig. 2.14 Particulate ATP and LPS concentrations versus depth for water samples collected in Gerlache Strait, Antarctica, over a 6-week period in 1989. ■ 31 October; ●, 7 November; ◆, 15 November; ▲, 19 November.

discussed in Section 2.6.6, the MA assay procedure is used primarily to estimate bacterial biomass in marine sediments and has not been applied routinely to pelagic habitats. This trend is partially a result of simpler alternative procedures for the estimation of bacterial biomass in the water column and partly due to assay sensitivity and specificity.

Muramic acid distributions in the Mediterranean Sea

The introduction of HPLC methodology has provided access to a more sensitive and accurate MA assay (Moriarty, 1983). This HPLC method was used by Mimura and Romano (1985) to measure MA concentrations in a variety of oceanic ecosystems, including the sea surface microlayer (0–100 μm depth), the upper water column and sediments in the Mediterranean Sea. Their results indicated a 20-fold enrichment of MA per bacterial cell in the microlayer relative to samples collected at a water depth of 0.5 m ($5.3 \pm 2.2 \times 10^{-10}$ vs. $2.5 \pm 0.93 \times 10^{-11}$ µg/cell), and a 33-fold enrichment relative to deeper water column samples (1–50 m). These results suggest that bacteria in the microlayer were, on average, larger than those found deeper in the water column or, alternatively, that detrital MA was enriched in the microlayer. A regression analysis of MA concentration versus bacterial numbers for all 62 water samples yielded a mean of $1.6 \pm 0.68 \times 10^{-10}$ µg MA per bacterial cell, which is equivalent to 3.2 µg MA per mg of bacterial C, assuming a mean cell volume of 0.25 μm^3 and a C to dry weight ratio of 0.46 (Mimura and Romano, 1985). This value compares favor-

ably with that of 5–10 μg MA per mg C obtained by Moriarty (1977), based on an analysis of eight species of gram-negative marine bacteria. Because gram-positive bacteria contain more MA, the results obtained by Mimura and Romano (1985) suggest that most of the bacteria in their Mediterranean Sea study region are gram-negative.

In a 6.4-km-long transect away from a municipal sewage outfall site, the MA in surface sediments decreased from 250 μg/g dry weight of sediment to <10 μg/g dry weight. Even at the station having the lowest biomass, the bacterial C in the surface sediment layer was 10^3–10^4 times more concentrated than in the upper water column (0.1–1 μg MA per liter of water; Mimura and Romano, 1985). The results of this sediment and water column comparison, and all others like it, emphasize the importance of the seawater–sediment interface as a habitat of generally enriched bacterial (and microbial) biomass.

2.6.9 NUCLEIC ACIDS

In a treatise on molecular methods in ocean sciences, we would certainly be remiss not to mention applications of nucleic acids as signature compounds for estimation of microbial biomass. Particulate DNA can be concentrated by vacuum filtration and measured using any one of several colorimetric or fluorometric assay procedures (summarized by Karl and Bailiff, 1989). Pioneering research efforts to employ DNA measurements for total microbial biomass estimation in the sea began in the late 1960s with the quantitative laboratory and field studies of Holm-Hansen and colleagues (Holm-Hansen *et al.*, 1968; Holm-Hansen, 1969a). During these initial investigations it was established that a large proportion of particulate DNA is associated with nonliving organic matter (Holm-Hansen, 1969a), thereby contradicting one of the basic assumptions for the use of biomarker compounds to estimate microbial biomass. Subsequent studies confirmed the presence of a large pool of detrital DNA, both in particulate and in dissolved fractions (Karl and Winn, 1984; DeFlaun *et al.*, 1986). For samples collected at an oligotrophic North Pacific station, Karl and Bailiff (1989) reported higher concentrations of dissolved DNA than particulate DNA, and dissolved RNA to dissolved DNA ratios similar to those found in growing microorganisms (particulate RNA:DNA ranging from 3 to 10).

Despite these observations of large concentrations of detrital nucleic acids, the use of DNA and RNA as biomass indicators has great appeal. First, nucleic acids are ubiquitous macromolecules with important metabolic functions. Second, there is a highly conserved quota of DNA in microbial cells, and therefore a relatively constant C:DNA ratio (Holm-Hansen, 1969b). Furthermore, because the RNA content of cells varies predictably with growth rate, additional metabolic information can be derived from particulate RNA:DNA ratios in natural samples (Karl, 1981). Finally, the emergent methods for estimating rates of (heterotrophic) bacterial DNA synthesis by [^{3}H]thymidine incorporation (Fuhrman and Azam, 1980) or total microbial DNA synthesis by [^{3}H]adenine incorporation (Karl, 1981) can be used in conjunction with measurements of particulate DNA to predict specific growth rate without the need for uncertain DNA to C conversion factors. The key, therefore, is to develop a method to distinguish living DNA from detrital, or nonviable, DNA in water or sediment samples collected from the marine environment.

Winn and Karl (1986) proposed a novel approach for separating cell-associated and detrital DNA pools. The method relies upon a comparison of the specific radioactivity (radioactivity per unit mass) of the total microbial DNA pool with that of the intracellular dAMP pool following the addition of [^{3}H]adenine (a nucleic acid precursor assimilated by most microorganisms), as well as an independently derived estimate of specific

growth rate. If the entire particulate DNA pool is associated with viable, replicating microbial cells, then the specific radioactivity of total DNA should be one-half of the value measured for the specific radioactivity of the immediate precursor (dAMP), after exactly one doubling time. The difference, if any, between the measured and predicted DNA pool specific radioactivities is a direct measure of nonreplicating DNA. Application of this method to water samples collected in the tropical Pacific Ocean indicated that >90% of the measured DNA in surface waters was nonliving.

In principle, this DNA discrimination procedure could be conducted with $^{32}PO_4^{3-}$ (to label nucleic acid precursor pools and products) or with any other tracer molecule known to label DNA, provided that the DNA precursor pool(s) can be isolated and measured. A subsequent improvement allows the investigator to estimate specific growth rate from the same ^{3}[H]adenine sample incubation, thereby reducing the overall effort (Karl *et al.*, 1987). The DNA-based microbial biomass method would, therefore, involve: (1) determination of total particulate DNA, (2) determination of nonliving particulate DNA and, by difference, viable cell DNA and (3) extrapolation of cell-associated DNA to microbial biomass carbon, using laboratory-derived relationships. Until now this approach has not been suggested as a potential protocol for microbial biomass estimation.

Finally, the use of ribosomal RNA (rRNA) as a phylogenetic biomarker is thoroughly discussed in Chapter 1 and will not be repeated here. The ecological interest in RNA sequence analysis is primarily for evolutionary studies rather than carbon-system dynamics. Nevertheless, it should be emphasized that the taxonomic information derived from rRNA sequence analysis is critical to contemporary issues of marine biodiversity and, as such, is relevant to many unresolved ecological problems.

2.7 DIRECT COMPARISONS OF TECHNIQUES TO ESTIMATE MICROBIAL BIOMASS

Carbon is traditionally used in ecological studies as the basic unit of living biomass, production and flux among trophic levels. Unfortunately, few methods in microbial ecology measure carbon pools or rates of carbon turnover directly. Consequently, one must rely on the validity of the laboratory-derived conversion factors to translate the various indirect measurements of microbial biomass and metabolism to a more universal ecological metric.

Because microbial biomass in the sea cannot be measured directly, it is difficult to assess the precision of our indirect field-derived estimates. Furthermore, there are no commercially available reference standards that can be used to determine biomass measurement accuracy. Consequently, it is imperative that the underlying assay assumptions and extrapolation factors for each method be reviewed periodically and, if necessary, revised.

One approach for establishing greater confidence in the estimation of microbial biomass is to compare results obtained by independent measurement techniques. These results can be used to constrain certain biomass components and to improve data interpretation. For example, total microbial biomass extrapolated from direct microscopic enumeration with cell sizing (and subsequent conversion to carbon) or biochemical analysis (e.g. of membrane phospholipids) should not exceed the total POC (living plus nonliving) as determined by direct, chemical oxidation procedures. Furthermore, the estimation of total microbial biomass (e.g. by ATP) should always equal or exceed biomass estimates of specific subcomponents of the community (e.g. chl *a* for photoautotrophs or LPS for gram-negative bacteria). These checks and mass balances are an essential component of microbial ecology and are used to judge the reliability of the field-collected data.

Watson and Hobbie (1979) compared three independent microbial biomass estimates on samples from the coast of southwest Africa. The techniques employed were: (1) ATP (total microbial biomass), (2) AO direct counts (total bacterial biomass) and (3) cell-bound LPS (total gram-negative bacterial biomass, including cyanobacteria). Several interesting trends were observed (Table 2.6). Within the surface ocean (0–100 m), the average microbial C biomass extrapolated from LPS determinations (C_{LPS}) was, on average, 40% greater than that derived from the direct count method (C_{DCM}; i.e. $C_{LPS}/C_{DCM} = 1.40 \pm 0.41$, $n = 9$). Unfortunately, ATP measurements in the euphotic zone were not reported. Assuming for the moment that both assay procedures behaved 'as advertised', these results are consistent with the expected presence of a substantial population of cyanobacteria that, in theory, would contribute only to the C_{LPS} determination. However, in practice it is difficult to distinguish autotrophic from heterotrophic bacteria using AO epifluorescence microscopy (Campbell *et al.*, 1994) so the reported discrepancy may simply be a result of an improper selection of extrapolation factors or some other systematic inaccuracy (note that the range of C_{LPS}/C_{DCM} is from 0.82 to 1.92). Nevertheless, the agreement between these methods is remarkable considering that they are based on independent criteria.

Below the euphotic zone (150–1800 m), the microbial biomass extrapolated from LPS was again larger than that based on DCM ($C_{LPS}/C_{DCM} = 1.32 \pm 0.52$, $n = 12$, range 0.83–2.75). However, given the variability observed, it is fair to conclude that the two biomass estimations were indistinguishable. In the absence of a reference standard, we cannot comment on the accuracy of either method. ATP concentrations for these same water samples (C_{ATP}) indicated an essentially equal microbial biomass ($C_{ATP}/C_{LPS} = 1.15 \pm 0.39$, $n = 12$, range 0.67–1.86). Important implications derived from these observations are: (1) microbial biomass below the euphotic zone is dominated by gram-negative Bacteria (on average 85% of the total microbial biomass) with a relatively minor contribution from Archaea and Eucarya, (2) in these intermediate ocean depths all Bacteria that are detectable by biochemical or microscopic techniques are alive (i.e. they contain ATP) and are, therefore, most likely indigenous populations, and (3) the independent extrapolation factors commonly used for these indirect biomass estimations appear to be robust when applied to this open-ocean ecosystem.

Balkwill *et al.* (1988) also have compared the efficacy of several independent methods of microbial biomass estimation including membrane lipids, cell wall constituents, ATP and direct counts. The model ecosystem they selected was an uncontaminated subsurface aquifer sediment with microbial biomass levels and nutrient fluxes not unlike those of deep-sea marine sediments. Based upon direct microscopic observations, the microbial community was dominated by coccobacillary bacteria of uniform size and was, therefore, considered to be an excellent natural microbial assemblage for methods intercomparison. Their biochemical analysis of this community is one of the most comprehensive comparative studies of microbial biomass undertaken to date.

All of the independent methods employed gave similar microbial biomass values when expressed as dry weight of cells (Table 2.7). For example, the dry weight extrapolated from the direct count method (DW_{DCM}) was nearly identical to dry weight based on ATP determinations (DW_{ATP}), supporting the suggestion that bacteria dominate the microbial community. All of the other biochemical indices (membrane lipids, LPS, MA) yielded higher microbial biomass estimations, ranging from less than two-fold greater for lipid phosphate to more than four-fold greater for LPS. This result may be a manifestation of the longer post-mortem turnover times of these compounds, relative to ATP, especially in this

Table 2.6 Microbial biomass estimation, expressed as mg C per m^3, extrapolated from measurements of ATP, LPS and direct counts of heterotrophic bacteria. The samples were collected off southwest Africa on cruise 93/3 of the R/V *Atlantis II*

Water depth (m)	*ATP method*[a]	*LPS method*[b]	*Direct count method*[c]
10	—[d]	22.9	14
20	—	24.8	12.9
30	—	19.6	10.6
40	—	22.7	16.7
50	—	11.8	12.3
60	—	9.1	11.1
70	—	13.1	7.7
80	—	7.4	5.2
100	—	5.1	5.4
150	6.2	4.4	4.2
200	3.0	4.4	3.6
300	2.0	2.0	1.9
400	1.4	1.8	1.7
500	1.8	1.6	1.1
600	1.2	1.8	1.2
700	1.9	1.4	1.2
800	1.6	1.2	1.4
900	1.5	1.9	2.3
1000	1.3	1.2	1.0
1400	1.8	1.1	0.4
1800	1.3	0.7	0.4

Source: Watson and Hobbie (1979).
[a]Based on an extrapolation factor of ATP × 250.
[b]Based on an extrapolation factor of LPS × 6.35.
[c]Based on an extrapolation factor of 10 fg (10×10^{-15} g) C per cell.
[d]ATP not reported for the upper 100 m of the water column.

ecosystem characterized by low biomass and low metabolic activity. On the other hand, convergence of the five biochemical estimations on a biomass value approximately 2–3 times greater than either the direct microscopic method or ATP could be a result of an underestimation of biomass by these two latter methods.

An entirely different approach was adopted by Christian and Karl (1994) in an attempt to constrain the values used for extrapolation of biomarker concentrations to estimates of microbial cell carbon (see also Section 2.8). Their approach employed a least-squares inverse method to minimize the residual sum of squares for a three-component matrix of indicator ratios (C:chl *a*, C:bacteria and C:ATP). This method can be used to obtain 'best fit' values for two of the three unknowns (the third must be known or assumed). The researchers apportioned the total microbial biomass carbon (i.e. ATP × 250) into photoautotrophic and heterotrophic biomass compartments for euphotic zone water samples from Station ALOHA. The best fit solutions indicated that the photoautotrophic microbial assemblage decreased from approximately 40–50% of the total in the near surface waters, to 5.3% at a depth of 175 m (Christian and Karl, 1994). Another conclusion from this study is that the biochemical indicator ratios may be variables rather than constants. Significant changes in the best-fit solutions were observed as a function of both

Table 2.7 Microbial biomass estimation extrapolated from measurements of ATP, membrane lipids, LPS, MA and direct counts. The sediment samples were collected from an uncontaminated aquifer

Method	*Concentration (nmol/g dry wt sediment)*	*Extrapolation factor used (μmol/g dry wt cells)*	*Biomass equivalence (μg cells/g dry wt sediment)*
Direct count (DC)	—	—	1.3 ± 1.1[a]
Membrane lipids			
Phospholipid fatty acids	0.35 ± 0.08	100	3.5 ± 0.1
Lipid phosphate	0.11 ± 0.03	50	2.2 + 0.05
Glycerol phosphate	0.22 ± 0.09	50	4.4 ± 0.15
LPS[b]	0.09 ± 0.04	15	6.0 ± 1.5
Muramic acid (MA)	0.22 ± 0.1	58.5	3.7 ± 2.5
ATP	1.39 ± 0.42[c]	1.7[d]	1.4 ± 0.8[e]

Source: Balkwill *et al.* (1988).
[a]Assumes 2×10^{13} cells/g dry wt of cells, mean cell volume = 1.07 μm^3 and mass = 1.72×10^{-11} g/cell.
[b]Measured as lipid A hydroxyl fatty acids.
[c]ATP value given in ng ATP/g dry wt sediment.
[d]ATP conversion factor is 1.7 ng ATP per 10^7 cells.
[e]Assumes 10^7 cells = 1.72 μg.

water depth and season (Christian and Karl, 1994). These conclusions add complexity and uncertainty to the previous use of extrapolation 'constants' in marine microbial ecology, and re-emphasize the magnitude of the 'microbial biomass challenge'.

2.8 MICROBIAL BIOMASS DISTRIBUTIONS AT STATION ALOHA

Systematic, long-term studies of selected aquatic and terrestrial habitats have yielded significant contributions to earth and ocean sciences. However, time-series observations of biogeochemical and microbiological variables in the ocean are rare. Repeated oceanographic measurements are imperative to understand natural variability in ecosystem processes that exhibit slow or irregular change or that are driven by episodic phenomena. These changes are not visible from a single foray into the field. Time-series studies are also ideally suited to document complex natural phenomena that are under the combined influence of physical, chemical and biological controls.

The subtropical gyres of the world ocean are extensive, coherent regions that occupy approximately 40% of the surface of the earth. The subtropical gyre of the North Pacific Ocean, delimited from approximately 15°N to 35°N longitude and 135°E to 135°W latitude, is the largest circulation feature on our planet (Sverdrup *et al.*, 1946). The gyre is a remote habitat that has been undersampled relative to the equatorial and coastal regions of the North Pacific.

In 1988, a multidisciplinary deep-water oceanographic station was established at a site north of Oahu, Hawaii, with the intent of establishing a long-term (>20 years) database on oceanic variability (Karl and Lukas, 1996). The primary objective of the Hawaii Ocean Time-series (HOT) program is to obtain high-quality measurements of selected oceanographic properties, including: water mass structure, dynamic height, currents, dissolved and particulate chemical constituents, microbiological processes and particulate matter fluxes. Ideally, the suite of time-series parameters provides the data necessary for calibration, validation and refinement of existing biogeochemical models. More importantly, these data sets improve our understanding of microbial processes in the sea and elucidate the processes that govern the fluxes of carbon into and from the oceans.

The time-series data available through the

HOT program (>60 cruises between October 1988 and December 1995) can be used to examine the distributions and abundances of microorganisms in the upper 1000 m of the water column and to constrain carbon-pool inventories (Table 2.8 and Fig. 2.15). As expected, the total carbon inventory in these surface waters is dominated by inorganic carbon (>95% of the total for the 0–50 m depth stratum). Within the mesopelagic zone (200–1000 m), the proportion of inorganic carbon is even greater due to net CO_2 production from *in situ* utilization of particulate and dissolved organic carbon pools (Table 2.8).

With regard to the organic carbon inventory, detritus is the dominant pool regardless of water depth (Table 2.8; compare dissolved and particulate organic matter pools with microbial biomass). Even within the POC pool, detrital C exceeds biomass C by factors ranging from 3 (for 0–50 m depth stratum) to 8.5 (for 200–1000 m depth stratum). This dominance of largely uncharacterized, nonliving particulate organic matter is the focus of many contemporary investigations of the marine carbon cycle.

Microbial biomass in the upper euphotic zone (0–50 m) averages 33% of the POC and <1% of the TOC (Table 2.8 and Fig. 2.15). Photoautotrophic microorganisms account for 50% of the microbial biomass near the surface (0–50 m) and 31% in the lower portion of the euphotic zone (50–200 m). Heterotrophic and chemolithotrophic bacteria account for the remaining microbial biomass. It is encouraging to see the convergence of the sum of these independent biomass estimates derived from pigment-biomarker data (chl *a*) plus DNA-

Table 2.8 Euphotic zone (0–200 m) carbon inventories for the oligotrophic North Pacific Ocean from data collected during the Hawaii Ocean Time-series (HOT) program[a]

Carbon reservoir	*Depth range (m)*	*Method*[b]	*Average concentration (per m³)*[c]	*Total carbon*[d] *(g C/m²)*
Dissolved inorganic carbon	0–50	Coulometry	24.1 g (±0.1; n = 53)	1 205
	50–200		24.6 g (±0.1; n = 51)	3 690
	200–1000		27.2 g (±0.3; n = 48)	21 760
Dissolved organic carbon	0–50	High-temperature	1.12 g (±0.12; n = 24)	56
	50–200	combustion-oxidation	0.93 g (±0.07; n = 24)	140
	200–1000		0.57 g (±0.04; n = 23)	456
Particulate organic carbon	0–50	High-temperature	27 mg (±5.2; n = 61)	1.35
	50–200	combustion	17 mg (±3.7; n = 61)	2.55
	200–1000		5.1 mg (±1.6; n = 60)	4.08
Total microbial biomass	0–50	ATP	36 µg (±15; n = 62)	0.45
	50–200		24 µg (±11; n = 62)	0.90
	200–1000		2.4 µg (±1.2; n = 62)	0.48
Phototrophic microorganisms	0–50	chl *a*	93 µg (±30; n = 62)	0.23
	50–200		126 µg (±33; n = 62)	0.28
Heterotrophic bacteria	0–50	Flow cytometry	5×10^{11} cells (±1.3; n = 50)	0.25
	50–200		3.3×10^{11} cells (±0.9; n = 50)	0.50
	200–1000		7×10^{10} cells (±3.5; n = 37)	0.56

[a]HOT program data are available through the Internet, using anonymous file transfer protocol (ftp): mana.soest.hawaii.edu.
[b]HOT program methods are available on the World Wide Web: http://hahana.soest.hawaii.edu.
[c]Average concentration for specified depth stratum in units shown.
[d]Based on the extrapolation factors: 0–50 m C:chl *a* = 50, 50–200 m C:chl *a* = 15 (Christian and Karl, 1994); 10 fg C per heterotrophic bacterial cell (Christian and Karl, 1994) and C:ATP ratio of 250 (Karl, 1980).

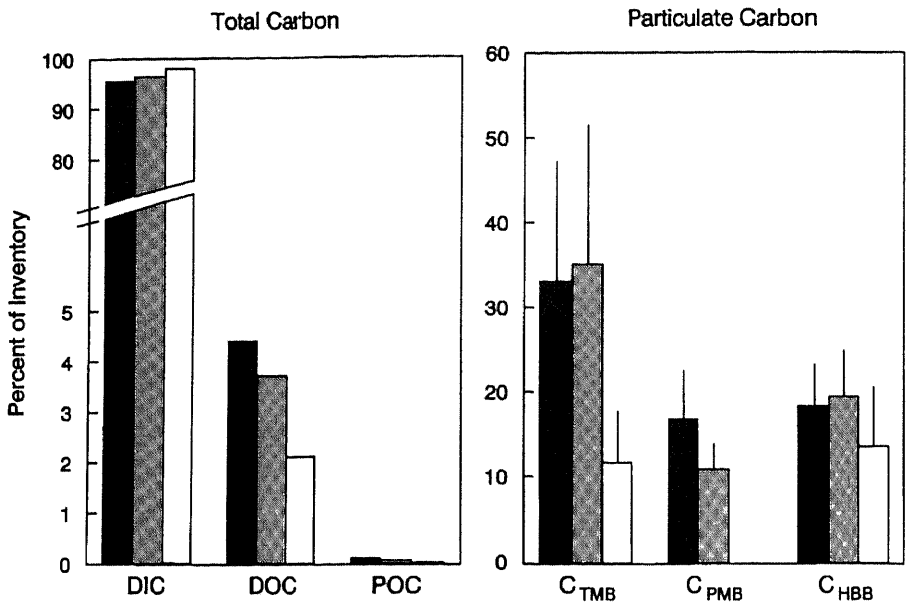


Fig. 2.15 Relative distributions of carbon in various pools for the upper water column at Station ALOHA for three specific depth horizons. (Left) DIC, DOC and POC as percentage of total carbon (inorganic plus organic) pool; total carbon pool = 1263 g/m² (0–50 m), 3833 g/m² (50–200 m) and 22 221 g/m² (200–1000 m). (Right) Microbial biomass as a percentage of POC pool. Total microbial biomass (C_{TMB}) based on ATP, phototrophic microbial biomass (C_{PMB}) based on chl *a* and heterotrophic bacterial biomass (C_{HBB}) based on flow cytometry. Solid, 0–50 m; shaded, 50–200 m; clear, 200–1000 m.

stained particle enumeration (flow cytometry) for phototrophic and heterotrophic microorganisms, respectively, and ATP values for total microbial biomass.

In the mesopelagic zone, heterotrophic bacteria dominate the total microbial biomass (Table 2.8 and Fig. 2.15). Within the precision of our estimations, bacterial biomass between 200 and 1000 m determined by flow cytometry (0.56 ± 0.28 g C per m²) is indistinguishable from total microbial biomass determined by ATP measurements (0.48 ± 0.24 g C/m²). While the various particulate living and nonliving carbon pools at Station ALOHA appear to be fairly well constrained by this 7-year data set, we know much less about the rates of exchange among the pools or the magnitude of the energy fluxes. These are challenges for the next decade.

2.9 METABOLIC ACTIVITY, ENERGY FLUX AND GRAVITATIONAL SETTLING OF MICROBIAL BIOMASS

The bulk density of most particulate organic matter in the sea and that of nearly all living microorganisms exceeds the average density of surface water (≥1.02 g/cm³). This fact suggests a net downward flux of microbial biomass by gravitational settling. Microbial cells alter their sinking speed by changes in morphology (shape, size, colony formation), by lipid storage or gas vacuolation, by regulation of the ionic composition of the cytoplasm or by motility (Smayda, 1970). However, in the highly turbulent oceanic regime, vertical position may be determined largely by water motion rather than by strategic movement of the microorganism.

Cell sinking has been studied most widely in planktonic Eucarya (Smayda, 1970, 1971). For solitary cells under laboratory conditions, there is a positive relationship between sinking rate and cell diameter, with sinking rates of 0.1–1 m/day for 10 μm-sized cells. The average sinking rates of heterogeneous subtropical photoautotrophic microbes (3–102 μm) range between 0.3–0.8 m/day and 1–2 m/day for the 3–20 μm and 20–102 μm size fractions, respectively (Bienfang, 1980). Senescent cells sink, on average, 3–4 times faster than growing cells, and dead cells sink fastest (Smayda, 1971).

Aggregate formation in the sea can lead to changes in both size and density, and can also affect sinking rates. Likewise, the association with or inclusion into larger organic or inorganic particles can lead to an accelerated flux of microbial biomass in the sea. The egestion of viable phototrophic microorganisms with crustacean fecal pellets, especially during bloom conditions, can increase the net downward flux of living microorganisms by orders of magnitude compared to intact single cells. This process is believed to be responsible for the observations of competent, pigmented Eucarya at abyssal ocean depths (Smayda, 1971).

Oceanographers recognize two classes of particles in seawater, broadly characterized as suspended and sinking. The former class dominates the standing stock of particulate matter in the ocean and the latter class dominates the exchange between the surface waters and greater ocean depths (McCave, 1975). The downward vertical flux of particulate organic matter (POM) in the open ocean exhibits a nonlinear decrease with increasing water depth, and greater than 75% of the net POM loss occurs in the upper 500 m of the water column. Because most sinking particles contain viable, metabolically active microorganisms, the downward flux of particulate matter is an important process for the vertical transfer of microbial biomass within the water column.

The first attempt to measure the net downward flux of microbial biomass in the sea was conducted using free-drifting particle-interceptor traps (i.e. sediment traps) to collect settling particulate matter and an *in situ* ATP extraction to estimate the biomass C associated with the collected particles (Fellows *et al.*, 1981). These experiments revealed that the living microorganisms associated with sinking particulate matter can be a significant portion of the microbial biomass, especially in habitats below the euphotic zone. Subsequent field experiments confirmed the importance of the downward flux of microbial biomass (based on ATP) and further revealed the rapid production and cell division of the biomass collected in traps when incubated under *in situ* conditions (Karl and Knauer, 1984). We currently believe that a large percentage of the annual POM flux from the euphotic zone (perhaps as much as 1–2 Gt C annually) may be exported in the form of microbial biomass (Karl *et al.*, 1988a; Taylor and Karl, 1991). This revised view of the flux of microbial biomass in the sea replaces the historical view of the 'rain of detrital carbon'. Still unresolved, however, is (1) the relationship between these particle-associated sinking microorganisms and those suspended in the water column and (2) the relative importance of these two separate microbial biomass pools in the overall process of particle decomposition and deep-sea respiration (Karl *et al.*, 1988a; see also Chapter 1).

Two alternative processes can be invoked to explain the net loss of organic matter as particles sink. First, abiotic particle fragmentation (conversion of sinking particles to smaller nonsinking particles) and solubilization (conversion of sinking particles to dissolved constituents) are important processes in controlling the net loss of particulate organic matter from the upper regions of the sea. Once the organic matter has been removed from the rapidly sinking particles, the microbial inhabitants of the upper mesopelagic zone (150–500 m) can oxidize

suspended particulate or dissolved organic matter with net consumption of oxygen, production of DIC and the regeneration of nitrate and phosphate. Although this model makes heterotrophic microorganisms ultimately responsible for the remineralization processes, the site of active microbial growth and decomposition is shifted from microbes attached to rapidly sinking particles to the microbial populations which are either free-living in the water column or attached to suspended (nonsinking) particulate matter. An alternative model invokes particle ingestion by mid-water zooplankton and micronekton as the mechanism responsible for controlling the flux of POM. Decomposition of the particulate matter would then take place within the intestinal tracts of these animals, most likely as a result of the activities of psychrophilic and barophilic bacterial populations. These two decomposition models are not mutually exclusive (Karl *et al.*, 1988a).

2.10 MICROBIAL BIOMASS INVENTORY FOR THE GLOBAL OCEAN

A primary objective of marine microbial ecology is to ascertain the global distributions of biomass and to understand the physical, chemical and biological controls thereof. With regard to the global distribution of microbial biomass, we have a general understanding, but fall short of our ultimate goal. This challenging research will continue to provide interesting data for at least several decades.

As discussed in previous sections of this chapter, there are significant regional and temporal variations in the biomass of pelagic microorganisms, whether expressed as total microbial biomass or as a select subcomponent of the total. For example, total microbial biomass (i.e. ATP) decreases on transects from the continental shelf to the open ocean and with depth below the euphotic zone. There are also substantial seasonal and interannual variations in water column microbial biomass, especially for photoautotrophs in high-latitude regions. By contrast, the biomass of microorganisms in the abyssal ocean appears to be constant irrespective of location or season.

These summary data can be used to estimate the total biomass of microorganisms in the global ocean above the sediment–water interface by dividing the ocean into representative regions (Table 2.9). Although the accuracy of this global extrapolation is unknown, we can compare these values to independent measurements of the detrital organic carbon pool and to estimates of global-ocean productivity, export and respiration. Like the quality of a fine wine, the accuracy of these inventories and fluxes is expected to improve with time.

Several interesting trends are revealed by this analysis. First, our results indicate that the global ocean sustains a microbial biomass between 0.6 and 1.9 Gt C, distributed primarily in the open ocean (approximately 75% of the total microbial biomass is in the oceanic province) and primarily at depths greater than 100 m (Table 2.9). Our estimate of total microbial biomass compares favorably with values derived by other, less direct, means. For example, Ajtay *et al.* (1970) presented a comprehensive summary of terrestrial and oceanic biomass estimations and concluded that plankton, bacteria and protozoa in the marine environment together represent approximately 1.6 Gt C, a value remarkably similar to our ATP-based estimate of global ocean biomass.

The large abundance of microorganisms in the open ocean relative to coastal areas is consistent with global patterns of primary production and particle export (Karl *et al.*, 1996). These results derive from the fact that the volume of the oceanic province is much larger than that of the neritic province, rather than being a result of higher biomass or productivity per unit volume (Table 2.9). Certainly, these distributions and abundances of marine microorganisms must have important implications for oceanic food production and global biogeochemical cycles.

Another important result of our analysis is that the total microbial biomass in waters >100 m deep is approximately equal to the biomass in the upper 0–100 m of the global ocean. If we assume that approximately half of the microbial biomass in the epipelagic zone of the open ocean is heterotrophic bacteria and that heterotrophic bacteria dominate microbial biomass within the subeuphotic zone (see Table 2.8), then we are left with the very interesting conclusion that there is a greater biomass of bacteria beneath the euphotic zone than within it. Given the importance of microbial photoautotrophic processes in the upper euphotic zone (0–100 m) as the primary source of carbon and energy for the remainder of the water column, the relatively large microbial biomass supported in waters >100 m deep may come as a surprise. An even more enigmatic result is the high concentration of microbial biomass in the mesopelagic zone (200–1000 m) of the ocean (i.e. approximately 25% of the global ocean total; Table 2.9). This region of the water column supports the active decomposition of organic matter and regeneration of inorganic nutrients required to sustain net primary productivity in the euphotic zone. If we accept that the annual global export production in the oceanic province is 3.5 Gt C (Karl *et al.*, 1996), then the global microbial biomass of the subeuphotic zone, estimated to be between 0.18 and 0.54 Gt C, could be sustained with a doubling time of about 70 days if carbon assimilation efficiency approached 50%. On the other hand, there could be a large range of growth rates from a rapidly growing subcomponent through resting or even dying populations. This region of the water between the sunlit surface waters and the dark abyss can be appropriately termed the 'twilight zone' for reasons other than absolute light levels.

Table 2.9 Oceanic microbial biomass inventory extrapolated from regional ATP distribution

Region[a]	*Depth range (m)*	*Total regional area and volume*[b] *Area* ($\times 10^8$ km^2)	*Volume* ($\times 10^6$ km^3)	*Range of ATP concentrations*[c] (μg ATP m^{-3})	*Range of total regional biomass*[d] ($\times 10^{12}$ g C)	*Percent of global ocean total*
Neritic province						
Upper region	0–100	0.36	3.6	150–450	135–405	21.2
Lower region	100–200	0.36	3.6	40–120	36–108	5.7
Oceanic province						
Epipelagic zone						
upper region	0–100	3.26	32.6	25–75	204–611	32.0
lower region	100–200	3.26	32.6	10–30	82–245	12.8
Mesopelagic zone	200–1000	3.26	316	2–6	158–474	24.8
Bathypelagic zone	1000–4000	3.19	805	0.1–0.3	20–60	3.1
Abyssopelagic zone	>4000	2.1	183	0.05–0.15	2.3–6.9	0.4
Global ocean						
Euphotic zone						
upper region	0–100	3.62	36	25–450	339–1016	53
lower region	100–200	3.62	36	10–120	118–353	19
Aphotic zone	>200	3.26	1304	0.05–6	180–541	28
Total	0–10 000	3.62	1376	0.05–450	637–1910	100

[a] Based on classification scheme of Hedgpeth (1957), where neritic is defined as the coastal ocean above the continental shelf and oceanic refers to waters beyond the shelf break.
[b] Based on total ocean surface area of 3.62×10^8 km^2, total ocean volume of 1376×10^6 km^3 (Press and Siever, 1978) and ocean depth distribution data of Jannasch and Wirsen (1977).
[c] Seasonally averaged values over the depth range indicated; minimum and maximum estimates are used to reflect global variations observed; from numerous sources including a summary of more than 50 ocean profiles (Karl, 1980).
[d] Based on microbial C:ATP ratio of 250 (Karl, 1980).

2.11 CONTROLS ON MICROBIAL BIOMASS IN THE SEA

The instantaneous biomass of microorganisms in any marine ecosystem is a result of a complex balance between production and removal processes. For photoautotrophic microbial populations, biomass production is controlled by the availability of light and the distribution of macronutrients (C, N, P, S), trace elements and other essential growth factors (e.g. vitamins). Heterotrophic microorganisms have similar requirements (except for light) in addition to their need for reduced carbon substrates. Biomass limitation by resource availability is termed bottom-up regulation. Predatory control, whether through higher trophic-level grazing processes or viral infection and lysis, is termed top-down regulation. Despite their common usage in microbial ecology, these concepts are oversimplifications of complex, microbial food-web interactions (Fig. 2.16).

The electromagnetic radiation derived from the sun, or solar flux at the outer edge of the Earth's atmosphere, is estimated to be 2 cal/cm^2 per min, a value which varies diurnally, seasonally and with location on the Earth (Kormondy, 1969). Approximately 42% of this incoming radiation is reflected back by clouds and dust and an additional 10% is absorbed or scattered by substances in the atmosphere. Of the energy that finally reaches the ocean, a large portion is either reflected back from the sea surface or otherwise removed by diffuse attenuation. Theoretical calculations of gross photosynthetic production in the sea suggest an upper limit of 190 Gt C per year if N and P are not limiting (Vishniac, 1971). Two recent estimates, based on regional measurements of primary carbon fixation (^{14}C uptake method), suggest global ocean production between 51 and 66 Gt C per year (Martin *et al.*, 1987; Karl *et al.*, 1996), or approximately 30% of the theoretical maximum based on first principles of solar-energy flux and cellular bioenergetics. Because there is uncertainty over whether the ^{14}C method measures gross or net production, the concordance between these two independent estimations of global ocean photoautotrophic biomass production is somewhat reassuring.

A large portion (85–90%) of the organic matter produced in the epipelagic zone is consumed or recycled locally, with the remainder available for export to the aphotic zone. Because biological systems conform to the second law of thermodynamics, this flux of reduced carbon from the euphotic zone (approximately 5–7.4 Gt C per year) sets an upper limit on production of heterotrophic microbial biomass in the subeuphotic zone. The result of this calculation is dependent upon the carbon-assimilation efficiency (biomass produced per mass of carbon consumed) used, which for bacteria ranges from approximately 10% to 60% (Vézina and Platt, 1988). A prediction of this simple analysis is that the production of biomass in the subeuphotic zone cannot exceed a maximum of about 10% of the euphotic zone's production, under steady-state conditions. If the average specific-growth rate of the subeuphotic zone populations is similar to that measured for the surface waters, then the total biomass in each habitat would provide a reasonable estimate of energy flux. More likely, however, the specific-growth rates of the aphotic zone microbial assemblages will be much lower, in which case the microbial biomass may be uncoupled from metabolic activity or biomass production rates.

One approach to the study of microbial biomass control is to examine empirical relationships between limiting concentrations of resources (e.g. nitrate or phosphate for photoautotrophs or available DOC for heterotrophic bacteria) and population abundances. These cross-ecosystem analyses support several predictions that derive from our generalized microbial food-web hypothsis, including: (1) evidence of strong resource control of heterotrophic bacterial populations based on a significant positive correlation

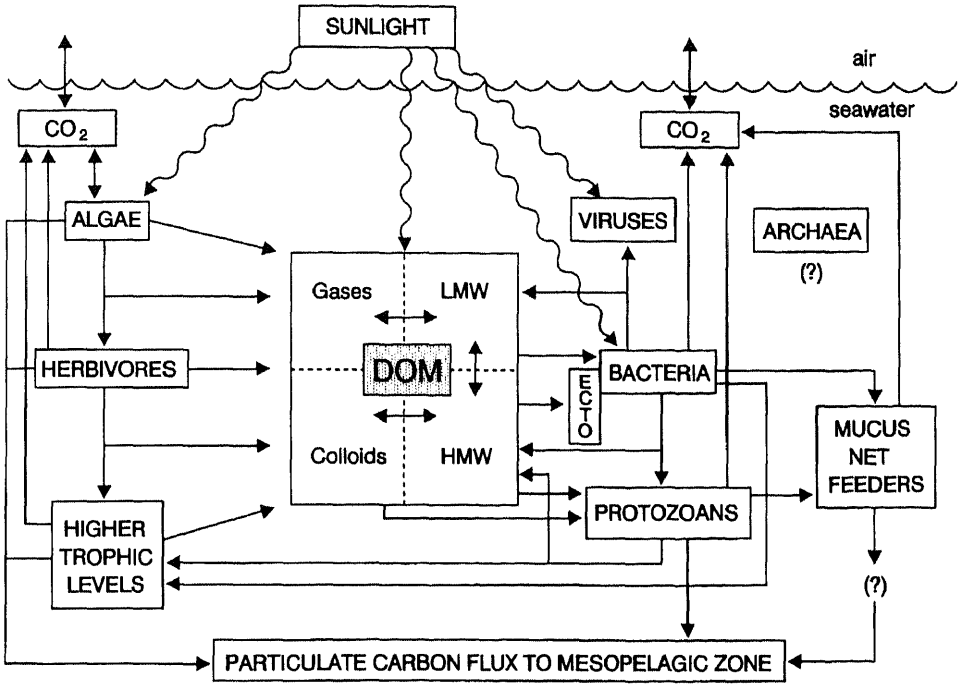


Fig. 2.16 Schematic representation of the oceanic food web showing, on left, the classical pathway of carbon and energy flow through the photosynthetic Eucarya, to herbivores and on to the higher trophic levels. Depicted on the right is the present view of the microbial food web, which utilizes energy stored in the large detrital carbon pool to produce microbial biomass that can re-enter the biogenic carbon cycle. Cell-associated ectoenzymes (Ecto) enable Bacteria to utilize high molecular weight (HMW) DOC. Also shown in the microbial web are the virus particles and the Archaea. At the present time we have only a rudimentary knowledge of the role of Archaea in the oceanic food web. Shown at the bottom of this diagram is the downward flux of particulate carbon (and energy) which is now thought to fuel most subeuphotic zone processes. (Adapted from Karl, 1994.)

between bacterial abundances and chl *a* concentrations (Bird and Kalff, 1984) and a strong positive correlation between heterotrophic bacterial production and photoautotrophic production (Cole *et al.*, 1988) and (2) a potential top-down limitation as a result of viral lysis (Maranger and Bird, 1995). Despite the fact that heterotrophic bacteria are actively grazed by protozoans, it has been difficult to establish any systematic relationship between the abundance of heterotrophic bacteria and that of heterotrophic nanoflagellates (Gasol and Vaqué, 1993). Lack of a relationship may be a consequence of the large detrital resource (POC and, perhaps, high molecular weight DOC) available to protozoans. One major criticism of these empirical, static analyses is that abundances rather than fluxes are compared, leading to a tenuous dynamical extrapolation. Furthermore, the roles of respiration and natural mortality as potential controls on the biomass of microbial populations in the sea are not well described in our current models of the microbial food web (Ducklow, 1994).

2.12 SUMMARY

When confronted with a large number of molecular approaches to estimate microbial biomass, one has to exercise judgment based

on the nature of the sample materials, the required precision and accuracy, as well as other logistical and financial considerations. Many of these molecular techniques, though straightforward, require a certain level of experience and care. However, with a modest fiscal investment and practice, microbial biomass estimation can become a routine tool in marine microbial ecology. Application of these methods, either singly or as redundant analyses, provides useful data that can help unravel mysteries of microbial life in the sea and contribute to our expanding knowledge of oceanic biogeochemical processes.

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