

Assessing the roles of nitrogen fixing microorganisms in subsurface environments

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Microbial nitrogen fixation (diazotrophy) is responsible for converting atmospheric dinitrogen gas (N₂) into biologically reactive ammonia (NH₃). Diazotrophs are highly specialized microbes that are ubiquitous in marine, terrestrial, and freshwater surficial ecosystems. As such, nitrogen fixation is an important process in these surface environments, as it replenishes nitrogen lost through denitrification and anammox processes. While diazotrophy is important, few studies have focused on identifying and characterizing diazotroph biodiversity in subsurface ecosystems. Depending on the system or formation, labile subsurface nitrogen may be limiting and could ultimately hinder the productivity of the microbial communities. Thus, nitrogen fixation may be underappreciated and could alleviate nitrogen limitations in subsurface systems. The molybdenum-dependent nitrogenase is encoded by three protein subunits: NifH, NifD, and NifK. The *nifH* gene is widely used as a marker for nitrogen fixation. However, the presence of pseudo-*nifH* genes (e.g., homologs of the *nifH* that have been adapted for other functions) can greatly overestimate the presence of nitrogen fixation, especially in anoxic environments. Furthermore, primer-based amplification studies suffer from biases that systematically exclude certain taxa and thus underestimate diazotroph diversity and relative abundance. Therefore, we chose to look for novel diazotrophs using metagenomes and metagenome-assembled genomes (MAGs) available through the Census of Deep Life program, as metagenomes are not reliant on PCR amplification. We observed a large disparity between the number of NifH and NifDK proteins, highlighting the overestimation of nitrogen fixation and the importance of identifying pseudo-*nifH* genes prior to downstream analysis. While many of the sequences we recovered were similar to previously known groups, several were novel, highlighting the biases observed with PCR-based approaches. Finally, using a metabolic annotation approach with known genomes, we highlight the putative metabolisms for many of these novel groups and gain deeper insight into their ecology across diverse subsurface environments. Together, this work suggests that undescribed diazotrophs are lurking in the subsurface and we need a focused effort to understand their contributions to the nitrogen cycle.