



To: parties interested in the live-dead analysis of plankton organisms

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Unlike animals with a heartbeat, establishing the live-dead status in plants and especially in unicellular microscopic plankton, is difficult. Theoretically the only method that unequivocally establishes microscopic cell death is the complete disintegration of cellular compounds: nucleus, plastids and the cell membrane. However, a cell that has not yet disintegrated may be alive, dead or somewhere in between -zombie plankton- and there is no single method to measure the absolute live-dead status in an intact cell with certainty. For instance, a motile organism can be regarded alive when motility is observed but is it dead when its motility has stopped? My argument is that time needs to be included as a factor in gauging the live-dead status of microscopic plankton.

A number of techniques take advantage of physiological changes in a cell during the process of dying. Before a cell disintegrates its membrane will become permeable and this process can be measured using certain stains (e.g. DIBAC and SYTOX). In the stage during or preceding membrane permeabilization, physiological changes occur such as a reduction in enzyme activity or primary production in the case of algae. These reductions can also be measured: enzyme activity by e.g. the FDA stain and primary production by CO<sub>2</sub>-uptake and fluorescence methods. The outcome of such measurements is evaluated by using the same technique on populations of exponentially growing (alive) and dead (killed) cells. A combination of different techniques can be used, as in IMO-type (G8) BWTS testing, to evaluate the concentration of living cells in a water sample.

It would be practical if a single method would be capable of gauging cell death. Indeed, there are indications that a negative environmental trigger can start a cascade of cellular degradation processes that inevitably lead to cell death. This so-called Programmed Cell Death or PCD has been studied in a number of unicellular plankton species using specific assays that detect the various PCD-stages en route to cell death. Unfortunately, at the moment it is not clear if PCD analysis is applicable in measuring the live-dead status in the wide variety of unicellular plankton.

Depending on the nature and strength of the negative environmental trigger (heat, toxic compound, UV) it takes a cell time to go from fully alive to completely dead. Establishing PCD in a cell would indicate its imminent death, but in addition to its applicability, a simple routine PCD assay is currently not available. Instead, one or more of the above mentioned physiological techniques are used, that measure certain stages in the process of dying. A much simpler method is to wait till cell disintegration has occurred. In fact, this is the old "die-out" method that requires counting the decreasing number of still intact cells in a population over time. One potential drawback of die-out assays is their duration that depends on the cells' disintegration rates.

Time is an important variable in measuring cell death. All BWTS test protocols make use of time in establishing treatment efficacy. Firstly, treated ballast water has to be stored for at

least one (ETV) up to five (G8) days. Secondly, samples from discharged ballast water may need 4-5 hours to count using the FDA-CMFDA method (ETV protocol) and up to 6 hours for the (non-specified) techniques is allowed in G8. The possibility of applying various techniques in G8 lead to different assays being used by the test facilities and in each test potential evaluation difficulties occur if assays give disparate results. It is understandable that a single live-dead assay is therefore preferred. However, if different negative triggers result in a difference in mortality rates that are not captured by a single technique then this technique should be improved or time should be included as a factor in the evaluation of the organism's live-dead status.

Interestingly, the ETV protocol acknowledges that "Techniques and standardized methods for the enumeration and viability analyses of protists remain an active area of investigation." If more analysis time would be allowed not only the "die-out" method but also the "grow-out" method, which has the potential of gauging the ecologically relevant viability the living organisms, may become feasible.

I hope that continued research will lead to rapid, simple and unequivocal tests to gauge the live-dead status of unicellular microscopic plankton.

Yours sincerely,



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