Nitrous oxide (N₂O) is an unwanted byproduct during biological nitrogen removal processes in wastewater. To establish strategies for N₂O mitigation, a better understanding of production mechanisms and their controls is required. A novel stable isotope labeling approach using ¹⁵N and ¹⁸O was applied to investigate pathways and controls of N₂O production by biomass taken from a full-scale nitritation-anammox reactor. The experiments showed that heterotrophic denitrification was a negligible source of N₂O under oxic conditions (≥0.2 mg O₂ L⁻¹). Both hydroxylamine oxidation and nitrifier denitrification contributed substantially to N₂O accumulation across a wide range of conditions with varying concentrations of O₂, NH₄⁺, and NO₂⁻. The O₂ concentration exerted the strongest control on net N₂O production with both production pathways stimulated by low O₂, independent of NO₂⁻ concentrations. The stimulation of N₂O production from hydroxylamine oxidation at low O₂ was unexpected and suggests that more than one enzymatic pathway may be involved in this process. N₂O production by hydroxylamine oxidation was further stimulated by NH₄⁺, whereas nitrifier denitrification at low O₂ levels was stimulated by NO₂⁻ at levels as low as 0.2 mM. Our study shows that ¹⁵N and ¹⁸O isotope labeling is a useful approach for direct quantification of N₂O production pathways applicable to diverse environments.