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Linking metabolic state to N_2O isotope signatures in bacterial denitrifiers using position-specific and clumped isotopes

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N_2O is a potent greenhouse gas whose isotopic composition is widely used to infer microbial production pathways in the nitrogen cycle. Interpretation of N_2O isotope signatures produced during denitrification commonly assumes that these signatures reflect fixed, enzyme-specific endmembers. In particular, $\delta^{18}O_{N2O}$ and N_2O site preference (SP) are widely interpreted as diagnostic parameters, reflecting differential oxygen isotope exchange during nitrite reduction (NirS versus NirK) and nitrogen–nitrogen bond formation during NO reduction by NorB. However, because these interpretations are derived from a limited range of experimental conditions, they may not be robust to changes in microbial metabolic state, potentially conflating enzyme identity, pathway expression, and cellular regulation.

We investigated N_2O production by two closely related, NosZ-lacking denitrifiers, *Pseudomonas aureofaciens* (NirK) and *Pseudomonas chlororaphis* (NirS), across experiments designed to probe distinct physiological states. Real-time $\delta^{15}N_{N2O}$, $\delta^{18}O_{N2O}$, and SP were measured using quantum cascade laser absorption spectroscopy (QCLAS) and complemented by isotope ratio mass spectrometry (IRMS) analysis of accumulated N_2O.

Oxygen isotope exchange between N_2O precursors and water varied strongly with physiological state. *P. chlororaphis* (NirS) exhibited consistently high O-atom exchange (65–77%), whereas *P. aureofaciens* (NirK) showed pronounced dependence on experimental conditions, resulting in exchange rates ranging from minimal (2–4%) to nearly complete O-atom exchange (38–100%). Consequently, $\delta^{18}O_{N2O}$ did not reliably reflect the expected enzyme-specific distinction between NirK and NirS, with NirS generally associated with higher oxygen isotope exchange than NirK, and under some conditions exhibited the opposite pattern. Time-resolved SP showed transient early elevations (10–20‰), consistent with contributions from alternative NO reduction pathways distinct from NorB. These dynamics indicate that conventional N_2O isotope parameters do not uniquely resolve enzyme identity independently of metabolic and pathway regulation.

To address this limitation, we extended the framework to include clumped N_2O isotopes quantified by QCLAS. We evaluated the combined behavior of $\Delta^{14}N^{15}N^{18}O$, $\Delta^{14}N^{15}N^{16}O$, and $\Delta^{15}N^{15}N^{16}O$, which provide complementary sensitivity to N–O bond formation and cleavage, oxygen isotope exchange, and N–N bond formation during NO reduction. Our results demonstrate that clumped N_2O isotopes provide a mechanistic framework to resolve ambiguities inherent to conventional isotope approaches, thereby disentangling effects of enzyme identity, pathway expression, and metabolic state.