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Supplement of

A novel laser-based spectroscopic method reveals the isotopic signatures of nitrous oxide produced by eukaryotic and prokaryotic phototrophs in darkness

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Section S1. Denitrifiers cultures

Denitrifying inocula were cultivated in closed Duran bottles containing simulated domestic wastewater (**Table S1**). These reactors were seeded with a pond sediment suspension (Massey University) and continuously mixed using magnetic agitation at 100 rpm. Once denitrification was confirmed (via nitrate monitoring), the reactors were operated as sequential batch reactors by gradually increasing the feeding rate to reach a hydraulic retention time (HRT) of 10 days. This operation was achieved by i) turning the agitation off and letting biomass to settle for 1 hour before ii) removing 100 ml of culture broth using a syringe (via a sample port) and 3) injecting 100 ml of fresh medium before resuming agitation. The settling period was discontinued once a strong denitrifying ability was evidenced to prevent the excessive accumulation of dead biomass.

Table S1: Denitrification medium

Solution/salt	Composition (g/L)	mL added/L
Organic solution	Starch (2.1), milk powder (2), dried yeast (0.9), soy oil (0.5)/L, peptone (0.3)	100
NO_3	KNO_3 (14.43)	120
Mg	$MgCl_2 \cdot 4H_2O$ (4.19)	100
Phosphate buffer	K_2HPO_4 (2.178) and KH_2PO_4 (0.612)	560
Ca	$CaCl_2 \cdot 2H_2O$ (1.33)	100
Trace element 1	$FeSO_4$ $7H_2O$ (9) and EDTA (6)	10
Trace element 2	EDTA (15), $ZnSO_4 7H_2O$ (0.43), $CoCl_2.6H_2O$ (0.24), $MnCl_2 4H_2O$ (0.99), $CuSO_4.5H_2O$ (0.25), $NaMoO_4 2H_2O$ (0.22), $NiCl_2 6H_2O$ (0.19), H_3BO_3 (0.014)	10

Section S2. Analytical blind test using isotope analysis to determine fractions of N_2O from two biological sources in a gas mixture

Aliquots of N_2O produced by denitrifiers (60 %) and *Chlorella vulgaris* (40 %) were mixed following the incubation experiments described in the manuscript. N_2O was analysed has described in the manuscript. Using the isotope measurement of N_2O from the pure cultures, the fractions of each N_2O source were calculated in a blind experiment, to test for the robustness of the method developed.

The fractions determined using all measured tracers $\delta^{15}N\alpha$, $\delta^{15}N\beta\alpha$, $\delta^{15}N$(bulk) and especially SP-N_2O are in good agreement with the fractions used during the preparation (**Figure S1**), except for $\delta^{18}O$ (not shown), where the initial values of denitrifiers and *C. vulgaris* are too similar for differentiation with our analysis.

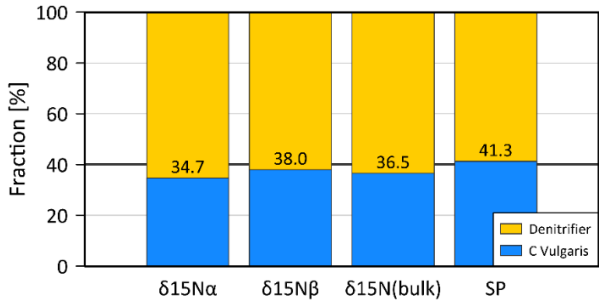

Figure S1: Bar plot showing fractions of N_2O from *Chlorella vulgaris* and denitrifiers synthetic gas mixture prepared during incubation experiments. Fractions are calculated based on isotope ratios measured in pure N_2O from the respective culture. The thick black line shows the target in this blind test.

Section S3. N_2O and isotopomers measurements bias due to N_2O amount and cell pressure dependence

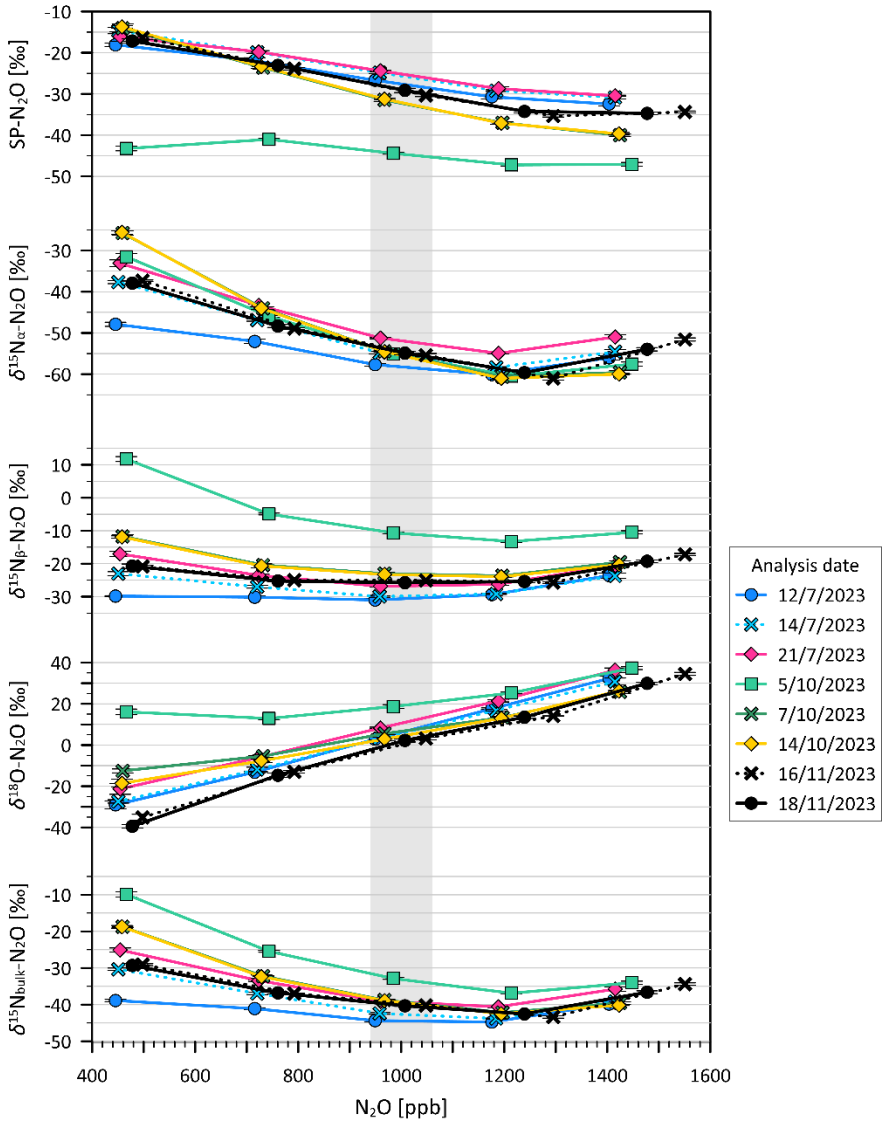

Figure S2. Day-to-day variation in isotopomer measurements due to N_2O amount dependence. This was determined with USGS51-in-air at the start of each measurement sequence. Error bars indicate the standard deviation (1 σ) of the isotope measurements at the respective N_2O level. The grey bar indicates the typical N_2O mole fraction range of samples and reference gases.

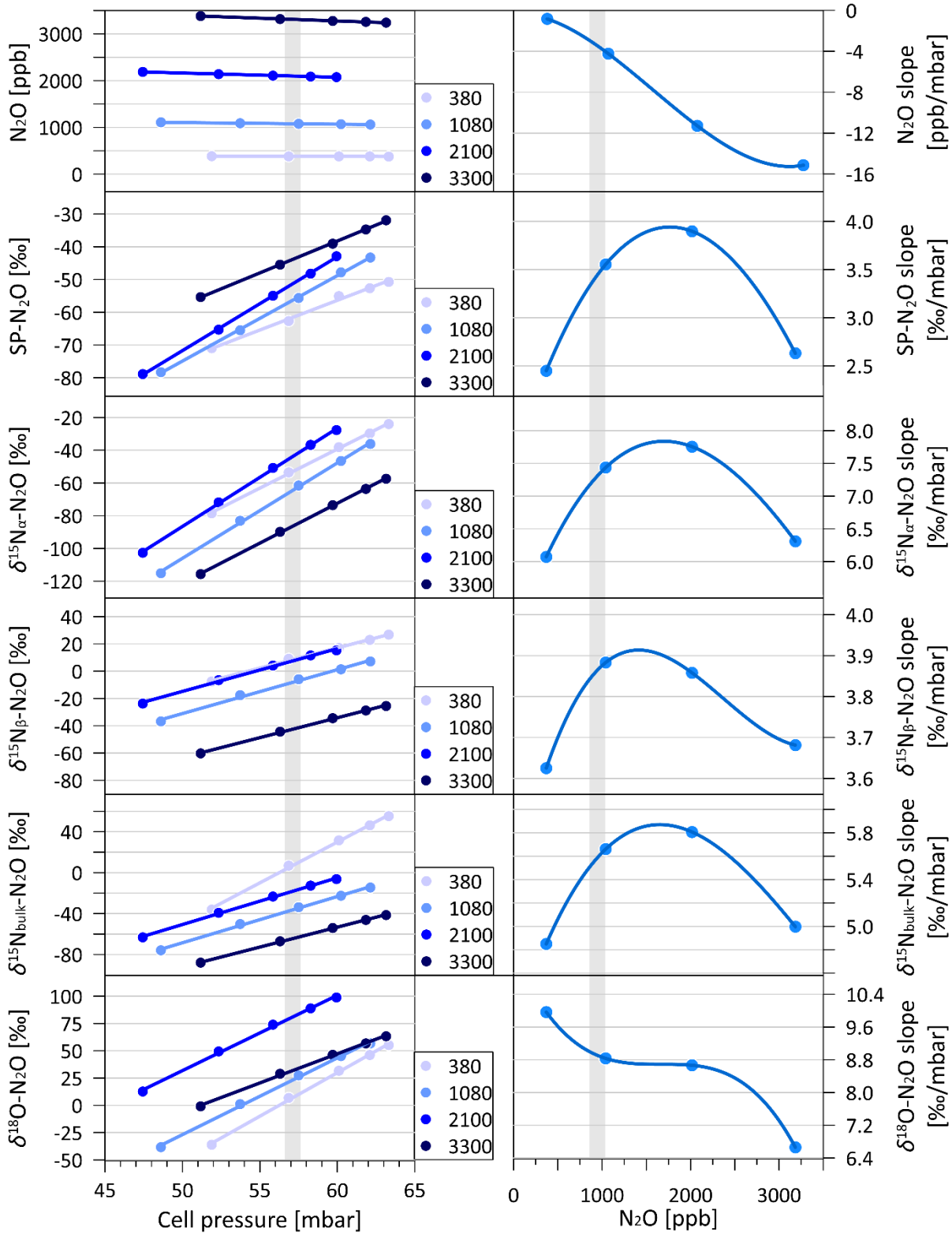

Figure S3: Left - Analyser response dependence on cell pressure changes at N_2O mole fractions of 0.33, 1.08, 2.1 and 3.3 ppm. The response is linear across the typical measurement range (grey box). Right - Polynomial fits of the pressure dependence on N_2O or isotopomers. The slope of the linear response change varies with N_2O mole fractions following a 3rd order polynomial.

Section S4. Measurements sequence followed

Block #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Gas	**WT**	**USGS51-in-air**	**WT**	**USGS52-in-air**	**WT**	**USGS51-in-air**	**S1**	**USGS51-in-air**	**USGS51-in-air**	**S2**	**USGS51-in-air**	**WT**	**USGS51-in-air**	**S3**	**USGS51-in-air**	**USGS51-in-air**	**S4**	**USGS51-in-air**	**WT**
N_2O amount	1080	400-1500	1080	400-1500	1080	match S1	1000	match S1	match S2	1000	match S2	1080	match S3	1000	match S3	match S4	1000	match S4	1080
purpose	drift	N_2O amount SP assignment	drift	N_2O amount SP assignment	drift	S1 drift correction		S1 drift correction	S2 drift correction		S2 drift correction	drift	S3 drift correction		S3 drift correction	S4 drift correction		S4 drift correction	drift
n repetitions	5	5 x 5	5	5 x 5	5	10	10	10	10	10	10	5	10	10	10	10	10	10	5

Figure S4: The measurement sequence combines blocks of measurements of air from the working tank (1080), the USGS51-in-air and USGS52-in-air standards used to define the correction for the N_2O amount effect as well as scaling for a two-point calibration of SP-N_2O values, up to four samples (S1-S4) and bracketing blocks of USGS51-in-air measurements with N_2O amounts that match the N_2O amount in their respective sample.