Supplementary Information

**Title:** Nitrous Oxide (N\textsubscript{2}O) production in axenic *Chlorella vulgaris* microalgae cultures: evidence, putative pathways, and potential environmental impacts.

**Authors:** Benoit Guieysse, Maxence Plouviez, Marie Coihac, Laurent Cazali

S1 – N\textsubscript{2}O emissions during *C. vulgaris* cultivation in a 50 L photobioreactor

S2 - PCR analysis

S3 - Effect of nitrite addition on N\textsubscript{2}O emissions during *C. vulgaris* cultivation in a 50 L photobioreactor

S4 - Effect of NR repression, L-arginine and LNNA on N\textsubscript{2}O production by *C. vulgaris*

S5 - Impact of cell age on N\textsubscript{2}O production from *C. vulgaris* cultures

S6 - N\textsubscript{2}O emission during outdoor *C. vulgaris* cultivation.

8 Figures

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C. vulgaris was cultivated in buffered BG 11 medium containing (g L$^{-1}$ of distilled water) NaNO$_3$ (1.5), K$_2$HPO$_4$ (3.1), KH$_2$PO$_4$ (1.52), MgSO$_4$$\cdot$7H$_2$O (0.075), CaCl$_2$$\cdot$2H$_2$O (0.036), citric acid (0.006), ammonium ferric citrate (0.006), EDTA (0.001), and 1 mL of a trace element solution containing (g L$^{-1}$ of distilled water) H$_3$BO$_3$ (2.86), MnCl$_2$$\cdot$4H$_2$O (1.81), ZnSO$_4$$\cdot$7H$_2$O (0.222), CuSO$_4$$\cdot$5H$_2$O (0.079), Na$_2$MoO$_4$$\cdot$2H$_2$O (0.39), and Co(NO$_3$)$_2$$\cdot$6H$_2$O (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$_2$O 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$_2$O specific production rate averaged 24 ± 10 nmol N$_2$O g-DW$^{-1}$ h$^{-1}$ during the monitoring period (p = 0.05, n = 10).
Figure S1-1: N₂O 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. N₂O specific production averaged 58 ± 23 nmol N₂O g-DW⁻¹ h⁻¹ during the monitoring period (p = 0.05, n = 8).
**Figure S1-2**: N$_2$O production (diamonds), and *C. vulgaris* concentration (triangles) in a 50 L column photobioreactor submitted to continuous illumination.

References:

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\(_2\)O-producing denitrifying bacteria were isolated from local soil and their potential for N\(_2\)O 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\(_2\)O 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°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 Diagnostics), 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>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Sequence</th>
<th>Exp. size (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>norB</td>
<td>norB1F</td>
<td>CGNGARTTYCTSGARCC</td>
<td>670</td>
<td>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)</td>
</tr>
<tr>
<td>norB</td>
<td>norB8R</td>
<td>CRTLGCVCRCRWAGAAYGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cnorB</td>
<td>cnorBF</td>
<td>GACAAGNNNTACTGAGTTGT</td>
<td>389</td>
<td></td>
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<tr>
<td>cnorB</td>
<td>cnorBR</td>
<td>GAANCCANACNCCNGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>qnorB</td>
<td>qnorBF</td>
<td>GGNCAVCARGGNTAYGA</td>
<td>262</td>
<td></td>
</tr>
<tr>
<td>qnorB</td>
<td>qnorBR</td>
<td>ACCCANAGRTGNACNACGACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rbcl</td>
<td>rbclAF</td>
<td>ATGTCACCACAAACAGAGACTAAAGC</td>
<td>1200</td>
<td>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)</td>
</tr>
<tr>
<td>16S</td>
<td>F27</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
<td>1000 – 1500</td>
<td>94°C – 3 min [94°C - 30 s, 50°C - 30 s, 72°C – 90 s] × 35 cycles, 72°C – 5 min, 10 °C hold (Giovannoni, 1991)</td>
</tr>
<tr>
<td>1492R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Archaea</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amoA</td>
<td>arch-AmoAF</td>
<td>5′-STAATGGTCTGGCTTAGACG-3′</td>
<td>635</td>
<td>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)</td>
</tr>
<tr>
<td>amoA</td>
<td>arch-AmoAR</td>
<td>5′-GCGGCCATCCATCGTGATGA-3′</td>
<td></td>
<td></td>
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<tr>
<td>16S</td>
<td>340F</td>
<td>CCCTAYGGGGYGACGACC</td>
<td>660</td>
<td>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)</td>
</tr>
<tr>
<td>1000R</td>
<td></td>
<td></td>
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</table>
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).

<table>
<thead>
<tr>
<th></th>
<th>norB</th>
<th>cnorB</th>
<th>qnorB</th>
<th>amoA</th>
<th>Rbcl</th>
</tr>
</thead>
<tbody>
<tr>
<td>pure Chlorella</td>
<td>N</td>
<td>N*</td>
<td>N</td>
<td>N</td>
<td>Y* (1.2KB)</td>
</tr>
<tr>
<td>Chlorella plus antibiotics</td>
<td>N</td>
<td>N*</td>
<td>N</td>
<td>N</td>
<td>Y* (1.2KB)</td>
</tr>
<tr>
<td>Chlorella plus bacteria</td>
<td>Y</td>
<td>Y*</td>
<td>Y</td>
<td>N</td>
<td>Y* (1.7KB)</td>
</tr>
<tr>
<td>Bacteria</td>
<td>Y</td>
<td>Y*</td>
<td>Y</td>
<td>N</td>
<td>Y* (1.7KB)</td>
</tr>
<tr>
<td>Negative control</td>
<td>N</td>
<td>N*</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

* 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 (~1.7 kb) 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 N₂O 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**


S3 - Effect of nitrite addition on N₂O emissions during *C. vulgaris* cultivation in a 50 L photobioreactor

N₂O 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, N₂O 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 N₂O 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**: N₂O 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 NH₄⁺-laden BG11 medium and inoculated. Nitrite was added at t = 3 d.
S4 - Effect of NR repression, L-arginine and LNNA on N₂O 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 (MoO₄²⁻) was replaced by orthotungstate (WO₄²⁻) at the same relative concentration (0.253 mg Na₂WO₄ L⁻¹) as described by Deng et al. (1989). Following cultivation during 4 days, each algae culture was withdrawn, washed, and tested for N₂O emissions in its respective medium as described above (darkness). Figure S4-1 shows the specific rate of N₂O 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 N₂O 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 N₂O 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 N₂O emissions.

**Figure S4-2:** Specific rate of N₂O 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$_2$O 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$_2$O production.

![Figure S5-1: Influence of cell age on N$_2$O 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).](image)

As can be seen in Figure S5-1, the initial rate of N$_2$O specific emissions was inversely correlated with cell age.
S6 - N₂O emission during outdoor *C. vulgaris* cultivation.

The 50 L photobioreactor described in S1 was operated outdoor and supplied with CO₂-enriched air (3% of CO₂) 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, N₂O specific production was found to range from 4.4 – 32.3 nmol g DW⁻¹ h⁻¹.

![N₂O production during *C. vulgaris* cultivation in a 50 L closed photobioreactor operated outdoors (Day 44 of cultivation, May 12/2011; Palmerston North, New Zealand).](image)

To estimate the potential impact of N₂O 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\(_2\)O emission ranged from 1.38 - 10.1 kg N\(_2\)O-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\(_2\)O accounts for 1.96 – 14.4 g CO\(_2\)-equivalent MJ\(^{-1}\) fuel (N\(_2\)O-CO\(_2\) equivalent of 298 g/g).

Reference: