Probing the single-cell activity of *Alcanivorax venustensis* during deep-sea oil degradation

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Marine oil spills originating from leaking ships, drilling rigs, and anthropogenic activities have detrimental effects on various levels of the marine ecosystem. This form of pollution, which mainly consists of liquid petroleum hydrocarbons, affects the biota via direct contact with the contaminants, as well as via the disturbances caused by the oil spill cleanup process. A considerable amount of spilled oil is dissimilated by active microbial degradation. The *Alcanivorax genus*, usually found in marine environments has been shown to proliferate in these contaminated environments. The capability of the Alcanivorax to dissimilate oil compounds (e.g. acyclic saturated hydrocarbons) into harmless compounds presents an as-of-yet untapped potential for both bioremediation and biotechnology applications (e.g., anti-oil spill agent and biopolymer production). While Alcanivorax taxa are frequently found at oil spills at the ocean's surface, there are questions about Alcanivorax' oil degrading capacity at greater depths where oil can precipitate and accumulate. There exists only limited information on the in-situ activity of Alcanivorax taxa during the biodegradation process at elevated hydrostatic pressures.

In this study, we optimized a single cell activity approach called Bioorthogonal Non-canonical Amino Acid Tagging (BONCAT) for studying *Alcanivorax venustensis*, a recently isolated culture able to withstand higher hydrostatic pressures. The BONCAT procedure labels active cells through an in vivo incubation with synthetic l-methionine analogs (e.g., l-homopropargyglycine (HPG) or l-azidohomoalanine (AHA)), which become incorporated in the protein content of the cells, and can be made fluorescent via a Click-it reaction. This protocol was integrated with SYBR Green I as a counterstain for labeling all cells, and was then used to probe the alkane degradation activity of *A. venustensis* at elevated hydrostatic pressures (i.e. 10 bar, 20 bar) for an incubation period of 72 hours using high-pressure reactor systems. Counterstaining with SYBR green I enabled us to make a distinction between active and non-active cells. The protocol optimization consisted of varying the click-it dye concentration from 0.01 to 10 μM and testing different cell fixation methods (no fixation, EtOH PFA, and glutaraldehyde fixation). The final protocol was compatible with both flow cytometric and microscopic analysis and consisted of a 10 μM dye concentration, combined with a sequential ethanol fixation procedure. It was also crucial to incorporate an ultrasonication step as *Alcanivorax venustensis* created aggregates during cultivation. The controls, consisting of treatments with 150 μM mL-1 chloramphenicol to inhibit protein synthesis, and a sample incubated without methionine analogues resulted in no labeled cells, as verified by flow cytometric and microscopic analysis. During incubation at elevated hydrostatic pressures, preliminary data suggests that *A. venustensis* exhibited a lower activity at higher pressures. These results will now be further complemented by gene expression analyses to associate the translational activity of *A. venustensis* with its gene expression.

Keywords: Oil degradation; *Alcanivorax venustensis*; Bacterial activity; BONCAT; Flow cytometry