Unraveling microbial nitrate turnover in a polluted fractured limestone aquifer using metagenomics

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The long residence time of groundwater in aquifers may allow for a slow microbial transformation of critical pollutants such as nitrate. However, little is known about the *in-situ* microbial key players involved in nitrate turnover in fractured limestone aquifers. Our study site, the middle Triassic fractured limestone aquifer in the Ammer catchment in Southern-Germany is greatly impacted by anthropogenic activity and shows variable groundwater nitrate concentrations, with several monitoring wells above the guideline value of 50 mg/L and others <1 mg/L. In one of these low nitrate wells, the formation of a well-developed biofilm in the fractured rock was observed. The aims of this study were (i) to characterize the isotope (hydro)geochemistry of the well, (ii) to identify microbial key players involved in nitrate turnover, and (iii) to use metagenome assembled genomes to determine the metabolic potential of planktonic- and biofilm-associated microbes. The results from $^{3}H/^{3}He$ dating, nitrate isotopes ($\delta^{15}N$ and $\delta^{18}O$), and geochemical analyses of the groundwater provided first indications that microbial denitrification is an important process in this oligotrophic ecosystem. Mineralogical analysis of the limestone revealed the heterogeneous distribution of pyrite (5.6-6.3 mg/g) in the rock, presenting a potential electron donor for nitrate turnover. In support of this, 16S rRNA gene sequence analysis of the microbial communities showed a high abundance of chemolithoautotrophs that have the potential to couple the oxidation of reduced iron (e.g. *Gallionella, Sideroxydans*) and sulfur (e.g. *Sulfurimonas, Sulfuricurvum*) to nitrate reduction. Ongoing metagenomic analysis will determine the metabolic potential of the microbial key players, testing our hypothesis that nitrate removal occurs through a series of redox transformations involving microbial key players and metabolic handoffs. Our findings provide the first insights into *in-situ* microbial nitrate turnover in a fractured limestone aquifer and show the potential for pyrite-dependent nitrate reduction.