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

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Abstract

Using antibiotic assays and genomic analysis, this study demonstrates nitrous oxide (N_2O) is generated from axenic *C. vulgaris* cultures. In batch assays, this production is magnified under conditions favoring 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. 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

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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 *Nannochloropsis 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 characterized 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; Hatzenpichler, 2012) 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).

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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 Sect. 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 assay, 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 4.4×10^3 rpm 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

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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} of 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

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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 Genomic analysis using PCR

Based on the protocol used by Fagerstone et al. (2011), a genomic analysis was conducted to detect bacterial 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} of and 25 mg streptomycin L^{-1} were added 3 days after inoculation) were transferred in DNA-free Eppendorph 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 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 as described in Sect. S2 using the following primers: norB, cnorB and qnorB primer pairs to amplify fragments of genes encoding for NOR; 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; and a "universal" 16S primer pair for bacteria. The rbcl amplification was performed as a positive control to show that there was enough DNA in the samples to be amplified. N_2O production in the presence of 12 mM nitrite under darkness was also confirmed in all cultures in batch assays.

2.6 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 illu-

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mination at 260 W m^{-2} (average of 40 measurements taken at 5 different heights and 8 different angular positions, Light adapter EXTECH 401020, using a 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}.

2.7 Analysis

N_2O concentration in gas samples was quantified using as chromatography (Shimadzu GC-2010, Shimadzu, Japan) equipped with a 1000 µL sample loop (380 °C), a 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 and nitrite concentration was quantified by ionic chromatography.

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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}, $p = 0.05$, $n = 10$, Sect. S1). Batch assays using axenic *C. vulgaris* cultures were therefore conducted to confirm this initial finding and indeed showed N_2O 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 confirmed by Qian et al. (2012) for streptomycin (see further discussion below). Genomic analysis using PCR did not reveal the presence of nor-genes or bacterial 16s rDNA in the algae cultures, regardless of whether *C. vulgaris* was cultivated in the presence of antibiotics (Sect. S2). In addition, considering the very low N_2O/NH_4^+ yields associated with N_2O

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generation by ammonium-oxidizing archaea (AOA) (0.0002 mole mole^{-1} under oxia, Löscher et al., 2012), the amount of NH_4^+ initially supplied (22 µmoles) could not sustain the N_2O production recorded in the positive controls (9.66 µmoles after 30 h). 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, and is further 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 (Sect. 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

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4-fold, suggesting this enzyme played a key role in N_2O production (Sect. S4). The influence of nitrite concentration on N_2O production shown on Fig. 3a can be described by a sigmoid plot, which often characterizes multimeric enzymes such as NR (Ricard and Cornish-Bowden, 1987). The linear correlation between algal biomass concentration and N_2O production seen on Fig. 3b 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 characterized by an initial "build-up" phase of 4 h followed by a period of "linear" evolution lasting more than 24 h (Fig. 4). Cell age (3–7 d) significantly impacted the initial rate (0–4 h) of N_2O production but, besides for 3 days-old cells, had no apparent impact on N_2O specific production rates during the linear phase (Sect. S4). This observation, together with the rapid response in N_2O production to the nitrite addition during cultivation in a closed photobioreactor (Sect. 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 NADH is available) but NiR,

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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 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; Shreiber 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 characterized in the cyanobacterium *Synechocystis* (Büsch et al., 2002). The genome of *C. vulgaris* may therefore encode for a similar function and further research is needed to confirm this hypothesis.

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

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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 (Sect. S4).

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

A "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 normal 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. 2–4). 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 recognized 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 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)

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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 (Shreiber 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, although the mechanisms are still unclear (Hatzenpicher, 2012). From their respective studies on *Nannochloropsis 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

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strongly suggests that nitrite intracellular accumulation and its reduction by NR trigger N_2O production in *C. vulgaris*.

The putative pathways shown in Fig. 5 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 nitrifiers, 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. 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 illumination in the absence of exogenous nitrite (Sect. S1) and when the same reactor was operated outdoors ($12 \pm 6\,\mathrm{nmol\,N_2O\,g\,DW^{-1}\,h^{-1}}$, $p = 0.05$, $n = 10$; Sect. 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 at-

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tenuation at high cell densities when nitrate was used as N-source (dissolved nitrite was detected at 0.15–0.80 mM, while N_2O was not released in the 50 L reactor operated indoor when ammonium was used as N-source, Sects. S1 and S3). Based on the outdoor rates reported here, *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) (see Sect. S6 for detailed calculations) would release 1.38–10.1 kg N_2O-N ha^{-1} yr^{-1}. 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 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 favoring 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 minimize N_2O emission during microalgae cultivation.

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Supplementary material related to this article is available online at: http://www.biogeosciences-discuss.net/10/9739/2013/bgd-10-9739-2013-supplement.pdf.

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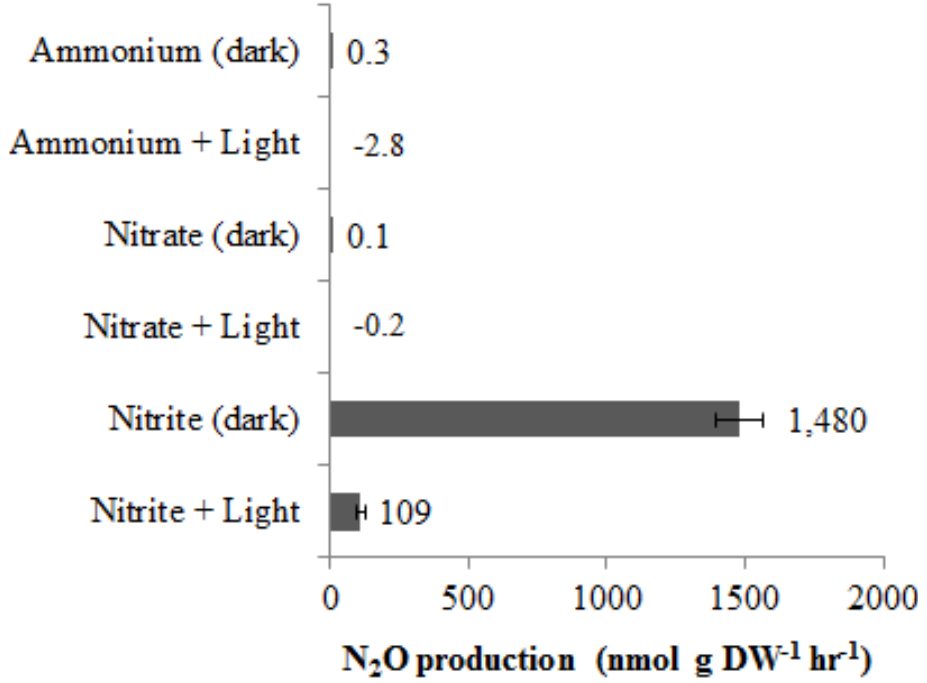

Fig. 1. Influence of nitrogen source and illumination on specific N_2O production by *C. vulgaris* (0.22 g DW L^{-1}). The rates shown represent averages from duplicates ± error and were determined between 2.9 and 21.5 h after nitrogen addition (12 mM).

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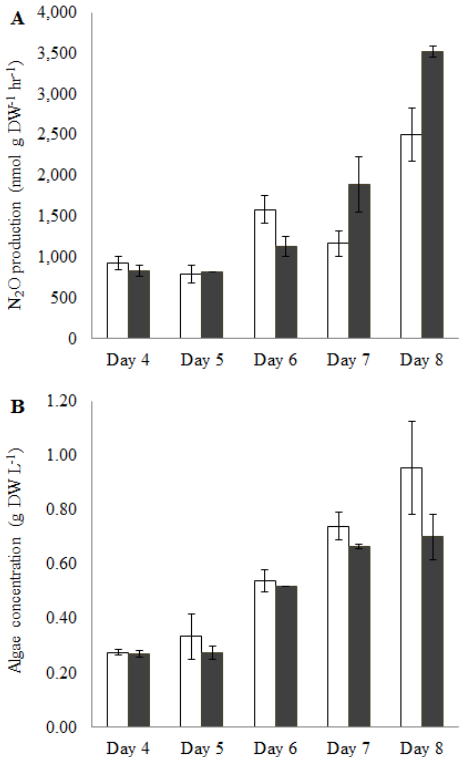

Fig. 2. Evolution of specific N_2O production **(A)** and biomass concentration **(B)** in antibiotic-laden (grey bars) and antibiotic-free (while bars) *C. vulgaris* cultures. The rates shown represent averages from duplicates and were determined between 4 and 24 h after nitrite addition (12 mM).

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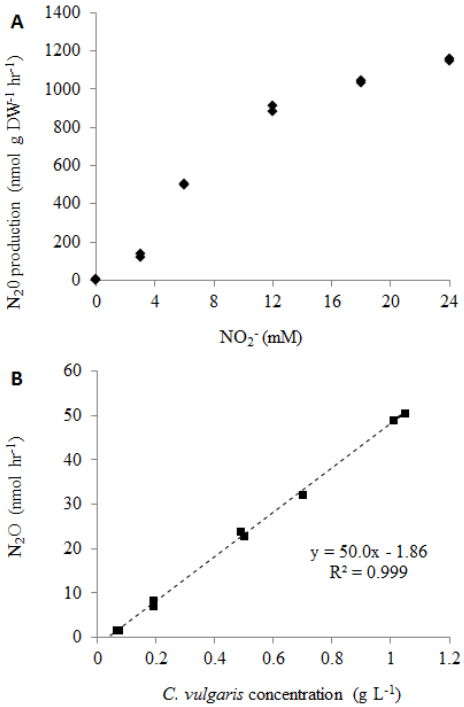

Fig. 3. Influence of nitrite concentration **(A)** and *C. vulgaris* concentration **(B)** on specific N_2O production from *C. vulgaris* cultures incubated in darkness ($n = 2$). When nitrite concentration was varied, *C. vulgaris* concentration was 0.58 g L^{-1}; when algae concentration was varied, 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).

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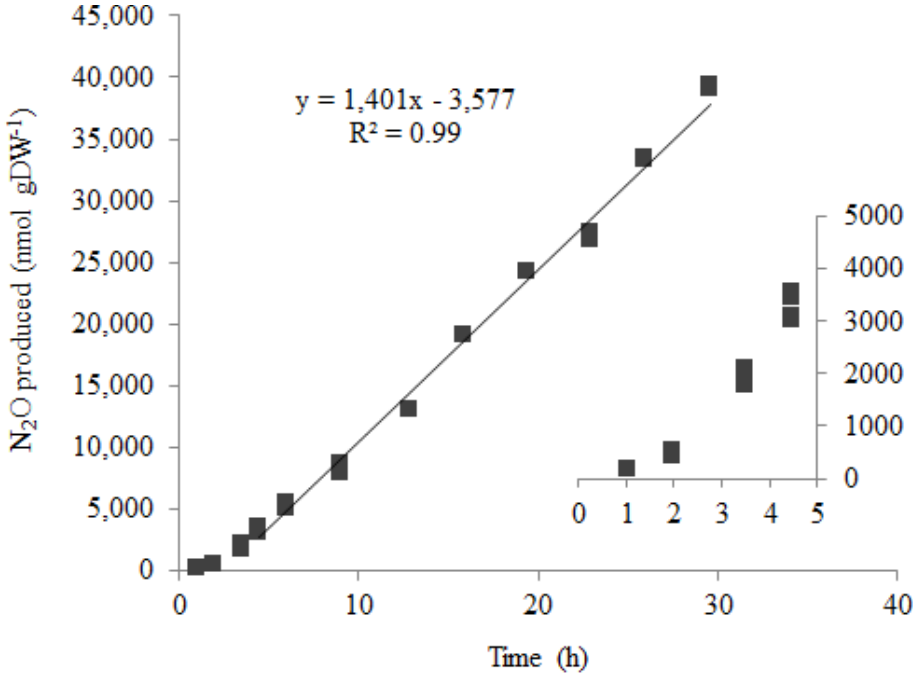

Fig. 4. Time change of N_2O production (nmol g DW^{-1}) in *C. vulgaris* supplied with 12 mM nitrite as sole exogenous nitrogen source and incubated in the dark. The plain line represents the linear regressing over 4.5–29.5 h. Insert shows initial build-up phase. The data shown is a combined plot of duplicates (e.g. not the average) from 3 independent experiments.

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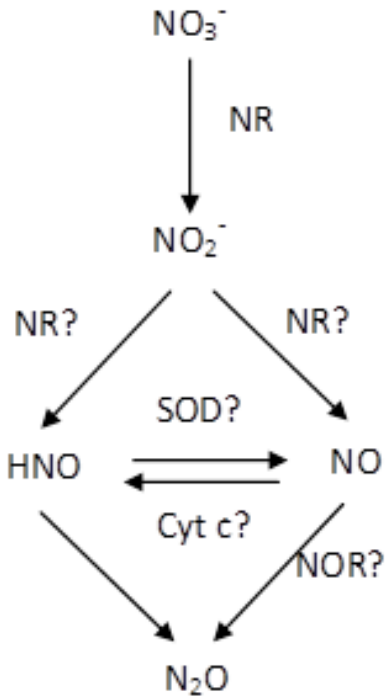

Fig. 5. Putative pathways of N_2O formation in *C. vulgaris* (NO_3^- = nitrate, NO_2^- = nitrite, NO = nitric oxide, HNO = nytroxyl, NR = nitrate reductase, NiR = nitrite reductase, SOD = superoxide dismutase, NOR = NO reductase, Cyt c = cytochrome c oxidase).

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