Supplementary Information

Title: Nitrous Oxide (N_2O) production in axenic *Chlorella vulgaris* microalgae cultures: evidence, putative pathways, and potential environmental impacts.

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S1 – N_2O emissions during *C. vulgaris* cultivation in a 50 L photobioreactor

S2 - PCR analysis

S3 - Effect of nitrite addition on N_2O emissions during *C. vulgaris* cultivation in a 50 L photobioreactor

S4 - Effect of NR repression, L-arginine and LNNA on N_2O production by *C. vulgaris*

S5 - Impact of cell age on N_2O production from *C. vulgaris* cultures

S6 - N_2O emission during outdoor *C. vulgaris* cultivation.

8 Figures

2 Tables

15 Pages

S1 – N_2O emissions during *C. vulgaris* cultivation in a 50 L photobioreactor

C. vulgaris was cultivated in buffered BG 11 medium containing (g L^{-1} of distilled water) $NaNO_3$ (1.5), K_2HPO_4 (3.1), KH_2PO_4 (1.52), $MgSO_4 \cdot 7H_2O$ (0.075), $CaCl_2 \cdot 2H_2O$ (0.036), citric acid (0.006), ammonium ferric citrate (0.006), EDTA (0.001), and 1 mL of a trace element solution containing (g L^{-1} of distilled water) H_3BO_3 (2.86), $MnCl_2 \cdot 4H_2O$ (1.81), $ZnSO_4 \cdot 7H_2O$ (0.222), $CuSO_4 \cdot 5H_2O$ (0.079), $Na_2MoO_4 \cdot 2H_2O$ (0.39), and $Co(NO_3)_2 \cdot 6H_2O$ (0.0494).

C. vulgaris was then inoculated in a column photobioreactor filled with 50 L of buffered BG11 medium. The reactor design, operation, and monitoring were described by Béchet et al. (2013) with the difference that this reactor was operated indoors and was therefore submitted to artificially continuous illumination at 260 W m^{-2} (average of 40 measurements taken at 5 different heights and 8 different angular positions using a EXTECH 401020 light multimeter adaptor, conversion factor of 2.7 W PAR m^{-2} per klux) using 16 light tubes (Philips TLD 58W/865 Cool Daylight). Nitrite concentration was measured by ionic chromatography and N_2O concentration in inlet and outlet samples were measured by GC-ECD. To provide mixing and CO_2, moisturized air enriched at 2.5% CO_2 was continuously bubbled at the base of the fluid column at a flow of 1.2 L min^{-1}. As can be seen in Figure S1-1, N_2O specific production rate averaged 24 ± 10 nmol N_2O g-DW^{-1} h^{-1} during the monitoring period ($p = 0.05$, $n = 10$).

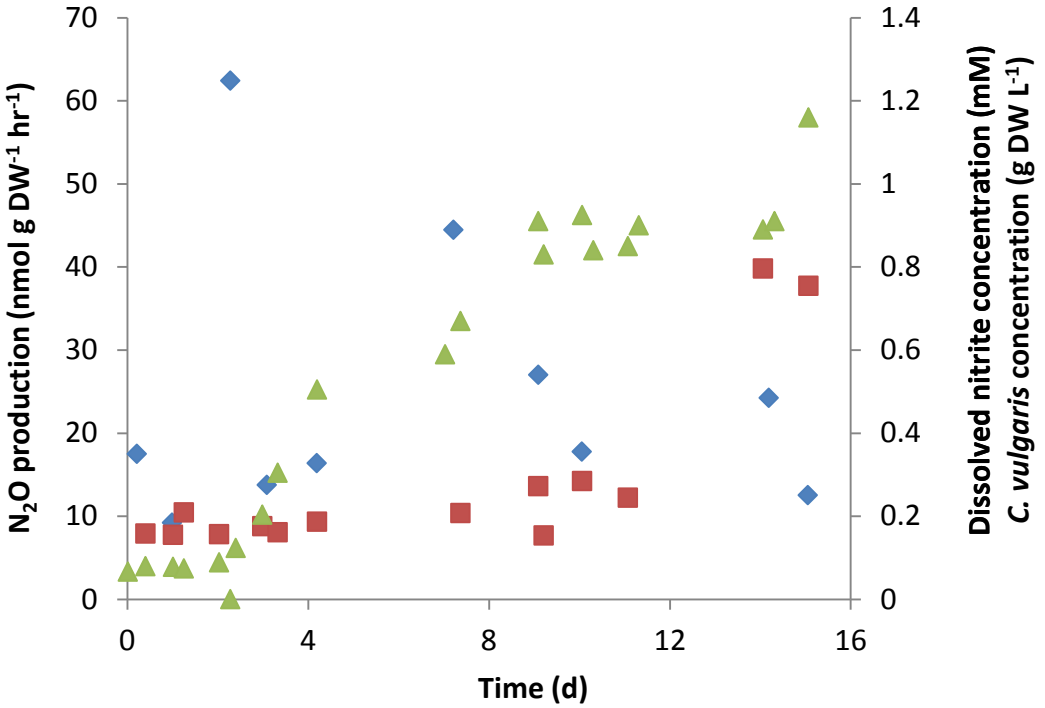


Figure S1-1: N_2O production (diamonds), dissolved nitrite concentration (squares) and *C. vulgaris* concentration (triangles) in a 50L column photobioreactor submitted to continuous illumination.

This experiment was repeated and the results are shown in Figure S1-2. N_2O specific production averaged 58 ± 23 nmol N_2O g-DW^{-1} h^{-1} during the monitoring period ($p = 0.05$, $n = 8$).

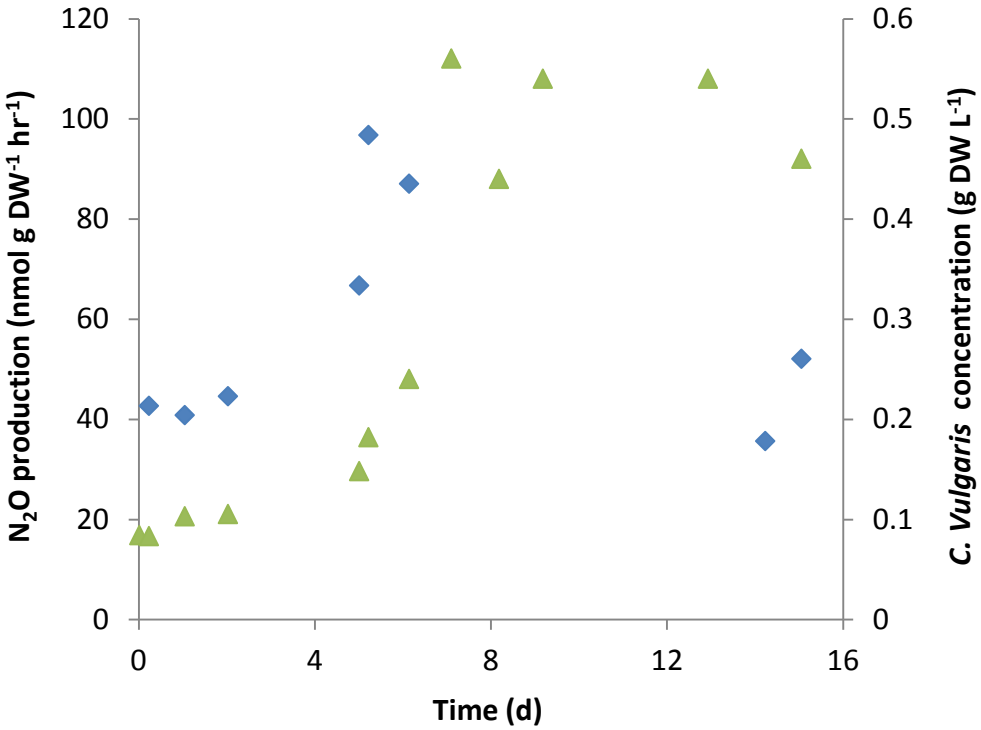


Figure S1-2: N_2O production (diamonds), and *C. vulgaris* concentration (triangles) in a 50 L column photobioreactor submitted to continuous illumination.

References:

Béchet, Q.; Muñoz, R.; Shilton, A.; Guieysse, B. Outdoor cultivation of temperature-tolerant Chlorella sorokiniana in a column photobioreactor under low power-input. *Biotechnol. Bioeng*, 110 (1), 118-126, 2013.

S2 - PCR analysis

C. vulgaris was phototrophically-grown in 'normal' and 'antibiotic-laden' buffered BG11 where 100 mg penicillin L^{-1} of and 25 mg streptomycin L^{-1} were added 4 days after inoculation. To determine if the protocol was indeed able to detect bacterial genes within algae cultures, N_2O-producing denitrifying bacteria were isolated from local soil and their potential for N_2O production was confirmed in batch assays.

A sample (1.7 ml) was taken from each of the following cultures: pure chlorella (Sample 1); chlorella plus antibiotics (Sample 2); chlorella plus denitrifying bacteria (50:50 vol, Sample 3); denitrifying bacteria (Sample 4). N_2O production in the presence of 12 mM nitrite under darkness was confirmed in all cultures in batch assays. Each sample was split over two tubes and spun at 13,000 g in a bench top microcentrifuge for 5 min to pellet the algae and bacteria. The supernatant was removed and the samples were stored at - 80^{o}C. One set of samples was extracted using the Epicentre Water Metagenomic DNA Extraction Kit ^{TM} according to the manufacturer's protocol. After extraction, a band of high molecular weight genomic DNA was visible on an agarose gel for Samples 3 and 4. No band was visible for Samples 1 and 2 (a low yield of DNA is often common with alga). PCR was carried out on the extracted genomic DNA using the primers and amplification conditions in Table S2-1 . Each PCR reaction contained 1X Buffer with 1.5 mM $MgCl_2$ (Roche Diagnostics), 250 μM each dNTP, 10 pmol of each primer, 2 μl of template DNA and 1U Taq polymerase (Roche Diagnoatics), in a final volume of 20 μl. Following PCR, 5 μl of reaction mix was analyzed on an agarose gel (1 or 2% (w/v) agarose in 1XTris-acetate –EDTA buffer) and visualized using SYBR-SAFE (Invitrogen) on a gel documentation system (BIORAD).

Table S2-1: Primers and conditions using during PCR analysis

Gene	Primers	Sequence	Exp. size (bp)	PCR conditions
Bacteria				
norB	norB1F	CGNGARTTYCTSGARCARCC	670	95°C - 5 min [95°C - 30 s, 54°C - 45 s, 68°C – 45 s] × 35 cycles 68°C -7 min, 10°C hold. (Fagerstone et al., 2011)
	norB8R	CRTADGCVCCRWAGAAVGC		
cnorB	cnorBF	GACAAGNNNTACTGGTGGT	389	
	cnorBR	GAANCCCCANACNCCNGC		
qnorB	qnorBF	GGNCAYCARGGNTAYGA	262	
	qnorBR	ACCCANAGRTGNACNACCCACCA		
rbcl	rbclAF	ATGTCACCACAAACAGAGACTAAAGC	1200	94°C - 3 min [94 °C – 30 s, 53°C – 30 s, 72°C – 90 sec] × 35 cycles 72°C – 5 min, 10°C hold (Hasebe et al., 1994)
	rbclAR	GCAGCAGCTAGTTCCGGGCTCCA		
16S	F27	AGAGTTTGATCCTGGCTCAG	1000 – 1500	94 ºC – 3 min [94ºC - 30 s, 50ºC - 30 s, 72ºC – 90 s] × 35 cycles, 72ºC – 5 min, 10 ºC hold (Giovannoni, 1991)
	1492R	TACGGYTACCTTGTTACGACGAC		
Archaea				
amoA	arch-AmoAF	5′-STAATGGTCTGGCTTAGACG-3′	635	95°C - 5 min [94°C - 45 s, 53°C - 60 s, 72°C – 60 s] × 30 cycles 72°C -15 min, 10°C hold. (Francis et al., 2005)
	arch-AmoAR	5′-GCGGCCATCCATCTGTATGT-3′		
16S	340F	CCCTAYGGGGYGCASCAG	660	98°C - 2 min [95°C - 30 s, 57°C - 30 s, 72°C – 90 s] × 30 cycles 72°C -7 min, 10°C hold. (Gantner et al., 2011)
	1000R	GGCCATGCACYWCYTCTC		

The norB, cnorB and qnorB primer pairs amplify fragments of gene encoding for bacterial NOR (NO-reductase) and the amoA primer pair specifically amplifies a fragment of gene encoding for ammonia monooxygenase in archaea. The rbcl primer pair amplifies a fragment of the large subunit of the ribulose-1,5-bisphosphate carboxylase oxygenase gene which is present on the chloroplast in algae, plants and cyanobacteria and in the genomes of some bacteria. The rbcl amplification was performed as a positive control to show that there was enough DNA in the samples to be amplified. The 16S primer pairs amplify a region of the 16S rDNA in bacteria or archaea and are considered a 'universal' primer pairs for bacteria and archaea, respectively. A negative control was included for each primer pair, this reaction contains all components except the template DNA.

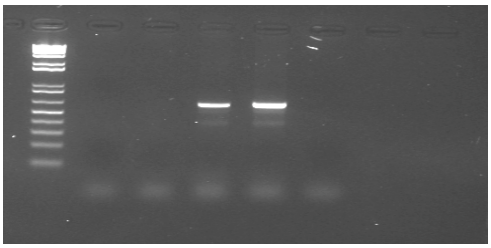
Table S2-2: Results from genomic analysis (Y indicates there was a band of the expected size, N means no band of the expected size).

	norB	cnorB	qnorB	amoA	Rbcl
pure Chlorella	N	N*	N	N	Y* (1.2KB)
Chlorella plus antibiotics	N	N*	N	N	Y* (1.2KB)
Chlorella plus bacteria	Y	Y*	Y	N	Y* (1.7KB)
Bacteria	Y	Y*	Y	N	Y* (1.7KB)
Negative control	N	N*	N	N	N

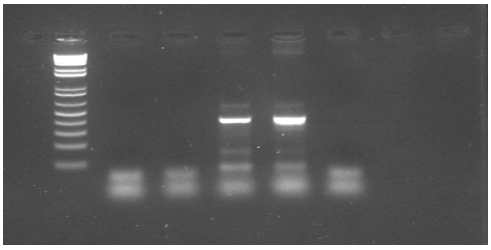
* an asterisk indicates the presence of other bands which are PCR artifacts or primer dimers.

Results from PCR analysis are shown in Figure S2-1. A PCR product of the expected size was seen for each of the denitrification primer pairs in Samples 3 and 4. No PCR products were ever visible for any of the three denitrification genes in Samples 1 and 2 (Figure S2-1A,

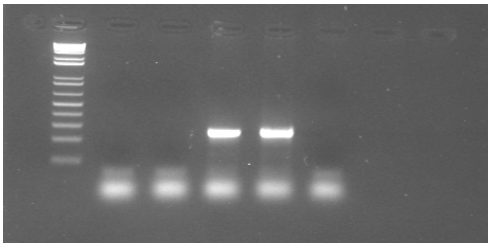
S2-1B, S2-1C) in replicate amplifications. A PCR product of the expected size (1.2 kb) for the rbcl primers was visible on a gel in samples 1 and 2. Samples 3 and 4 also produced a product from these primers but it was much larger (~ 1.7kb) and was therefore more likely a product from a bacterial genome than a product from a chloroplast gene (Figure S2-1D). When the 4 samples were amplified using the 16S primer pair, a dominant band of ~1.5 kb , with a light smear down to ~1 kb was seen in samples 3 and 4. There was no apparent trace of bacterial PCR products in the algae Samples 1 and 2, which confirms that N_2O emissions in these samples was caused by algae rather than associated bacteria. No PCR products were obtained for any of the samples using the archaea-specific primers (Arch-amoAF/R and 16S 340F/1000R).



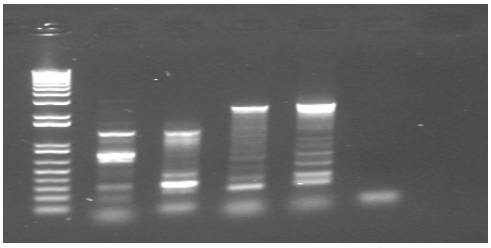
A .norB on 2% (w/v) agarose, in 1XTAE



B. cnorB on 2% (w/v) agarose, in 1XTAE



C. qnorB on 2% (w/v) agarose, in 1XTAE



D. rbcl on 1% (w/v) agarose, in 1XTAE

Figure S2-1: Results of PCR analysis viewed under UV on agarose gels. Left to right: 1kb+Ladder, algae (Sample 1), algae + antibiotics (Sample 2), algae + bacteria (Sample 3), bacteria (Sample 4), negative control.

References

Fagerstone, K. D.; Quinn, J. C.; Bradley, T. H.; De Long, S. K.; Marchese, A. J. Quantitative measurement of direct nitrous oxide emissions from microalgae cultivation. *Environ. Sci. Technol*. 45 (21), 9449-9456, 2011.

Francis, C. A.; Roberts, K. J.; Beman, J. M.; Santoro, A. E. Oakley, B. B. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc Natl Acad Sci U S A.*102 (41), 14683–14688, 2005.

Gantner, S.; Andersson, A. F.; Alonso-Sáez, L.; Bertislon, S. Novel primers for 16S rRNA-based archaeal community analysis in environmental samples. J. *Microbiol. Methods.*, 84, 12-18, 2001.

Hasebe, M.; Omori, T.; Nakazawa, M.; Sano ,T.; Kato, M.; Iwatzuki, K. rbcL gene sequences provide evidence for the evolutionary lineages of Leptosporangiate ferns. *Proc. Natl. Acad . Sci. USA.* 91, 5730 –5734, 1994.

Giovannoni, S. J. Nucleic Acid Techniques in Bacterial Systematics, in *The Polymerase Chain Reaction*: Stackerbrandt, E., Goodfellow, M. (eds), John Wiley and Sons. Chichester, pp 177-203, 1991**.**

S3 - Effect of nitrite addition on N_2O emissions during *C. vulgaris* cultivation in a 50 L photobioreactor

N_2O emission in response to nitrite addition was confirmed in the 50 L pilot scale reactor described in S1 when ammonium was initially used as the sole N-source. As can be seen in Figure S3-1, N_2O release was not detected prior to nitrite injection (13.4 mM) whereas large emissions were recorded after injection. In light of the putative pathway for N_2O synthesis proposed in this manuscript, these emissions can be explained by the fact nitrate reductase (NR) can remain active in the presence of ammonium.

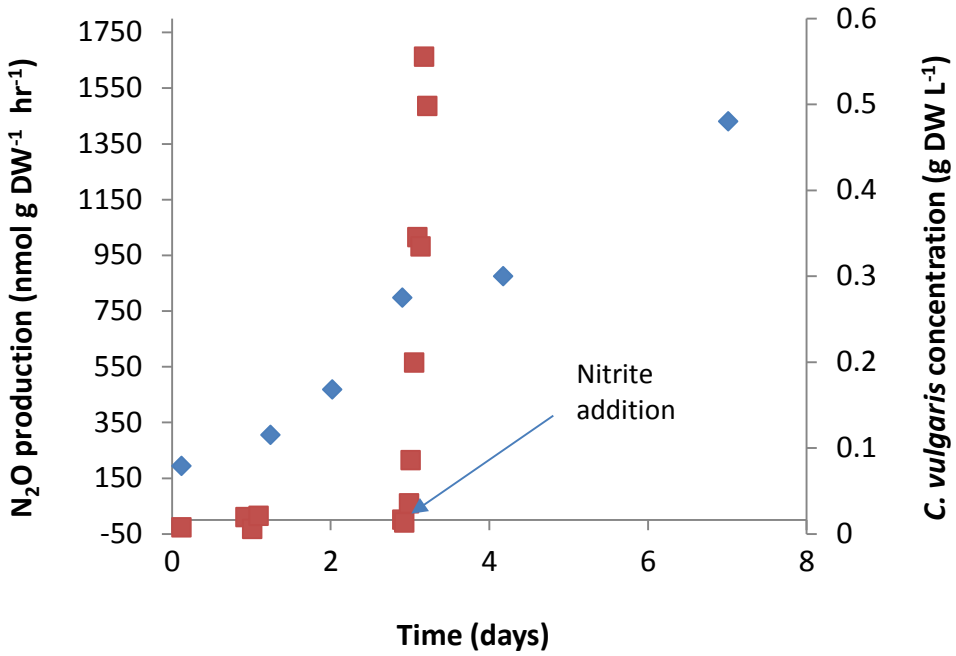


Figure S3-1: N_2O production during *C. vulgaris* cultivation in a 50 L column photobioreactor operated under continuous artificial illumination. A t = 0 d, the reactor was filled with NH_4^+-laden BG11 medium and inoculated. Nitrite was added at t = 3 d.

S4 - Effect of NR repression, L-arginine and LNNA on N_2O production by *C. vulgaris*

After 10 days of cultivation, aliquots of phototrophically-grown algae were centrifuged and re-suspended either in 'normal' buffered BG11 or in buffered BG11 where molybdate (MoO_4^{2-}) was replaced by orthotungstate (WO_4^{2-}) at the same relative concentration (0.253 mg Na_2WO_4 L^{-1}) as described by Deng et al. (1989). Following cultivation during 4 days, each algae culture was withdrawn, washed, and tested for N_2O emissions in its respective medium as described above (darkness). Figure S4-1 shows the specific rate of N_2O emissions (nmol g DW^{-1} h^{-1}) from 1.5 to 4.5 h after transfer into sealed flasks and/or nitrite addition.

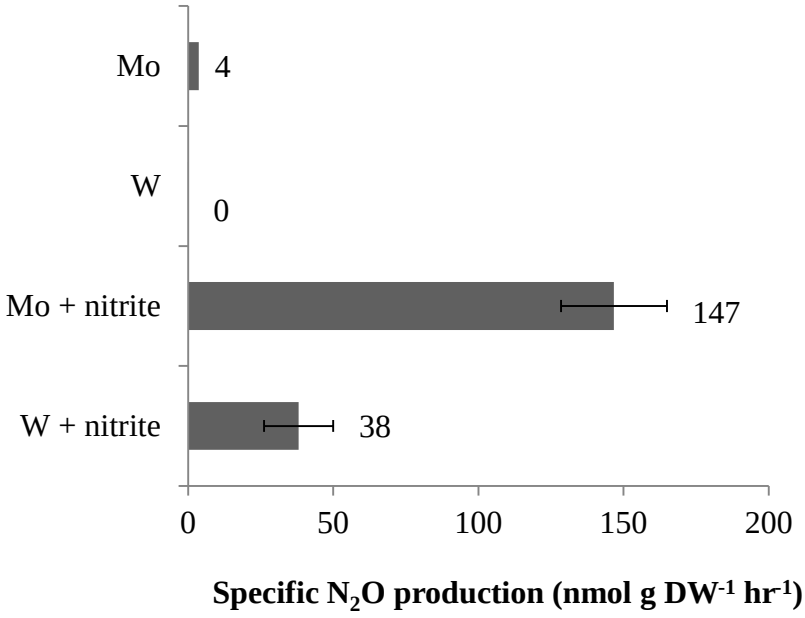


Figure S4-1: Specific rate of N_2O production from 1.5 - 4.5 h after transfer into sealed flasks and/or nitrite addition (1.34 mM), algae concentration of 0.26 g L^{-1} (W test) and 0.28 (Mo test). Error bars show min/max values from duplicates.

Figure S4-2 shows the impact of NOS (NO synthase) substrate arginine and NOS-inhibitor Nω-nitro-L-arginine (LNNA) on N_2O production by *C. vulgaris* in the presence of nitrite. As

can be seen, there is no significant impact of L-arginine and LNNA addition on N_2O emissions.

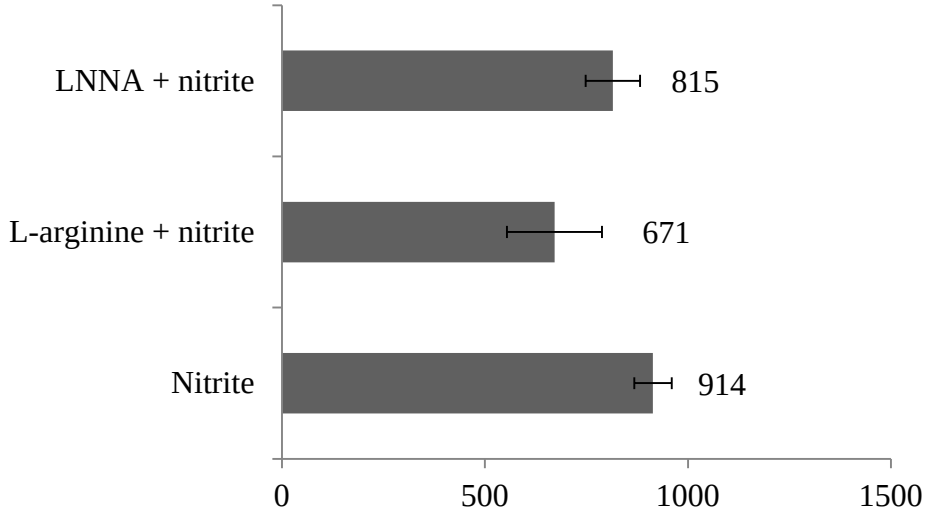


Figure S4-2: Specific rate of N_2O production from 2 - 4 hours after nitrite addition in closed serum flasks supplied with *C. vulgaris* (0.15 g DW L^{-1}), nitrite (10 mM), LNNA (10 mM) and L-arginine (10 mM).

S5 - Impact of cell age on N_2O production from *C. vulgaris* cultures

Phototrophically grown axenic *C. vulgaris* was aseptically inoculated into 250 mL E-flasks containing 125 mL of buffered BG11 and cultivated under continuous illumination and agitation. After 3, 4, 5, 6, or 7 days cultivation, algae were withdrawn from several flasks, mixed, centrifuged, and the biomass pellets were mixed and re-suspended with N-free buffered BG11 to 0.1 or 0.2 g DW L^{-1}. Then, 50 mL of *C vulgaris* suspension and 2 mL of freshly prepared 12 mM NO_2^- were transferred into 120 mL glass flasks and incubated at 25 ± 2°C under continuous agitation (180 rpm) in darkness. Gas samples were withdrawn 4, 7, and 24 h after nitrite addition to quantify N_2O production.

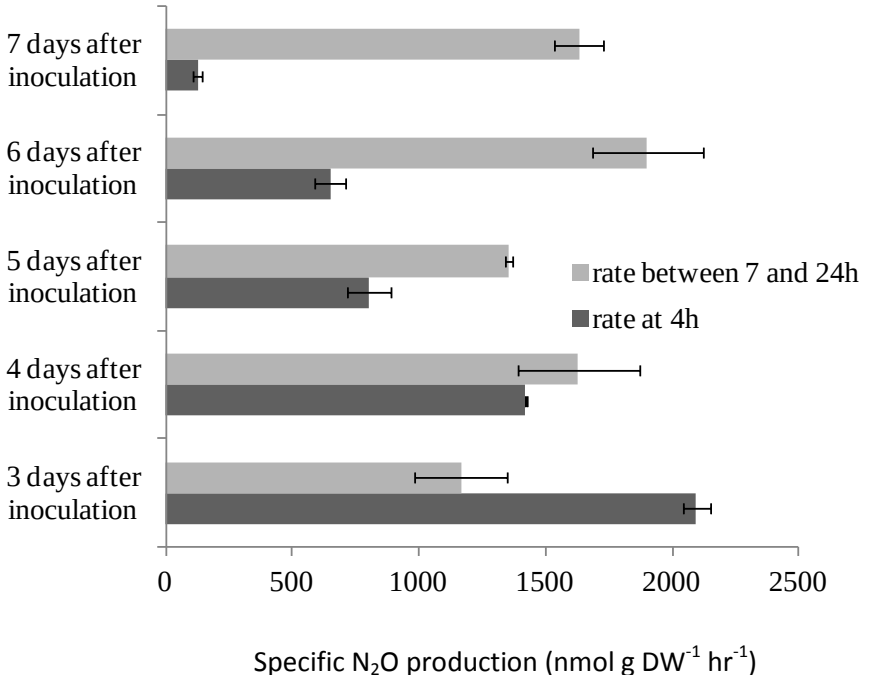


Figure S5-1: Influence of cell age on N_2O production from *C. vulgaris* cultures (12 mM nitrite, darkness, data show the average from experiments performed at 0.2 and 0.1 g algae DW L^{-1}, error bars represents max-min values).

As can be seen in Figure S5-1, the initial rate of N_2O specific emissions was inversely correlated with cell age.

S6 - N_2O emission during outdoor *C. vulgaris* cultivation.

The 50 L photobioreactor described in S1 was operated outdoor and supplied with CO_2-enriched air (3% of CO_2) at 1 L min^{-1}. The reactor was operated in a batch mode for 6 days followed by fed-batch mode for 37 days by daily replacing 5 L of algae culture with 5 L of fresh buffered BG11 medium. The algal concentration was 0.7 - 0.8 g L^{-1}. As can be seen in Figure S6-1, N_2O specific production was found to range from 4.4 – 32.3 nmol g DW^{-1} h^{-1}.

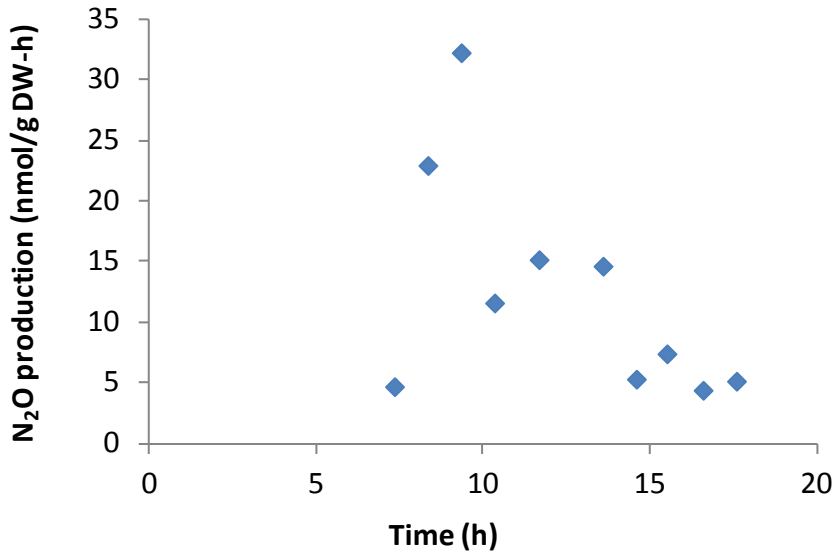


Figure S6-1: N_2O production during C. vulgaris cultivation in a 50 L closed photobioreactor operated outdoors (Day 44 of cultivation, May 12/2011; Palmerston North, New Zealand).

To estimate the potential impact of N_2O from 'realistic' algae cultivation systems we used data from Guieysse et al. (2013) who simulated algae cultivation in open ponds under various climate, design and operational regimes. As a representative scenario, we selected the case when *C. vulgaris* is cultivated in a 0.25 m deep open pond located near Merced, California (representative of a Mediterranean climate, yearly solar irradiance of 6.59 GJ m^{-2} yr^{-1}) and operated at an average hydraulic retention of time of 7 days. Assuming *C. vulgaris* grows at an average net efficiency of 2.5% and has an average heat value of 24.7 kJ g DW^{-1}

(corresponding to a lipid content of 25%) yields a yearly productivity of 0.165 GJ m^{-2} yr^{-1} (18.3 g m^{-2} d^{-1}) and an average algae concentration of 512 g m^{-3} (see Guieysse et al. 2013 for references, validation and further discussion). Based on the specific production rates reported above, N_2O emission ranged from 1.38 - 10.1 kg N_2O-N ha^{-1} yr^{-1}. If we then assume that 80% of the energy found in the biomass-lipids is recovered as biofuel (e.g. 20% of the total biochemical energy photosynthesized), the GHG equivalent of N_2O accounts for 1.96 – 14.4 g CO_2-equivalent MJ^{-1} fuel (N_2O-CO_2 equivalent of 298 g/g).

Reference:

Guieysse, B.; Béchet. Q.; Shilton, A. Variability and uncertainty in water demand and water footprint assessments of fresh algae cultivation based on case studies from five climatic regions. *Bioresour. Technol*. 128, 317–323, 2013.