Biogeosciences, 10, 6737–6746, 2013
www.biogeosciences.net/10/6737/2013/
doi:10.5194/bg-10-6737-2013

Biogeosciences Open Access

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

B. Guieysse, M. Plouviez, M. Coilhac, and L. Cazali

School of Engineering and Advanced Technology, Massey University, Private Bag 11 222, Palmerston North 4442, New Zealand

Correspondence to: B. Guieysse (b.j.guieysse@massey.ac.nz)

Received: 21 May 2013 – Published in Biogeosciences Discuss.: 17 June 2013
Revised: 11 September 2013 – Accepted: 13 September 2013 – Published: 25 October 2013

Abstract. Using antibiotic assays and genomic analysis, this study demonstrates nitrous oxide (N_2O) is generated from axenic *Chlorella vulgaris* cultures. In batch assays, this production is magnified under conditions favouring intracellular nitrite accumulation, but repressed when nitrate reductase (NR) activity is inhibited. These observations suggest N_2O formation in *C. vulgaris* might proceed via NR-mediated nitrite reduction into nitric oxide (NO) acting as N_2O precursor via a pathway similar to N_2O formation in bacterial denitrifiers, although NO reduction to N_2O under oxia remains unproven in plant cells. Alternatively, NR may reduce nitrite to nitroxyl (HNO), the latter being known to dimerize to N_2O under oxia. Regardless of the precursor considered, an NR-mediated nitrite reduction pathway provides a unifying explanation for correlations reported between N_2O emissions from algae-based ecosystems and NR activity, nitrate concentration, nitrite concentration, and photosynthesis repression. Moreover, these results indicate microalgae-mediated N_2O formation might significantly contribute to N_2O emissions in algae-based ecosystems (e.g. 1.38–10.1 kg N_2O-N $ha^{-1}\,yr^{-1}$ in a 0.25 m deep raceway pond operated under Mediterranean climatic conditions). These findings have profound implications for the life cycle analysis of algae biotechnologies and our understanding of the global biogeochemical nitrogen cycle.

1 Introduction

Nitrous oxide (N_2O) is a major ozone-depleting atmospheric pollutant and greenhouse gas (Ravishankara et al., 2009; EPA, 2010). The production of this compound from microalgal and cyanobacterial cultures (henceforth referred to as "algae" for simplicity) was demonstrated more than 25 yr ago (Weathers, 1984; Weathers and Niedzielski, 1986) and has been suspected to cause measurable N_2O emissions in various aquatic environments (Twining et al., 2007; Mengis et al., 1997; Wang et al., 2006; Oudot et al., 1990; Florez-Leiva et al., 2010). This mechanism is nevertheless often challenged as a significant source of N_2O in algae-based ecosystems and many authors have attributed these emissions to associated bacteria (Law et al., 1993; Morell et al., 2001; Ni and Zhu, 2001; Harter et al., 2013; Ferrón et al., 2012). Yet, to our knowledge, only Fagerstone et al. (2011) have hitherto evidenced a bacterial origin to N_2O emissions in algal cultures by showing N_2O production stopped when *Nannochloropsis salina* was supplied with bacterial antibiotics and by detecting bacterial genes encoding for nitric oxide reductase (NOR) in the culture (this enzyme is associated with N_2O production during bacterial denitrification). This finding does not rule out the possible occurrence, or co-occurrence, of algal N_2O production in non-axenic algae cultures for several reasons: First, eukaryotic microalgae are commonly found in symbiotic relationship with bacteria (Croft el al., 2005) meaning microalgae might cease to synthesize N_2O when their prokaryotic symbiotes are impacted by, for example, antibiotics. Second, algae have unequal abilities to synthesize N_2O (Weathers, 1984) so findings on

N. salina cultures cannot be extrapolated to other systems. Third, the detection of a gene does not warrant the putative enzyme it codes is synthesized, active, and quantitatively significant.

It therefore appears that the possibility of algal-mediated N_2O production and its significance remain opened to discussion. This potential N_2O source must be characterised because it could challenge the general consensus that bacteria and archaea are the main biotic source of atmospheric N_2O (EPA, 2010; Schreiber et al., 2009; Codispoti, 2010; Williams and Crutzen, 2010; Löscher et al., 2012; Santoro et al., 2011) and has profound implications to algae biotechnologies, whose attractiveness largely relies upon a belief that microalgae-based products and services have intrinsically low carbon footprints (Pienkos and Darzins, 2009; Wijffels and Barbosa, 2010). With this perspective, the objectives of this study were to (1) demonstrate N_2O production in axenic *Chlorella vulgaris* cultures; (2) propose a putative pathway to N_2O formation for this species; and (3) determine critical areas for further investigation. *C. vulgaris* was selected for being well studied and representative of algae species with high commercial potential (Spolaore et al., 2006). *Chlorella* spp. are unicellular green microalgae and belong to the phylum *Chlorophyta*. They are ubiquitous in the environment and have been extensively studied due to their fast growth rate (Kay, 1991; Richmond, 2004).

2 Materials and methods

2.1 Algae cultivation

Unless otherwise stated, *C. vulgaris* was cultivated in a BG 11 medium (Andersen, 2005) with modifications detailed in Supplement S1. These cultures were incubated at 25 °C in a 0.5 % CO_2 enriched atmosphere under continuous orbital agitation (180 rpm) and illumination at 21 W of Photosynthetically Active Radiation (PAR) m^{-2} at the culture surface (using five 18 W Polylux coolwhite tubes located 30 cm over the E-flasks) for 2 weeks before being stored at 4 °C in darkness. Axenic *C. vulgaris* colonies were maintained onto sterilized solid medium containing (in $g\,L^{-1}$ of buffered BG 11) bacto agar (10), dextrose (5) and yeast extract (0.5). Prior to testing N_2O production in batch assays, 8 mL of axenic algae were aseptically inoculated into 250 mL E-flasks containing 125 mL of buffered BG11 and phototrophically-grown under continuous illumination and agitation as described above.

2.2 Influence of N-source, nitrite concentration and kinetics

Immediately before testing, 200 mL of 5–8 days old phototrophically-grown axenic algae were withdrawn from several flasks, mixed, and transferred into 50 mL centrifugation tubes. Following centrifugation at 2900 g for 3.5 min, supernatants were discarded and the biomass pellets were mixed and re-suspended with N-free buffered BG11 to the desired final concentration (0.2–0.7 g DW L^{-1}). Then, 50 mL of *C. vulgaris* suspension and 2 mL of freshly prepared 0.3 M NO_2^-, NO_3^-, or NH_4^+ stock solutions were transferred into 120 mL glass flasks (final concentration of 12 mM). The flasks were immediately sealed with rubber septa and aluminum caps ("time 0" of experiment) and incubated at 25 ± 2 °C under continuous agitation (180 rpm) in darkness or under continuous illumination (82 W PAR m^{-2}). A similar protocol was used to quantify the impact of nitrite concentration (3–24 mM) in darkness and N_2O production kinetics in the presence of 12 mM of nitrite in darkness. Unless otherwise stated, experiments were done in duplicates and gas samples of 5 mL were periodically withdrawn from the flasks using a disposable syringe to quantify N_2O production. When significant N_2O emissions were recorded in the presence of algae, abiotic N_2O emissions were quantified under similar conditions in algae-free medium and always found to be negligible. Batch assays in darkness in the presence of 12 mM nitrite were repeatedly conducted to serve as positive controls.

2.3 Nitrate Reductase (NR) inhibition

After 10 days of cultivation, aliquots of phototrophically-grown algae were centrifuged and re-suspended in either "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 previously described (Deng et al., 1989). Following cultivation during 4 days, each algae culture was withdrawn, washed, and tested for N_2O production in the presence of nitrite in its respective medium as described above (darkness).

2.4 Antibiotic assays

Antibiotic-assays were conducted to verify N_2O emissions from *Chlorella* cultures were not caused by associated bacteria. Penicillin G potassium (purity > 99 %) and streptomycin sulfate (purity > 99 %) were purchased from Serva and Merck, respectively, and dissolved in buffered N-free BG 11 to a final concentration of 10 $g\,L^{-1}$ and 2.5 $g\,L^{-1}$, respectively. Using these stock solutions and based on the protocol used by Fagerstone et al. (2011), 100 mg penicillin L^{-1} and 25 mg streptomycin L^{-1} were added to phototrophic *C. vulgaris* cultures 4 days after inoculation with a single colony under aseptic conditions, followed by an additional supply of 25 mg penicillin L^{-1} and 5 mg streptomycin L^{-1} twice a day from days 5 to 8. From day 4, 25 mL aliquots were daily withdrawn under sterile conditions and the algae cell concentration (DW) was quantified. The samples were then centrifuged, resuspended in 25 mL of N-free medium and tested for N_2O production in batch assay under darkness in the presence of 12 mM nitrite as described above. N_2O concentration in the gas headspace was quantified after 4 and 24 h.

2.5 Polymerase Chain Reaction (PCR) analysis

PCR analysis was conducted to detect bacterial and archaeal genes in the algae cultures. Aliquots of 7 days old algae cultures phototrophically-grown in "normal" and "antibiotic-laden" modified BG11 medium (100 mg penicillin L^{-1} and 25 mg streptomycin L^{-1} were added 3 days after inoculation) were transferred in DNA-free Eppendorf tubes (N_2O production in darkness in the presence of nitrite was confirmed in both cultures prior to sampling). 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. The DNA from aliquots of algal, bacterial, and mixed bacterial/algal cultures (50/50 vol : vol) was then extracted and amplified using: (i) norB, cnorB and qnorB primer pairs to amplify fragments of genes encoding for NOR in bacteria (Fargerstone et al., 2011); (ii) a "universal" 16S primer pair for bacteria (Giovannoni, 1991); (iii) a amoA primer pair to amplify a fragment of gene encoding for ammonia monooxygenase in archaea (Francis et al., 2005); (iv) a "universal" 16S primer pair for archaea (Gantner et al., 2011); and (v) a rbcl primer pair to amplify a fragment of the large subunit of the ribulose-1,5-bisphosphate carboxylase oxygenase gene which is present in the chloroplast of algae, plants and cyanobacteria and in the genomes of some bacteria (Hasebe et al., 1994). The PCR conditions associated with each primer pair are detailed in S2. The rbcl amplification was performed as a positive control to show that there was enough DNA in the samples to be amplified.

2.6 Effect of nitrite addition on N_2O emissions during *C. vulgaris* cultivation in a closed photobioreactor

C. vulgaris was cultivated in buffered BG11 medium in a 4 L (working volume) cylindrical closed photobioreactor (0.195 m diameter, 0.134 m working height) continuously illuminated with 14 light bulbs (OSRAM DULUXSTAR 23W/865 LUMINUX Daylight) supplying 89 W m^{-2} (as PAR) at the reactor surface (average from 12 measurements taken at 3 height and 4 angular, conversion factor of 2.7 W PAR m^{-2} per klux) and supplied with moisturized air (0.4 L min^{-1}) enriched in CO_2 (40 mL min^{-1}). The reactor was operated in a batch mode for 8 days under continuous illumination until the lights were turned off and the reactor was covered with aluminum foil. N_2O concentration was then periodically monitored in the gas influent and effluent.

2.7 N_2O emissions during pilot-scale outdoor cultivation

C. vulgaris was 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). The reactor was first operated indoors under continuous artificial 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). 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}. The same reactor was operated outdoor and supplied with CO_2-enriched air (3 % of CO_2) at 1 L min^{-1}. In this case, the reactor was operated in a batch mode for 6 days following inoculation before being operated in a fed-batch mode for 37 days by daily replacing 5 L of algae culture with 5 L of fresh buffered BG11 medium. Algal concentration was thus maintained to 0.7–0.8 g L^{-1}.

2.8 Analysis

N_2O concentration in gas samples was quantified using gas chromatography (Shimadzu GC-2010, Shimadzu, Japan) using a 1000 µL sample loop (380 °C), an Alltech Porapak QS 80/100 column (70 °C, Sigma-Aldrich, USA) and an electron conductivity detector (315 °C). N_2 was used as carrier gas (30 mL min^{-1}) and a CH_4/Ar mixture (10.3 : 89.7 vol : vol) was used as make-up gas (10 mL min^{-1}). The results herein presented show the total amount of N_2O produced in the flasks assuming the dissolved N_2O concentration in the aqueous phase was at equilibrium with the gas phase (Henry constant of 0.025 mol kg^{-1} bar^{-1}, National Institute of Standards and Technology, USA). N_2O losses and pressure changes caused by sampling were accounted for.

To quantify algae density as dried weight (DW) concentration (g L^{-1}), a known volume of algae culture was filtered on a pre-weighted glass-microfiber disc (Sartorius-Stedim Biotech, Germany). The filter was then rinsed with distilled water and dried at 105 °C for one hour before being weighted again. The DW concentration was calculated by weight difference. Nitrite concentration was quantified using a Dionex ICS-2000 ionic chromatography system (Dionex Corporation, Sunnyvale, USA) equipped with an AS-50 autosampler (25 µL sample loop), a DS6 conductivity detector, and a ASRS-300 suppressor from the same manufacturer. Samples were eluted at 30 °C through a Dionex IonPac AG11-HC guard column and a Dionex IonPac AS11-HC (4 × 250 mm) column using 32 mM KOH at 1 mL min^{-1}.

3 Results and discussion

3.1 Antibiotic assays and genomic analysis demonstrate N_2O production from axenic *C. vulgaris* cultures

We first detected N_2O production while cultivating *C. vulgaris* indoors in a column photobioreactor (24 ± 10 nmol N_2O g DW^{-1} h^{-1}, $p = 0.05$, $n = 10$, Supplement S1). Batch assays using axenic *C. vulgaris* cultures were conducted to confirm this initial finding and indeed showed N_2O

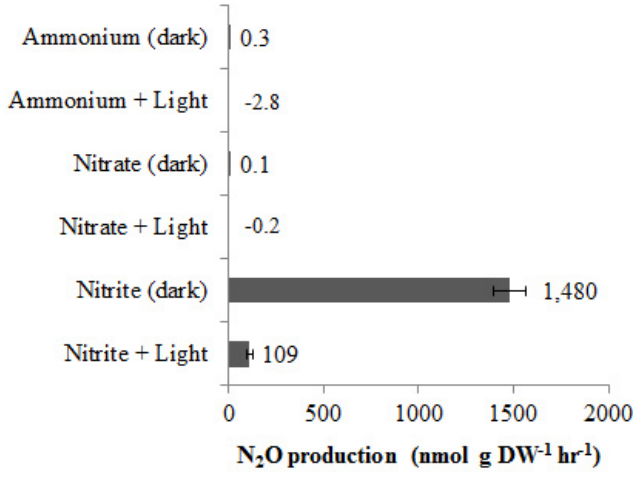

Fig. 1. Influence of nitrogen source and illumination on specific N_2O production by *C. vulgaris* (0.22 g $DW^{-1}\,L^{-1}$). The rates shown represent averages from duplicates ± error and were determined between 2.9 and 21.5 h after nitrogen addition (12 mM).

production was biologically-mediated (abiotic N_2O emissions were negligible under the experimental conditions tested) and magnified when the algae were incubated in darkness and supplied with nitrite as sole exogenous N-source (Fig. 1; *C. vulgaris* does not assimilate N_2). The variation in the rates recorded from duplicates was generally small, being around 15 % of the average (Figs. 1 and 2a). The rates of N_2O emissions depended on the pre-cultivation conditions (see discussion below) and when positive controls were rigorously repeated (12 mM nitrite, darkness, 25 °C, 5.5 days old algae cells), the error was estimated to 11 % ($n = 9$, $p = 0.05$, tests conducted by 2 different operators over 1 yr) showing the use of duplicate was satisfactory (as shown in Figs. 3 and 4). The impact of nitrite and light on N_2O emissions can therefore be considered as significant.

Axenic algae cultures are notoriously difficult to obtain and maintain so antibiotic assays and genomic analysis were used to confirm *C. vulgaris* were indeed responsible for N_2O production under the experimental conditions tested. As shown in Fig. 2a, *C. vulgaris* pre-incubation in the presence of antibiotics was followed by an increase in specific N_2O production rate. This increase in specific activity may be linked to the inhibitory effect of the antibiotics on algae growth evidenced in Fig. 2b and reported by Qian et al. (2012) for streptomycin (this inhibitory effect was only statistically significant after 5 days of exposure, on-tailed *t* test at $p = 0.05$, the average biomass concentration being then 27 % lower in the antibiotic-laden culture than in the control; see further discussion below). Genomic analysis using PCR did not reveal the presence of bacterial or archaeal genes in the algae cultures, regardless of whether *C. vulgaris* was cultivated in the presence of antibiotics (Supplement S2). The absence of ammonium oxidizing prokaryotes was expected as the addition of ammonium did not trigger N_2O emissions (Fig. 1). Moreover, there was not enough ammonium in the flasks (< 1 µmole, Paz-Yepes et al., 2007) to sustain the levels of N_2O production recorded via nitrification (up to 9.66 µmoles after 30 h), especially considering the very low N_2O / NH_4^+ yields associated with N_2O generation during ammonium oxidation (Santoro et al., 2011; Vajrala et al., 2013). An additional control conducted under anaerobic conditions showed no significant impact of oxygen concentration on N_2O emission in the presence of nitrite under darkness (data not shown). These results demonstrate *C. vulgaris* indeed generated N_2O in the presence of nitrite under darkness, which validates the earlier findings from Weathers (1984) who did not verify the possible involvement of associated microorganisms. The discrepancy between these findings and those of Fagerstone et al. (2011) could be explained by differences in the abilities of the algae tested and their associated microorganisms (as discussed below).

3.2 N_2O emissions are magnified when photosynthesis is repressed and nitrite is added

As stated above, N_2O production by *C. vulgaris* was considerably magnified when the algae were incubated in the dark and supplied with nitrite as sole exogenous N-source (Fig. 1). The impact of photosynthesis repression on N_2O production was confirmed by cultivating *C. vulgaris* under artificial illumination (82 W m^{-2}) in the presence 10 µM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) as specific photosystem II inhibitor (yielding 672 ± 125 nmol N_2O g $DW^{-1}\,h^{-1}$ during 2–4 h after exposition). These observations corroborate the results of Weathers (1984) and Weathers and Niedzielski (1986) who linked N_2O production by microalgae and cyanobacteria to nitrite reduction in the dark and reported similar *C. vulgaris* emission rates of 958–1833 nmol N_2O g $DW^{-1}\,h^{-1}$ over 24 h under darkness following nitrite addition at 43 mM. The positive effect of nitrite on N_2O generation was confirmed when nitrite was added to algae actively grown in closed photobioreactors (Fig. 3 and S3).

C. vulgaris pre-cultivation in the presence of tungstate as specific NR-inhibitor (Deng et al., 1989; Li and Bishop, 2004; Vega et al., 1971) repressed N_2O production nearly 4-fold, suggesting this enzyme played a key role in N_2O production (Supplement S4). The influence of nitrite concentration on N_2O production shown on Fig. 4a can be described by a sigmoid plot, which often characterises multimeric enzymes such as NR (Ricard and Cornish-Bowden, 1987). The linear correlation between algal biomass concentration and N_2O production seen on Fig. 4b confirmed the biotic origin of N_2O in these cultures and provides a rationale for expression these emissions as specific rates. The kinetics of N_2O production in batch assays were characterised by an initial "build-up" phase of 4 h followed by a period of "linear" evolution lasting more than 24 h (Fig. 5). Cell age

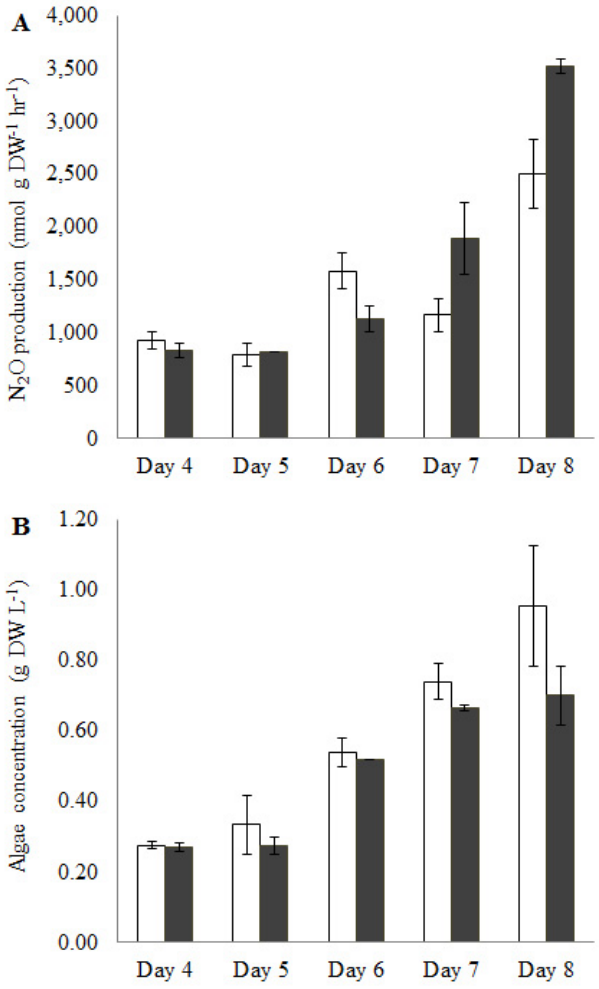

Fig. 2. Evolution of specific N_2O production **(A)** and biomass concentration **(B)** in antibiotic-laden (grey bars) and antibiotic-free (white bars) *C. vulgaris* cultures. The rates shown represent averages from duplicates and were determined between 4 and 24 h after nitrite addition (12 mM).

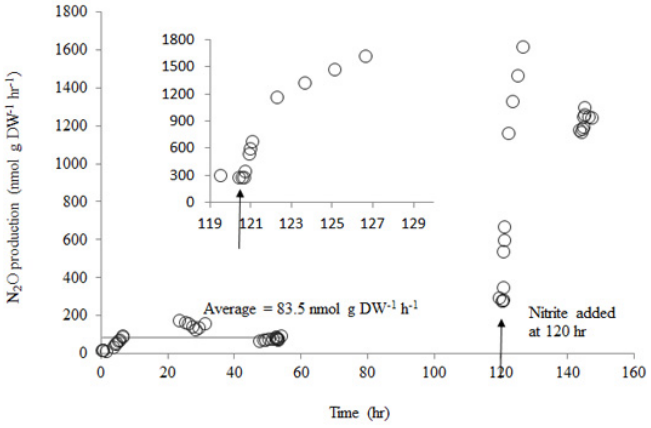

Fig. 3. Changes in N_2O specific production rate (nmol g DW^{-1} h^{-1}) in a 4 L column photobioreactor. Illumination was turned off at $t = 0$ and $NaNO_2$ (1.38 g) was added after 120 h of monitoring. The concentration of algae was 1.26–1.56 g L^{-1}.

(3–7 d) significantly impacted the initial rate (0–4 h) of N_2O production but, besides for 3 day-old cells, had no apparent impact on N_2O specific production rates during the linear phase (Supplement S5). This observation, together with the rapid response in N_2O production to the nitrite addition during cultivation in a closed photobioreactor (Fig. 3 and S3), suggests the build-up phase was associated with changes in enzyme activities rather than mass transfer limitations (i.e. the rates measured during the linear phase represents the actual metabolic rates).

3.3 A possible NO-pathway to N_2O formation in axenic *C. vulgaris*

In plants, nitrate assimilation is carried out by NR, which catalyzes the reduction of nitrate (NO_3^-) into nitrite (NO_2^-) using NAD(P)H as electron donor (Eq. 1), and Nitrite Reductase (NiR), which catalyzes the reduction of nitrite into ammonium (NH_4^+) using reduced ferredoxin (Fd_{red}) as electron donor (Eq. 2).

$$NO_3^- + NAD(P)H + H^+ \rightarrow NO_2^- + NAD(P)^+ + H_2O \quad (1)$$

$$NO_2^- + 6Fd_{red} + 8H^+ \rightarrow NH_4^+ + 2H_2O + 6Fe_{ox} \quad (2)$$

A key difference between these two enzymes in plant cells is that NR has an ancient origin whereas NiR was imported during endosymbiosis (Stolz and Basu, 2002). Consequently, NR is found in the cytoplasm and can reduce nitrate into nitrite even when algal cells are exposed to darkness (when cytoplasmic NAD(P)H is available) but NiR, which is found in the chloroplast, cannot carry out nitrite reduction if photosynthesis does not regenerate Fd_{red}. As demonstrated in *Chlamydomonas reinhardtii* (Sakihama et al., 2002) NiR repression causes nitrite accumulation in the cytoplasm and its reduction into nitric oxide (NO) by NR (Eq. 3).

$$NO_2^- + 0.5NAD(P)H + 1.5H^+ \rightarrow NO + 0.5NAD(P)^+ + H_2O \quad (3)$$

As described above, the involvement of NR in N_2O production in *C. vulgaris* cultures was confirmed by pre-incubating the algae in the presence of tungstate. NO generation via NR-mediated nitrite reduction is well documented in higher plants (Gupta et al., 2011) and NO may therefore act as a precursor for N_2O formation in algae cells via a pathway similar to N_2O formation in bacterial denitrifiers (Hino et al., 2010). This mechanism would explain the impact of nitrite and light supply on N_2O production. It is however unclear if and how NO can be reduced into N_2O under oxic conditions in plant cells. Yet, evidence of

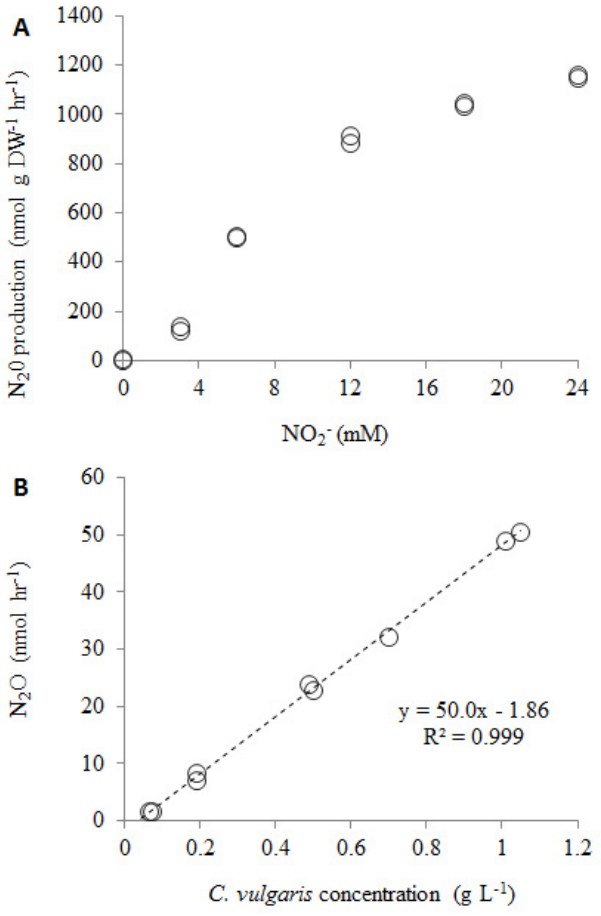

Fig. 4. Influence of nitrite concentration **(A)** and *C. vulgaris* concentration **(B)** on specific N_2O production from *C. vulgaris* cultures incubated in darkness ($n = 2$). When nitrite concentration was varied, *C. vulgaris* concentration was 0.58 g L^{-1}, when algae concentration was varied, nitrite concentration was 12 mM. The rates shown represent data from duplicates (e.g. not the average) and were determined between 1.5 and 3.5 h after nitrite addition (12 mM).

NOR-mediated NO reduction into N_2O under aerobic conditions has been reported in denitrifying bacteria and fungi (Bell and Ferguson, 1991; Morley et al., 2008; Schreiber et al., 2009; Wrage et al., 2001). Moreover, it has been proposed that the denitrifying system of certain fungi is a remnant of the protomitochondrion (Takaya and Shoun, 2000) and a gene encoding a NOR with putative NO-detoxification function has been characterised in the cyanobacterium *Synechocystis* (Büsch et al., 2002). To support this hypothesis, the protein sequence of *Chlamydomonas reinhardtii* NOR (NCBI accession: XP_001700272.1) was searched against the *Chlorella variabilis* NC64A genome assembly (Blanc et al., 2010, Plant Cell) using tblastn. A significant hit was found on assembly scaffold CHLNCscaffold_7 (Expect $= 1 \times 10^{-18}$) between positions 397077 to 397262 bp. Furthermore, blastp analysis enabled to identify a *Chlorella variabilis* hypothetical protein CHLNCDRAFT_51513 (accession: EFN56743.1) that is highly identical to the *Chlamydomonas* NOR (Expect $= 1 \times 10^{-152}$; 57 % identity). Interestingly, the NOR of *Chlamydomonas reinhardtii* has a high similarity with the NOR of *Fusarium oxysporum* (NCBI) and N_2O emission in the presence of nitrite was confirmed in *Chlamydomonas reinhardtii* batch culture (data not shown).

It should be noted that tungstate can have non-specific effects in plant cells (Xiong et al., 2012) and that there exist other pathways to NO formation in plant cells than NR-mediated nitrite reduction (Gupta et al., 2011). Hence, under a scenario where NR is necessary to generate nitrite but not to reduce it into a putative N_2O precursor (Tischner et al., 2004), an apparent repression of N_2O production by tungstate could be caused by the absence of cytoplasmic nitrite production rather than the repression of nitrite reduction into N_2O precursors. This was however unlikely in this study since nitrite was added to the culture broth when NR was repressed by tungstate. Certain plant cells also contain NO synthases (NOS) capable of releasing NO during L-arginine oxidation but neither the addition of L-arginine nor the use of the specific NOS inhibitor Nω-nitro-L-arginine impacted N_2O production under the experimental conditions studied (Supplement S4).

3.4 An alternative nytroxyl (HNO) pathway to N_2O formation in *C. vulgaris*

An "HNO-pathway" has long been hypothesized to explain N_2O generation in microalgae (Cohen and Gordon, 1978). This pathway involves the formation of nytroxyl (HNO being the dominant form of HNO / NO^- in cells at physiological pH, Miranda et al., 2003) during nitrite reduction by NR in a reaction that mimics the 2-electron reduction of nitrate into nitrite by this enzyme (compare Eqs. 2, 3, and 4). This mechanism also provides a simple explanation to N_2O production under oxia as Miranda et al. (2003) hypothesized that a quantitatively significant fraction of HNO could dimerize to N_2O in hydrophobic cell areas (Eq. 5).

$$NO_2^- + NAD(P)H + H^+ \rightarrow NO^- + NAD(P)^+ + H_2O \quad (4)$$

$$2HNO \rightarrow N_2O + H_2O \quad (5)$$

The significance of HNO biochemistry has only recently been recognised and remains poorly understood (Fukuto et al., 2005), which could explain why this compound is not often considered as possible N_2O precursor. Yet, Ishimura et al. (2005) showed NO^- was released during L-arginine conversion by NOS and that a portion of this NO^- was dimerized into N_2O under oxia in vitro. Similarly, Schmidt et al. (1996) demonstrated that NO generation during L-arginine oxidation by NOS actually involved the generation of NO^- and its subsequent reduction into NO by superoxide dismutase (SOD). These authors therefore hypothesized

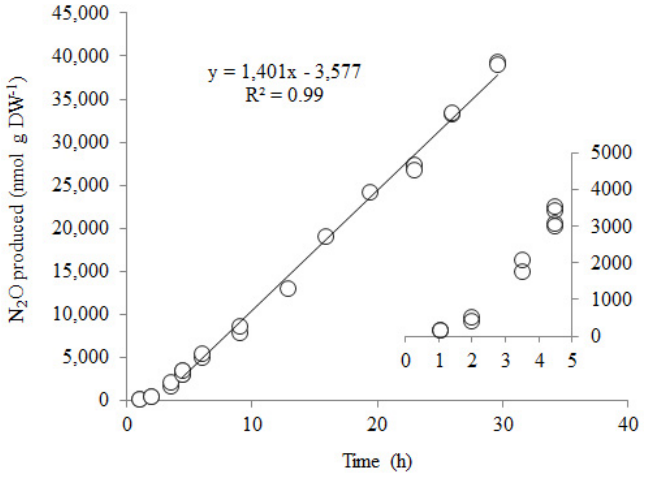

Fig. 5. Time change of N_2O production (nmol g DW^{-1}) in *C. vulgaris* supplied with 12 mM nitrite as sole exogenous nitrogen source and incubated in the dark. The plain line represents the linear regressing over 4.5–29.5 h. Insert shows initial build-up phase. The data shown is a combined plot of duplicates (e.g. not the average) from 3 independent experiments.

N_2O could arise directly via NO^- dimerization or indirectly from NO reduction by NOS acting as NOR. A rapid turnover of HNO into NO and N_2O would explain a correlation between N_2O emission and the "apparent" NO generation from NR-nitrite reduction in plants. Moreover, Sharpe and Cooper (1998) described a Bovine cytochrome *c* oxidase capable of reducing NO into NO^- in vitro under oxia and N_2O can be released via the reaction of HNO with NO (Beckman and Koppenol, 1996). HNO can also induce the formation of NH_2OH (Arnelle and Stamler, 1995; Schmidt et al., 1996), which itself is used as electron acceptor in the reduction of NO into N_2O during nitrifier denitrification (Schreiber et al., 2009). Hence, the "HNO" and "NO" pathways herein described might be parts of a more complex mechanism leading to N_2O production. NO and HNO are highly reactive and take part in numerous cellular functions and the putative enzymes involved (NR, NOR) are capable of catalyzing a broad range of biological reactions (Miranda, 2005; Planchet and Kaiser, 2006). Further research is therefore needed to confirm these species as N_2O precursors.

3.5 Significance and future work

Bacteria can generate N_2O during denitrification under hypoxia (i.e. low-oxygen environment) or anoxia, heterotrophic nitrification under normoxia or hypoxia, autotrophic nitrification under normoxia, and nitrifier denitrification under hypoxia (Wrage et al., 2001). In addition, there is now clear evidence that AOA cause significant N_2O emissions in certain environments, especially under hypoxia (Hatzenpicher, 2012; Santoro et al., 2011; Vajrala et al., 2013). From their respective studies on *N. salina* and *Dunaliella salina*, both Fagerstone et al. (2011) and Harter et al. (2013) concluded that N_2O emissions were more likely caused by bacterial denitrification in low-oxygen micro-environments (e.g. biofilm) than by algal-mediated synthesis or microbial nitrification. The presence of anaerobic zones is however unlikely in well-mixed photobioreactors because the entire volume of 1.0–1.4 mm diameter microbial flocs has been shown to remain oxic during biological wastewater treatment, even under conditions of incomplete oxygen saturation and high oxygen uptake (Li and Bishop, 2004). Furthermore, ammonium oxidizing bacteria (AOB) are not expected to thrive under the conditions normally found during algae cultivation conditions (Harter et al., 2013). Finally, AOA-mediated N_2O synthesis is currently only suspected to be significant in oligotrophic low-oxygen environment. By contrast, the evidence herein provided strongly suggests that nitrite intracellular accumulation and its reduction by NR trigger N_2O production in *C. vulgaris*.

The putative pathways shown in Fig. 6 can explain the positive correlations reported between N_2O production and NR activity (Goshima et al., 1999), nitrate concentration (Ferrón et al., 2012; Fagerstone et al., 2011), nitrite concentration (Weather, 1984), and photosynthesis repression (Weather, 1984; Fagerstone et al., 2011; Law et al., 1993) in algae cultures. This pathway could also explain why Harter et al. (2013) reported higher N_2O emissions when NO was supplied as N-source rather than nitrate. These authors proposed N_2O was generated during NO detoxification by bacterial denitrifiers, which is interesting since NR-mediated NO production might be involved in plant stress response (Gupta et al., 2010). The possibility of stress-induced NO-mediated N_2O emission is consistent with our observation that exposure to antibiotics repressed *C. vulgaris* phototrophic growth in agreement with the findings from Qian et al. (2012), but increased the specific rate of N_2O production by this microorganism (an additional experiment showed biomass concentration was 81 % lower in the antibiotic-laden culture than in the control after 7 days of exposure, and followed by a 4 fold increase in the specific rate of N_2O emissions). There is therefore strong experimental support to the theory herein presented. The experimental data disclosed and the proposed pathways also show N_2O production by *C. vulgaris* is controlled by two transient parameters during cultivation outdoors: nitrite concentration and light supply. N_2O production may therefore go undetected in monitoring studies that do not have sufficient temporal resolution (Wang et al., 2010). Our results also indicate the attribution of the origins of N_2O emissions will be challenging in mixed cultures given that the bacterial, archaeal and algal pathways potentially involve similar precursors and enzymes (Hatzenpicher, 2012; Wrage et al., 2001).

As can be seen from Fig. 1, N_2O production is not expected from nitrite-free *C. vulgaris* cultures, regardless the conditions of light supply. Yet, N_2O emissions were recorded during *C. vulgaris* cultivation under continuously

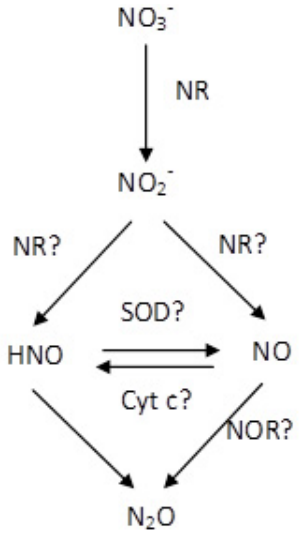

Fig. 6. Putative pathways of N_2O formation in *C. vulgaris* (NO_3^- = nitrate, NO_2^- = nitrite, NO = nitric oxide, HNO = nytroxyl, NR = nitrate reductase, SOD = superoxide dismutase, NOR = NO reductase, Cyt c = cytochrome c oxidase).

illumination in the absence of exogenous nitrite (Supplement S1) and when the same reactor was operated outdoors (12 ± 6 nmol N_2O g DW^{-1} h^{-1}, $p = 0.05$, $n = 10$; S6). In both cases, the dissolved oxygen concentration in the reactor was always close to saturation. These emissions were inputted to the intracellular accumulation of nitrite triggered by strong light attenuation at high cell densities when nitrate was used as N-source (dissolved nitrite was detected at 0.15–0.80 mM when nitrate was used as N-source in the 50 L photobioreactor, while N_2O was not released in same reactor operated indoor when ammonium was used as *N* source, S1 and S3). Based on the outdoor rates reported in the absence of exogenous nitrite, *C. vulgaris* cultivation in a 0.25 m deep raceway pond operated at 512 g algae DW m^{-3} in a Mediterranean climate (Guieysse et al., 2013) would release 1.38–10.1 kg N_2O-N ha^{-1} yr^{-1} (i.e. 1.3–31.5 ton CO_2 equivalent ha^{-1} yr^{-1} for a production of approx. 68 ton algae-DW ha^{-1} yr^{-1}, see Supplement S6 for detailed calculations). By comparison, emissions from terrestrial energy crops range from 1.9–9.2 kg N_2O-N ha^{-1} yr^{-1}, against less than 0.7 kg N_2O-N ha^{-1} yr^{-1} for natural vegetation (Smeets et al., 2009). The productivity of algae (e.g. 18.6 g dried weight m^{-2} d^{-1} in the example above) is typically higher than the productivity of terrestrial plants and if we assume that 80 % of the energy found in the algal lipids is recovered as biofuel in the example above, N_2O emission could generate a carbon footprint of 1.96–14.4 g CO_2–equivalent MJ $fuel^{-1}$, which is considerably higher than the negative value of -16.54 g CO_2–equivalent MJ $fuel^{-1}$ reported by Batan et al. (2010) for algae biodiesel production. Although this carbon footprint is lower than the WWF guideline of 30 g CO_2–equivalent MJ $fuel^{-1}$ cited by Smeets et al. (2009), N_2O emissions could be higher under operational conditions favouring intracellular nitrite accumulation and/or when different species are cultivated. We therefore recommend N_2O emissions should be monitored during algae cultivation and systematically accounted for in the life cycle analysis (LCA) of algal-based products and services. Future research is critically needed to (1) confirm the putative pathway, possibly using a model algae which genome has been fully sequenced in order to identify and track the expression of the genes potentially involved; (2) further study the possible impact of stress on N_2O emissions by microalgae; (3) establish the ozone depletion potential of N_2O production as this potentially significant impact (Lane and Lant, 2012; Revell et al., 2012) has not yet been of considered in the LCA of algae biotechnologies; and (4) determine if N_2O is generated by other algae species and if targeted operation can minimise N_2O emission during microalgae cultivation.

As of today, the microalgae biotechnology industry is most likely too small to have a significant impact on the global N_2O cycle (we do not speculate, however, on the potential significance of algal N_2O emissions from natural ecosystems). The significance of these emissions must be evaluated within the context of recent massive investments to develop new capacities for mass algae production. Hence, there is a unique opportunity to raise awareness and tackle this potential issue before it becomes globally significant. Based on the batch assays, the use of ammonium as sole N-source has the potential to mitigate N_2O emissions from axenic *C. vulgaris* culture. This was confirmed in non-axenic continuous *C. vulgaris* cultures at laboratory scale (manuscript under preparation). We have also observed that not all species of algae release N_2O under the experimental conditions described in the paper (unpublished data). Algae selection may therefore provide another means of mitigation.

Supplementary material related to this article is available online at http://www.biogeosciences.net/10/6737/2013/bg-10-6737-2013-supplement.pdf.

Acknowledgements. This research was supported by Massey University and the Marsden Fund Council from New Zealand Government Funding, administrated by the Royal Society of New Zealand. Trish McLenachan is gratefully acknowledged for performing the PCR analysis and David Chagne, Plant and Food Research New Zealand, is gratefully acknowledged for the genome analysis. The authors also wish to thank Andy Shilton, Chris Pratt, Quentin Béchet, Gerald Pinauld, Fanny Schlemmer and Quentin Frigeri for their assistance in various aspects of this work.

Edited by: K. Küsel

References

Andersen, R. A.: Algal culturing techniques, Elsevier Academic press, Singapore, 2005.

Arnelle, D. R. and Stamler, J. S.: NO^+, NO·, and NO^- donation by S-nitrosothiols: Implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation, Arch. Biochem. Biophys, 318, 279–285, 1995.

Batan, L., Quinn, J., Willson, B., and Bradley, T.: Net energy and greenhouse gas emission evaluation of biodiesel derived from microalgae, Environ. Sci. Technol, 44, 7975–7980, 2010.

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

Beckman, J. S. and Koppenol, W. H.: Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly, Am. J. Physiol, 271, C1424–1437, 1996.

Bell, L. C. and Ferguson, S. J.: Nitric and nitrous oxide reductases are active under aerobic conditions in cells of *Thiosphaera pantotropha*, Biochem. J., 273, 423–427, 1991.

Blanc, G., Duncan, G., Agarkova, I., Borodovsky, M., Gurnon, J., Kuo, A., Lindquist, E., Lucas, S., Pangilinan, J., Polle, J., Salamov, A., Terry, A., Yamada, T., Dunigan, D. D., Grigoriev, I. V., Claverie, J. M., and Van Etten, J. L.: The *Chlorella variabilis* NC64A genome reveals adaptation to photosymbiosis, coevolution with viruses, and cryptic sex, Plant cell, 22, 2943–2955, 2010.

Büsch, A., Friedrich, B., and Cramm, R.: Characterisation of the norB Gene encoding nitric oxide reductase, in the nondenitrifying cyanobacterium *Synechocystis* sp. Strain PCC6803, Appl. Environ. Microbiol, 68, 668–672, 2002.

Codispoti, L. A.: Interesting times for marine N_2O, Science, 327, 1339–1340, 2010.

Cohen, Y. and Gordon, L. I.: Nitrous oxide in the oxygen minimum of the eastern tropical North Pacific: evidence for its consumption during denitrification and possible mechanisms for its production, Deep-Sea Res., 25, 509–524, 1978.

Croft, M. T., Lawrence, A. D., Raux-Deery, E., Warren, M. J., and Smith, A. G.: Algae acquire vitamin B12 through a symbiotic relationship with bacteria, Nature, 438, 90–93, 2005.

Deng, M., Moureaux, T., and Caboche, M.: Tungstate, a molybdate analog inactivating nitrate reductase, deregulates the expression of the nitrate reductase structural gene, Plant Physiol, 91, 304–309, 1989.

Fagerstone, K. D., Quinn, J. C., Bradley, T. H., De Long, S. K., and Marchese, A. J.: Quantitative measurement of direct nitrous oxide emissions from microalgae cultivation, Environ. Sci. Technol, 45, 9449–9456, 2011.

Ferrón, S., Ho, D. T., Johnson, Z. I., and Huntley, M. E.: Air–water fluxes of N_2O and CH_4 during microalgae (*Staurosira* sp.) cultivation in an open raceway pond, Environ. Sci. Technol, 46, 10842–10848, 2012.

Florez-Leiva, L., Tarifeño, E., Cornejo, M., Kiene, R., and Farías, L.: High production of nitrous oxide (N_2O), methane (CH_4) and dimethylsulphoniopropionate (DMSP) in a massive marine phytoplankton culture, Biogeosciences Discuss., 7, 6705–6723, doi:10.5194/bgd-7-6705-2010, 2010.

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

Fukuto, J. M., Switzer, C. H., Miranda, K. M., and Wink, D. A.: Nitroxyl (HNO), Chemistry, Biochemistry, and Pharmacology, Annu. Rev. Pharmacol. Toxicol, 45, 335–355, 2005.

Gantner, S., Andersson, A. F., Alonso-Sáez, L., and Bertislon, S.: Novel primers for 16S rRNA-based archaeal community analysis in environmental samples, J. Microbiol. Methods, 84, 12–18, 2011.

Giovannoni, S. J.: Nucleic acid techniques in bacterial systematics, in: The Polymerase Chain Reaction, edited by: Stackerbrandt, E. and Goodfellow, M., John Wiley and Sons. Chichester, 177–203, 1991.

Goshima, N., Mukai, T., Suemori, M., Takakashi, M., Caboche, M., and Morikawa, H.: Emission of nitrous oxide (N_2O) from transgenic tobacco expressing antisense NiR mRNA, The Plant J., 19, 75–80, 1999.

Guieysse, B., Béchet. Q., and 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.

Gupta, K. J., Fernie, A. R., Kaiser, W. M., and van Dongen, J. T.: On the origins of nitric oxide, Trends Plant. Sci., 16, 160–168, 2011.

Harter, T., Bossier, P., Verreth, J., Bodé, S., Van der Ha, D., Debeer, A. E., Boon, N., Boeckx, P., Vyverman, W., and Nevejan, N.: Carbon and nitrogen mass balance during flue gas treatment with *Dunaliella salina* cultures, J. Appl. Phycol., 25, 359–368, 2013.

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

Hatzenpichler, R.: Diversity, physiology and niche differentition of ammonia-oxidizing archaea, Appl. Environ. Microbiol, 78, 7501–7510, 2012.

Hino, T., Matsumoto, Y., Nagano, S., Sugimoto, H., Fukumori, Y., Muratam T., Iwata, S., and Shiro, Y.: Structural basis of biological N_2O generation by bacterial nitric oxide reductase, Science, 330, 1666–1670, 2010.

Ishimura, Y., Gao, Y. T., Panda, S. P., Roman, L. J., Masters, B. S. S., and Weintraub, S. T.: Detection of nitrous oxide in the neuronal nitric oxide synthase reaction by gas chromatography – mass spectrometry, Biochem. Biophys. Res. Commun, 338, 543–549, 2005.

Kay, R. A.: Microalgae as food and supplement, Crit. Rev. Food Sci. Nutr., 30, 555–573, 1991.

Lane, J. and Lant, P.: Including N_2O in ozone depletion models for LCA, Int. J. Life Cy. Assess, 17, 252–257, 2012.

Law, C. S., Rees, A. P., and Owens, N. J. P.: Nitrous oxide production by estuarine epiphyton, Limnol. Oceanogr, 38, 435–441, 1993.

Li, B. and Bishop, P. L.: Micro-profiles of activated sludge floc determined using microelectrodes, Water Res, 38, 1248–1258, 2004.

Löscher, C. R., Kock, A., Könneke, M., LaRoche, J., Bange, H. W., and Schmitz, R. A.: Production of oceanic nitrous oxide by ammonia-oxidizing archaea, Biogeosciences, 9, 2419–2429, doi:10.5194/bg-9-2419-2012, 2012.

Mengis, M., Gachterm R., and Wehrli, B.: Sources and sinks of nitrous oxide (N_2O) in deep lakes, Biogeochem, 38, 281–301, 1997.

Miranda, K. M.: The chemistry of nitroxyl (HNO) and implications in biology, Coord. Chem. Rev, 249, 433–455, 2005.

Miranda, K. M., Paolocci, N., Katori, T., Thomas, D. D., Ford, E., Bartberger, M. D., Espey, M. G., Kass, D. A., Feelisch, M., Fukuto, J. M., and Wink, D. A.: A biochemical rationale for the discrete behavior of nitroxyl and nitric oxide in the cardiovascular system, Proc. Nat. Acad. Sci. USA, 100, 9196–9201, 2003.

Morell, J. M., Capella, J., Mercado, A., Bauzá, J., and Corredor, J. E.: Nitrous oxide fluxes in the Caribbean and tropical Atlantic waters: evidence for near surface production, Mar. Chem, 74, 131–143, 2001.

Morley, N., Baggs, E. M., Dörsch, P., and Bakken, L.: Production of NO, N_2O and N_2 by extracted soil bacteria, regulation by NO_2^- and O_2 concentrations, FEMS Microbiol. Ecol, 65, 102–112, 2008.

Ni, W. Z. and Zhu, Z. L.: Gaseous nitrogen losses and nitrous oxide emission from a flooded paddy soil affected by illumination and copper addition, Biol. Fertil. Soils, 34, 460–462, 2001.

Oudot, C., Andrie, C., and Montel, Y.: Nitrous oxide production in the tropical Atlantic ocean, Deep-Sea Res, 37, 183–202, 1990.

Paz-Yepes, J., Herrero, A., and Flores, E.: The NtcA-regulated amtB gene is necessary for full methylammonium uptake activity in the cyanobacterium *synechococcus* elongates, J. Bacteriol, 189, 7791–7798, 2007.

Pienkos, P. T. and Darzins, A.: The promise and challenges of microalgal-derived biofuels, Biofuels Bioprod, Biorefining, 3, 431–440, 2009.

Planchet, E. and Kaiser, W. M.: Nitric oxide production in plants, Plant Signaling Behav, 1, 46–51, 2006.

Qian, H., Li, J., Pan, X., Sun, Z., Ye, C., Jin, G., and Fu, Z.: Effects of streptomycin on growth of algae *Chlorella vulgaris* and *Microcystis aeruginosa*, Environ. Toxicol., 27, 229–237, 2012.

Ravishankara, A. R., Daniel, J. S., and Portmann, R. W.: Nitrous oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st century, Science, 326, 123–125, 2009.

Revell, L. E., Bodeker, G. E., Huck, P. E., and Williamson, B. E.: Impacts of the production and consumption of biofuels on stratospheric ozone, Geophys. Res. Lett, 39, L10804, doi:10.1029/2012GL051546, 2012.

Ricard, J. and Cornish-Bowden, A.: Co-operative and allosteric enzymes: 20 years on, Eur. J. Biochem, 166, 255–272, 1987.

Richmond, A.: Handbook of microalgal culture: biotechnology and applied phycology, Blackwell Publishing, UK, 2004.

Sakihama, Y., Nakamura, S., and Yamasaki, H.: Nitric oxide production mediated by nitrate reductase in the green alga *Chlamydomonas reinhardtii*: an alternative NO production pathway in photosynthetic organisms, Plant Cell Physiol., 43, 290–297, 2002.

Santoro, A. E., Buchwald, C., McIlvin, M. R., and Casciotti, K. L.: Isotopic signature of N_2O produced by marine ammonia-oxidizing archaea, Science, 333, 1282–1285, 2011.

Schmidt, H. H. H. W., Hofmann, H., Schindler, U., Shutenko, Z., Cunningham, D. D., and Feelisch, M.: No •NO from NO synthase, Proc. Natl. Acad. Sci. USA, 94, 14492–14497, 1996.

Schreiber, F., Loeffler, B., Polerecky, L., Kuypers, M. M., and de Beer, D.: Mechanisms of transient nitric oxide and nitrous oxide production in a complex biofilm, ISME J., 3, 1301–1313, 2009.

Sharpe, M. A and Cooper, C. E.: Reactions of nitric oxide with mitochondrial cytochrome c: a novel mechanism for the formation of nitroxyl anion and peroxynitrite, Biochem. J., 332, 9–19, 1998.

Smeets, E. M. W., Bouwman, L. F., Stehfest, E., van Vuuren, D. P., and Posthuma, A.: Contribution of N_2O to the greenhouse gas balance of first-generation biofuels, Glob. Change Biol, 15, 1–23, 2009.

Spolaore, P., Joannis-Cassan, C., Duran, E., and Isambert, A.: Commercial applications of microalgae, J. Biosci. Bioeng, 101, 87–96, 2006.

Stolz, J. F. and Basu, P.: Evolution of nitrate reductases: molecular and structural variations on a common function, Chem. Bio. Chem., 3, 198–206, 2002.

Takaya, N. and Shoun, H.: Nitric oxide reduction, the last step in denitrification by *Fusarium oxysporum*, is obligatory mediated by cytochrom P450nor, Mol. Gen. Genet, 263, 342–348, 2000.

Tischner, R., Planchet, E., and Kaiser, W. M.: Mitochondrial electron transport as a source for nitric oxide in the unicellular green algae *Chlorella sorokiniana*, FEBS Lett, 576, 151–155, 2004.

Twining, B. S., Mylon, S. E., and Benoit, G.: Potential role of copper availability in nitrous oxide accumulation in a temperate lake, Limnol. Oceanogr, 52, 1354–1366, 2007.

US EPA: Methane and nitrous oxide emissions from natural sources, US EPA, 430-R-10-001, 2010.

Vajrala, N., Martens-Habbena, W., Sayavedra-Sota, L. A., Schauer, A., Bottomley, P. J., Stahl, D. A., and Arp, D. J.: Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea, Proc. Natl. Aca. Sci. USA, 110, 1006–1011, 2013.

Vega, J. M., Herrera, J., Aparicio, P. J., Paneque, A., and Losada, M.: Role of molybdenum in nitrate reduction by *Chlorella*, Plant. Physiol., 48, 294–299, 1971.

Wang, H., Wang, W., Yin, C., Wang, Y., and Lu, J.: Littoral zones as the hotspots of nitrous oxide (N_2O) emission in a hyper-eutrophic lake in China, Atmos. Environ., 40, 5522–5527, 2006.

Wang, S., Yeager, K. M., Wan, G., Liu, C., Tao, F., and Fan, C.: Short term field observation of nitrous oxide saturations in Lake Taihu, China: The need for high temporal resolution studies, J. Environ. Qual, 39, 1858–1863, 2010.

Weathers, P. J.: N_2O evolution by green algae, Appl. Environ. Microbiol., 48, 1251–1253, 1984.

Weathers, P. J. and Niedzielski, J. J.: Nitrous oxide production by cyanobacteria, Arch. Microbiol, 146, 204–206, 1986.

Wijffels, R. H. and Barbosa, M. J.: An outlook on microalgal biofuels. Science, 329, 796–799, 2010.

Williams, J. and Crutzen, P. J.: Nitrous Oxide from aquaculture, Nat. Geosci, 3, 143 pp., 2010.

Wrage, N., Velthof, G. L., van Beusichem, M. L., and Oenema, O.: Role of nitrifier denitrification in the production of nitrous oxide, Soil Biol. Biochem, 33, 1723–1732, 2001.

Xiong, J., Fu, G., Yang, Y., Zhu, C., and Tao, L.: Tungstate: is it really a specific nitrate reductase inhibitor in plant nitric oxide research?, J. Exp. Biol, 63, 33–41, 2012.