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PHYSIOLOGICAL STRUCTURE AND SINGLE-CELL ACTIVITY IN MARINE BACTERIOPLANKTON

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INTRODUCTION

Generations of microbiologists have pondered over the issue of bacterial activity and whether most bacteria in the environment are “active,” “inactive,” or “dormant” (Stevenson 1978). This is a question with clear practical implications, in terms of food, sanitary, and clinical microbiology, as well as profound ecological implications. The issue started to be debated in earnest within the aquatic microbial community roughly at the time researchers were developing new methods to estimate the total abundance of aquatic bacteria, since some of the first stains used were thought to respond differently depending on the RNA content of single bacterial cells (see, e.g., Francisco et al. 1973). It was not until bacteria were recognized as being not only very abundant, but also active, that the notion of microbes as important partners in the marine food web gained widespread recognition (Pomeroy 1974). The early studies using radiotracer incorporation showed surprisingly high rates of bacterial

biomass production and of DNA replication, so clearly there had to be active bacterial cells in the oceans. The question then arose as to how this activity was distributed among the 10^5 – 10^6 cells that are on average present in 1 mL of ocean water (Hoppe 1976; Stevenson 1978). Did all these cells have roughly the same level of metabolic activity, or was the bulk of bacterioplankton activity concentrated in a few key players? Marine microbial ecology has gone a long way since those early studies, but the question is as valid and relevant today as it was back then.

That complex aquatic bacterial assemblages harbor a wide range of single-cell metabolic activities as well as of physiological states is neither surprising nor much contested nowadays. Yet, in spite of the simplicity of this basic premise, the actual description and quantification of this metabolic and physiological heterogeneity have proven elusive, and its underlying mechanisms, as well as its ecological and evolutionary consequences, have been very difficult to address in practice (Mason et al. 1986; Koch 1997; Kell et al. 1998; Smith and del Giorgio 2003). Under the term “bacterial single-cell activity,” various authors throughout the years have lumped together a number of very different cellular processes, including cell division rate, single and complex substrate uptake, respiratory activity, protein synthesis, intracellular enzyme activity, membrane potential and polarity, membrane integrity, ultrastructural characteristics, nucleic acid and other macromolecular contents, motility, and the list continues. While all these different aspects of cell structure, metabolism, and physiology are surely connected, the links that exist between them are complex, with feedbacks, compensatory mechanisms, and nonlinear responses, so that information on any particular cell function does not necessarily allow inferences about the others (Smith and del Giorgio 2003). In this chapter, we refer to the ensemble of these single-cell characteristics and processes as the “physiological structure” of bacterial assemblages. As we hope to make evident in this chapter, this structure can be neither described nor understood on the basis of any one single aspect of cell function.

In spite of the very incomplete descriptions of the physiological structure of bacterioplankton that we currently have, some patterns are emerging: the evidence converges to suggest that while many bacterial cells appear to be intact, many of these do not seem to have detectable levels of activity, at least with the methods that are currently available. In addition, in all assemblages studied to date, there seems to be a non-negligible proportion of cells that are either damaged or dead. The presence of significant numbers of these cells in almost all assemblages studied begs the question: Why are there so many apparently inactive, damaged, and dead cells in all aquatic systems? What are the factors that induce cell inactivation and damage? How can these cells persist in the assemblage, and what is their role in community functioning and the maintenance of genetic diversity? How and when does individual cell activity relate to phylogenetic composition? Recent technical and conceptual developments are providing the tools to at least begin to address some of these questions.

In this chapter, we review the current state of knowledge and the technical and conceptual progress that has been made in the past two decades in the study of the single-cell characteristics and of the physiological structure of marine bacterioplankton. There are five main issues concerning the physiological structure of marine

bacterial assemblages that we address in this chapter: (1) the approaches used to probe various aspects of bacterial activity and physiology at the single cell level; (2) the connections that exist between these various cell functions; (3) the environmental and biological factors that influence the different aspects of bacterial physiology and metabolism; (4) the empirical findings concerning the distribution of single-cell bacterial activity and the physiological structure of marine bacterioplankton assemblages; and (5) the biogeochemical, ecological, and evolutionary significance of the resulting patterns of cellular activity. It is the goal of this chapter to provide a conceptual framework to help interpret and integrate the data that have been generated in the last few years concerning the physiological structure of bacterioplankton assemblages.

DISTRIBUTION OF PHYSIOLOGICAL STATES IN BACTERIOPLANKTON ASSEMBLAGES

The Concept of “Physiological Structure” of Bacterioplankton Assemblages

The physiological structure of bacterioplankton is the distribution of cells in different physiological categories within the assemblage. The physiological structure is akin to the “phylogenetic structure” of an assemblage, except that the classification of organisms based on “phylogenetic units” is replaced by one based on “physiological state units.” The challenge in microbial ecology has precisely been how to define these units. Metabolic activity and cell physiology are generally not discreet variables—they are rather expressed as a continuum that is impossible to capture by any single method. Cells must thus be grouped into artificial categories that are essentially operational, depending directly on the characteristics and thresholds of the methods used (see below).

The physiological structure of bacterioplankton, that is, the distribution within the assemblage of cells in different physiologic categories, is regulated by two major groups of factors. On the one hand, there are the environmental and phylogenetic factors that influence the level of activity and physiological response of individual bacterial cells. It is unlikely that cells from different bacterial strains and taxa respond identically to environmental stimuli, and since in any bacterioplankton assemblage there are many coexisting bacterial phylotypes, for any combination of environmental factors (e.g., salinity, and rate of supply and nature of the organic substrates and nutrients) there must necessarily be a diversity of metabolic and physiological responses.

The second major aspect of the regulation of the physiological structure of bacterioplankton is imposed by factors that regulate not the activity but the loss and persistence of these different categories of cells in the assemblage. For example, there is now evidence that protists may selectively graze on the more active cells (del Giorgio et al. 1996), and that viruses may preferentially infect cells that are growing and have higher levels of metabolism (Weinbauer 2004), such that these cells at the high end of the activity spectrum may be subjected to higher removal

rates than the average cell in the assemblage. Thus, the proportion of these cells in a given assemblage is not a simple function of the rates of cell division and activation, but rather depends as well on the loss rates. Likewise, because of their size, and also the relative recalcitrance of their cellular components (see, e.g., McCarthy et al. 1998), dead or inactive bacteria may remain suspended in plankton for long periods and thus accumulate in the assemblage even if the rates at which they are generated are low.

There are no doubt interactions and feedbacks between these two levels of control of physiological structure. For example, viral infection and the resulting bacterial lysis transfers cells from the active to the injured or dead pool, but, in doing so, releases organic carbon and nutrients that may induce the activation of dormant cells, or enhances the growth rates of cells that are already active. It is thus the net balance between levels of regulation (i.e., rates of activation, inactivation, or death) and selective removal, persistence, or loss that determines the actual proportion of cells in different physiological categories found in aquatic bacterioplankton assemblages. We focus on the description of the physiological structure, as well as on these different aspects of regulation in the following sections.

The distribution of cells into different physiological categories is termed the “**physiological structure**” of bacterioplankton:

- Within a bacterial assemblage, there is a continuum of activity: from dead to highly active cells.
- The categories used to describe the physiological structure are operational, and depend on the methods used.
- The physiological structure is related, albeit in complex ways, to the size structure of the community, as well as to the phylogenetic structure.
- The physiological structure is dynamic; that is, the proportions of cells in various physiological states may vary on short time scales and small spatial scales.

Starvation, Dormancy, and Viability in Marine Bacterioplankton

One issue at the center of the concept of the physiological structure concerns the different adaptive strategies developed by aquatic bacteria to cope with a dilute and fluctuating environment, and the cellular physiological states associated with these strategies. In particular, and as we will see below, the evidence to date suggests that, in all aquatic ecosystems, there is a fraction, often large, of bacterial cells that do not have measurable metabolic activity but that nevertheless retain cellular integrity. The marine microbial literature abounds in references to “dormant,” “latent,” “starved,” “quiescent,” and “inactive” bacteria to describe these cells. These terms are often used quite loosely to denote absence or low levels of bacterial activity, but dormancy, starvation response, and slow growth are not synonymous, because they are associated with different life strategies and ecological consequences.

Starvation, that is, the presence of organic substrates below the threshold of the uptake capacities or of the minimum cell requirements, is the most obvious cause for the inactivation of bacterial cells in aquatic ecosystems, and is the one that has been traditionally invoked to explain differences in cell activity within and among assemblages (Kjelleberg et al. 1993; Morita 1997). The physiological, molecular, and genetic basis of the starvation response has been studied mostly in laboratory cultures of *Vibrio* spp. and *Escherichia coli* (Morita 1997; Kjelleberg et al. 1993; Koch 1997), and a handful of marine isolates (Morita 1997), including the marine bacterium *Sphingomonas* sp. (Fegatella and Cavicchioli 2000). All cultured strains explored to date appear to have some systematic response to starvation, regulated by several genes, which involves a complex series of morphological as well as molecular and physiological changes: reduction in size and changes in cell shape, changes in macromolecular composition, as well as the synthesis of specific proteins, changes in nucleic acid structure, and cell miniturisation (Nyström et al. 1992; Ihssen and Egli 2005). Some of the changes are geared to maximizing substrate uptake; others are to enable the cell to quickly resume synthesis and growth if conditions become favorable; and yet others are to protect the cell against cell damage or degradation in the absence of sufficient energy and carbon supply; there is also protection against possible sudden carbon surfeit that could have negative consequences to the cells (Koch 1997). In addition, the starvation-related changes may confer protection against other stresses, such as ultraviolet (UV) or temperature shock, viral infection, and even protistan grazing and digestion (Nyström et al. 1992), and this cross-protection does not necessarily exist for cells that have low levels of metabolism and are simply growing slowly.

Dormancy, on the other hand, is a response to stress, not just to carbon starvation, but also to osmotic, temperature, water, and other types of environmental stresses, also involving structural and biochemical changes that function to keep the cell in a state of suspended animation (Koch 1997). In this regard, dormancy could be viewed as the final and most extreme stage of the starvation response (Kjelleberg et al. 1993). Bacteria in the dormant state do not undergo cell division, and function at very low metabolic rates or have complete metabolic arrest (Kjelleberg et al. 1993; Barer and Harwood 1999). When environmental conditions change and become favorable, dormant bacteria can (in theory) resume cell division. Dormancy involves adaptive mechanisms, but not morphological differentiation such as sporulation, and cells may remain in a dormant state for extended periods of time without significant loss of cellular integrity (Roszak and Colwell 1987; Barer and Harwood 1999). As with the starvation-related process, dormant cells may have increased resistance to a range of environmental stresses (Kell et al. 1998).

Aspects of cell dormancy are related to the concept of cell culturability. In traditional microbiology, the capacity of cells to multiply in artificial media was used as the main criteria for bacterial viability. This definition had to be revised when it became clear that there were cells that lost their ability to grow in culture but nevertheless remained alive in a state that was later termed "viable but not culturable" (VBNC) (Roszak and Colwell 1987). A VBNC cell is one that persists intact in the environment, but that does not divide in culture. The cell must retain cellular integrity, an intact membrane, RNA and DNA, and potential for protein synthesis;

a VBNC cell may also show measurable levels of cellular activity in terms of substrate uptake, respiration, or protein turnover (Gribbon and Barer 1995; Kell et al. 1998). Gram-negative bacteria, such as *Vibrio cholerae*, *V. vulnificus*, and *E. coli*, have been reported to enter this VBNC state, from which they are able to return to a culturable state under certain conditions (Colwell 2000; Oliver 2000). There has been debate as to whether the VBNC state actually exists in nature or if it is the result of experimental conditions (Kell et al. 1998; Oliver 2000). The importance of this concept from a sanitary and clinical viewpoint is the realization that culturability cannot be used as the ultimate indicator of cellular viability or survival. Kell et al. (1998) point out that dormancy refers to cells that have negligible activity but that are ultimately culturable, whereas VBNC cells are metabolically active but nonculturable, but this criterion can only be applied to strains that are easily culturable in the laboratory. In this regard, aquatic microbial ecologists recognized long ago that although most marine bacteria cannot be cultured, they should not be considered dead or non-viable (see, e.g., Staley and Konopka 1985; Amann et al. 1990).

Finally, many marine bacterial isolates are naturally slow-growing under ambient conditions (Schut et al. 1997; Eguchi et al. 2001; Rappé et al. 2002), even under optimum conditions. Slow growth can therefore be both a stage in the response to extreme starvation and an adaptive strategy of marine bacteria. In either case, growth (even slow growth) requires the operation and maintenance of a large number of cellular processes, including transport and biosynthesis systems, and there are thermodynamic constraints on how slowly these systems can effectively operate (Chesbro et al. 1990). For example, at very low substrate and energy fluxes, there is a danger associated with incomplete synthesis of macromolecules, or the degradation of newly synthesized compounds, with negative effects on cell growth and survival (Koch 1997). In pure culture, responses to extreme carbon starvation are heterogeneous, with some cells taking up substrate and continuing slow synthesis and growth, and others not, and instead entering dormancy, suggesting that there is a choice of energy expenditure even in situations of extreme lack of substrates or unfavorable environmental conditions (Koch 1997).

What are then the costs and benefits associated with entering dormancy, as opposed to the strategy of maintaining low growth rates and dividing until thermodynamically and physically impossible? The latter strategy implies cell injury and death if unfavorable conditions persist; the former implies decreased cell death over extended exposure to unfavorable conditions but increased expenditures in terms of the dormancy response. This question has recently been explored through modeling. Bär et al. (2002) modeled the outcome of two cyanobacterial populations that are capable or incapable of becoming dormant in response to lack of water. They concluded that under a constant supply of water (even if the supply is extremely low), the dormant strategy does not provide any benefit, and it can, instead, decrease the chances of survival of the population. Konopka (2004) also modeled two bacterial populations, one entering dormancy and the other not making this transition, and found that the cells capable of entering a dormant state would be favored only in situations where the periodicity of substrate inputs is long compared with the minimum doubling time of the bacterium. These results point to the fact that

intermittency and microscale patchiness of resource supply probably play as large a role as the actual rate of supply in terms of shaping bacterial responses in aquatic ecosystems.

The starvation response and the processes leading to cell dormancy profoundly alter the physical and biochemical properties of cells relative to slow-growing cells, and this in turn influences how the cell interacts with both its physical and biological environments. Whether bacterioplankton cells are intrinsically slow-growing, in a starvation/survival mode, or dormant *sensu stricto* is not just a methodological or philosophical issue but rather one that has significant ecological consequences. In practice, however, it is at present difficult, if not impossible, to differentiate in natural communities intrinsically slow-growing cells from those that are undergoing a starvation response. In addition, it is also difficult to differentiate truly dormant cells from those that have low levels of metabolic activity. In the rest of this chapter, we will refer to “inactive” cells to encompass these different physiological categories, acknowledging that this represents a major oversimplification that needs to be corrected in the future.

Dormancy, starvation-survival, latency, slow growth, and inactivity are often used interchangeably to denote low levels of cellular activity in marine bacteria, but these terms are not synonyms and refer to different states:

- Under conditions of extreme substrate and energy deprivation, marine bacteria may undergo a “**starvation**” response.
- The **starvation** response is regulated by specific genes, and involves cell miniaturization and profound changes in macromolecular composition, with the synthesis of specialized protective proteins.
- Prolonged starvation may lead to cell “**dormancy**,” which is a state of complete metabolic arrest that allows long-term survival under unfavorable conditions. Cells in a dormant state are more resistant to environmental stresses than active cells.
- There are costs and benefits associated with entering dormancy as opposed to maintaining a slow level of metabolic activity and growth as a response to low substrate availability.
- Resource patchiness and temporal variability play a major role in shaping the survival strategies of marine bacteria, whether it be slow growth, starvation response, or dormancy.
- Microbiologists have further described the **viable but nonculturable (VBNC)** state, based on the inability of strains to grow in artificial media.
- The VBNC state is not analogous to cell dormancy, because in the former the cells may have significant metabolic activity, whereas in the latter cells have no measurable metabolic activity but retain the capacity to reproduce on plates. Since most marine bacteria cannot be grown in plates even if actively growing in the ocean, this concept has little applicability to the ecology of naturally occurring marine bacteria.

DESCRIBING THE PHYSIOLOGICAL STRUCTURE OF BACTERIOPLANKTON

A number of cellular characteristics are indicative of the physiological state of the cell and of its level of metabolic activity (Fig. 8.1). In this section, we describe the most ecologically relevant indicators of cell physiology, and the analytical tools that are used to assess these indicators. We then propose a series of operational categories based on these measurements that can be used to describe the physiological structure of bacterioplankton.

Single-Cell Properties and Methodological Approaches

There are increasing numbers of techniques available to probe the activity and characteristics of single bacterioplankton cells. We refer the reader to excellent reviews for technical details (Joux and Lebaron 2000; Brehm-Stecher and Johnson 2004). Here we focus on the adaptation and application of these techniques to the study of bacterioplankton communities. A list of techniques and probes that have been used to assess bacterial single cell characteristics in bacterioplankton can be found in Table 8.1. These techniques target different categories of cell function: (1) morphological integrity; (2) DNA quantification and status; (3) respiration activity; (4) the status of the membrane; (5) the internal enzymatic capacity; (6) the uptake of organic substances; (7) the synthesis of DNA; (8) cell growth; (9) the amount of rRNA (see Fig. 8.1).

Morphological Integrity Marine bacteria that are intact and functional are generally surrounded by a capsular layer, while damaged cells do not have this capsule (Heissenberger et al. 1996). The capsule may play a number of roles in the functioning of the cell: It may prevent viral adhesion, or act as a grazing deterrent (Storedegger and Herndl 2002). Bacteria growing in culture become more hydrophobic, and the capsule may be involved in the increased capacity to attach to

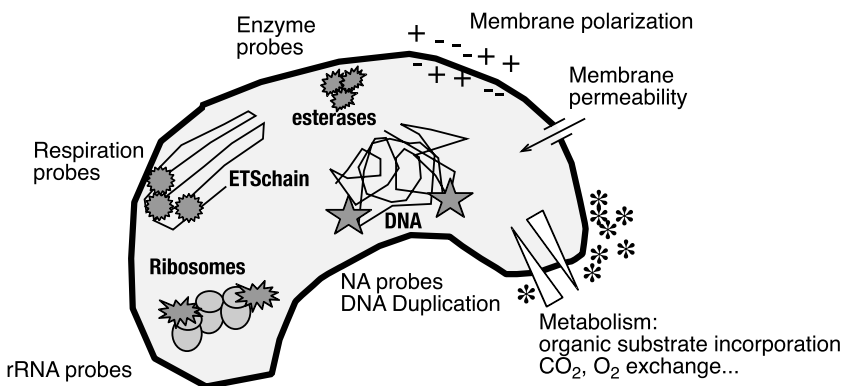


Figure 8.1 Schematic representation of the cellular components targeted by the different activity probes listed and categorized in Table 8.1.

TABLE 8.1 Activity Probes Used for Monitoring Bacterial Viability^a

Probe(s) ^b	Mode of Action	Method ^c	Key References
<i>Respiration</i>			
INT	Reduction by dehydrogenases	OM	Zimmerman et al. (1978)
CTC, XTT	Indicator of respiratory-chain activity	EF, FC, Spect	del Giorgio et al. (1997)
<i>Membrane Polarization and Membrane Potential (including dye-exclusion probes)</i>			
EthBr, Eth-D2	Excluded by living cells	EF/FC	Schumann et al. (2003)
PI	Excluded by living cells	EF/FC	Williams et al. (1998)
Rh123	Accumulated in live cells	FC	Davey et al. (1999)
Calcefluor white, Tinopal	Excluded by living cells	FC	Mason et al. (1995)
CBS-X, . . .			
Carbocyanines (cationic) (JC-1, DiOC ₆ (3), DiOC ₂ (3))	Excluded by living cells	FC	Mason et al. (1995)
Oxonols (anionic) (DiBac ₄ (3), Oxonol V, VI)	Excluded by living cells	FC	del Giorgio and Bouvier (2002)
TOPRO-1, TOPRO-3	Excluded by living cells	EF/FC	Maranger et al. (2002)
Sytox Green	Excluded by living cells	EF/FC	Lebaron et al. (1998)
<i>Intracellular Enzymes</i>			
FDG	β-galactosidase activity	FC	Nir et al. (1990)
FDA and its derivatives: 6CFDA, calcein blue AM, SFDA, BCECF_AM; Chemchrome B, V6	Cleaved by intracellular enzymes	EF/SPC/FC	Quinn (1984) and Catala et al. (1999)

(Continued)

TABLE 8.1 Continued

Probe(s) ^b	Mode of Action	Method ^c	Key References
CellTracker Green CMFDA	Esterases	EF	Schumann et al. (2003)
CMAC-Leu	Aminopeptidases	EF	Schumann et al. (2003)
<i>Nucleic Acid State</i>			
AO	Different color when linked to DNA or other things	FC	Darzynkiewicz and Kapuscinski (1990)
DAPI (or SG1) destaining	Eliminates staining of non-DNA	EF	Zweifel and Hagström (1995)
Syto13, SybrGreen, TOTO TOPRO	Separates at least two populations based on DNA/RNA content	FC	Robertson and Button (1989), Li et al. (1995)
<i>Double/Multiple Staining Protocols</i>			
Double "live/dead" stains BacLight, NADS, ...	Live/dead	EF/FC	Joux et al. (1997a), Grégori et al. (2001)
FDA/PI; BCECF-AM/PI	Activity/dead	FC	Yamaguchi and Nasu (1997)
EUB FISH probes/PI/DAPI	Active/dead/inactive/recently dead/live but inactive	EF	Williams et al. (1998), Howard-Jones et al. (2001)
<i>Cell Division/Growth</i>			
DVC	Live cells elongate when in presence of antibiotics	EF/FC	Kogure et al. (1979), Joux and Lebaron (1997)
Microcolony formation	Capability of producing a small colony	OM/EF	Jannasch and Jones (1959), Simu et al. (2005)
BrdU immunodetection	Incorporated into DNA instead of TdR	EF	Urbach et al. (1999), Pernthaler et al. (2002)

Miscellaneous

c-SNARE-1 AM 16S rRNA probes	Intracellular pH Detection of ribosomes	FC EF/FC	Leyval et al. (1997) Amann et al. (1990), Bouvier and del Giorgio (2003)
AAs, glucose, thymidine MAR	Organic substrate incorporation	EF	Hoppe (1976), Tabor and Neihof (1984), Pedrós-Alió and Newell (1989), Karner and Fuhrman (1997), Cottrell and Kirchman (2000)

^aUpdated from Table 3 in Gasol and del Giorgio (2000). An expanded version of this table can be found in the web appendix (www.icm.csic.es/bio/personal/gasol/chapterKirchmanbook).

^bINT, 2-(*p*-iodophenyl)-3-*p*-(nitrophenyl)-5-phenyltetrazolium chloride; AO, acridine orange; DAPI, 4',-6-diamidino-2-phenylindole; EthBr, ethidium bromide; SG1, SybrGreen 1; CTC, 5-cyano-2,3-ditolyltetrazoliumchloride; XTT, sodium 3'-[1-(phenylamino)-carbonyl]-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate; Eth-D2, ethidium homodimer-2; PI, propidium iodide; Rh123, rhodamine123; DiBAC₄(3), bis(1,2-dibutylbarbituric acid) trimethine oxonol; DIOC, diethylloxacarboyanine; DiOC₆(3), 3,3'-dihexyloxacarboyanine iodide; 6CFDA, 6-carboxyfluorescein diacetate; FDG, fluorescein -galactopyranose; FDA, fluorescein diacetate; CMAC-Leu, 7-amino-4-chloromethylcoumarin, 1-leucine amide, hydrochloride; CMFDA, 5-chloromethylfluorescein diacetate; BCECF-AM, 2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; MAR, microautoradiography; DVC, direct viable count; BrdU, 5-bromo-2'-deoxyuridine; NADS, nucleic acid double-staining protocol.

^cEF, epifluorescence microscopy; FC, flow cytometry; SPC, solid-phase cytometry; OM, optical microscopy; Spect, spectrophotometry.

surfaces (van Loodsrecht et al. 1987). Production of a capsule might be an indication of nutrient limitation (van Loodsrecht et al. 1987) and could also be a mechanism to enhance nutrient and substrate acquisition. Damaged and dead cells usually lack a capsule, show shrunken or partially deteriorated membranes, and may lack intracellular structures such as cytoplasm and ribosomes (Heissenberger et al. 1996). The capsules can be observed by transmission electron microscopy (TEM), or by a simpler microscopic method using Congo Red or Maneval's stain of cells transferred from filters onto a gelatin-covered slide (Storedegger and Herndl 2001).

Cellular Composition The ionic composition of the bacterial cells can give hints of relative activity (Fagerbakke et al. 1999), and also the macromolecular composition of bacterial cells is not fixed but rather varies as a function of cell size, activity, physiological state, and even phylogenetic composition (Kjelleberg et al. 1993). There is a wide plasticity in composition within a given bacterial organism, which is evident, for example, during the starvation process (Roszak and Colwell 1987; Kjelleberg et al. 1993).

Some of these properties can be examined by flow cytometry. The amount of intracellular nucleic acids is determined from the fluorescence of stains that attach preferentially to DNA, RNA or both. While DAPI, and to a lesser extent, acridine orange, are the standard fluorochromes for determining bacterial abundance in plankton samples using epifluorescence, a new generation of blue-excitable stains, including Syto13, PicoGreen, TOTO, SybrGreen, and SybrGold, are increasingly being used, particularly in combination with flow cytometry. The latter technique allows the simultaneous determination of bacterial abundance and of the amount of fluorescence emitted, and thus the relative nucleic acid content, of individual cells (del Giorgio et al. 1996; Gasol and del Giorgio 2000).

One of the most interesting findings is that bacterioplankton cells tend to cluster into distinct fractions based on differences in the individual cell fluorescence (related to the nucleic acid content) and in the side and forward light scatter signal (see, e.g., Li et al. 1995). There are at least two major fractions: cells with high nucleic acid content (HNA cells) and cells with low nucleic acid content (LNA cells) (Robertson and Button 1989; Li et al. 1995; Gasol et al. 1999; Gasol and del Giorgio 2000; Lebaron et al. 2002). These cytometric populations are almost always present regardless of the sample and of the different protocols, stains, and type of cytometer used, suggesting that these fractions are not methodological artifacts (Bouvier et al. 2007). The general trend that emerges is that the HNA cells appear to be not only larger (Troussellier et al. 1999) but also more active, with higher growth rates than the LNA cells, and that changes in total bacterial abundance are often associated with changes in this fraction (Gasol and del Giorgio 2000; Lebaron et al. 2002). Several studies have supported the idea that HNA bacteria are the high-growth component of bacterial assemblages (Gasol et al. 1999; Troussellier et al. 1999; Gasol and del Giorgio 2000; Lebaron et al. 2001; Seymour et al. 2004), because they increase in abundance in mesocosm or dilution experiments, while LNA bacteria do not (Gasol et al. 1999; Zubkov et al. 2004).

However, other studies have shown that LNA also develop in marine dilution experiments (Jochem et al. 2004; Longnecker et al. 2006). Cell sorting experiments have yielded mixed results, some showing higher cell-specific activities of the HNA fraction (Servais et al. 1999; Lebaron et al. 2001, 2002), others showing substantial activity in the LNA fraction as well (Zubkov et al. 2004; Longnecker et al. 2005). The evidence to date suggests that the simple dichotomy of HNA = active and LNA = inactive is an oversimplification (Bouvier et al. 2007), and that LNA cannot routinely be considered to be inactive or dead.

Nucleoid Zweifel and Hagström (1995) proposed a de-staining procedure that stains with DAPI only those cells with a compacted nucleoid (NucC, nucleoid-containing cells). The standard DAPI procedure, they claimed, stains the DNA and also other cellular components. An isopropanol rinse following DAPI staining removes the nonspecific staining and leaves only the stain associated with DNA, which appears as a yellowish spot. Choi et al. (1996) proposed some modifications to the protocol of Zweifel and Hagström, yielding higher counts of nucleoid-containing cells.

Electron Transport Activity Probes The activity of the respiratory enzymes can be assayed using tetrazolium salts as indicators of bacterial respiration; the electrons produced from the electron transport chain in actively respiring bacteria reduce these salts from a soluble form to a dense, insoluble, cell-localized precipitate. These compounds compete with oxygen as electron acceptors. Depending on the type of salt used, the precipitate is colored or fluorescent. The first application of the method was with the probe 2-(*p*-iodophenyl)-3 (phenyl)-5-phenyltetrazolium chloride (INT) (Zimmerman et al. 1978). Rodriguez et al. (1992) introduced a modification of the method, based on the fluorogenic tetrazolium dye 5-cyano-2,3-ditolyltetrazolium chloride (CTC), which is reduced to a red-fluorescent formazan molecule that can be detected both with epifluorescence microscopy and with flow cytometry (del Giorgio et al. 1997; Sieracki et al. 1999; Sherr et al. 1999a). The techniques were initially thought to be applicable only to aerobic bacteria, but in anaerobic conditions, particularly during fermentation, the methods seems to work well (Smith and McFeters 1997), although nonbiological reduction may occur in sediments and other reducing environments (Smith and McFeters 1997).

Some authors have criticized the CTC method, on the basis of the potential cellular toxicity of the CTC, or because of the low numbers of CTC-positive cells often recorded in field studies (Ullrich et al. 1999; Servais et al. 2001). Ullrich et al. (1996) and Servais et al. (2001) both showed inhibitory effects of CTC on bacterial respiration and metabolic activity. On the other hand, Epstein and Rossel (1995) reported that CTC-stained and nonstained benthic bacteria grew equally well and that the ciliate *Cyclidium* sp. can survive on a diet of CTC-stained bacteria. Based on flow-cytometric sorting of red-fluorescent particles (assumed to be CTC-labeled bacteria) after incorporation of a radioactive tracer, Servais et al. (2001) concluded that the CTC dye is not suitable for the detection and enumeration

of active bacteria, since the apparent contribution of these cells to total leucine uptake was relatively small, and similar results were later reported by Longnecker et al. (2005). However, recent flow-cytometric observations suggest that part of the discrepancy might be due to the fact that very active cells might explode as the result of the CTC-formazan granule formation, and free the granule to the medium. Cell sorting based only on red fluorescence of the granules would miss the most active cells, as those would have been destroyed before sorting (Gasol and Aristegui 2007). Overall, it would appear that the CTC method targets the cells with the highest respiration rates (Sherr et al. 2001; Smith and del Giorgio 2003; Sieracki et al. 1999).

Membrane Integrity and Functionality The bacterial cell membrane plays a key role in the functioning of the cell, and the state of the membrane thus provides much information on the general physiological condition of the cell (Brehm-Stecher and Johnson 2004). Several aspects of membrane function are of ecological interest, such as membrane integrity, energization and polarity, and the existence of pH and ionic gradients. Membrane potential and membrane integrity are two aspects of cellular function that should be a priori strongly linked, but studies have shown that this expectation is not always borne out, because the loss of membrane integrity is not always accompanied by loss of potential, and vice versa (Novo et al. 2000; del Giorgio and Bouvier 2002).

There are a number of different approaches to probe the state of the cell membrane (Table 8.1). Cell injury and damage results in increased membrane permeability that can be assessed using exclusion stains that bind to nucleic acids, such as propidium iodide (PI), TOPRO, or SYTOX Green (Veldhuis et al. 2001; Roth et al. 1997; Maranger et al. 2002). Because of their molecular weight and structure, these dyes can only enter cells with damaged or “leaky” membranes, and cannot enter cells with intact membranes (Haugland 2005). Cell injury and death is accompanied by loss of membrane potential and polarization. The latter can be assessed with the use of oxonols, negatively charged molecules that are excluded by cells with polarized membranes (Jepras et al. 1995). For example, DiBAC₄(3) is an anionic membrane potential-sensitive dye that enters cells with depolarized plasma membranes and then binds to lipid-containing intracellular material. It has been used to differentiate live and dead cells (Mason et al. 1995) and to assess the physiological changes of bacteria along salinity gradients (del Giorgio and Bouvier 2002).

Intracellular Enzymatic Activity Various fluorescein esters have already been used in bacterial viability assays, including fluorescein diacetate (FDA: Chrzanowski et al. 1984; Diaper et al. 1992), carboxyfluorescein diacetate (CFDA: Dive et al. 1988; Miskin et al. 1998), and Chemchrome B (Clarke and Pinder 1998) and CMFDA (Schumann et al. 2003). These esters are uncharged and nonfluorescent, and are passively transported into cells. Internal enzymes (esterases) then hydrolyze them to fluorescein derivatives, which are charged and fluorescent. In viable cells, the membrane is impermeable to the charged molecules, which, therefore, can accumulate intracellularly and be detected by fluorescence. Organisms with leaky membranes

do not retain the fluorescein. Thus, these assays target two aspects of cell function: intracellular enzymatic activity and membrane permeability. The method has some problems: many bacteria are unable to transport FDA, the fluorescence emission tends to be weak, and intracellular esterase activity is not always directly coupled to respiration (Diaper et al. 1992).

Uptake of Organic Substances Microautoradiography (MAR) is one of the earliest single-cell methods; the first reports of its use in aquatic microbial ecology were published in 1959 (Saunders 1959), and Brock (1967) used it to quantify the growth rate of a conspicuous freshwater bacterium. It was used in marine samples as early as 1976 (Hollibaugh 1976; Hoppe 1976). In this technique, microbial assemblages are incubated with a radiolabeled substrate and cells are then placed in contact with an autoradiographic emulsion, and subsequent exposure of the emulsion to the radioactive emissions produces silver grain deposits around the cells that are radioactive. Amino acids, acetate, glucose, along with leucine and thymidine were used in the initial studies, but recent studies have expanded to other substrates, such as chitin, *N*-acetylglucosamine, and even $^{14}\text{CO}_2$. Grain density may provide further information on the level of substrate uptake by the individual cells (Cottrell and Kirchman 2003; Sintes and Herndl 2006). In 1999, two independent studies showed that it was possible to combine this technique with fluorescence in situ hybridization (FISH), providing the first insights into the in situ single-cell activity of specific bacterial groups. A number of acronyms have been proposed for variations of the same approach, including MAR-FISH (microautoradiography–fluorescence in situ hybridization: Lee et al. 1999), STAR-FISH (substrate tracking autoradiography–fluorescence in situ hybridization: Ouverney and Fuhrman 1999), and Micro-FISH (microautoradiography–fluorescence in situ hybridization: Cottrell and Kirchman 2000).

DNA Synthesis and Replication An alternative to using radioactive compounds for measuring bacterial DNA synthesis is to use 5-bromo-2'-deoxyuridine (BrdU), which is a thymidine analog. Cells that are synthesizing DNA, and thus in the process of cell division, incorporate BrdU into DNA, and can be detected by immunofluorescence using anti-bromodeoxyuridine monoclonal antibodies or isolated by immunochemical capture using antibody-coated paramagnetic beads (Urbach et al. 1999; Borneman 1999; Hamasaki et al. 2007). This protocol has been also combined with CARD-FISH to allow the identification of individual growing cells (Pernthaler et al. 2002).

Direct Viable Count (DVC) Method The direct viable count method was first described by Kogure et al. (1979) and was based on incubating samples with the antibiotic nalidixic acid, which inhibits cell division. Active cells continue to grow but not to divide, elongating in the process, and these elongated cells can be detected microscopically (or by flow cytometry). The method has been used extensively to assess activity and viability of bacteria both in culture and in the environment (Roszak and Colwell 1987; Kogure et al. 1987; Yokomaku et al. 2000). The treatment

generating elongated bacteria now includes the addition of glycine (Yokomaku et al. 2000) and an antibiotic cocktail (Joux and Lebaron 1997).

There are currently a variety of techniques that target key categories of bacterial function and physiology at the single-cell level:

- **Morphological integrity**, including the presence of capsules and intracellular contents (determined using transmission electron microscopy)
- **Macromolecular composition**, including the cell-specific DNA and RNA contents using flow cytometry and fluorescence in situ hybridization (FISH)
- **Respiratory activity**, determined using redox-sensitive dyes such as CTC, combined with microscopy or flow cytometry
- **Status of the cell membrane**, using exclusion dyes such as PI or TOPRO, and potential-sensitive dyes such as oxonols, combined with flow cytometry
- **Internal enzymatic capacity**, determining internal esterase or galactosidase activities
- **Substrate uptake**, using radiolabeled organic molecules
- **Synthesis and replication of DNA**, using microautoradiography or the incorporation of nucleotide analogs such as BrdU and immunofluorescence
- **Cellular growth**, by inhibiting cell division and following the resulting cell elongation using microscopy or flow cytometry
- **Mixed approaches**, which combine two or more of the above in a single protocol

Combined Approaches There are increasing attempts to combine approaches, and a number of recent combinations have been proposed. Molecular probes introduced the BacLight Live/Dead kit, which is a mixture of a DNA stain (Syto9) that permeates into cells and an exclusion stain (PI). Both are added at the same time and interact so that live cells are stained green and dead cells are stained red. These cells can be counted by epifluorescence microscopy or flow cytometry. The latter allows the separation of cells into categories: green, green plus orange-red, and orange-red cells, which correspond to live, damaged, and dead cells, respectively (Grégori et al. 2001). Recently, Manini and Danovaro (2006) have suggested combining EthD-2 and PI in sediments. EthD-2 has a much higher (i.e., double) molecular weight, and is thus a good candidate to overcome problems encountered with the use of propidium iodide (PI).

Other combination approaches have also been proposed. Williams et al. (1998) combined staining with PI (as a membrane integrity marker), FISH, and DAPI; this protocol differentiates cells that are dead (positive signal due to propidium iodide (PI+), and a negative FISH signal (FISH-), cells that are dead but had been active until recently (PI+, FISH+), cells that are alive and active (PI-, FISH+), and cells that are inactive but not dead (PI-, FISH-). It is, in theory, possible to discriminate dormant bacteria from the difference in the number of NucC

cells, and the number of “live” cells determined by a probe that does not permeate membranes (see, e.g., Gasol et al. 1999; Luna et al. 2002). Manini and Danovaro (2006) proposed the destaining of SybrGreen I in a way similar to the destaining of DAPI. They combined this procedure with staining by PI or EthD-2, which allows dead cells (without a visible nucleoid region) to be distinguished from red-stained bacteria with a still-visible nucleoid (NucC) region. These cells (PI+, NucC+) are considered to be either cells that died recently or cells competent for transformation (having large “holes” in the membrane to allow DNA entry). Pirker et al. (2005) combined MAR and PI, to simultaneously assess the metabolic activity and viability of individual bacterioplankton cells in the North Sea.

Operational Categories of Single-Cell Activity

There has been much debate concerning the effectiveness of some of the methods described above, particularly in the context of natural bacterial communities characterized by low levels of activity and also by relatively mild sources of stress, at least compared to laboratory conditions (Ullrich et al. 1996; Karner and Fuhrman 1997; Kell et al. 1998; Colwell 2000; Smith and del Giorgio 2003). The current methods only allow us to place bacteria in very broad physiological categories, that is, active or inactive in substrate uptake, or having and intact or injured membranes. The categories themselves are often not well defined, because all the methods have thresholds of detection, and the thresholds themselves vary with the different protocols used and sample conditions. The physiological categories obtained are thus operational and in no way absolute (Smith and del Giorgio 2003). In addition, there is likely a large range of activity or physiological states within each of these categories. For example, within the cells that are scored positive for substrate uptake, there is probably a large range in the actual rates of substrate uptake (see, e.g., Sintes and Herndl 2006). Likewise, there is often a large range in the level of red fluorescence of cells that are scored as positive to CTC reduction within any given sample (Sherr et al. 1999a; Posch et al. 1997).

There are several approaches that link individual cell activity to phylogenetic composition, including the following:

- **Flow sorting of specific cell fractions** (radiolabeled with substrates or stained with specific dyes) followed by molecular analysis of the DNA of the sorted fractions
- **Autoradiography** (radiolabeled substrate uptake) combined with FISH to determine the identity of substrate-active cells (Micro-FISH, MARFISH)
- Detection of DNA-synthesizing cells through the **incorporation of BrdU followed by immunocytochemical** detection of the target cells with further DNA analysis, or FISH identification

Universe of DAPI+ particles

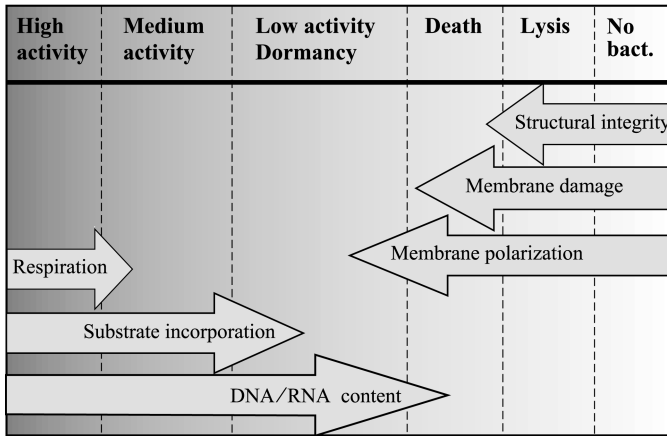


Figure 8.2 Conceptualization of a continuum of physiological states that is targeted at different levels by different methods. The DAPI-positive particles can in theory be classified into the upper-line categories, but can also be classified depending on each method sensitivity and target cellular process.

Other problems arise from the fact that there is not always a tight correlation between categories. For example, a dormant cell may be intact and viable but show no signs of activity whatsoever, whereas a cell that is badly damaged may still show measurable signs of remnant metabolism. In this regard, Smith and del Giorgio (2003) suggested that the fact that current methods target different cell functions and have different intrinsic thresholds, and are thus often not comparable, should be viewed as an advantage rather than as an obstacle, because when taken collectively they provide complementary information on the physiological structure of bacterioplankton. Figure 8.2 offers a simplified view of how the different categories of methods cover different areas of the physiological spectrum of bacterioplankton assemblages. These approaches should be viewed not as independent but rather as providing a nested structure, which allows one to differentiate at least five basic categories of cells: (1) intact, active and growing; (2) intact, active but without apparent growth; (3) intact but no apparent sign of activity; (4) injured, with or without signs of activity; (5) dead. These categories roughly agree with those proposed by other authors (Barer 1997; Kell et al. 1998; Weichart 2000; Nebe-von Caron et al. 2000). Dormant cells would fall somewhere within categories 2 and 3, whereas extremely slow growing cells might fall in categories 2, 3, and 4. We use this basic classification to describe the existing data in the sections below.

Regulation of Physiological Structure of Marine Bacterioplankton

In this section, we briefly review some of the main factors that influence the level of metabolic activity and the physiological state of marine bacterioplankton cells, as well as the factors that influence the persistence and loss of the different physiological

fractions of the community. Chapter 10 explores the factors that influence growth at the community level. Here, we focus on the factors that influence the physiological state and the metabolic activity of single bacterial cells.

Factors Influencing Physiological State of Bacterial Cells in Marine Ecosystems

Slow growth and dormancy are certainly not the only physiological states of interest from an ecological point of view. Cells are subject to a wide range of environmental characteristics that either enhance metabolic activity or are deleterious and generate stress, and that may ultimately cause cell death. The response of cells to stress depends greatly on what cellular function is investigated and might vary specifically (Joux et al. 1997a; Agogué et al. 2005). For example, exposure to heat or UV radiation had very different impacts on various cellular functions of *E. coli* (Fiksdal and Tryland 1999). López-Amorós et al. (1995a, b) found that stress and mortality caused by starvation did not result in an increase in membrane permeability, and was thus difficult to detect using traditional exclusion stains. Likewise, the capacity to detect bacteria using FISH is extremely sensitive to osmotic stress and to carbon starvation, but not to cold and other types of physical and chemical stress (Tolker-Nielsen et al. 1997; Oda et al. 2000). There is thus no single or simple response to stress, and loss of a certain function does not necessarily imply loss or degradation of all other functions, except in the most extreme cases.

The availability of energy, mainly in the form of organic carbon (or as certain inorganic compounds or light for some bacteria), is perhaps one of the key factors influencing bacterial single-cell activity in aquatic ecosystems (Kjelleberg et al. 1993; Morita 1997; Azam 1998; and see Chapter 10). As discussed above, the response of cultured bacteria to extreme carbon limitation has been extensively studied, but the question remains: What is starvation from the viewpoint of a marine bacterium, and is extreme starvation the most common state for marine bacteria? Although much of the ocean is extremely dilute and oligotrophic, this environment is still not analogous to the conditions of complete nutrient deprivation created in laboratory cultures used to study the starvation response. (Fegatella and Cavicchioli 2000). Substrate concentrations in marine waters are indeed extremely low, due in part to low supply rates, and but also as the result of high turnover of these substrates, precisely due to extremely efficient bacterial consumption. It has often been assumed that bacteria in oligotrophic marine systems oscillate between scenarios of feast or famine (Azam 1998) in terms of substrate availability, but the reality for most oceanic bacteria lies probably somewhere in between. In this regard, Ferenci (2001) recently introduced the notion of “hungry” bacteria: those that experience suboptimal resource conditions that are somewhere between feast and extreme famine. Hungry bacteria develop a whole set of adaptations that are in fact distinct from those that characterize the more extreme starvation response.

We do not know to what extent marine bacteria are “hungry” as opposed to “starved,” but it is clear that there is no absolute threshold of carbon limitation/starvation that applies to all bacterial types, so a given level of organic substrate

availability will trigger the starvation response in some bacteria and not in others (Ferenci 2001). There is increasing evidence that phylotypes within broad phylogenetic groupings may share functional and metabolic traits such as patterns of substrate utilization or intrinsic levels of cellular activity (Cottrell and Kirchman 2000; Zubkov et al. 2001; Yokokawa and Nagata 2005).

Exposure to high substrate concentrations can cause cell death in cells that are dormant and in starvation-survival mode (Koch 1997). In fact, typical oligotrophic marine bacteria, such as *Sphingomonas* spp. (Schut et al. 1997) and *Pelagibacter* (Rappé et al. 2002), are unable to grow in rich media, most likely due to an inability to deal with high respiration rates and the associated oxidative stress. In marine environments bacterial cells need to cope not only with low ambient concentrations of organic substrates and nutrients, but also, and perhaps more importantly, with highly intermittent supply at very different spatial and temporal scales. Some of these fluctuations may exceed the protective mechanisms of the cells, with deleterious or lethal consequences to the microorganisms (Koch 1997).

Cells lacking key inorganic nutrients might still consume and respire organic matter, but biomass accumulation and cell division may be severely impeded under nutrient starvation. This should be reflected in the distribution of bacterial cells with different physiological states, as cells would be actively respiring but unable to synthesize nucleic acids. It has been noted that while carbon starvation often leads to cell miniaturization (Morita 1997), acute nutrient limitation may lead to an increase in cell mass (Kjelleberg et al. 1993). Elemental composition may shift according to the physiological state of cells. For example, bacteria typically have more nitrogen and phosphorus per unit carbon than algae, but unicellular cyanobacteria often have higher carbon in relation to heterotrophic microorganisms, and this has been interpreted as storage of carbon under nutrient deficiency (Heldal et al. 2003). Carbon availability associated with inorganic nutrient limitation can translate into the accumulation of excess reserve polymers, greatly increasing cell size. In the “Winnie-the-Pooh” hypothesis (Thingstad et al. 2005), increase in cell size might be seen as a strategy to “dominate” the system and simultaneously optimize uptake and minimize predation.

There are a number of direct as well as indirect effects of light, and particularly UV, on bacterial physiology. Direct effects include damage to DNA, RNA, and cell membranes (Herndl et al. 1993; Fiksdal and Tryland 1999). Indirect effects include UV-mediated generation of toxic radicals and hydrogen (Xenopoulos and Bird 1997); increased substrate availability through photochemical degradation of refractory dissolved organic carbon (DOC) into more bioavailable forms (Kieber et al. 1989; Moran and Zepp 1997); reduction in viral abundance and infectivity (Suttle and Chen 1992; Noble and Fuhrman 1997; Wilhelm et al. 1998), perhaps countered by induction of the lytic cycle in lysogenic bacteria (Freifelder 1987; Maranger et al. 2002); and declines in the rate of flagellate grazing on bacteria and thus influence on the loss rates of bacteria (Sommaruga et al. 1996). Not all bacterioplankton groups seem to be equally susceptible to UV damage (Joux et al. 1999; Arrieta et al. 2000; Agogué et al. 2005; Alonso-Sáez et al. 2006), and UV appears to have a greater effect on the more active cells (Rae and Vincent 1998; Maranger et al. 2002; Alonso-Sáez et al. 2006). Cell-specific activity seems to be

more susceptible to UV damage than membrane structure and potential (Alonso-Sáez et al. 2006).

Membranes, nucleic acids, and certain enzymes are sites prone to damage by heat (Teixeira et al. 1997; Fiksdal and Tryland 1999), but a typical marine bacterium will be rarely exposed to very high temperatures. Cold shock has been shown to generate sublethal injuries and force cells into a dormant state (see, e.g., Kjelleberg et al. 1987; Roszak and Colwell 1987; Munro et al. 1989). Whatever the direct mechanism involved at low temperatures, the general pattern is that more cells enter dormancy, and there are fewer cells capable of forming colonies or reducing CTC (see, e.g., Smith et al. 1994). Temperature and organic carbon availability interact, such that the inhibitory effects of temperature are modulated in the presence of higher substrate concentrations (see, e.g., Wiebe et al. 1992; Pomeroy and Wiebe 2001).

Osmotic shock has also been shown to generate sublethal injuries and force cells into dormancy. The capacity to detect bacteria by FISH, for example, has been shown to be extremely sensitive to osmotic stress (Tolker-Nielsen et al. 1997; Oda et al. 2000). del Giorgio and Bouvier (2002) found in the 0–10 salinity range of the Choptank estuary a dramatic decline in hybridization with FISH probes, a decrease in bacterial production and growth efficiency, and an increase in cells with depolarized and injured membranes. Painchaud et al. (1995) had already identified salinity as one of the main factors related to cell mortality in the St. Lawrence estuary. Changes in the structure and functioning of the microbial food web along gradients of salinity (Schultz and Ducklow 2000; del Giorgio and Bouvier 2002; Bouvier and del Giorgio 2002; Casamayor et al. 2002; Gasol et al. 2004; Kirchman et al. 2005) indicate that this parameter is a variable structuring both the composition and physiology of bacterioplankton.

Finally, there are a wide range of chemical substances that may influence bacterial activity and physiology in marine waters. These include antibiotic and toxic compounds released by bacteria (Long and Azam 2001) and by other planktonic organisms (Adolph et al. 2004), allelopathic substances (Gross 2003), or substances associated with quorum sensing (Miller and Bassler 2001). Contaminants may also interfere with marine bacterial activity (Price et al. 1986). Other factors may influence bacterial physiology, such as barometric pressure, low oxygen, or even localized anoxia (e.g. in particles or guts), and turbulence (e.g. Malits et al. 2004), but, as far as we know, their effects have not been well explored to date.

Factors Influencing Loss and Persistence of Physiological Fractions

Grazing Predation is a major loss mechanism of bacterial cells in all marine systems (see Chapter 11). There is now strong evidence that protistan grazing can be highly selective in terms of the physiological state of the cells. In general, cells that are more active tend to be selectively cropped by protistan grazers (del Giorgio et al. 1996; Šimek et al. 1997; Pernthaler et al. 1997; Tadonlécé et al. 2005). The underlying basis of this selectivity is partly cell size, since there is in general a positive relationship between cell size and activity in bacterioplankton

(Gasol et al. 1995; and see below), but is not restricted to it. Grazing preferentially removes active cells, but may produce injured and dead cells, through incomplete digestion in protistan vacuoles and passage through the gut of metazoans. Incomplete feeding also results in cell fragments (Nagata and Kirchman 2000; Nagata 2000a), or incompletely digested egesta (picopellets, Nagata 2000b) that can potentially be counted as normal bacteria by standard protocols.

Cell Size Small size may facilitate survival by being a refuge from predation (Jürgens and Güde 1994). Several empirical studies have shown a preference of larger bacterial prey by cultured and natural assemblages of flagellates (Andersson et al. 1986; González et al. 1990; Jürgens and Güde 1994) and ciliates (Fenchel 1980; Epstein and Shiaris 1992; Šimek et al. 1994). Size, thus, can have a large impact on the fate of bacterial production because it determines susceptibility to grazers and viruses (see also Chapter 11).

Viral Lysis Viral infection has been proposed as the main mechanism generating empty or “ghost” bacterial cells that retain external structure but lack internal material (Heissenberger et al. 1996). There is now increasing evidence that viral infection is selective, towards cells that are either more sensitive, or with higher growth and metabolic rates (Waterbury and Valois 1993; Middelboe et al. 2001; Bouvier and del Giorgio 2007), and, like grazing, viral lysis preferentially removes cells from the most active categories and moves them to the dead or detrital categories. About 35 percent of all bacteria may be infected at a given time, and lysogeny may influence the growth rate and the metabolism of the host cells (Weinbauer 2004). There are feedbacks between processes, so that environmental stress may trigger the lytic cycle in lysogenic bacteria (Weinbauer 2004).

Cell Motility This is another factor that plays a role in both cellular activity and loss. Motility is widespread in marine bacteria (Blackburn et al. 1998; Grossart et al. 2001). It allows a bacterium to track patches of organic matter (Grossart et al. 2001), and, in an environment with a complex microscale structure (sensu Azam 1998), motility should increase the chance of growth and survival (Blackburn et al. 1997). On the other hand, motility increases the probability of encounter with a phage (Bratbak et al. 1992) or with a protist (González et al. 1993; Monger et al. 1999), although very fast bacteria may also be able to avoid predation (Matz and Jürgens 2001).

Cell Degradation The persistence of dead and injured cells in the water column has not been well studied (Mason et al. 1986). Due to their size, these cells remain suspended, and, due to the relative recalcitrance of their membrane components (see, e.g., McCarthy et al. 1998), dead and injured cells, as well as cell fragments, may persist for a long time (Mason et al. 1986). Remains of bacterial cell membranes and cellular fragments have been proposed as the main source of dissolved organic nitrogen in the ocean (McCarthy et al. 1998). The potential interaction between abiotic factors (UV, temperature, water chemistry) and grazing in determining the loss of dead and injured cells has not been well explored to date.

Regulation of the physiological structure of bacterioplankton communities

has three main components:

- *Environmental factors* that influence the individual level of metabolic activity and cell integrity and damage, such as substrate and nutrient availability, UV, and temperature
- *Physical and biological factors* that influence the persistence and loss of the various physiological fractions, such as selective grazing and viral infection, and selective degradation
- *Intrinsic phylogenetic characteristics* that modulate the response of different bacterial strains to the above factors

DISTRIBUTION OF SINGLE-CELL CHARACTERISTICS IN MARINE BACTERIOPLANKTON ASSEMBLAGES

In this section, we discuss the data generated over the past three decades concerning the distribution of single-cell characteristics in marine bacterioplankton assemblages. We have assembled a comprehensive dataset based on published and unpublished observations of bacterioplankton single-cell activity and physiological state from studies that have applied some of the techniques listed in Table 8.1.

Distribution of Single-Cell Activity and Physiological States in Marine Bacterioplankton

For each category of assay, we present the average proportion of cells scored as positive relative to the total for all the studies that have applied the technique, together with the range of values obtained in the different studies as well as the resulting standard error and distribution quartiles (Fig. 8.3). Most of the published studies have applied a single technique, although there are a handful of marine studies that have combined several approaches. The latter have been summarized in Table 8.2 and are discussed in the following section.

Figure 8.3 shows a clear ordination of methods based on the average proportion of cells scored as positive when applied to marine bacterioplankton assemblages. Although almost all techniques yield a wide range of results, most results for any given technique cluster in a specific range (as evidenced from the standard error around the mean). At one end of the spectrum, methods that target activity tend to yield relative low proportions of reactive cells. For example, researchers find on average less than 20 percent of CTC-positive, esterase-positive, and DVC-positive cells in marine samples. At the other extreme are methods that target membrane integrity or macromolecular composition, such as BacLight, FISH (RNA) or high-DNA cells, which fall consistently in the range between 40 and 60 percent of the total cells. Other approaches, such as MAR (substrate uptake) and ultrastructural analysis

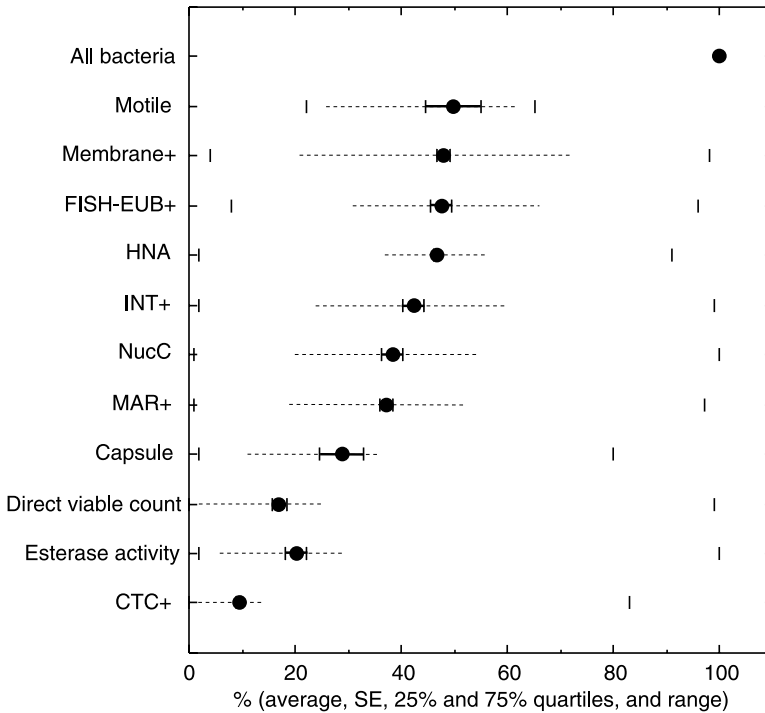


Figure 8.3 The percentage of reactive cells determined by different methods for marine plankton communities (database available at www.icm.csic.es/bio/personal/gasol/chapterKirchmanbook). The average (filled-in circles), standard error (SE: solid lines), range between the 25 and 75 percent quartiles (dotted lines), and ranges of values (spaces between the two vertical lines) are presented. “Membrane +” indicates all membrane polarity and potential probes, including double-staining protocols.

(presence of capsule), generally fall within the range of 20–40 percent. These results would suggest that, on average, marine bacterioplankton assemblages have a relatively high proportion (>50 percent) of cells that appear to have intact membranes and ultrastructural integrity, and detectable levels of DNA and RNA, but that only a fraction of these apparently intact and healthy cells are active in substrate uptake, and that an even smaller fraction have high levels of metabolic activity, expressed as cell growth, cell division, or detectable respiratory activity. Moreover, these results suggest that, on average, a nontrivial proportion (>20 percent) of marine bacterioplankton cells may be either injured or dead.

The pattern in Figure 8.3 further suggests that certain aspects of cell physiology may be more coupled than others. For example, DNA and RNA contents and membrane integrity result in similar proportions of positive cells, whereas respiratory, enzymatic, and substrate uptake activity appear generally clustered in the lower end of the spectrum. The fact that a relatively large fraction of intact cells appears to have extremely low or nondetectable metabolic rates agrees well with recent estimates

TABLE 8.2 Overview of Studies Comparing Different Activity Protocols

		Site	Method (and %) ^a			
<i>Marine Studies</i>						
Hoppe (1976)	Kiel Bay	AO >	MAR+ >	cfu		
		100	41	1		
Meyer-Reil (1978)	Kiel Bay	AO >	INT+ >	cfu		
		100	31	2		
Kogure et al. (1979)	Pacific Ocean	AO >	DVC >	cfu		
		100	7	0.0001		
Tabor and Neihof (1984)	Chesapeake Bay	AO >	INT+ ≈	MAR+ >	DVC	
		100	46	43	32	
Newell et al. (1986)	Chesapeake Bay	TC >	MAR+ ≈	INT+ >	DVC	
		100	61	56	35	
Choi et al. (1996)	Oregon coast	DAPI >	NucC >	Live+ >	CTC+	
		100	25	14	7.5	
		100	44	21	19	
Karner and Fuhrman (1997)	California coastal	DAPI >	FISH >	MAR+ >	NucC >	
		100	56	49	29	
Joux and Lebaron (1997)	Mediterranean coast	DAPI >	DVC >	CTC+ >	CFU	
		100	12	3	0.8	
Gasol et al. (1999)	Mediterranean coast	DAPI >	Live+	NucC >	HNA	
		100	70	68	60	
Catala et al. (1999)	Mediterranean coast	DAPI >	Esterase+ >	DVC ~	CTC >	
		100	8	6	5	
				1	cfu	

(Continued)

TABLE 8.2 Continued

	Site		Method (and %) ^a			
Bernard et al. (2000b)	Mediterranean coast	SG>	CTC+≈	cfu		
		100	2	1.5		
Grossart et al. (2001)	California coast	DAPI>	Motile>	NucC>	CTC+	
		100	60	33	9	
Sherr et al. (2002)	Atlantic shelf	DAPI>	NucC>	CTC+		
		100	51	6		
Storedegger and Herndl (2002)	North Sea	DAPI>	Capsule>	CTC		
		100	35	19		
Schumann et al. (2003)	Estuarine Baltic Sea	DAPI>	PI->	Esterase+	CTC+	
		100	74	12	10	
		100	67	8	15	
Schumann et al. (2003)	Global	DAPI>	EthD2->	Sytox-~	PI->	leu AMP
		100	87	71	70	6
Yakimov et al. (2004)	Ross Sea	DAPI>	FISH+>	CTC+		
		100	87	11		
		100	18	2		
Davidson et al. (2004)	Tasmanian coastal	DAPI>	PI->	MPN>	CFDA+≈	CTC+
		100	30	23	13	13
Zampino et al. (2004)	Mediterranean coast	DAPI>	Live+≈	DVC≈	CTC+≈	cfu
		100	4	4	2	1
Pearce et al. (2007)	Antarctic coast	DAPI>	Esterase+>	Live+		
		100	37	14		

Other Aquatic Studies

Maki and Remsen (1981)	Lakes	AO >	INT+ ≈	DVC		
		100	10	8		
Quinn (1984)	Lakes	AO >	INT+ ≈	DVC >	FDA+ >	cfu
		100	26	22	5	0.4
Porter et al. (1995)	Lake Windermere	AO >	CFDA ~	CTC >	DVC >	cfu
		100	6	4	0.6	0.2
	River Mersey	AO >	Chem B >	DVC >	cfu	
		100	80	71	56	
Yamaguchi and Nasu (1997)	Rivers	DAPI >	Esterase+ >	CTC+		
		100	63	25		
Kawai et al. (1999)	Purified water	DAPI >	6CFDA >	DVC ≈	CTC+ >	μcol >
		100	20	5	5	1
Yokomaku et al. (2000)	River water	DAPI >	Esterase+ ~	DVC >	CTC+ >	cfu
		100	34	17-45	20	12
Berman et al. (2001)	Lake Kinneret	DAPI >	Live+ ≈	NucC ~	CTC+	
		100	9	8	5	
Vollertsen et al. (2001)	Wastewater	AO >	DAPI >	Live+ >	MAR+ >	CTC+
		100	75	60	40	20
Luna et al. (2002)	Marine sediments	AO >	Live+ >	NucC >	DVC	
		100	28	4	2	
Schumann et al. (2003)	Freshwater	DAPI >	PI- >	Esterase+	CTC+	
		100	65	9	11	
Freese et al. (2006)	River	DAPI >	FISH+ ≈	Membr+ >	Esterase+ >	CTC+
		100	29	27	5	2

^aThe methods are ordered according to the percentage of the reference value (first column). A qualitative appreciation is given by symbols =, ≈ or > (indicating equal, similar or higher value). Acronyms are as in Table 8.1, with the addition of cfu, colony-forming units; MAR, microautoradiography; SG, SybrGreen; AB, antibody-positive; leu-AMP, leucine aminopeptidase; B-Gal: β-galactosidase; μ-col, microcolony formation.

of the proportion of marine bacterioplankton cells that are dividing, based on the incorporation of BrdU, which are in the range of less than 1 to 10 percent in coastal assemblages (Urbach et al. 1999; Borneman 1999; Pernthaler et al. 2002).

Simultaneous Determination of Several Aspects of Single-Cell Activity and Physiology

The average distribution of single-cell characteristics presented in Figure 8.3 is derived mostly from studies that have applied individual techniques in different systems, and thus the pattern could be driven to some extent by biases in terms of the origin and characteristics of the samples. To what extent does the average distribution of single-cell characteristics presented in Figure 8.3 represent the real sequence of physiological states that could be found within a single sample if multiple techniques were applied simultaneously? In Table 8.2, we have summarized the marine studies that combined and compared several approaches in the same samples. For reference, we have included in this table some additional studies that simultaneously assessed multiple aspects of single-cell activity in other aquatic ecosystems.

The data summarized in Table 8.2 show a pattern of distribution of single-cell characteristics within individual samples that is similar to the average cross-sample pattern shown in Figure 8.3. Most studies that combined several approaches found relatively high proportions of cells that appear intact in terms of membrane integrity, ultrastructure, and macromolecular component, but a much smaller fraction that appears active in terms of respiration, intracellular enzyme activity, or substrate uptake. Some of these studies found some coherence between the different aspects of single-cell physiology. For example, Karner and Fuhrman (1997) found that NucC values were similar to those from autoradiography and FISH, although these two aspects of cell function were not significantly correlated. Likewise, Gasol et al. (1999) found that the number of HNA bacteria measured by flow cytometry was similar to the number of NucC cells, and the rates of change of both groups were similar. Davidson et al. (2004) and Schumann et al. (2003) found similar number of CTC-positive and CFDA-positive or esterase-active cells, suggesting that these aspects of cell function might be more tightly coupled.

Some of these studies have also revealed apparent discrepancies. For example, Karner and Fuhrman (1997) reported extremely low numbers of CTC-positive cells, in spite of relatively high proportions of MAR and FISH-positive cells. Likewise, Grossart et al. (2001) found that between 22 and 64 percent of bacteria were motile in coastal marine sample. Larger cells were more motile than smaller cells, and there was a correlation between colony-forming units (CFU) and percentage of cells that were motile, but this percentage was about twofold higher than the percentage of NucC-containing cells, and about sixfold higher than the proportion of CTC-positive cells. Since motile bacteria must be respiring, these differences must be due to the different thresholds of detection of each method. Interestingly, Grossart et al. (2001) also found that motile and CTC-positive percentages tended to converge (91 percent for CTC-positive and 85 percent for motile cells) upon the

addition of organic substrates, suggesting greater uncoupling of these processes under growth-limiting conditions.

Some of these apparent discrepancies may be methodological, but others may be a reflection of the complexity of the coupling between various aspects of cellular function. A number of laboratory and regrowth studies have revealed complex patterns of response to starvation and stress at the single-cell level. For example, Joux et al. (1997b) described the response of a marine bacterium to prolonged starvation, in which total cell counts and DNA content remained relatively stable during the first 2 months of starvation, whereas there was a clear sequence of changes in other aspects of cell functioning, starting with loss of culturability, followed by a decline in respiratory and enzymatic capacity, and ending in a loss of membrane integrity. Others have shown similar responses following exposure to a variety of stresses in pure bacterial cultures (Fiksdal and Tryland 1999; Caro et al. 1999; Suller and Lloyd 1999; Maalej et al. 2004). These studies collectively confirm that there is a hierarchy of physiological states in plankton assemblages, such that, in practical terms, scoring a cell as “live” does not imply activity, and, conversely, that scoring a cell as “dead” or “injured” does not imply complete inactivity.

Patterns in Distribution of Single-Cell Activity and Physiology Along Marine Gradients

Figure 8.4 shows the relationship between the abundance of seven specific bacterial fractions (MAR-positive, CTC-positive, HNA, membrane-intact (membrane-positive), membrane-impaired, DVC, NucC and esterase-positive) and total bacterial abundance. These relationships include all the individual data points that were used to construct Figure 8.3. The relationships extend over three orders of magnitude in total bacterial abundance (10^5 – 10^7 cells/mL), thus covering much of the range in bacterial abundance in aquatic waters. Figure 8.4 suggests different patterns in the relative abundance of these different fractions: the log-slopes of the HNA and membrane-positive relationships are not significantly different from unity, suggesting that although there is a large degree of scatter in each of these relationships, the relative contribution of these fractions does not change systematically along gradients of increasing bacterial abundance and thus of total system productivity. In contrast, the log-slopes of the CTC-positive, DVC, NucC and MAR-positive relationships are significantly greater than unity, suggesting that the relative importance of these fractions tends to increase with increasing overall abundance and probably system productivity. The proportion of esterase-positive cells tends to decrease with total bacterial abundance, but the database is not large and seems to be biased towards nutrient-rich aquatic ecosystems.

There appears to be a relative constancy in the proportion of intact versus damaged cells, and of cells with high nucleic acid contents and nucleoid, and a correlation between these fractions. Further, the relative constancy of these fractions suggests that the processes that result in bacterial cell death and damage, as well as in distribution of DNA, are not only present along the entire marine gradient, but are exerting a similar effect throughout. In contrast, the apparent increase in metabolically active

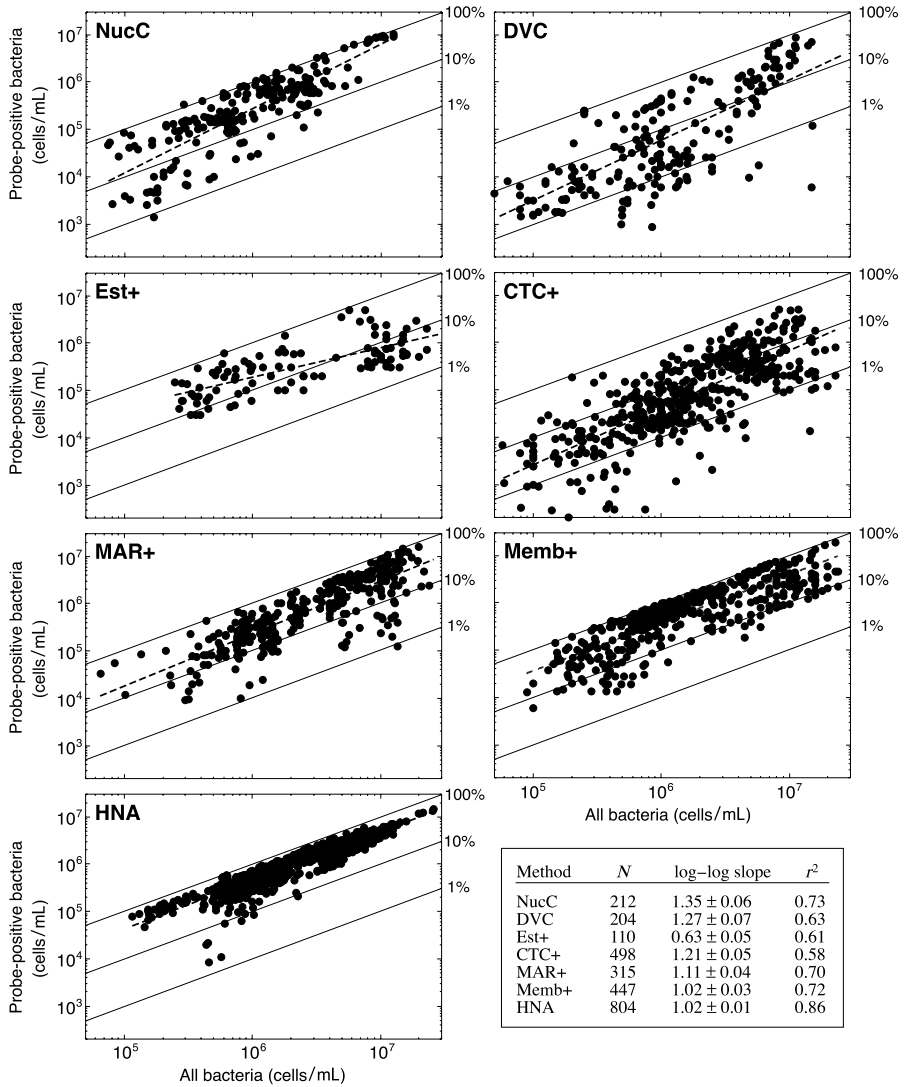


Figure 8.4 Total bacterial abundance and abundance of reactive bacteria as estimated with the most frequently used activity probes or methodologies. The dashed lines are the regression lines. Est+ = esterase+; Memb+ = Membrane+.

bacteria suggests that, along this same gradient, some processes, probably linked to substrate availability, enhance single-cell activity. For example, in any given system, the proportion of CTC-positive bacteria tends to be higher for particle-associated than for free-living bacteria (Sherr et al. 1999a, 2002; Yager et al. 2001). Further, these patterns agree with previous studies reporting that both the number of

CTC-positive cells and the percentage of CTC-positive cells increase with system productivity (del Giorgio and Scarborough 1995; Lovejoy et al. 1996; Søndergaard and Danielsen 2001), and that the abundance of CTC-positive cells is correlated with bacterial production (del Giorgio et al. 1997; Lovejoy et al. 1996; Sherr et al. 1999a) and cell growth (Choi et al. 1996, 1999; Sherr et al. 1999b). The apparent increase in the proportion of highly active cells could also potentially be due to a covariation with other physical factors, such as temperature, which may vary independently from nutrient and substrate availability. However, there are reports of extremely high numbers of substrate-reactive and percentage of CTC-positive cells in very cold but nutrient-rich waters (Yager et al. 2001; Schumman et al. 2003; Freese et al. 2006), so temperature itself does not appear to be the main reason for the low apparent proportion of highly active cells in low-productivity systems. Arístegui et al. (2005) found 8 percent of CTC-positive prokaryotes in mesopelagic samples, a fraction similar to that in much warmer surface waters. In addition, the formazan granules of CTC-positive cells in the mesopelagic zone were 30 percent larger than those in cells at the surface, suggesting that the per cell respiration rate was higher in the mesopelagic zone. Likewise, Herndl et al. (2005) reported relatively high proportions of active *Archaea* in deep oceanic waters.

To summarize, our analysis suggests that the relative proportion of intact and live cells does not vary significantly across marine gradients, but that the proportion of active cells (in terms of respiration, enzyme activity, or substrate uptake) tends to increase along the same gradients. These observations suggest (1) that the proportion of inactive or dormant cells declines systematically along a gradient of increasing bacterial abundance and thus of system productivity, (2) that cell death and injury may be regulated by factors that are relatively independent from those that regulate cell activation and inactivation, and (3) that there may be changes in the selective loss processes of specific physiological fractions along these gradients, and in particular, a relatively higher removal of the more active cells in the least productive systems.

Distribution of Activity and Growth Among Bacterial Size Classes

Standard allometric relationships suggest that the smallest bacterial cells should have the highest specific metabolic and growth rates, and early results supported this pattern (Fuhrman 1981), but later studies have shown evidence that the smaller cells tend to have lower specific metabolic rates (Billen et al. 1990; Bjørnsen et al. 1989). For example, Gasol et al. (1995) showed that most CTC-positive cells in a coastal marine assemblage were in the medium to large size range. Their calculations of specific growth rates for each of seven size classes between 0.008 and 0.30 μm^3 showed that growth rates increased from about 0.3/d to 1.4/d and biomass turnover times to decrease with increasing cell size, from about 2.1 days for small bacteria down to 0.5 days for larger bacteria. Others have also observed a relationship between cell size and the capacity to reduce CTC (Søndergaard and Danielsen

2001), and Bernard et al. (2000b) and Lebaron et al. (2002) confirmed this pattern by measuring tritiated leucine incorporation rates by bacteria sorted according to size using flow cytometry. Cottrell and Kirchman (2004) also showed that larger bacteria were more likely to be taking up thymidine than smaller bacteria.

Distribution of Activity Across and Within Major Phylogenetic Groups

An increasing number of studies have attempted to determine the phylogenetic affiliation of cells in different physiological states. Several of these have used cell sorting in order to isolate cells or clone genes, or have used FISH or DNA fingerprinting to analyze specific fractions, such as HNA and LNA cells, or CTC-positive cells. Results have been somewhat contradictory. Some indicate that these fractions are not phylogenetically distinct (Bernard et al. 2000a; Servais et al. 2003; Fuchs et al. 2005; Longnecker et al. 2005), while others suggest that the composition might differ between fractions (Bernard et al. 2000b). Mary et al. (2006) suggested that most LNA cells in open ocean environments belong to the SAR11 clade, and that these cells do not appear in the HNA fraction. Other studies have combined BrdU incorporation with FISH to assess the composition of substrate-active or dividing cells. For example, Pernthaler et al. (2002) found that only 3 percent of total cells incorporated BrdU, indicating a very small fraction of dividing cells, at least in early autumn in the North Sea. Use of group-specific probes allowed the identification of the dividing cells as belonging to three main populations: SAR86, *Roseobacter*, and *Alteromonas*. Interestingly, the SAR86 group of the *Gammaproteobacteria* accounted for over 50 percent of BrdU-positive bacteria. Hamasaki et al. (2007) combined BrdU uptake and immunocapture followed by DGGE in coastal marine samples, and found that the composition of the BrdU-incorporating cells was substantially different from that of the total community, suggesting that numerically dominant phylotypes may not necessarily be those that are highly active and growing.

An increasing number of studies have used MAR-FISH to explore the phylogenetic affiliation of cells that are active in substrate uptake. We have collected some of the recently published results using this approach (Fig. 8.5), selecting the substrates most likely being universally incorporated, and analyzing their use by the most abundant groups of marine bacteria. These results show (1) a wide range in the proportion of cells active in substrate uptake within each of the major bacterial groups, and (2) major differences in the proportion of cells apparently active in substrate uptake within a given group, depending on the substrate utilized. Certain substrates, such as thymidine, generally lead to relatively low fraction of active cells in most groups, but most other substrates that have been tested, such as amino acids and glucose, result in an extremely wide range of values, both for the same group in different samples and for different phylogenetic groups within the same sample. A portion of this variability is no doubt methodological and related to the sensitivity of the FISH and MAR protocol used (Bouvier and del Giorgio 2003),

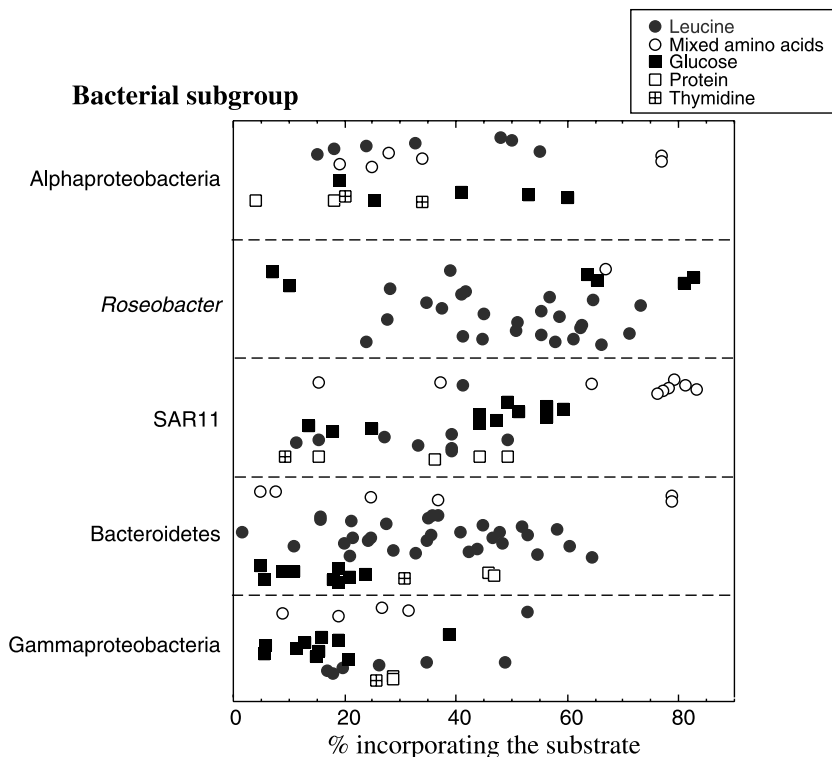


Figure 8.5 Some published MAR-FISH data showing the percentage of cells of the major bacterial groups (*Alpha*- and *Gammaproteobacteria*, and *Bacteroidetes*, and the alphaproteobacterial subgroups SAR11 and *Roseobacter*) active in assimilating different substrates.

but often the same protocol will yield a wide range in the apparent proportion of cells capable of substrate uptake. Studies that have simultaneously assessed the uptake of several substrates have shown that within the major bacterial groups the proportion of cells that take up each substrate varies greatly (Alonso-Sáez and Gasol 2007), so the relatively low percentage of substrate-active bacteria often reported may, in fact, reflect metabolic specialization rather than low metabolic activity. Nevertheless, most reported values of MAR-FISH are below 60 percent, regardless of the substrate used, suggesting that at any given time a relatively large fraction of all major bacterial phylogenetic groups may not be active in the uptake of a specific substrate. This in turn suggests that within major phylogenetic groups there may be at any given time a range of cellular metabolic states, and that activation, inactivation, dormancy, and/or injury or death operate at lower phylogenetic levels than those targeted by most MAR-FISH protocols.

What is the Link Between Single-Cell Activity and Phylogenetic Affiliation?

- MAR-FISH analyses show that in most cases there is a mixture of cells that are active and inactive in substrate uptake within any given bacterial group (see, e.g., Fig. 8.5), suggesting that the level of single-cell activity is not intrinsic but rather that members of the same group may express very different levels of activity depending on their microenvironment and of their immediate history.
- This scenario would further suggest that resource microheterogeneity may play a key role in determining the distribution of activity within bacterial assemblages.
- Alternatively, the heterogeneity of single-cell activity detected within broad phylogenetic groups may indicate that within these groups there is a wide range of genetic diversity that is expressed as a wide range in metabolic responses of different cells to the same set of environmental conditions.
- This establishes two extreme scenarios: (1) the physiological structure is entirely due to environmental heterogeneity, microscale patchiness, and temporal variability; (2) physiological heterogeneity is due entirely to genetic and phenotypic diversity. Where along this gradient lie natural bacterioplankton assemblages is still a matter of study.

Dynamics of Single-Cell Activity and Physiological States

The dynamics of cell activation, inactivation, injury, and death are not well understood for marine bacterioplankton assemblages. Cells that are apparently inactive and are thus not scored positive in activity assays can quickly activate following changes in resources and physical conditions. For example, Choi et al. (1996) found in a starved laboratory culture resupplied with nutrients that 100 percent of cells without visible nucleoid developed yellow-fluorescing nucleoids before cell abundance began to increase. Likewise, Choi et al. (1999) reported that substrate additions to a natural marine assemblage resulted in rapid increases in the proportion of CTC-positive cells, from low ambient values (<10 percent), to over 70 percent under enriched conditions, without any significant change in cell abundance. These studies suggest rapid activation upon a change in the resource environment of bacterial cells that are either dormant or have extremely low levels of activity. There is also evidence in situ of rapid changes in the distribution of activity within the bacterioplankton assemblages. There were large and rapid increases in the proportion of CTC-positive bacteria as a response to algal blooms in arctic waters, even at low temperatures (Yager et al. 2001). Gasol et al. (1998) and Hagström et al. (2001) observed diel cycles of NucC-containing cell abundance in several oceanic stations, with increases during the night. The relatively large (14–86 percent) shifts in NucC numbers recorded during the diel cycles suggests that at least some non-NucC bacteria were capable of turning on and becoming NucC-positive on short time scales.

The higher proportion of NucC cells found at night could be an indication of membrane changes and/or DNA synthesis and thus of cell division; the lower proportion of NucC cells during the day could in turn be the result of strong selective grazing or of cell inactivation.

The dynamics in the physiological structure of bacterioplankton assemblages result from a combination of changes in the physiological states of individual cells and of loss processes that selectively influence the different fractions, such as protistan grazing, but these processes are difficult to discern in ambient waters. The dynamics of the physiological structure may be better understood through the manipulation of the resource conditions or the grazing pressure, or both. Figure 8.6 shows an example of an experiment with a coastal marine bacterioplankton assemblage in 100 L tanks. Bacteria initially grew fast, and for some days became decoupled from their protistan grazers. Initially, protistan density was extremely low, and bacterial abundance quickly increased over the first few hours or days, but total abundance dropped steeply as heterotrophic nanoflagellate abundance increased (Fig. 8.6a). At the start of the incubation, the proportions of “live” and CTC-positive cells were low (<50 and <20 percent, respectively), similar to in situ values, but both increased sharply, exceeding 60 percent. The relative abundance of both live and CTC-positive cells dropped sharply to low levels similar to initial

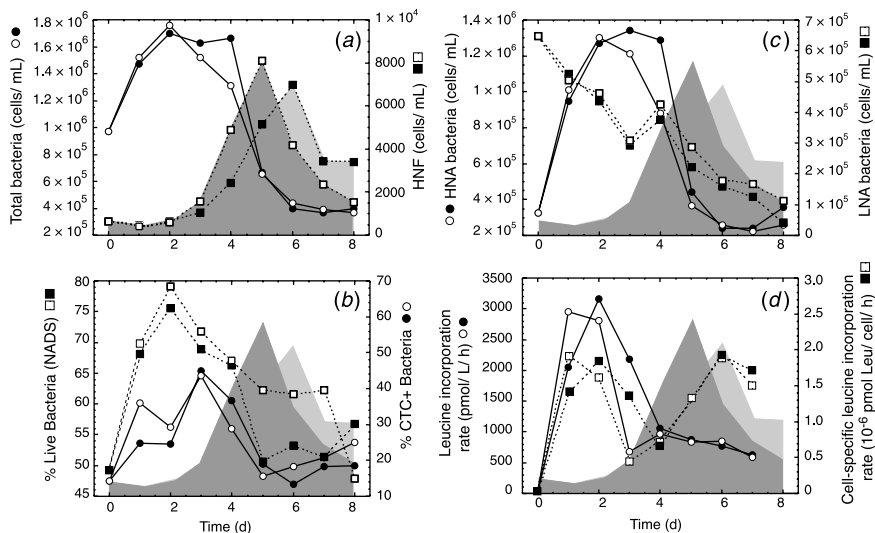


Figure 8.6 Example of the change in bacterial abundance, leucine incorporation rate, flagellate abundance, and some cell-specific indicators of bacterial metabolic status in two replicate (closed and open symbols) laboratory microcosms with Mediterranean coastal waters. (a) Total bacterial and flagellate abundance. (b) Percentage of CTC-positive and membrane-positive bacteria (“live”, determined with the NADS protocol). (c) HNA and LNA bacteria. (d) Total leucine incorporation rate and cell-specific leucine incorporation rate.

values, concomitant with the increase in protistan density (Fig. 8.6*b*). HNA cells followed a similar pattern in density as the total number of cells, whereas LNA cells showed a completely different dynamics during the incubation, apparently independent of protistan grazing (Fig. 8.6*c*). The highest proportions of LNA cells occurred at the onset of the incubation, and at the peak of protistan grazing, which corresponded to the lowest proportions of live, CTC-positive, and HNA cells. Total leucine incorporation followed well the patterns in total and HNA abundance, but cell-specific leucine followed closely the pattern in the percentage of CTC-positive cells (Fig. 8.6*d*).

These experiments suggest the following: (1) in the absence of grazing, there is evidence for both cell activation and multiplication; (2) under these circumstances, dormant and inactive cells that were originally in the assemblage are diluted, leading to high proportions of active cells; (3) protistan grazing removes all categories of cells, but preferentially those that are active, alive, and with HNA contents, thus impacting not only the total abundance but the proportion of these various fractions; (4) some bacterial fractions, such as LNA cells, are not selectively removed, and can thus persist in the assemblage, even under high grazing pressure; (5) the variations in cell-specific metabolic activity at the community level result not only from changes in the overall level of activity of all the cells, but also from changes in the relative distribution of highly active versus less active cells; (6) even dilute marine waters can harbor bacterial assemblages with relatively high proportions of active cells. It is difficult to extrapolate from these experiments to natural conditions, but the observations of generally low proportions of active and dividing cells, high proportions of LNA and dead/injured cells, and relatively high protistan abundance in ambient marine waters would suggest that microbial communities *in situ* resemble more a regrowth assemblage after 4–5 days of incubation (Fig. 8.6) than either the initial or final conditions found in these experiments.

That even extremely oligotrophic waters may support high proportions of highly active cells was also reported by del Giorgio et al. (1996) in experiments carried out in coastal Mediterranean waters. In these experiments, the ambient bacterial assemblages were incubated *in situ* in dialysis bags with and without protistan grazers, and both the total abundance and the abundance of CTC-positive cells were followed. Similar to what was observed above, the results from these dialysis bag experiments also revealed large increases in the percentage of CTC-positive cells in the absence of grazing, and a strong negative relationship between the percentage of CTC-positive cells and protistan density in the various treatments (del Giorgio et al. 1996). Perhaps more interestingly, these experiments allowed us to explore in more detail the processes underlying the changes in total bacterial abundance, growth rate and production.

If only total abundance is considered (as is usually the case in most studies), the results suggested a relatively modest net growth rate and grazing rate for the entire assemblage (0.08/d and $-0.4/d$) (Fig. 8.7). If, alternatively, one considers not just total abundance but also the dynamics of CTC-positive cells, the scenario that emerged was much more complex: most of the growth of the assemblage appeared

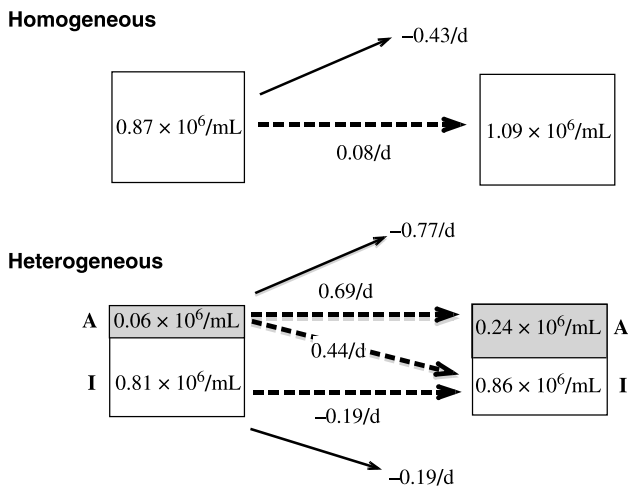


Figure 8.7 Comparison of an approach that considers the populations homogeneous with one that considers them heterogeneously formed by an active (A) and an inactive (I) pool. The values in the boxes are bacterial abundances, and the values with the arrows are rates of change (growth), of loss to predators (arrows pointing away from the boxes), or of transformation from the A to the I pool. The model used in the original publication (del Giorgio et al. 1996) assumed that no cells could move from the I to the A pool.

due to the multiplication of a very small, highly active fraction that was present at the onset of the experimental incubations; this fraction had much higher growth rates ($0.7/\text{d}$) than those estimated for the bulk assemblage, and also had much higher predation loss rates ($-0.8/\text{d}$) (Fig. 8.7). In addition, modeling also indicated that there was not only selective removal of the highly active cells during the course of the experiment, but also inactivation of at least a portion of these newly formed cells (at a rate of $0.4/\text{d}$), which then fed the pool of inactive bacteria and allowed this pool to remain relatively constant in numbers (Fig. 8.7). Although the two scenarios shown in Figure 8.7 share the same starting conditions and result in the same final total bacterial abundance, the one that takes into consideration the internal heterogeneity within the bacterial assemblages, in spite of its simplicity, provides a more realistic depiction of the complex interactions, mechanisms, and controls that underlie the changes at the community level, than the one that considers the assemblage as being homogenous.

ECOLOGICAL IMPLICATIONS OF PATTERNS IN BACTERIOPLANKTON SINGLE-CELL ACTIVITY

The evidence from studies that have targeted single-cell activity suggests that bacterioplankton assemblages should be considered a dynamic mosaic of activity, and that

there may be as much variability in cell-specific metabolism within any single bacterioplankton assemblage as there is in the average activity between assemblages along environmental gradients and between ecosystems (ultraoligotrophic gyres to highly productive coastal and estuarine regimes). In brief, the evidence suggests that (1) there are dead and injured cells in all assemblages assayed so far, (2) there are significant numbers of cells that appear to be dormant, (3) the abundance as well as the proportion of active cells increases with system productivity, (4) the metabolic and growth rates of the active fraction also tend to increase, and (5) the loss processes affect these different fractions very differently. We explore possible ecological consequences of these patterns in the sections below.

Community Versus Individual Cell Growth and Metabolic Rates

The concepts of specific production, growth rate, and biomass turnover are generally applied in aquatic microbial ecology at the population and more often at the community level. Total metabolism is scaled to total bacterial abundance, and thus the resulting growth and turnover rates represent an average for the various phylogenetic and physiological groups that comprise the assemblage. These bulk growth and specific production rates have been the focus of research for the past three decades, and much has been learnt in terms of the magnitude and regulation of bacterial growth (reviewed in Chapters 9 and 10). Bacterial abundance is by far one of the least variable parameters in oceanography, and variations in bacterial abundance in general explain very little of the patterns in bulk metabolism and growth. On the other hand, there is an overall range of at least three to four orders of magnitude in the average specific growth rates (estimated as total bacterial production divided by total abundance), and variation in specific respiration (bacterial respiration divided by abundance) is somewhat smaller; the ranges for both are even greater if deep ocean assemblages are considered.

The evidence presented in the previous sections would suggest that at least a portion of this range in the average specific activity across communities is due to changes in the proportion of different physiological fractions. The key question is to what extent this large range in cell-specific activity is due to shifts in the specific activity of the entire assemblage or to shifts in the relative proportion of cells in different physiological states. For example, we could hypothesize a bacterioplankton assemblage composed of cells that are mostly dormant and in metabolic arrest, except for a small fraction of cells that upon encounter of local patches of higher substrate concentration quickly resumes growth and divide. Bulk approaches would rightly conclude that biomass doubling time of this assemblage is extremely long, and that growth rates for the entire assemblage are therefore very low, and yet these estimates would mask the fact that the division rate of the cells that are actually dividing may be on the order of a few days or even hours.

The average growth rates (μ) for an assemblage are the net result of the contributions of the various physiological fractions. In the simplest of scenarios, such as

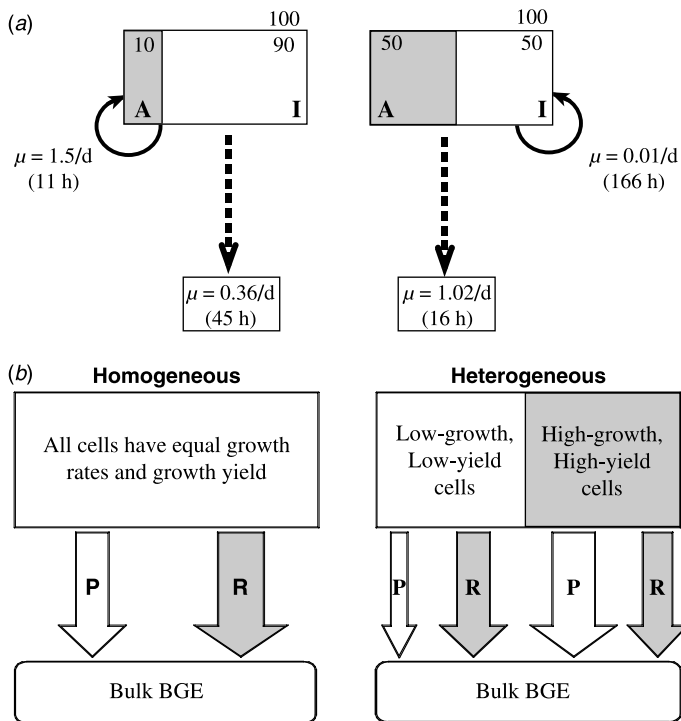


Figure 8.8 (a) Simulation of the bulk growth rate of a bacterial assemblage formed by different proportions of very active (A, growing with a growth rate 1.5/d) and not very active (I, with growth rate 0.01/d) bacterial cells. (b) The bacterial growth efficiency (BGE) of a bacterial assemblage that is homogeneous or is composed of two subpopulations: one growing with a high yield and another with a lower yield.

the one presented in Figure 8.8a, cells are distributed into two physiological categories, one fast-growing (in our example $\mu = 1.5/d$), and the other slow-growing (i.e., $\mu = 0.01/d$). The growth rates measured for the entire assemblage could thus vary over two orders of magnitude, from 0.01/d (all slow-growing cells) to 1.5/d (all fast-growing cells), and this large variation in community growth rates could be generated with no changes in cell density, and no changes in the actual levels of activity within each fraction. This scenario could be extended to the more realistic situation of multiple coexisting physiological categories, all varying not in their level of activity but rather in their contribution to total biomass and activity. Under this alternative scenario, bacterial assemblages have cells along roughly the same range of specific production (i.e., from dormant to highly active), and what differs between assemblages are not the actual levels of activity but rather the proportion of components with different activity.

Community Versus Population or Single-Cell Based Growth Estimates

- Scaling total bacterial activity (i.e., biomass production or cell division) to the entire community or to the active (and growing) portion of the community has profound consequences on the resulting patterns of bacterial growth.
- Low community-level growth rates may result from low overall growth rate within the assemblage, or from the coexistence of a small number of highly active cells within a large background of extremely slow-growing and dormant bacteria.
- Current information suggests that both scenarios coexist in marine bacterioplankton communities: the dominant groups often have intrinsically low growth rates, but the real growth rates of the active fractions are probably greatly underestimated by dilution with large numbers of inactive bacteria.
- Field and experimental studies have shown that the dynamics observed at the community level in total production and growth and biomass turnover rates are not the result of just changes in overall abundance and growth rate, but rather are underlain by complex shifts in the distribution of activity and physiological states within the assemblage.

Linking the Distribution of Single-Cell Parameters and the Bulk Assemblage Response

There are several ways by which bacterioplankton assemblages can shift their overall metabolism and growth rates: (1) there can simply be more or fewer cells doing the same thing; (2) the cell number may not change substantially, but all the cells in the assemblage may shift up or down their metabolic rate; (3) the number may not change substantially, but the proportions of cells in different physiological states may vary. Although this is not explicitly stated, the underlying assumption in many aquatic microbial studies is that variations in total community metabolism or growth are the result of some combination of scenarios 1 and 2, and scenario 3 has seldom been considered as an alternative, yet the evidence presented in the previous sections would suggest that this may in fact be one of the key underlying mechanisms in the patterns of bacterial metabolism in marine systems.

There have been relatively few studies that have attempted to link the patterns in the distribution of single cell activity with the patterns of bulk community metabolism. Several authors have shown that the rates of bacterial production and respiration relate more closely to the abundance of CTC-positive cells and HNA cells than to total counts (Lovejoy et al. 1996; del Giorgio et al. 1997; Smith 1998; Sherr et al. 1999a). Both the electron transport system measure of respiration and the CTC count of respiring bacteria were found to be positively correlated in at least one field study (Berman et al. 2001), and estuarine bacterial respiration was also strongly correlated to the number of CTC-positive cells (Smith 1998). In other studies, total red CTC fluorescence correlated to CO₂ production in mixed bacterial assemblages in aerobic bioreactors (Cook and Garland 1997), and there was

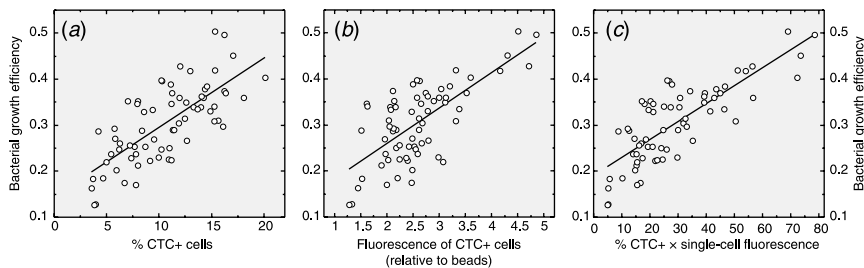


Figure 8.9 Relationship between percentage of CTC-positive cells and bacterial growth efficiency (BGE) of the bulk assemblage (a), average fluorescence of the CTC-positive cells (b), and a combination of both parameters (c). The data are from various estuaries and lakes in Quebec and eastern United States. Equations are: (a) $BGE = 0.14 + 0.015 \text{ CTC}\%$, $r^2 = 0.53$, $N = 88$; (b) $BGE = 0.10 + 0.077F$, $r^2 = 0.49$, $N = 88$; (c) $BGE = 0.19 + 0.00389 \text{ \%CTC} \times F$, $r^2 = 0.64$, $N = 88$ where F = fluorescence.

coherence between the patterns in CTC-positive cell abundance and CO_2 production in *E. coli* cultures under varying conditions (Créach et al. 2003).

In theory, not only growth rate, but also other key processes, such as bacterial growth efficiency (BGE), should be linked to the distribution of growth and activity within the community. For example, Figure 8.8b shows two scenarios: one community where all cells have similar growth rates and growth efficiencies and thus contribute equally to production and respiration, and another community that is composed of groups with different growth rates and growth efficiencies. In this case, the community growth rate and BGE are a function of the proportions of each physiological group. This latter scenario was explored by del Giorgio and Newell (in preparation), who hypothesized that highly active, CTC-positive bacteria have both a high growth rate and higher growth efficiency. This hypothesis was tested with coastal and salt marsh bacterioplankton communities with a wide range in both bulk bacterial growth rate and growth efficiency. In this study, BGE was positively correlated with the proportion of CTC-positive cells (Fig. 8.9a), suggesting that these cells may have higher BGE than less active, CTC-negative cells, and that community BGE was the direct result of the relative contributions of these groups. Furthermore, these authors found that BGE was also positively related to the fluorescence of the CTC cells (orange or red: Fig. 8.9b), which is itself an index of single-cell metabolic activity. These results further suggest that even within the CTC-positive cells there is a range of single-cell activity and that this may also be linked to their individual growth rate and efficiency. The best predictor of BGE in these communities was in fact a combination of both the percentage of CTC-positive cells and their average fluorescence (Fig. 8.9c), exemplifying the interaction between the proportion of cells in different physiological states, and the level of activity within these categories, and their roles in shaping metabolism at the community level.

Ecological Role of Different Physiological Fractions

There are some conceptual as well as practical differences between the ecology of microbes and that of other organisms. Marine and terrestrial ecologists working on

eukaryotes and metazoans do not include dead or injured organisms in population and community budgets, growth rate estimations, or genetic models. In animal populations, predators generally do not attack and consume dead prey, and it is often the slowest and smallest that are more vulnerable. Things are quite different within microbial assemblages: dead and dying cells persist in the environment, and current techniques target not only the active fractions but also the inactive and even the injured and dead cell pools (cf. Pedrós-Alió 2006), so the latter are included in budgets and calculations. Poor health and small size actually represent refuges in the microbial world. Many of the cells we enumerate in marine samples are there not by virtue of their growth but because they have avoided predation and thus reflect inefficient removal mechanisms. On the other hand, the cells that do grow are probably maintained at low levels through selective grazing and viral infection (see, e.g., Bouvier and del Giorgio 2007), with episodic “blooms” (see, e.g., Beardsley et al. 2003), and yet these cells are probably responsible for much of the carbon processing even if present at very low densities.

The ecological notion of different bacterial pools, some very abundant but turning over very slowly, others much less abundant but turning over very rapidly, is in many ways analogous to the biogeochemical notion of coexistence of different DOC pools (see Chapter 7): a large refractory pool that turns over slowly and thus persists in the ecosystem, and a small, highly labile pool that turns over rapidly and never builds up. In the same way that each of these DOC pools plays a role in the global carbon dynamics, so do each of the bacterial fractions in the functioning of bacterioplankton assemblages. It has been hypothesized that the more recalcitrant pools of DOC play a stabilizing role in ecosystem function, by fueling a slow but continuous background metabolism (del Giorgio and Williams 2005). Do the dormant and slow-growing bacterial fractions similarly play a role in stabilizing the functioning of microbial food webs?

CONCLUDING REMARKS

The physiological structure probably plays a central role in the maintenance of bacterial function and diversity in marine ecosystems, and as such it is not accidental but rather is an adaptive characteristic of bacterioplankton. In the oligotrophic, extremely heterogeneous and fluctuating marine environment, cell persistence is almost as important as cell growth. Marine microbial ecology has traditionally focused on the magnitude and regulation of bacterial production, growth, and loss at the community level, and much less on the issue of persistence and its related processes at the single-cell level. The dynamics observed at the community level in total bacterial abundance, production, and average growth and biomass turnover rates are underlain by complex shifts in the distribution of activity and physiological states within the assemblage. The physiological structure is a reflection not just of environmental conditions, but also of key biological interactions within microbial food webs, and the physiological states assumed by bacteria allow growth when possible, and persistence when necessary, thus playing a key role in both shaping community metabolism, and maintaining microbial genetic diversity.

SUMMARY

1. Marine bacterioplankton assemblage have relative large proportions of cells with extremely low metabolic rates, and which are dormant, injured or dead.
2. The low proportion of measurably active bacteria observed in many marine assemblages is not simply the result of oligotrophy and resource limitation. Selective removal processes, such as protistan grazing and viral infection, influence the physiological structure of bacterioplankton.
3. The range in physiological states reflects both the large physiological and metabolic versatility that characterizes individual marine bacterial strains and the high genetic and phenotypic diversity of bacterioplankton communities.
4. The proportion of highly active cells increases, and that of inactive or dormant cells declines systematically, along gradients of increasing system productivity.
5. The selective loss processes of specific physiological fractions change along these gradients, and, in particular, there is a relatively higher removal of the more active cells in the least productive systems. Cell injury and death may be regulated by factors that are relatively independent of those that regulate cell activation and inactivation.
6. Single-cell variations in physiological state may in fact be the predominant strategy both to cope with scarce and fluctuating resources and to escape from predation, viral infection, and other types of negative biological interactions. Low activity and eventually dormancy are part of long-term survival and protection strategies, whereas high metabolic activity increases the potential for growth, but also vulnerability to predation and death.

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