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The biosynthesis of nitrous oxide in the green alga *Chlamydomonas reinhardtii*

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SUMMARY

Over the last decades, several studies have reported emissions of nitrous oxide (N_2O) from microalgal cultures and aquatic ecosystems characterized by a high level of algal activity (e.g. eutrophic lakes). As N_2O is a potent greenhouse gas and an ozone-depleting pollutant, these findings suggest that large-scale cultivation of microalgae (and possibly, natural eutrophic ecosystems) could have a significant environmental impact. Using the model unicellular microalga *Chlamydomonas reinhardtii*, this study was conducted to investigate the molecular basis of microalgal N_2O synthesis. We report that *C. reinhardtii* supplied with nitrite (NO_2^-) under aerobic conditions can reduce NO_2^- into nitric oxide (NO) using either a mitochondrial cytochrome *c* oxidase (COX) or a dual enzymatic system of nitrate reductase (NR) and amidoxime-reducing component, and that NO is subsequently reduced into N_2O by the enzyme NO reductase (NOR). Based on experimental evidence and published literature, we hypothesize that when nitrate (NO_3^-) is the main Nitrogen source and the intracellular concentration of NO_2^- is low (i.e. under physiological conditions), microalgal N_2O synthesis involves the reduction of NO_3^- to NO_2^- by NR followed by the reduction of NO_2^- to NO by the dual system involving NR. This microalgal N_2O pathway has broad implications for environmental science and algal biology because the pathway of NO_3^- assimilation is conserved among microalgae, and because its regulation may involve NO.

Keywords: climate change, greenhouse gases, microalgae, nitric oxide, nitrite response, nitrous oxide.

INTRODUCTION

Nitrous oxide (N_2O) is a potent greenhouse gas and a major ozone-depleting pollutant (Ravishankara *et al.*, 2009) that can be emitted from algal cultures (Weathers, 1984; Weathers and Niedzielski, 1986), including verified axenic cultures (Guieysse *et al.*, 2013) and eutrophic ecosystems characterized by a high level of primary activity (Mengis *et al.*, 1997; Wang *et al.*, 2006). Although little is known about N_2O synthesis in microalgae, nitrite (NO_2^-) has long been suspected to be a substrate (Weathers, 1984; Weathers and Niedzielski, 1986). Guieysse *et al.* (2013) later proposed that *Chlorella vulgaris* synthesizes N_2O via the reduction of NO_2^- to nitric oxide (NO) or nitroxyl (HNO) by nitrate reductase (NR), followed by the reduction of NO to N_2O by a NO reductase (NOR) or the spontaneous dimerization of HNO to N_2O. However, if NO can indeed be reduced to N_2O under oxia, several alternative scenarios for NO generation could also lead to N_2O synthesis. Firstly, a dual system of NR and the amidoxime-reducing component NOFNiR (NO-forming nitrite reductase) was recently shown to mediate NO production (both *in vitro* and *in vivo*) in *Chlamydomonas* when the intracellular concentration of NO_2^- increases in the presence of NO_3^- (Chamizo-Ampudia *et al.*, 2016). Secondly, the reduction of NO_2^- to NO could be catalyzed *in vitro* and under anoxia by molybdoenzymes similar to the xanthine oxidase/dehydrogenase found in animals (Maia and Moura, 2014) and vascular plants (Gupta *et al.*, 2011). Thirdly, NO_2^- can be reduced to NO by mitochondrial cytochrome *c* oxidase

(COX) and alternative oxidase (AOX), as previously proposed for plants (Planchet *et al.*, 2005; Gupta *et al.*, 2016) and the microalga *Chlorella sorokiniana* (Tischner *et al.*, 2004). Fourthly, L-arginine can be oxidized to NO by nitric oxide synthase (NOS) activity, described in plants, although the protein involved has not yet been identified (an animal-type NOS exist in several algae; Gupta *et al.*, 2011; Jeandroz *et al.*, 2016). Finally, NO_2^- can be reduced to NO by hemoglobin under hypoxic/anoxic conditions in vascular plants, algae and many cyanobacteria (Sturms *et al.*, 2011; Tiso *et al.*, 2012; Ciaccio *et al.*, 2015).

This study was conducted to determine the pathway of N_2O synthesis in microalgae, with the view that this knowledge is critical for the understanding of the nitrogen cycle in aquatic ecosystems (Weathers, 1984; Hayatsu *et al.*, 2010; Kamp *et al.*, 2013). This information will also allow better assessments of the environmental impacts of algal biotechnologies and anthropogenic eutrophication (Guieysse *et al.*, 2013). The model unicellular green microalga *Chlamydomonas reinhardtii* is especially suitable for investigating N_2O synthesis because: (i) nitrogen assimilation and regulation have been extensively studied in this species (Navarro *et al.*, 2000; Fernández and Galván, 2008; Schmollinger *et al.*, 2014; Park *et al.*, 2015; Sanz-Luque *et al.*, 2015a); (ii) the cellular reduction of NO_2^- to NO has already been shown (Sakihama *et al.*, 2002; Wei *et al.*, 2014; Chamizo-Ampudia *et al.*, 2016); and (iii) biological and genomic resources are available for this organism (Navarro *et al.*, 2000; Harris, 2001; Gonzalez-Ballester *et al.*, 2005; Pröschold *et al.*, 2005; Merchant *et al.*, 2007; *Chlamydomonas* Resource Centre, http://www.chlamycollection.org/). This alga was not known to synthesize N_2O prior to the current study, so this ability was first demonstrated in axenic cultures. The pathway(s) involved in N_2O synthesis was then determined using mutants and/or specific inhibitors, and these findings were elaborated using RNA sequencing analysis.

RESULTS

Nitrous oxide emissions from axenic *C. reinhardtii* cultures

While significant N_2O emissions (average 1770 ± 500 nmol g DW^{-1} after 24 h, $n = 41$; DW, dry weight) were recorded from *C. reinhardtii* 6145c cultures supplied with NO_2^- in the dark (Figure 1), N_2O production was negligible in autoclaved cultures or sterile medium (Table S1). The N_2O production recorded from *C. reinhardtii* cultures was unlikely to be a result of bacterial or archaeal contamination because sensitive PCR-based detection assays provided no evidence for the presence of these microorganisms (see Appendix S1 in the Supporting Information). These results conclusively demonstrate that *C. reinhardtii* can synthesize N_2O under oxia, which was unknown until now. The production of N_2O was confirmed for all *C. reinhardtii* strains tested, although the rates of emission were strain-dependent (Table S2). N_2O production was linearly correlated with microalgal biomass concentration (Figure S1), providing further evidence that biological processes in the alga were the source of N_2O. In the dark the kinetics of N_2O biosynthesis was characterized by an immediate and short period of N_2O production lasting for 1–4 h, followed by a phase of slow production lasting up to approximately 20 h of incubation, and a final phase of

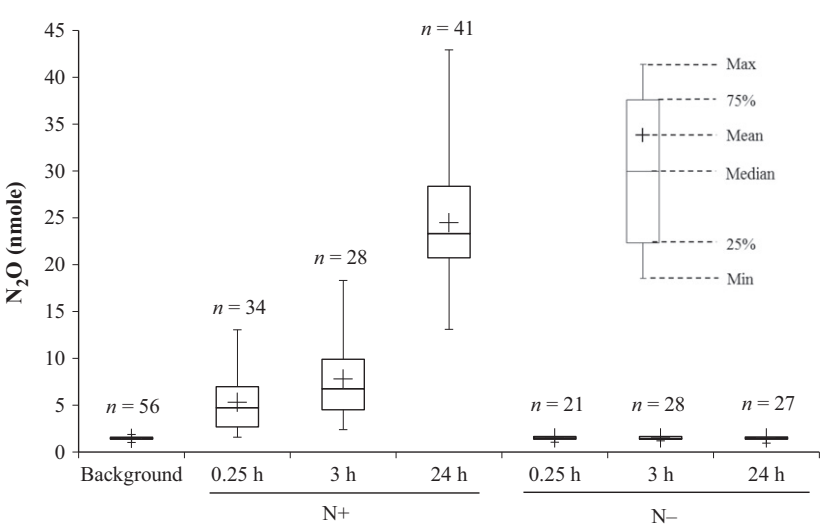

Figure 1. Box plot of N_2O production in cultures of *Chalmydomonas reinhardtii.*
Box plot of N_2O production (nmole) recorded in cultures of *C. reinhardtii* wild type 6145c supplied with 10 mM NO_2^- (shown as 'N+') and N-free cultures of 6145c (shown as 'N-') after 0.25, 3 and 24 h of incubation in the dark. The amount of N_2O initially present in flasks supplied with medium (no inoculum) is shown as 'background' and *n* represents the number of independent replicates.

vigorous production over the remaining duration of the experiment (Figure S2a). The initial short phase of N_2O production was not observed in culture incubated under illumination; here N_2O biosynthesis started approximately 20 h after the addition of NO_2^- (Figure S2b). The implications of the kinetics, and the influence of light on N_2O production, are discussed below in the context of the potential pathways involved.

NO_2^- acts as substrate during microalgal N_2O synthesis

Supplying NO_3^- or NH_4^+ to the axenic *C. reinhardtii* 6145c cultures did not trigger significant synthesis of N_2O (Table S1), suggesting that this process depends on the availability of NO_2^- as a substrate. Supporting this idea, N_2O production in the dark was found to be linearly correlated to the extracellular concentration of NO_2^- (Figure 2). This strong association between the reduction of NO_2^- and the synthesis of N_2O is in agreement with previous studies (Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse *et al.*, 2013; Kamp *et al.*, 2013; Alcántara *et al.*, 2015). The NO_2^--independent synthesis of N_2O via conversion of L-arginine by NOS was unlikely because N_2O emissions were not observed in NO_2^--free cultures supplied with L-arginine and because N_2O production in NO_2^--laden cultures increased slightly in the presence of the NOS inhibitor *N*ω-nitro-L-arginine (L-NNA) (Table S1). These results are consistent with previous findings achieved over shorter durations (4 h) using *C. reinhardtii* (Sakihama *et al.*, 2002) and *C. vulgaris* (Guieysse *et al.*, 2013), and the fact that NOS appears to be absent in *C. reinhardtii* (Jeandroz *et al.*, 2016).

NO is a key intermediate during N_2O synthesis

Guieysse *et al.* (2013) suggested that NO is an intermediate during N_2O synthesis in *C. vulgaris*, and this compound is indeed known to be synthesized by *C. reinhardtii* and other microalgae (Mallick *et al.*, 1999, 2000; Sakihama *et al.*, 2002; Tischner *et al.*, 2004; Estevez and Puntarulo, 2005; Kim *et al.*, 2008; Chang *et al.*, 2013; Wei *et al.*, 2014; Sanz-Luque *et al.*, 2015b; Chamizo-Ampudia *et al.*, 2016). In *C. reinhardtii*, NO synthesis has been associated with nitrogen assimilation (Sanz-Luque *et al.*, 2013, 2015a) and both the hypoxic and the nitrogen stress response (Zhang and Mehta, 2008; Hemschemeier *et al.*, 2013; Wei *et al.*, 2014). In our study, the generation of green fluorescence in *C. reinhardtii* 6145c cells pre-incubated with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate) (inside cells, this permeable non-fluorescent chemical is converted into the non-permeable DAF-FM, and the latter reacts with NO to form a fluorescent derivative; see Appendix S2 for details) and subsequently exposed to NO_2^- suggests the reduction of NO_2^- caused synthesis of NO, in agreement with the results of Chamizo-Ampudia *et al.* (2016). This fluorescence was not observed when the NO scavenger bovine hemoglobin was added to the microalgal suspension before incubation with DAF-FM diacetate and supply of NO_2^- (see the full set of results from positive and negative controls in Appendix S2). Taken together these results demonstrate that the conditions triggering N_2O synthesis (Table 1) also trigger NO production in *C. reinhardtii*.

As HNO has also been proposed as a possible intermediate in algal N_2O synthesis via the reduction of NO_2^- (Guieysse *et al.*, 2013), an additional control was performed where *C. reinhardtii* 6145c cells pre-incubated with DAF-FM diacetate were supplied with Angeli's salt as a HNO donor (Appendix S2). The fluorophore DAF-FM is known to react with the NO oxidation products N_2O_3 and NO^+; however, the aqueous degradation of Angeli's salt releases NO_2^- and HNO under physiological conditions (Dutton *et al.*, 2004; Miranda *et al.*, 2005), but can also generate NO at low pH (Miranda *et al.*, 2005). The weak fluorescence reported in these experiments (Appendix S2) may therefore have been caused by 'indirect' NO_2^- supply causing NO synthesis, or direct NO synthesis in low-pH cellular compartments (Kurkdjian and Guern, 1989). While our results strongly suggest that NO was generated during N_2O synthesis, further work is needed to fully verify this hypothesis given that knowledge about the biochemistry of HNO production is still very limited (Fukuto *et al.*, 2005).

Immediate N_2O synthesis involves NR, but late synthesis involves other enzymes

The catalysis of the reduction of NO_2^- to NO by NR has long been suspected in plants and *C. reinhardtii* (Yamasaki, 2000; Rockel *et al.*, 2002; Sakihama *et al.*, 2002; Meyer *et al.*, 2005). More recently, this enzyme was shown to provide electrons to a NO-forming NiR, thereby enabling reduction of NO_2^- to NO by a dual NR-NOFNiR system of two molybdoenzymes (Chamizo-Ampudia *et al.*, 2016). In *C. vulgaris*, the involvement of NR during N_2O synthesis was evidenced by the repression of N_2O production in cells pre-cultivated with tungstate, an inhibitor of

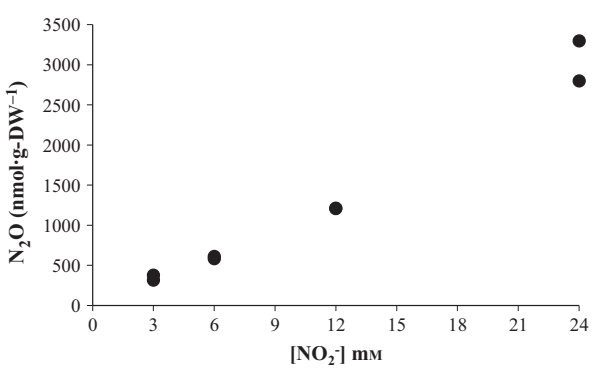

Figure 2. Impact of NO_2^- concentration on N_2O specific production (nmol g DW^{-1}) in duplicate cultures of *C. reinhardtii* 6145c (0.25 g L^{-1}) supplied with NO_2^- at different concentrations and incubated for 24 h in the dark. DW, dry weight.

Table 1 Total amount of N_2O (nmol ± standard error) and specific N_2O production (nmol g DW^{-1} ± standard error) in *Chlamydomonas reinhardtii* 6145c, 2929, M3, M4, 704 and amiCYP55 cultures supplied with NO_2^- (or NO_3^- for strains M3 and M4) and incubated in the dark (or illumination for strains M3 and M4)

				N_2O after 0.25 h		N_2O after 3 h		N_2O after 24 h	
Strain	Conditions	N source	Effector	Total (*n*)	Production (*n*)	Total (*n*)	Production (*n*)	Total (*n*)	Production (*n*)
6145c	Darkness	NO_2^-		5.30 ± 3.01 (34)[a]	300 ± 230 (34)	7.80 ± 4.43 (28)[a]	490 ± 345 (28)	24.5 ± 6.59 (41)[a]	1770 ± 500 (41)
	Darkness	NO_2^-	CN^-	1.59 ± 0.04 (3)	<LOQ	1.89 ± 0.31 (3)	<LOQ	3.78 ± 0.07 (3)	180 ± 20.0 (3)
2929	Darkness	NO_2^-		1.16 ± 0.25 (8)	<LOQ	2.30 ± 0.76 (8)	<LOQ	29.0 ± 9.20 (8)	2100 ± 700 (8)
	Darkness	NO_2^-	CN^-	–	–	2.71 ± 0.05 (2)[b]	<LOQ	3.16 ± 0.10 (2)	130 ± 20 (2)
M3	Darkness	NO_2^-		11.4 ± 0.26 (2)	800 ± 50 (2)	55.8 ± 24.1 (2)[b]	4200 ± 2000 (2)	75.6 ± 11.1 (2)	6000 ± 900 (2)
		NO_3^-		2.03 ± 0.35 (2)	<LOQ	1.97 ± 0.08 (2)[b]	<LOQ	4.06 ± 1.45 (2)	200 ± 110 (2)
	Illumination	NO_2^-		4.64 ± 0.78 (2)	250 ± 60.0 (2)	370 ± 8.20 (2)[b]	$(2.8 \pm 0.16) \times 10^4$ (2)	3480 ± 60.0 (2)	$[27.0 \pm 1.40] \times 10^4$ (2)
		NO_3^-		4.37 ± 0.00 (2)	230 ± 20.0 (2)	29.6 ± 3.42 (2)[b]	2180 ± 290 (2)	720 ± 71.0 (2)	$(5.60 \pm 0.61) \times 10^4$ (2)
M4	Darkness	NO_2^-		–	–	58.5 ± 0.16 (2)[b]	4400 ± 220 (2)	95.6 ± 6.56 (2)	7300 ± 600 (2)
		NO_3^-		2.04 ± 0.01 (2)	<LOQ	2.04 ± 0.01 (2)[b]	<LOQ	3.15 ± 0.70 (2)	130 ± 60 (2)
	Illumination	NO_2^-		81.9 ± 0.00 (2)	6240 ± 0.00 (2)	177 ± 11.5 (2)[b]	$(1.36 \pm 0.11) \times 10^4$ (2)	3700 ± 300 (2)	$(28.6 \pm 2.70) \times 10^4$ (2)
		NO_3^-		3.21 ± 0.09 (2)	137 ± 20.0 (2)	33.6 ± 0.92 (2)[b]	2500 ± 150 (2)	1830 ± 34.5 (2)	$14.2 \pm 0.76 \times 10^4$ (2)
704	Darkness	NO_2^-		2.03 ± 0.06 (3)	<LOQ	6.72 ± 0.99 (3)	410 ± 80.0 (3)	138 ± 9.42 (3)	$[1.06 \pm 0.9] \times 10^4$ (3)
amiCYP55	Darkness	NO_2^-		1.78 ± 0.02 (3)	<LOQ	2.17 ± 0.05 (3)	<LOQ	8.41 ± 2.40 (3)	540 ± 200 (3)

LOQ, limit of quantification.
[a]Values given in Figure 1 used to calculate N_2O production.
[b]After 6 h.

molybdoenzymes such as NR (Deng *et al.*, 1989). The hypothesis of NR-mediated N_2O synthesis was therefore tested in *C. reinhardtii*.

Tungstate is known to partially inhibit NR activity in *C. reinhardtii* (Llamas *et al.*, 2000), and pre-cultivation of the 6145c strain with this compound indeed caused partial repression of immediate N_2O production (Table S3). Interestingly, tungstate inhibits the terminal activity of NR without affecting the diaphorase-associated activity of this enzyme (Barea *et al.*, 1976). Tungstate is therefore likely to have inhibited immediate N_2O synthesis by inhibiting the NOFNiR activity of the dual molybdoenzyme system catalyzing NO synthesis (Chamizo-Ampudia *et al.*, 2011, 2016). To confirm this, N_2O emission was recorded during the cultivation of NR-deficient *C. reinhardtii* 2929 (Table 1). Immediate N_2O production was not recorded in NO_2^--replete cultures of the NR-deficient strain, although similar 0–24 h N_2O emissions were recorded in the 2929 mutant (2100 ± 700 nmol g DW^{-1}) and the 6145c control strain (1770 ± 500 nmol g DW^{-1}) after 24 h of incubation. Furthermore, fluorescence was not observed in NO_2^--replete cultures of NR mutant 2929 pre-incubated with DAF-FM diacetate (this protocol could only be used following short-term exposure to NO_2^- due to the reactivity of DAF-FM diacetate; Appendix S2). These results indicate that N_2O synthesis involved the reduction of NO_2^- into NO by the dual system NR-NOFNiR immediately following the addition of NO_2^-, and is consistent with a different enzyme system being involved in the late N_2O response.

NR and NiR activities impact N_2O synthesis under supply of NO_3^-

NO_3^- is the main nitrogen source available to microalgae in many 'natural' environments (Raven and Giordano, 2013) and it is commonly used during commercial cultivation of microalgae (Borowitzka, 2005). NR and NiR sequentially catalyze the assimilatory reduction of NO_3^- to NO_2^- and NH_4^+, thereby contributing to regulation of the intracellular concentration of NO_2^-. As NO_2^- 'fuels' N_2O synthesis via NO, the activities of NR and NiR are likely to affect N_2O emissions under physiological conditions (when NO_3^- is the main N source and the intracellular concentration of NO_2^- is relatively low): this was demonstrated when the NiR-deficient mutants supplied with NO_3^- produced large amounts of N_2O under illumination (Table 1). These mutants could carry out the stoichiometric reduction of NO_3^- to NO_2^- because they possess a NR activated by light and NO_3^- (Navarro *et al.*, 2000; Kaiser and Huber, 2001), but could not reduce NO_2^- to NH_4^+. Nitrite is therefore likely to have over-accumulated in illuminated NiR-deficient cells (Navarro *et al.*, 2000), which resulted in increased turnover of NO boosting N_2O emissions. Unlike the case of *C. reinhardtii* 6145c NO_3^--replete cultures, where N_2O production was repressed in the dark (Table S1) as cells could not sustain reduction of NO_3^- to NO_2^-, NiR-deficient mutants supplied with NO_2^- could release N_2O in light and dark conditions (Table 1), further highlighting the role of NO_2^- as a substrate for N_2O synthesis. In agreement with past studies (Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse *et al.*, 2013; Kamp *et al.*, 2013), illumination repressed the immediate production of N_2O by *C. reinhardtii* supplied with NO_2^- (Table 1), probably due to the reduction of NO_2^- by NiR during photosynthesis (Guieysse *et al.*, 2013).

Late N_2O synthesis involves the reduction of NO_2^- to NO by mitochondrial COX

The reduction of NO_2^- via the mitochondrial electron transport chain was first demonstrated in the green microalga *C. sorokiniana* (Tischner *et al.*, 2004), and mitochondria are a known source of NO in higher plants (Planchet *et al.*, 2005; Igamberdiev *et al.*, 2014; Gupta *et al.*, 2016). Mitochondrial COX or AOX can reduce NO_2^- to NO under hypoxic and anoxic conditions (Tischner *et al.*, 2004; Planchet *et al.*, 2005; Gupta and Igamberdiev, 2011) and, at a low rate, under normoxia (Tischner *et al.*, 2004; Planchet *et al.*, 2005). As experimental evidence linking these mitochondrial enzymes to N_2O synthesis was previously lacking, the wild-type *C. reinhardtii* 6145c and the NR-deficient mutant *C. reinhardtii* 2929 were supplied with NO_2^- in the presence of CN^-, an inhibitor of heme-containing enzymes (Sakihama *et al.*, 2002; Tischner *et al.*, 2004) known to affect mitochondrial COX (Way, 1984) at concentrations lower than 5 mM (Gans and Wollman, 1995). This treatment with 2 mM CN^- resulted in the immediate inhibition of both N_2O (Table 1) and NO (Appendix S2), which could be explained by the fact that CN^- can also inhibit NR (Tischner *et al.*, 2004; Planchet *et al.*, 2005). However, and in contrast to chemical inhibition or repression of NR, N_2O synthesis was inhibited by 90% and 96% in the wild-type and NR mutant cultures, respectively, after 24 h of exposure to CN^- and NO_2^- (Table 1). Together, these results suggest that mitochondrial COX catalyzes the reduction of NO_2^- to NO during the late period of N_2O production.

Given that the synthesis of N_2O by *C. reinhardtii* 6145c was not completely eliminated following the addition of CN^-, AOX may also be partially responsible for the reduction of NO_2^- to NO. Tischner *et al.* (2004) indeed demonstrated that the *C. sorokiniana* NiR mutant continuously generated high levels of NO under anoxia and normoxia, and that this production could be catalysed by both COX and AOX. The AOX-mediated reduction of NO_2^- to NO has also been demonstrated in the model plant *Arabidopsis thaliana* (Gupta *et al.*, 2016). The potential involvement of AOX could not be experimentally dismissed because we found that the chemical reaction of the known AOX inhibitor salicylhydroxamic acid (SHAM) generated N_2O from NO_2^- (44 ± 8 nmol N_2O from abiotic tests incubated for

3 h). It should also be noted that NO_2^- is a known antidote to cyanide, and that NO may affect the inhibitory effect of CN^- (Leavesley *et al.*, 2008).

The synthesis of N_2O by *C. reinhardtii* involves the reduction of NO to N_2O by NOR

Although the concurrent synthesis of NO and N_2O in response to NO_2^- supply was repeatedly observed in *C. reinhardtii* cultures, NO is typically oxidized into nitrogen dioxide (NO_2) and other products (e.g. $ONOO^-$) under oxia (Murphy *et al.*, 1998; Lamattina *et al.*, 2003). *Chlamydomonas reinhardtii*, however, harbors the gene CYP55, which is similar to a fungal gene encoding a NOR (Chang *et al.*, 2011; Guieysse *et al.*, 2013; and further evidence below) that is capable of reducing NO into N_2O under hypoxia (Morozkina and Kurakov, 2007; Shoun *et al.*, 2012). When supplied with NO_2^-, a *C. reinhardtii* NOR knockdown mutant (amiCYP55 strain with the gene *CYP55* silenced using artificial micro-iRNA; Appendix S3) synthesized 70–90% less N_2O than its respective parent (Table 1), suggesting that wild-type *C. reinhardtii* synthesizes N_2O via NOR-mediated NO reduction under oxia. Interestingly, N_2O synthesis increased when nitrite-laden *C. reinhardtii* 6145c was incubated under anoxic conditions (Table S1). These results may be explained by the absence of competition between NO_2^- and O_2 for the COX reaction sites (Gupta *et al.*, 2005), as well as reduced rates of NO oxidation (Gupta *et al.*, 2016) favoring reduction of NO to N_2O by NOR. N_2O synthesis was not fully inhibited, probably due to the fact NOR expression was not fully repressed in the amiCYP55 strain (e.g. 54% of the wild-type strain 704).

RNA sequencing

To study the changes in expression of key enzymes potentially involved in N_2O synthesis in *C. reinhardtii* (e.g. NR, NOR, COXs), RNA sequencing (RNA-seq) was performed on cultures exposed to NO_2^- in the dark (treatment) and compared with NO_2^--free cultures grown under identical conditions (control). RNA-seq was carried out on triplicate samples collected at 0.25, 3 and 24 h after NO_2^- addition, based on the three phases of N_2O kinetics observed in the dark (Figure S2a). As shown in Table 2, genes encoding NR, NiR and NOR were not significantly differentially expressed (the adjusted *P*-value threshold was 0.05) between the treatments and the controls at any of the time points tested. Overall, the transcriptomics data were consistent with the biochemical evidence that reduction of NO_2^- to NO leading to 'late' N_2O synthesis is not mediated by NR. NR would only be needed immediately following NO_2^- supply. The nitrite response via transcriptional regulation of *NOR* may not have been triggered/required under the experimental conditions tested, but the data clearly provide evidence for the presence of abundant *NOR* transcripts (the average normalized read count across all samples for *NOR* was 3156, $n = 18$).

In contrast to the genes discussed above, *THB1* and *THB2* were significantly differentially expressed after 24 h of exposure to NO_2^- (Table 2). Plant hemoglobins are known to reduce NO_2^- to NO (Hoy and Hargrove, 2008; Gupta *et al.*, 2011) and a microalgal hemoglobin could catalyze this reaction under hypoxic/anoxic conditions (Sturms *et al.*, 2011), a mechanism thought to be related to either NO_2^- detoxification (Sturms *et al.*, 2011) or survival under anoxia (Hemschemeier *et al.*, 2013). *Chlamydomonas reinhardtii* possesses 12 truncated hemoglobins (THB1–12), and some of these enzymes can indeed reduce NO_2^- (Ciaccio *et al.*, 2015; Huwald *et al.*, 2015). *Chlamydomonas reinhardtii* THB1, which was significantly differentially expressed in the treatment cells after 3 and 24 h of exposure to NO_2^- [$\log_2$ fold change (FC) of 0.7 and 0.5], is also involved in NO signaling, NO_3^- assimilation, regulation of NR activity and, of particular relevance, NO scavenging under normoxia via its dioxygenase activity (Sanz-Luque *et al.*, 2015b). Consequently, during NO_3^- assimilation, THB1 may help to regulate intracellular accumulation of NO by promoting the transformation of NO into NO_3^- in parallel with the reduction of NO to N_2O. The change in transcript abundances for *THB1* (positive $\log_2$ FC) and *THB2* (negative $\log_2$ FC) agrees with the transcriptional NO response described by Sanz-Luque *et al.* (2015b), and therefore provides additional evidence of both the involvement of NO as a N_2O intermediate and the involvement of THBs in the response to NO synthesis.

Transcriptomic data also showed that while numerous transcripts for several *COX* genes could be identified, only one *COX* gene was significantly differentially expressed ($\log_2$ FC of −0.5) after 24 h of NO_2^- exposure (Table 2). As in the case of NOR, our failure to observe changes in transcript abundance of the *COX* genes suggests that transcription regulation might not have been required under the experimental conditions tested. In contrast, *AOX1* and *AOX2* were significantly differentially expressed ($\log_2$ FC of 0.5 and 1.4, respectively) after 24 h of exposure to NO_2^-. AOXs are regulatory enzymes that balance electron transport in mitochondria (Vanlerberghe and McIntosh, 1997). Considering that NO has been shown to induce *AOX* in *Arabidopsis* (Huang *et al.*, 2002), AOX synthesis could have been activated in response to the generation of NO in *C. reinhardtii*. Further analysis is still needed to evaluate the full metabolic effect of high NO_2^- in *C. reinhardtii* as 180, 740 and 3914 genes were significantly differentially expressed between control and treatment groups after 0.25, 3 and 24 h of NO_2^- exposure, respectively. The transcriptomic data also showed significantly lower transcript abundances of several eukaryotic initiation factors in NO_2^--replete cultures (Table S4), indicative of cells that have activated stress response pathways (Langland *et al.*,

Table 2 Log_2 fold expression changes (Log_2FC) between control and treatment groups of candidate genes potentially involved in microalgal N_2O synthesis. Numbers in parenthesis represent mean normalized counts

			Time (h) after exposure to NO_2^-		
Locus ID	Gene	Annotation	0.25	3	24
Cre09.g410950	*NIA1*	Nitrate reductase (NR)	0.0 (1)	0.0 (0)	0.0 (0)
Cre09.g410750	*NII 1*	Nitrite reductase (NiR)	−0.2 (2)	−0.4 (4)	−0.7 (1)
Cre01.g007950	*CYP55*	Nitric oxide reductase (NOR)	0.2 (5150)	0.3 (2974)	−0.1 (1487)
Cre09.g389089	*ARC*	Amidoxime reducing component	0.0 (10)	−0.2 (7)	−0.8 (15)
Cre14.g615400	*THB1*	Truncated hemoglobin	**0.7 (1142)**	0 (1142)	**0.5 (1052)**
Cre14.g615350	*THB2*	Truncated hemoglobin	**−0.6 (133)**	−0.3 (342)	**−1.4 (57)**
Cre03.g154350	*COX2a*	Cytochrome *c* oxidase subunit II	−0.1 (945)	−0.2 (534)	−0.4 (420)
Cre04.g221700	*COX3*	Cytochrome *c* oxidase subunit III	−0.1 (1583)	−0.1 (920)	**−0.5 (921)**
Cre03.g157700	*COX5c*	Cytochrome *c* oxidase subunit	−0.1 (1445)	−0.2 (824)	−0.3 (518)
Cre06.g304350	*MT-CO1*	Mitochondrial cytochrome *c* oxidase subunit 1	0.0 (2)	−0.3 (2)	0.4 (0)
Cre01.g049500	*COX2b*	Cytochrome *c* oxidase subunit	−0.1 (3801)	−0.2 (2409)	0.0 (1482)
Cre01.g055550	*COX11*	Involved in the insertion of copper into the CuB center of subunit Cox1p	−0.1 (2)	0.0 (1)	0.4 (3)
Cre12.g537450	*COX13*	Cytochrome *c* oxidase subunit 10	−0.1 (2219)	−0.1 (1462)	−0.2 (742)
Cre02.g082700	*COX15*	Cytochrome *c* oxidase assembly factor	0.2 (2)	0.0 (0)	0.5 (7)
Cre03.g165400	*COX16*	Cytochrome *c* oxidase assembly factor	0.0 (2)	0.0 (2)	−0.1 (5)
Cre05.g232850	*COX17*	Involved in the delivery of copper to mitochondria	0.2 (8)	−0.1 (5)	1.2 (7)
Cre16.g690200	*COX18*	Cytochrome *c* oxidase assembly factor	0.2 (4)	0.1 (4)	0.0 (4)
Cre10.g454550	*COX19*	Cytochrome *c* oxidase assembly factor	0.2 (21)	0.2 (8)	−0.1 (6)
Cre01.g069107	*COX191*	Involved in cytochrome *c* oxidase expression	0.1 (3)	0.0 (9)	0.4 (19)
Cre03.g213425	*COX23*	Cytochrome *c* oxidase assembly factor involved in copper homeostasis	−0.2 (49)	−0.4 (37)	0.0 (51)
Cre16.g69185	*COX90*	Cytochrome *c* oxidase subunit	−0.1 (910)	−0.2 (465)	0.0 (473)
Cre09.g395950	*AOX1*	Alternative oxidase	**0.5 (1050)**	−0.5 (575)	**0.5 (736)**
Cre03.g169550	*AOX2*	Alternative oxidase	−0.2 (17)	0.4 (6)	**1.4 (14)**

Log_2 fold expression change values shown in bold are significant based on a Benjamini–Hochberg adjusted *P*-value < 0.05.

1996; Hinnebusch, 1997; Roy *et al.*, 2010; Pakos-zebrucka *et al.*, 2016). The activation of these pathways possibly explains the large amount of differential expression we observed in our transcriptomic comparisons, and this complicates the identification of other genes potentially involved in this process. A detailed analysis of the RNA-seq study is currently being carried out to fully characterize the molecular responses to NO_2^- loading.

DISCUSSION

Biological implications

This study showed that axenic *C. reinhardtii* supplied with NO_2^- can synthesize N_2O under oxia via the reduction of NO_2^- to NO followed by the reduction of NO to N_2O. The reduction of NO_2^- to NO was initially catalyzed by the dual NR:NOFNiR enzyme system, but this activity rapidly ceased and was later substituted by the reduction of NO_2^- to NO by COX. Under physiological conditions, NO_3^- is the main source of nitrogen and the intracellular concentration of NO_2^- is low. The reduction of NO_3^- to NO_2^- by NR therefore provides both the nitrogen source for growth and, under specific circumstances, the substrate for NO biosynthesis by the dual NR:NOFNiR system (Chamizo-Ampudia *et al.*, 2016). Moderate production of NO would be expected to take place at moderate cytosolic NO_2^- concentrations and, under such conditions, the dioxygenase activity of THB1 could efficiently modulate the intracellular concentrations of NO, NO_2^- and NO_3^- (Sanz-Luque *et al.*, 2015b). In contrast, significant exposure to high intracellular concentrations of NO_2^- (as in this work) appears to cause COX to reduce significant amounts of NO_2^- to NO, as indicated in the model proposed in Figure 3. Both NO_2^- and NO are known to generate harmful products (Faure *et al.*, 1991; Beckman and Koppenol, 1996) and cells have developed mechanisms to prevent the intracellular accumulation of these molecules, such as the oxidation of NO into NO_3^- by THBs (Sanz-Luque *et al.*, 2015b) and the excretion of NO_2^- (Faure *et al.*, 1991; Navarro *et al.*, 2000). The reduction of NO_2^- to N_2O via NO could therefore provide algal cells that periodically experience exposure to anoxia or hypoxia (e.g. wastewater stabilization ponds) with a means to detoxify NO_2^- and NO. This activity maybe the fortuitous consequence of COX activity under oxia, or it may provide additional detoxification capacity in microalgae (i.e. NOR would be acting as a security valve eliminating NO). Interestingly, the yield of algal N_2O synthesis was lower under oxia (0.001–0.03% g N-N_2O synthesized per g N-input after 24 h

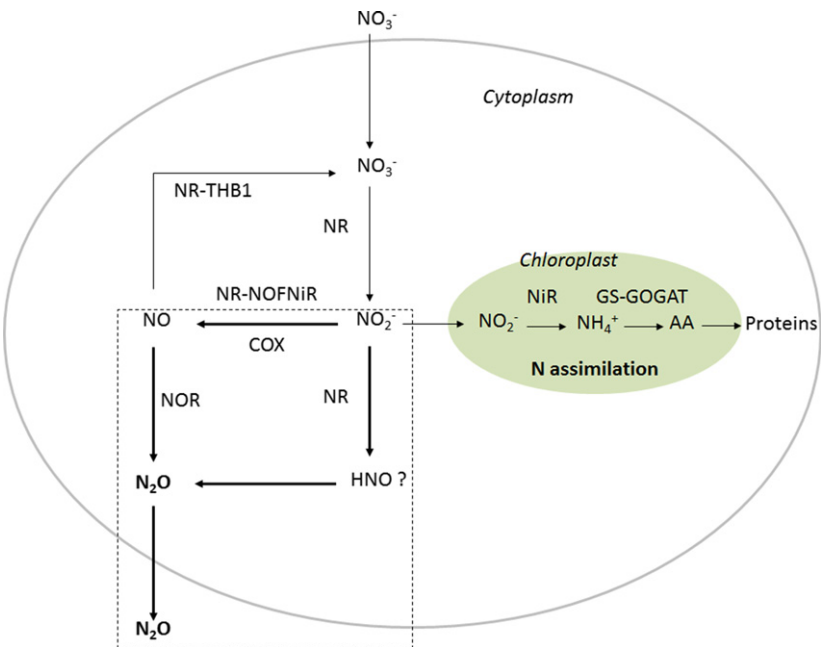

Figure 3. Proposed N_2O pathway in *Chlamydomonas reinhardtii*.
Abbreviations/symbols: NO_3^-, nitrate; NO_2^-, nitrite; NO, nitric oxide; HNO, nitroxyl; N_2O, nitrous oxide; NR, nitrate reductase; NiR, nitrite reductase; NR-NOFNiR, dual enzyme system NO-forming nitrite reductase; COX, cytochrome *c* oxidase; THB1, truncated hemoglobin; NH_4^+, ammonium; AA, amino acid; GS-GOGAT, glutamine synthetase–glutamine oxoglutarate amino transferase. [Colour figure can be viewed at wileyonlinelibrary.com]

of incubation in the dark for strain 6145c) than under anoxia (0.8% g N-N_2O synthesized per g N-input after 24 h of incubation in the dark for strain 6145c), suggesting the existence of a competitive NO metabolism under oxia (e.g. NO_x generation; Lamattina *et al.*, 2003). The low yield of N_2O synthesis under oxia also probably explains why the reduction of NO to N_2O has seldom been reported in past studies focusing on nitrogen metabolism in algae. Clearly, our understanding of the function and regulation of the enzymes and substrates involved in N_2O synthesis in microalgae remains limited. Nevertheless, the evidence presented here (such as the ability of *C. reinhardtii* to reduce NO to N_2O under oxia) provides new advances in algal biology in light of the importance of nitrate assimilation and NO metabolism in these organisms.

Environmental implications

As discussed above, the synthesis of N_2O under physiological conditions is linked to assimilation of NO_3^- in *C. reinhardtii* in a mechanism similar to the fungal N_2O denitrification pathway (Shoun *et al.*, 2012), with the significant difference that the microalgae carry out N_2O synthesis under oxia. This finding has broad implications, because the pathway of NO_3^- assimilation is conserved among microalgae (Sanz-Luque *et al.*, 2015a) and its regulation involves NO (de Montaigu *et al.*, 2010; Sanz-Luque *et al.*, 2013, 2015a). Moreover, several microalgal species have the ability to synthesize NO (Mallick *et al.*, 1999; Tischner *et al.*, 2004; Kim *et al.*, 2008; Kumar *et al.*, 2015) and/or N_2O (Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse *et al.*, 2013; Kamp *et al.*, 2013; Alcántara *et al.*, 2015), and *Chlorella variabilis* harbors a close homolog of the *Chlamydomonas NOR* gene (Guieysse *et al.*, 2013). These observations suggest that numerous species of microalgae could be capable of N_2O synthesis, which in turn could explain why correlations between primary productivity and N_2O emission rate have been reported for decades (Pierotti and Rasmussen, 1980; Outdot *et al.*, 1990; Mengis *et al.*, 1997; Wang *et al.*, 2006), even under very low exogenous NO_2^- concentrations (probably due to the intracellular generation of NO_2^- during the assimilation of NO_3^-; Pierotti and Rasmussen, 1980). Because microalgae are ubiquitous in the environment and often associated with anthropogenic pollution (e.g. algal blooms), the potential global significance of biosynthesis of N_2O by microalgae needs to be assessed. Fortunately, the evidence that mandates a broader recognition of the potential issue also provides guidance for mitigation strategies: distinct differences in species' synthetic abilities (Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse *et al.*, 2013; Kamp *et al.*, 2013) and the kinetics of microalgal N_2O emissions have been reported as, for example, nitrite-laden *C. vulgaris* was previously shown to constantly produce N_2O over 48 h of incubation in the dark (Guieysse *et al.*, 2013). These divergences indicate differences in pathways and regulatory mechanisms that, in turn, could be exploited to reduce N_2O emissions during algal cultivation.

EXPERIMENTAL PROCEDURES

Strains selection and maintenance

The wild type and mutant *C. reinhardtii* strains 6145c, 704, 409, 217, 112, 530, 124− and 125+ (Table S5) were used in this research. The amiCYP55 strain was created from strain 704 via

transformation with the vector pChlamiRNA3-CYP55 by following the protocol described by Molnar *et al.* (2009). Specific primers for artificial miRNA were amiForCYP55 and amiRevCYP55 (Appendix S3).

All strains were maintained on sterilized solid medium containing 15 g L^{-1} of agar agar in buffered TRIS–acetate–phosphate (TAP) medium (Harris, 2009), i.e. using NO_3^- as the N source (0.75 mM), and cultivated in TAP medium. The NR- and NiR-deficient mutants were cultivated in similar media where NH_4^+ was substituted for NO_3^- as the N source because these strains were unable to grow on NO_3^- and NO_2^-. Each week, 250-ml Erlenmeyer-flasks (E-flasks) were filled with 125 ml of autoclaved medium and inoculated with a single colony of *C. reinhardtii*. The microalgae were cultivated in a Minitron incubator (Infors HT, http://www.infors-ht.com/) at 25 ± 1°C under continuous agitation (180 rpm) and illumination [21 W of photosynthetically active radiation (PAR) m^{-2} at the culture surface, using five 15-W Sylvania GRO-LUX coolwhite tubes; https://www.sylvania.com/], and under an atmosphere of 2% (vol.) CO_2 in air to prevent CO_2 limitation. The E-flasks were closed with cotton tops and autoclaved with the medium prior to inoculation.

Nitrous oxide synthesis bioassays

N_2O production was quantified using gas chromatography as described in Guieysse *et al.* (2013) and the results presented herein show the total amount of N_2O produced in the flasks assuming equilibrium between aqueous and gaseous N_2O concentrations (considering N_2O losses and pressure changes; Appendix S4). Biomass concentration was quantified as cell dry weight (DW) using the protocol described by Béchet *et al.* (2015), while aqueous NO_3^- and NO_2^- concentrations were quantified using ionic chromatography, as described by Alcántara *et al.* (2015). Purity of the cultures was assessed by extracting and amplifying DNA using the primers and methods described by Guieysse *et al.* (2013). To provide algae cultures free of extracellular N, 25–50 ml of 5.5–10-day-old axenic *C. reinhardtii* cultures (described above) were centrifuged at 2900 ***g*** for 3.5 min, and the cell pellets were re-suspended in N-free and acetate-free TAP medium to reach a concentration of 0.25 g algae L^{-1} to serve as inocula in subsequent assays. Glass flasks were filled with N-free algal suspension, sealed with rubber septa and incubated for 24–48 h at 25 ± 1°C under continuous agitation (180 rpm) and either under continuous illumination (21 W PAR m^{-2}) or in the dark. The following experiments were thus conducted (see Tables S1 and S2 and corresponding results for further details):

1 *Chalmydomonas reinhardtii* 6145c cultures were supplied with different N sources (10 mM NO_2^-, NO_3^- or NH_4^+) or NO_2^- at various concentrations (2–24 mM).
2 Cultures of various *C. reinhardtii* strains were supplied with 10 mM NO_2^- in the dark.
3 Cultures of *C. reinhardtii* 6145c were pre-cultivated in modified TAP where molybdate (MoO_2^{4-}) was replaced by orthotungstate (WO_2^{4-}) to inhibit NR.
4 *Chlamydomonas reinhardtii* 6145c cultures were supplied with 10 mM of the NOS substrate L-arginine (≥ 98%; Sigma-Aldrich, http://www.sigmaaldrich.com/) and/or the NOS-inhibitor L-NNA (≥ 98%; Sigma-Aldrich).
5 Cultures of *C. reinhardtii* 6145c and *C. reinhardtii* NR mutant 2929 were supplied with cyanide (KCN ≥ 97%; Sigma-Aldrich) at 2 mM as a COX inhibitor.
6 Cultures of *C. reinhardtii* NiR mutant M3 and M4 were supplied with 10 mM NO_2^- or NO_3^- in the dark or under illumination.
7 Cultures of *C. reinhardtii* 704 and the NOR knockdown mutant amiCYP55 were supplied with 10 mM NO_2^- in the dark.

Over the duration of the study, a positive control (cultures of wild type 6145c supplied with 10 mM nitrite in the dark) was repeated 40 times (Figure 1). The 'background' N_2O level initially found in the flask was experimentally estimated to 1.45 ± 0.18 nmol (average ± SE; $n = 56$). Based on this value and the standard error of 57% recorded at $t = 0.25$ h (Figure 1), the limit of quantification was estimated to 2.9 nmol in the flasks (i.e. 200% of the background value). When N_2O production was higher than this threshold, specific production values (nmol g DW^{-1}) were calculated as the amount of N_2O produced a time t (nmole) minus the background level (1.45 nmole), divided by the initial cell concentration (g DW L^{-1}).

RNA sequencing assays

Aliquots of *C. reinhardtii* 6145c microalgal suspensions were withdrawn and poured into serum flasks incubated in the dark (centrifugation was avoided to prevent mechanical stress). Six flasks were thus incubated at constant temperature (25 ± 1°C) and agitation (180 rpm) for 1 h before NO_2^- (10 mM) was injected into three of the flasks, henceforth referred to as the 'treatments', and the remaining flasks were used as controls. Based on the N_2O kinetics recorded in the dark (see Results), gas and liquid samples were withdrawn from each flask after 1.25 (t1, 15 min after NO_2^- supply), 4 (t2, 3 h after NO_2^- supply) and 25 h (t3, 24 h after NO_2^- supply) of incubation to quantify N_2O and extract RNA (Appendix S5). This protocol was repeated three times to generate biological replicates for the RNA-seq experiment. The amount of N_2O recorded for each time point between triplicates over the three repeated experiments never exceeded 14% variability (for each time point $n = 9$, $P = 0.05$).

RNA was extracted using a plant NucleoSpin RNA kit (Macherey-Nagel, http://www.mn-net.com/). The presence of high-quality RNA was confirmed in each sample using an Agilent 2100 Bioanalyzer System (Agilent Technologies, http://www.agilent.com/). Illumina TruSeq stranded mRNA libraries were synthesized from poly-A purified RNA and the resulting libraries were sequenced using an Illumina HiSeq2500 machine (Otago Genome and Bioinformatics Facility, http://www.otago.ac.nz/genomics/). Adaptor sequences were removed from the 125-bp paired end of resulting reads using the software FASTQMCF (Aronesty, 2013) before quality trimming (with $P = 0.01$) using SolexaQA++ v.3.3 (Cox *et al.*, 2010). The reads were aligned to the *C. reinhardtii* v.5.5 genome model (Merchant *et al.*, 2007) using TOPHAT v.2.1.0 (Kim *et al.*, 2013). Read counts were generated from the alignments using htseq-count (Anders *et al.*, 2015) based on the *C. reinhardtii* v.5.5 genome model. Finally, differential expression analysis was performed on the dataset using the R-package DESeq2 (Love *et al.*, 2014) as described in the package vignette. The RNA-seq data have been deposited in the SRA archive under the number GSE90609.

Nitric oxide detection

The fluorescent dye sensitive to NO oxidation, DAF-FM diacetate (10 μM), was added to microalgal suspensions that were then incubated for 0.5–1 h in serum flasks (25°C, constant agitation at 180 rpm). Aliquots (5 ml) of these suspensions were withdrawn, centrifuged, re-suspended in the same volume of N-free medium and supplied with 10 mM NO_2^- in the darkness. The cell suspensions (200 μl) were then observed using a fluorescence microscope (Olympus BX51 microscope, http://www.olympus-ims.com/) equipped with a U-MWIBA2 filter block (excitation 460–490 nm,

emission 510–550 nm) (Chang *et al*., 2013). The specificity of DAF-FM for NO or HNO was tested using diethylamine NONOate (DEA NONOate ≥98%, Sigma-Aldrich) and Angeli's salt (HNO donor ≥99%; Sigma-Aldrich), respectively.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. N_2O synthesis is correlated to biomass concentration in *Chlamydomonas reinhardtii*.

Figure S2. Kinetics of N_2O synthesis by *Chlamydomonas reinhardtii* 6145c supplied with nitrite.

Table S1. N_2O synthesis by *Chlamydomonas reinhardtii* 6145c under various conditions.

Table S2. N_2O synthesis from various *Chlamydomonas reinhardtii* strains supplied with NO_2^- and incubated in the dark.

Table S3. N_2O synthesis of non- or tungstate-treated cells of *Chlamydomonas reinhardtii* 6145c cultures supplied with NO_2^- and incubated in the dark.

Table S4. Log_2 fold expression changes (Log2FC) between T3 control and T3 treatment for the 19 annotated eukaryotic initiation factor (EIF) genes. Numbers in parenthesis represent mean normalized counts.

Table S5. Wild-type and mutant strains used during this study.

Appendix S1. Polymerase chain reaction analysis.

Appendix S2. NO generation during N_2O synthesis in *Chlamydomonas reinhardtii*.

Appendix S3. NOR amistrains construction.

Appendix S4. N_2O quantification.

Appendix S5. RNA sequencing optimization and results summary.

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