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NOTE

Low fractions of active bacteria in natural aquatic communities?

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ABSTRACT: The notion that a significant fraction of individual cells within natural bacterial assemblages is not actively engaged in cellular metabolism, although not a new idea, remains fairly contentious. Different approaches for probing the metabolic activity of individual cells often yield widely divergent estimates of the proportion of active cells, with some methods suggesting very low levels of individual cell activity. We comment on 2 aspects of the current discussions regarding cell-specific activity in natural bacterioplankton. First, the apparent perception that most aquatic bacteria must be active is not uniformly supported by the data. In a systematic survey of the microautoradiography literature, for example, only 4 out of 23 studies reported a mean proportion of active cells in natural communities that was greater than 50%, and the mean across all such studies was 30%. Second, we propose that the problem of describing bacterioplankton single-cell activity is best approached from the viewpoint that there is a nested hierarchy of physiological states within bacterial communities. The lack of agreement among various methods points to the large range of criteria possible for describing metabolic activity in bacteria. In this regard, the discrete, and over-simplistic, notion of 'active' versus 'inactive' is not particularly useful and should be replaced by a conceptual model in which there exists a continuum of possible single-cell activities.

KEY WORDS: Bacteria · Cell-specific activity · Physiological diversity · Methodological approaches

An important goal of microbial ecology is to understand the variability in bacterial rate processes within, and across, aquatic communities. One frustration in this attempt has been the fact that such processes seldom relate well to total abundances of bacteria, which are typically rather invariant. This has led to the development of a number of different techniques for probing the activity of individual cells within the assemblage of total bacteria. Across a range of aquatic ecosystems, a non-trivial and often dominant fraction of the bacterial assemblage is typically unresponsive to at least some of these assays (Sherr et al. 1999, del Giorgio & Bouvier 2002). This suggests that either these methods are ineffective in describing bacterial activity, or that many bacterioplankton cells are indeed dormant or have very low levels of cellular activity.

The hypothesis that low levels of cellular activity within natural bacterial assemblages are not uncommon is not new. It has been over 2 decades since Stevenson (1978) first proposed that the physiological state of a significant portion of aquatic bacterioplankton can best be described as dormant. Indeed, there is substantial literature devoted to dormancy and the physiology of 'starvation survival' by bacteria (Morita 1997). Stevenson (1978) originally remarked that '[the] paucity of physiological data suggesting a dormant state for bacteria in the water column is not surprising: it borders on heresy to suggest that bacteria in this environment are not active.' While the number of published accounts of single-cell activity within the bacterioplankton has increased appreciably (Joux & Lebaron 2000), resistance to reports of large fractions of relatively inactive bacteria within natural communities continues to the present day.

One method in particular has been at the center of much of the recent debate over the distribution of cells with different levels of metabolic activity. It involves the use of the fluorogenic tetrazolium dye, 5-cyano-2,3 ditolyl tetrazolium chloride (CTC). In theory, bacterial cells that take up and reduce CTC to its fluorescent formazan (CTC+ cells) have an active electron transport system (ETS). As activity within the ETS is essential to cellular respiration (Packard 1985), these cells are thus thought to be 'actively respiring' (Rodriquez 1992). Although abundances of CTC+ cells in natural samples tend to be well correlated to measures of either bacterial production (e.g. del Giorgio et al. 1997, Sherr et al. 1999) or respiration (Smith 1998), the proportion of total cells scored as CTC+ tends to be low, generally less than 20% and sometimes less than just a few percent. It is precisely these low proportions that

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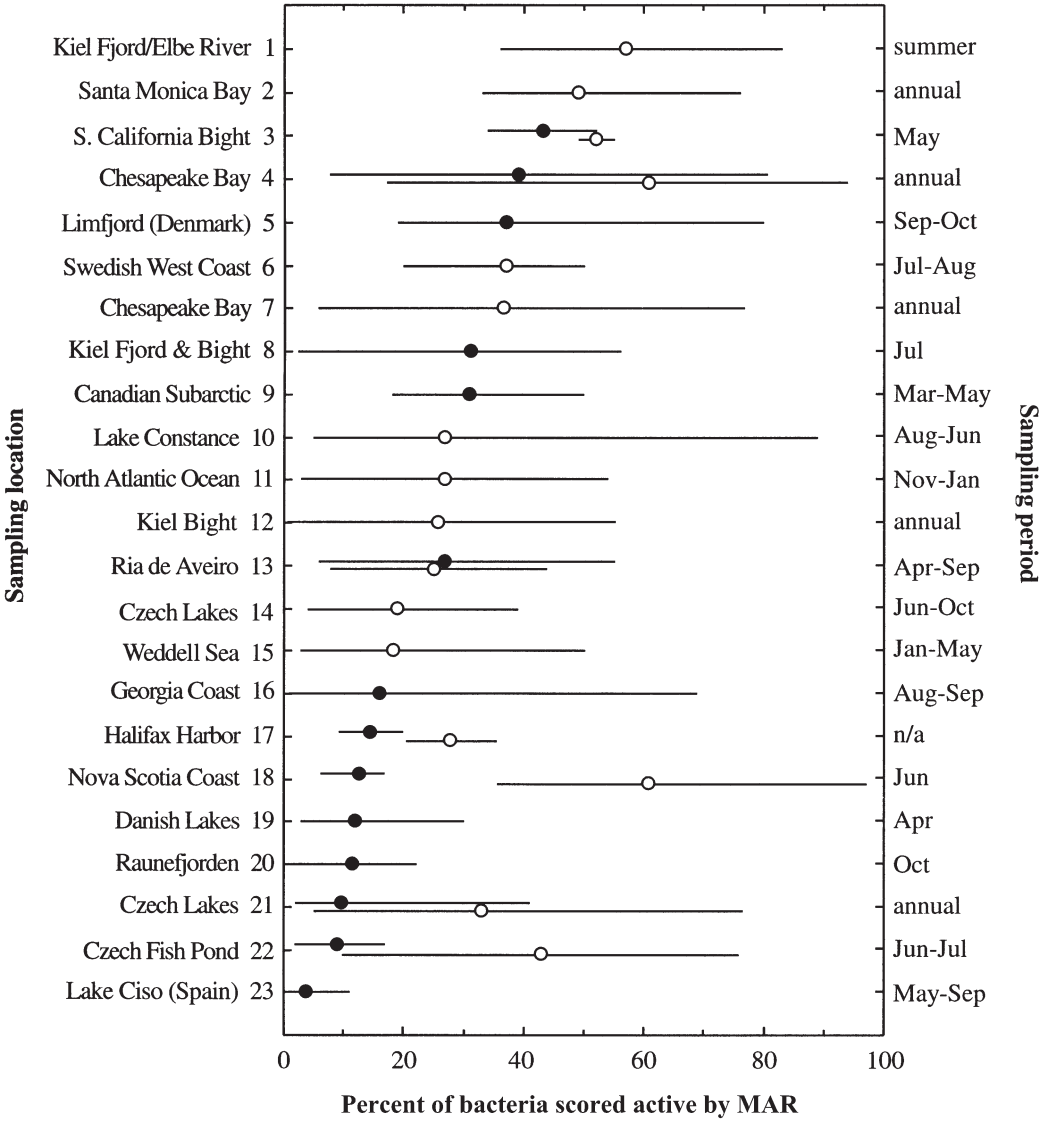

Fig. 1. Studies of natural bacterioplankton communities. Mean and range of % bacteria scored as active by microautoradiography (MAR). Open symbols are studies using amino acid(s) as tracer; closed symbols are studies using thymidine as tracer. Data sources are: 1. Ullrich et al. (1996); 2. Karner & Fuhrman (1997); 3. Fuhrman & Azam (1982); 4. Tabor & Neihof (1984); 5. Riemann et al. (1984); 6. Hermansson & Dahlback (1983); 7. Baily et al. (1983); 8. Meyer-Reil (1978); 9. Bunch & Harland (1990); 10. Simon (1985); 11. Hoppe & Gocke (1993) and Buck et al. (1996); 12. Hoppe (1977); 13. Almeida et al. (2001); 14. Nedoma et al. (1994); 15. Grossmann (1994); 16. Pedros-Alio & Newell (1989); 17. Novitsky (1983); 18. Douglas et al. (1987); 19. Marcussen et al. (1984); 20. Tuomi et al. (1995); 21. Straskrabova & Simek (1984); 22. Šimek et al. (1989); 23. Garcia-Cantizano et al. (1994)

have motivated much of the criticism of CTC as a means of determining activity in bacterial cells.

A defense of the CTC method is not the objective of this Note. While the use of this method has grown over the last several years (e.g. Sherr et al. 1999, Jugnia et al. 2000, Haglund et al. 2002), there have also been a number of studies highly critical of CTC as a means of distinguishing metabolically active cells (e.g. Ullrich et al. 1996, 1999, Karner & Fuhrman 1997, Servais et al. 2001). These latter studies have raised several legitimate methodological concerns about the use of CTC, and it could well be that the method is eventually improved, or even replaced by other more effective indices of single-cell activity. Instead, we would like to focus on 2 issues raised by the critics of CTC (e.g. Ullrich et al. 1999) that apply more generally to the problem of single-cell activity in bacterioplankton. The first is the statement that, as opposed to CTC, other more sensitive methods, such as microautoradiography, suggest that most natural bacterial cells are active.

Microautoradiography (MAR), one of the earliest single-cell methods developed (Hoppe 1976), is widely held as being the most sensitive technique for enumerating metabolically active bacteria (e.g. Karner & Fuhrman 1997, Ullrich et al. 1999). This method detects bacteria that are actively engaged in substrate uptake (as measured by a radiolabeled tracer, typically thymidine, an amino acid, or a mixture of amino acids), and therefore presumably active in growth and metabolism. The method is extremely sensitive because the uptake of minute amounts of radioactive substrate can be detected, so it is thought that in general, the threshold of detection of cell activity is also very low. Contrary to what is often assumed about MAR results, however, the number of cells active in substrate uptake seldom represents a dominant fraction of the total assemblage. A systematic survey of the MAR literature for the past 20 yr (Fig. 1) shows that, within natural bacterioplankton communities from a variety of freshwater and marine sites, only 4 of 23 studies reported a mean % active cells that was greater than 50%, and the average across all studies was 30%. The majority of these studies, it should be noted, were conducted in relatively productive lakes and coastal areas, so these data may be biased towards more active assemblages. Critics of the CTC method are right in pointing out that MAR detects more 'active' bacteria than CTC does, but MAR results certainly do not support the notion that most bacterioplankton cells are uniformly active and growing. What the MAR results do suggest is that there is a large heterogeneity, even within a given system, in the proportion of cells that are apparently active in substrate uptake.

The second issue that we would like to address, which is closely linked to the first one, is the intuition of many that reports of low fractions of 'active' bacteria must, *a priori*, be wrong, simply because the numbers are low. Corollaries of this intuition are: (1) that methods that result in high proportions of cells scored as positive must be intrinsically better as descriptors of single cell activity in natural bacterioplankton assemblages, and (2) bacterial single cell activity can be effectively described with a single such method. We would like to propose here that: (1) the fact that a method may account for a high proportion of cells does not necessarily imply that it provides a more effective description of the distribution of single-cell activity, and (2) the apparent lack of agreement between methods is the natural consequence of the diversity of physiological states in bacterioplankton, and should be viewed as an advantage rather than a problem.

As an example, one assay that can be used as an index of cell activity involves the detection of cells using oligonucleotide probes and fluorescence *in situ* hybridization (FISH). The capacity to detect bacterial cells using oligonucleotide probes and FISH is, in part, related to the cell-specific rRNA content (Kerkhof & Ward 1993, Ruimy et al. 1994), which itself has been suggested as an index of cell activity (Karner & Fuhrman 1997, Williams et al. 1998). Results of FISH in natural bacterial assemblages show a wide range in the proportion of cells that can be hybridized (less than 5% to over 100%, Table 1). A recent quantitative review of published reports (from over 60 papers) on the proportion of cells hybridized with the Eubacterial (EUB388) probe shows an average of 56% of bacterioplankton cells detected using FISH across aquatic ecosystems, and the level of cellular activity may explain at least a portion of this variation (Bouvier & del Giorgio in press). There have been, however, some recent advances in the FISH protocol, such as the use of polynucleotide probes (Pernthaler et al. 2002), which systematically yield close to 100% of target cells scored positive. The result of this increase in sensitivity of the FISH method is that these counts are much less dependent on variations in the physiological state of the cells. The fact that most cells can be accounted for by improved FISH protocols does not necessarily mean, however, that these approaches yield an effective description of the distribution of activity at the single cell level, because they do not allow the assessment of the large physiological diversity in bacterioplankton assemblages. At the other end of the spectrum, a method such as culturing, which usually scores less than 1% of all cells, is also less effective as a descriptor of single-cell activity.

What we would like to emphasize in this Note is that there is no inherent contradiction in the fact that FISH, MAR, CTC and other methods yield different results, and that this, in fact, is what should be expected based on what is known of bacterial growth in aquatic systems. Given the diversity of bacteria that

Table 1. Examples of studies that have used fluorescence *in situ* hybridization (FISH) in natural bacterioplankton assemblages, with the range in the proportion of cells hybridized with using a Eubacterial probe for different types of systems

System	% cells hybridized	Source
Lakes	29–70	Glöckner et al. (1999)
Lakes	31–78	Pernthaler et al. (1998)
Estuaries	51–>100	Cottrell & Kirchman (2000)
Estuaries	5–80	Bouvier & del Giorgio (2001)
Coastal ocean	22–78	Karner & Fuhrman (1997)
Coastal ocean	26–70	Fuchs et al. (2000)
Open ocean	39–96	Glöckner et al. (1999)
Open ocean	55–59	Eilers et al. (2000)

coexist in natural assemblages, and the fact that not all bacterial populations are growing at the same rates at a given time, it should not be surprising that there is the full range of metabolic states in a given assemblage. In this context, there is also no inherent problem with some methods yielding low proportions of 'reactive' cells, because it is not unrealistic to think that one or a few populations in the assemblage have much higher rates of activity than the rest. Perhaps the biggest criticism that can be targeted at CTC and other activity assays is precisely that most authors have applied these methods in isolation, and in most cases with the assumption that cells are either active or inactive depending on how they score with the technique in question. Such an either/or classification is clearly an over-simplification.

We believe that the problem of describing bacterioplankton single-cell activity should be approached from the viewpoint that there is a nested hierarchy of physiological states within bacterial communities. As such, the different proportions of active cell fractions reported by different methods may not necessarily be incompatible with one another. For example, if a study yields that over 80% in a given assemblage are reactive to FISH, another that 30% are responsive to MAR, and yet another that 5% are responsive to CTC or some other similar method, it would be erroneous to try to decide which of these numbers is the 'right' proportion of active cells in the assemblage. A more logical and meaningful interpretation would be that 80% of the cells have some level of activity (on the basis of ribosome content), that 37% of these may be actually taking up substrate at any significant rate based on MAR (although perhaps not all of them growing and dividing), and that 17% of these MAR-positive cells may have sufficiently high rates of metabolism to be scored positive with CTC.

One aspect of MAR methodology that is often overlooked, but germane to the arguments presented here, is the variability in number of silver grains per bacterium within a given autoradiogram. Briefly, cells are scored as active in MAR when their radioactivity causes the silver grains of a photographic emulsion to develop around the cell (see e.g. Fuhrman & Azam 1982 for details). Cells are then enumerated as either active (with silver grains) or inactive (without). The number of silver grains per bacterium within a given autoradiogram is, however, highly variable (e.g. Šimek et al. 1989, Grossman 1994). Since the number of silver grains per cell is a function of the amount of radiolabeled substrate incorporated by the cell, this variability is a reflection of the inherent variation in cell-specific metabolic activity present within the bacterial assemblage sampled. Returning to the notion of a nested hierarchy of physiological states, it may be possible that the cells that develop the most number of silver grains are the ones likely to be counted as active by methods such as CTC reduction. Likewise, the brightness of the fluorescent signal obtained with FISH is correlated to RNA content (Oda et al. 2000), and in any given sample it is typical to encounter a wide range of cell fluorescence intensities (del Giorgio & Bouvier 2002). Again, it may be possible that the CTC method detects only that fraction of cells that have the highest metabolism, and therefore the highest RNA content, and consequently the highest fluorescence intensities detected using FISH.

It is clear that all of the above will remain simple speculation unless we can quantitatively define the thresholds of detection and assign concrete levels of activity to each of the different indices that might be used in parallel. This remains perhaps one of the greatest challenges in this area of microbial ecology, but there has been some recent progress. For example, FISH has been successfully combined with MAR to assess the uptake of specific organic compounds by different phylogenetic bacterial groups (Ouverney & Fuhrman 1999, Cottrell & Kirchman 2000), but this approach may eventually be used as a double tracer of cell activity. There are also current attempts to combine MAR with CTC and other physiological probes (J. Gasol, E. & B. Sherr pers. comm.). Another very promising approach is the flow sorting of cells marked with various probes (Servais et al 2001, Zubkof et al. 2002).

We conclude that the current published data, including MAR and FISH results, do not support the notion that most bacterioplankton cells are uniformly active and growing. This is not to say that one must, therefore, accept the opposite conclusion, that most bacteria are inactive. Rather, these data, combined with other approaches of measuring activity at the single-cell level, should be interpreted within a conceptual model in which there is a continuum of metabolic states in bacterial communities. Different methods have different thresholds and metabolic 'targets' along this continuum, and the lack of agreement among them points to the large range of criteria possible for describing metabolic activity in bacterial communities. While it is clear that we have much to learn about the distribution of metabolic states *in situ*, it is unlikely that a single method can capture all the physiological diversity present in bacterioplankton assemblages. The discrete, and over-simplistic, notion of 'active' versus 'inactive' cells is not particularly useful in this context, however, and should be replaced by the notion of a continuum of single-cell activity, in which only a combination of different methods will likely yield an effective description of activity within bacterial assemblages.

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