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Characterizing the clumped isotope composition of microbially-produced H_2

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Discerning microbially produced H_2 from abiotic H_2 could enhance our ability to identify H_2 sources in the search of naturally occurring H_2 as an energy resource, and to study microbial modifications of H_2 stored in engineered crustal reservoirs. The molecular average deuterium content of H_2 has been used previously for identifying its sources and sinks, and recent studies suggest the multiply-substituted ('clumped') isotopologue, D_2, could provide additional constraints on molecular formation mechanisms and temperatures [1, 2]. Here we present the first clumped isotope measurements of biologically produced H_2. We measured the molecular average and clumped isotope composition of H_2 formed during fermentation in batch cultures of *Clostridium pasteurianum*; the H_2 was purified cryogenically and measured by the Thermo Fisher 253 Ultra high resolution IRMS. Our results show that microbially-produced H_2 expresses a consistent deviation from the equilibrium fractionation during exponential growth, for both the clumped and molecular average isotopic compositions, whereas during the stationary phase it conforms to near-equilibrium isotopic compositions at the culturing temperature. The specific combination of clumped and molecular average D contents we observed during the growth phase differs from equilibrium at any temperature and from simple kinetic fractionations such as diffusion; we suggest it may be a biologically regulated kinetic isotope signature unique to microbial growth. The shift from kinetic to equilibrium controls of isotopic composition moving from growth to stationary phase is consistent with theoretical calculation of the reversibility of the H_2 production reaction, because H_2 concentrations increase over growth, reaching and maintaining high levels during the stationary phase. It is plausible that in the environment, under lower H_2 concentrations, we would observe the distinctive, kinetic endmember during biological H_2 production. Since previous studies demonstrate that the clumped isotope composition of H_2 from natural reservoirs and hydrothermal vents reflects equilibrium values [2], the deviation from equilibrium during microbial growth can potentially be used to detect biological sources of subsurface H_2.

[1] Popa, M.E. *et al.* (2019) *Rapid Communications in Mass Spectrometry* 33(3), 239-251.

[2] Mangenot, X. *et al.* (2023), *Chemical Geology* 621, 121278.