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Abstract

How dead are “dead” microbes? A critical discussion.

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The problem

To know if a microbial cell is dead can be of crucial importance. However, is surprisingly difficult to verify its death. In almost all cases related to questions of hygienic water quality but also in the majority of scientific investigations about viability of microbial cells, culturability is the only parameter. Cells which are not able to form colonies or to grow in nutrient solutions are considered dead and can be neglected – they are off the radar of attention. The limitations of culturability are long known but still it has its merits, in particular for routine analysis, e.g., in drinking or process water monitoring, although many (but by far not all) people applying this method know that only the “tip of the iceberg” is detected. However, when it comes to critical situations, e.g., persistent and recurring contaminations of water systems with microorganisms of hygienic relevance, the cultivation criterion can become insufficient for localization and determination of successful elimination of contamination sources. In particular, the declaration of the safety of a water system after sanitation depends upon information about the reliable inactivation of contaminating microorganisms. The “coliform die-off” which has been reported from sewage ponds or after prolonged exposure to seawater is another example for the underestimation of the actual situation (Roszak and Colwell, 1987). In cases of industrial biofouling, it is frequently not the viable cells but the biomass itself which causes the problem (Flemming, 2002). Here, colony counts do not reflect the actual success of anti-fouling measures as “dead” organisms still remain and continue causing biofouling effects, usually recurring more or less shortly after biocide application. Relying on cfu data leads to many insufficient anti-fouling measures. All legal directives are based on colony counts, and in some areas, such as pharmaceutical production, no other method is allowed. It is long known that microorganisms which do not form colonies or grow in nutrient solutions still can be viable and return into their culturable state and may be “not so dead” after all (Colwell, 2001; Nyström, 2001). More recent definitions consider on membrane integrity, metabolism breakdown and their DNA destruction as death indicators. However, all depends upon the determination method.

Life below the level of culturability

How about the nonculturable rest? How fast and under which circumstances can they resuscitate? What are the mechanisms to regain viability? Viability can be demonstrated by a range of culture-independent methods which may reveal remaining cell integrity or physiological activity on various levels, resulting in the possible recovery of cells. The state of microorganisms after transition from cultivable to non cultivable is characterized by various descriptions such as “cryptobiotic”, injured, dormant, or “viable-but-non-culturable” (VBNC, Kell et al., 1998; Oliver, 2005). Inactivation of microorganisms can lead various levels of stress response, inducing

repair mechanisms which eventually lead to a state from which cells may recover and even become infectious again.

Some microbial life signs without growth

Even without growth, microorganisms can display quite a few life signs. And there are methods at hand to detect them, although it is really difficult to nominate a single parameter to declare cell death. Four (out of more) examples should illustrate this and demonstrate that more than one method has to be employed for this statement.

Cell wall integrity. One of the criteria of a viable cell is the integrity of the cell wall. This can be verified by using the "live/dead system" (Boulos et al., 1999), which has found wide application in microbiology. It is based on the impermeability of viable cell walls to the fluorescent dye propidium iodide (PI) or propidium monoazide (PMA, Nocker et al., 2007). It is crucial to calibrate the system as an excess of PI or PMA can lead to permeation even of viable cells due to variable susceptibility.

Cell elongation. Cells may still divide but not be able to form visible colonies. This can be visualized by adding nalidixic or pipmedinic acid which prevents cell division. As a consequence, dividing cells elongate and can be microscopically determined after fluorescence staining ("direct viable count", DVC, Kogure et al., 1979).

Formation of ribosomal RNA. Maintenance metabolism in microorganisms does not have to be connected to growth. Protein production as coded by rRNA may still be active. A suitable method to detect this is fluorescence in-situ hybridization (FISH), a method which has found wide and sophisticated application in microbiology. It is acknowledged that rRNA may still be present after sudden cell death, e.g., by disinfectant application. However, a positive FISH signal is part of the toolbox to determine microbial viability if interpreted in the context of the cell situation.

Fluorescence intensity of DNA-specific staining. A reasonable life sign is the presence of intact DNA. This was employed by fluorescence measurements of cells after chlorine exposure (Saby et al., 1997), using fluorescence decrease as a parameter for disinfection efficacy and cell death verification.

Conclusions

It is clear that the criterion of culturability cannot remain the only parameter to assess viability of microorganisms. Particularly in hygienically critical areas and encountering problems, further methods need to be taken into account, particularly, if the results are of public health relevance. Their employment can explain many cases of failed disinfection and sanitation. None of them is perfect but they should be part of a toolbox available for situations in which culturability alone is not sufficient. Time has come for a critical discussion about the role and relevance of non-culturable cells.

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