Stability and survival of VBNC cells - conceptual and practical implications

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ABSTRACT

Cold-stressed populations of the estuarine bacterium *Vibrio vulnificus* constitute model systems for the "viable but nonculturable" (VBNC) state, and are comprised of non-recoverable, injured, and fully culturable cells. The nonculturable cells display increased sonication resistance, but also substantial degradation of nucleic acids, raising the possibility that these "VBNC" cells are, in spite of their stability, in fact sublethally or lethally injured. As the standard criteria for viability of VBNC cells (cellular activity or uptake of substrate) are neither necessary nor sufficient criteria, recovery of these cells needs to be shown to prove their viability. Recoverable VBNC cells, however, are not truly nonculturable. In order to resolve this contradiction in terms, it is here proposed to apply the term "viable" only to recoverable cells, and the term "active but nonculturable" (ABNC) for non-recoverable (non-viable) cells such as lethally injured and senescent cells. Cells which recover only under specific "resuscitation" conditions are to be considered either sublethally injured or, if cellular activity is initially low, dormant. It is suggested that this classification allows the replacement of the "VBNC" category by a more consistent terminology.

The term "viable but nonculturable" (VBNC) has been coined to describe a state from which bacterial cells can not be recovered, but in which they maintain certain features of viable cells, such as cellular integrity and activity. It appears to be a common observation that bacteria enter such a "VBNC" state under environmental or laboratory conditions [19, 24, 29]. This "non-recoverable" state has often been interpreted to be a consequence of dormancy. It has to be pointed out, however, that dormancy is characterised by the absence of significant cellular activity, and that dormancy is by definition a reversible phenomenon - dormant cells are hence typically inactive and culturable [15]. Is it possible that "VBNC" cells dwell in a genetically determined "refractory" state other than dormancy in which cell division is blocked? Can some of the observations be due to mutations or infections with bacteriophages? Or are "VBNC" cells merely injured or debilitated by the exposure to stressful conditions and, if so, is this injury sublethal or lethal [19]?

These questions have tremendous significance for medical microbiology, epidemiology and general microbial ecology. It has been proposed that the discrepancy between plate counts and total counts in natural environments ("Great Plate Count Anomaly") may be partly explained by bacteria in a "VBNC state", and that this state may constitute an adaptive strategy of non-sporeforming bacteria allowing survival under adverse conditions [29]. If this were the case, VBNC cells of pathogenic bacteria would provide a potentially

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huge and dangerous reservoir for infection which evades detection by most standard methods [8, 24, 26, 28, 30, 34, 36].

What approaches and methods can be employed to shed light on this elusive issue? The methods normally used for the examination of "VBNC" cells are based on cellular activity or uptake of substrate, which are, however, neither necessary nor sufficient criteria for viability. Dormant cells, for example, may not display any detectable activity or uptake. Cells, which have acquired lethal DNA damage, on the other hand, may still display activity. Necessary viability criteria include cellular integrity, intact membrane barrier, presence of DNA and RNA, and potential for protein synthesis. But these are not sufficient criteria, as even lethally injured (non-viable) cells can still display remnants of such activity and integrity. For example, cells can stay morphologically intact even after autoclaving. The ability of a cell to recover and grow (as manifested by "culturability") is so far the only sufficient criterion for viability, but it may not be considered a necessary criterion. Some standard media and conditions, for example, may be unsuitable for a cell in a certain physiological state to initiate growth or to form visible colonies.

So far it has been impossible to devise reliable criteria for viability which are both necessary and sufficient. While use of necessary criteria as the basis of viability assays can create false-positive results, the use of sufficient criteria has the potential of leading to false-negative results. The choice of approach obviously depends on whether it is intended to estimate the maximum possible risk (thus including all "possibly viable" cells) or whether viability in the strict sense is determined (including only cells that have been proven to be viable). It is here suggested to use the term "viable" only for cells that have been unequivocally shown to be fully viable which, because of above considerations, means that they have to be recoverable. Thus, ultimately, for the practical purpose of detecting viable cells, culturability (defined as the ability to recover under at least one of any conditions, including resuscitation treatments) is proposed to have the status of a necessary criterion for viability.

Several groups of investigators have attempted to resolve the issue of whether VBNC cells are truly viable and whether they can be resuscitated [3-5, 7, 9, 10, 12, 13, 16-18, 21, 22, 24, 31, 33, 35]. One of the model organisms for the study of "VBNC phenomena" is the estuarine human pathogen *Vibrio vulnificus* [25]. Incubation of *V. vulnificus* under conditions mimicking cold estuarine environments leads to the formation of heterogeneous cell populations comprised of recoverable ("culturable") cells, and non-recoverable ("nonculturable") cells [25, 31, 33]. In this experimental system, the part of the population that remains culturable decreases during cold incubation, and contains a fraction of injured cells; the nonculturable cells can not grow in either liquid or solid media, and thus have to be considered "non-recoverable" in the absolute sense [31, 33].

The non-recoverable presumed VBNC cells, however, display improved sonication resistance as compared to growing cells, indicating increased cellular stability [31]. In fact, after several weeks of cold incubation, stability of non-recoverable cells is equal to that featured by (culturable) starved cells which are characterised by high autolysis-resistance. Protein synthesis is undetectable in "aged" nonculturable cells, and the nucleic acids of the majority of these cells are highly degraded, which might explain the loss of culturability [32].

The question of whether non-recoverable cells in the type of "VBNC state" displayed by cold-stressed *V. vulnificus* can be resuscitated has raised some controversy. While resuscitation by temperature shifts in laboratory media was initially claimed [20], it was later disproved [31, 33]. Two exciting reports on resuscitation in environmental chambers and in a mouse model [26, 27] raise the possibility that natural conditions may be able to "trigger" growth of VBNC cells of the organism. In the mouse model, however, only a small number of "young" VBNC cells could be demonstrated to retain infectivity. It has recently been reported that "VBNC" cells of *V. vulnificus* can also, under certain conditions, be resuscitated in the laboratory [34]. "VBNC" cells that are recoverable in laboratory media are, however, not truly nonculturable, and it is here proposed that they should instead be considered sublethally injured (viable AND culturable). If processes of injury are the main cause for "VBNC phenomena" - what are the mechanisms of injury? Is the type of DNA damage as observed in cold-stressed *V. vulnificus* one of the initial causes of injury, or is this a consequence of the loss of viability? What role can be attributed to processes such as "programmed cell death" or "cellular suicide" [6, 11, 23]?

How can the issue of injury be investigated? In addition to the obvious use of a range of different media (with high or low concentrations of salt, nutrient, agar etc.) two approaches have been employed in the case of *V. vulnificus*: sonication resistance and analysis of nucleic acids. While the increased mechanical stability of cold-incubated cells (see above, and [31]) points towards the fact that these cells may not be injured in the classical sense, the degradation of nucleic acids [32] indicates lethal injury during cold incubation. Thus the "VBNC state" may constitute a hitherto undescribed state of injury which is, superficially, highly stable. The survival of these "highly stable injured cells" could, however, be limited to short periods of time, after which they acquire lethal (e.g. suicidal) injuries. Recently, starved populations of *Salmonella typhimurium* have been analysed with an array of cytological techniques, demonstrating the heterogeneity and succession of cellular states with increasing degrees of injury in these populations [14].

Non-recoverable cells that have lost their viability may still play significant roles in ecology and epidemiology. Some toxins, for example, may be maintained or even produced in cells long after the ability for proliferation has been lost. Furthermore it is plausible that maintenance of cellular stability could allow for persistence of genetic material in the environment even if the organism itself has definitely lost its capability for propagation. This "surviving DNA" might serve as a pool of genetic traits that can be passed on to other organisms by transformation.

It is concluded that the concept of VBNC bacteria needs to be re-evaluated. In order to clarify the issue, it is here proposed to apply the term "viable" only to recoverable cells, the term "dormant" only for recoverable cells with low activity, and to use the term "non-culturable" in the strict sense, for cells that cannot be recovered under any conditions. Consequently, cells that are "active but non-culturable" ("ABNC") are non-recoverable and thus non-viable. Some of the cells hitherto addressed as "VBNC" may in fact dwell in a state of injury, in which they can retain viability (and restricted culturability) at least for limited periods. Surprisingly, cellular stability and integrity can be maintained well beyond viability, and are thus not reliable indicators for survival. Thus for the description of biological phenomena, it is proposed to classify cells as either actively growing, dormant, sublethally injured, or dead (or lethally injured), as these terms refer to true states of cellular

physiology. So far, the claim of the existence of a biologically defined "VBNC state" outside these categories remains to be substantiated [16].

More than two decades of research into "VBNC phenomena" have pointed out the possibility that cells that can not be recovered on standard media could play important roles in ecology and epidemiology. This has raised tremendous awareness of the issues of viability, culturability, detection methods and bacterial survival in general. A growing number of microbiologists, however, feel that lately this concept has created more confusion than progress [1, 2, 6, 16]. In particular, it appears possible that injury and subsequent cell death are the causes for some of the "VBNC phenomena" described so far. Thus there may be a justification for an initiative to discontinue the use of the term "VBNC", and to adhere to the terms "active", "culturable", "viable", and "dormant" in their strict sense [1, 16].

Obviously, the main concern of this discussion is not terminology; rather, it is hoped that clarity of concepts and language will bring about a better focus on relevant issues and will encourage research in key areas. These are, for example, the questions of what factors determine and limit viability, how microbial cells maintain viability during injury and dormancy, and what roles cellular suicide and programmed cell death play in the survival of bacteria in the environment. Based on this knowledge, it will be possible to develop suitable recovery protocols, reliable rapid detection methods and viability assays for microbial cells and populations.

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