



Article

Nitrous Oxide Emissions from Nitrite Are Highly Dependent on Nitrate Reductase in the Microalga *Chlamydomonas reinhardtii*

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Abstract: Nitrous oxide (N₂O) is a powerful greenhouse gas and an ozone-depleting compound whose synthesis and release have traditionally been ascribed to bacteria and fungi. Although plants and microalgae have been proposed as N₂O producers in recent decades, the proteins involved in this process have been only recently unveiled. In the green microalga *Chlamydomonas reinhardtii*, flavodiiron proteins (FLVs) and cytochrome P450 (CYP55) are two nitric oxide (NO) reductases responsible for N₂O synthesis in the chloroplast and mitochondria, respectively. However, the molecular mechanisms feeding these NO reductases are unknown. In this work, we use cavity ring-down spectroscopy to monitor N₂O and CO₂ in cultures of nitrite reductase mutants, which cannot grow on nitrate or nitrite and exhibit enhanced N₂O emissions. We show that these mutants constitute a very useful tool to study the rates and kinetics of N₂O release under different conditions and the metabolism of this greenhouse gas. Our results indicate that N₂O production, which was higher in the light than in the dark, requires nitrate reductase as the major provider of NO as substrate. Finally, we show that the presence of nitrate reductase impacts CO₂ emissions in both light and dark conditions, and we discuss the role of NO in the balance between CO₂ fixation and release.

Keywords: nitrous oxide emission; carbon dioxide emission; nitrate reductase; nitrite reductase mutants; nitric oxide; *Chlamydomonas*



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1. Introduction

Nitrous oxide (N₂O) is a greenhouse gas ~300-fold more potent than CO₂ and considered the dominant ozone-depleting chemical emitted in the 21st century [1–5]. In 2020, the atmospheric N₂O reached 333.2 ppb, which constitutes 123% of the pre-industrial (before 1750) levels, with the fastest-growing rate occurring in the past five decades [6–8]. N₂O emissions are released to the atmosphere from natural (~60%) and anthropogenic sources (~40%), including oceans, soils, biomass burning, fertilizers, and several industrial activities. N₂O emissions derived from human activities are dominated by nitrogen additions to crop plants [6,8]. In modern agriculture, the abundant supply of nitrogen fertilizers leads to excess nitrogen in the soil, and non-assimilated nitrogen can be emitted as N₂O to the atmosphere or lost as runoffs into aquatic ecosystems, causing their eutrophication [9,10]. Nitrification and denitrification are two well-documented biochemical processes that control N₂O emissions in terrestrial and aquatic ecosystems and are regulated by biological and environmental factors [8,11].

Bacteria and fungi are widely recognized as N₂O producers by the scientific community [1,11–14], but recently, plants and algae have also emerged as N₂O emitters. In the late 1970s, Hahn and Junge already hypothesized that phytoplankton and plants could release N₂O in the presence of nitrate (NO₃⁻) and nitrite (NO₂⁻) [15]. Several years later, this was demonstrated in microalgae [16,17] and plant leaves during photosynthesis [18–22]. Despite this, the intergovernmental agencies have not yet considered N₂O emissions by plants

and algae in the global budget [6,23]. Thus, understanding the molecular mechanisms associated with N_2O synthesis and their regulation under different environmental conditions is critical to assessing the actual contribution of plants and microalgae to atmospheric N_2O emissions.

The molecular players for N_2O synthesis are just starting to be studied in microalgae. *Chlamydomonas reinhardtii* (hereafter *Chlamydomonas*) is a unicellular, biflagellate, and green alga widely used as a model organism due to the numerous tools available to perform genetic and metabolic studies and its suitability for biotechnological applications [24,25]. Recently, two works have identified the flavodiiron proteins (FLVs) and cytochrome P450 (CYP55) as NO reductases (NORs) in *Chlamydomonas* [26,27]. The CrFLVs belong to a singular family of O_2 and NO reductases that are ubiquitous in oxygenic photoautotrophs, including cyanobacteria, the rhizarian *Paulinella chromatophora*, green algae, mosses, lycophytes, and gymnosperms, but are absent in angiosperms [28,29]. The *Chlamydomonas* CYP55 is a cytochrome p450 NO reductase closely related to fungal p450 nor members, which are considered biomarkers for N_2O production [14]. In *Chlamydomonas*, CYP55 and FLVs are proposed to be responsible for N_2O production in dark and light conditions, respectively [26,27]. In addition, light and dark N_2O emissions have been studied in different algal species and correlated with the presence of FLV and CYP55 genes in their genomes; algal species having only FLV (*Tetraselmis subcordiformis* and *Coccomyxa subellipsoidea*) seem to synthesize N_2O in the light but not in the dark. In contrast, algae lacking both CYP55 and FLV (*Galdieria sulphuraria*, *Porphyridium purpureum*, *Nannochloropsis gaditana*, *Phaeodactylum tricorutum*, and *Thalassiosira pseudonana*) do not reduce NO to N_2O , whereas those algal species containing both genes in their genome (*Chlamydomonas reinhardtii* and *Chlorella variabilis*) exhibit N_2O emissions in light and dark conditions [27].

Both FLV and CYP55 require NO as substrate in order to synthesize N_2O . Several NO synthesis pathways have been proposed in photosynthetic organisms. The most characterized mechanism entails the reduction of NO_2^- to NO in a process mediated by the cytosolic NO_3^- reductase (NR) in microalgae and plants [17,30–33]. These NRs are typical eukaryotic and nitrogen assimilatory enzymes that form homodimers containing FAD, heme b_{557} , and molybdenum cofactor as prosthetic groups [34,35]. These cofactors allow the sequential electron transfer from NAD(P)H to the molybdenum cofactor, the final electron donor for NO_3^- reduction. The formed NO_2^- is assimilated in the chloroplast by the following actions of NO_2^- reductase (NiR) and glutamine synthetase (GS) [36]. In *Chlamydomonas*, the NR-dependent NO synthesis requires the protein partner ARC (aka. NOFNiR), a molybdoenzyme that accepts electrons from the NR heme group to reduce NO_2^- to NO [35,37]. Moreover, the *Chlamydomonas* NR can also donate electrons to the truncated hemoglobin THB1 to scavenge NO and produce NO_3^- by deoxygenation [38,39]. Thus, NR has a central role comprising the recently named NO_3^- - NO_3^- cycle [29].

In this work, we use previously isolated *Chlamydomonas* NO_2^- reductase mutants, which cannot assimilate NO_2^- , as a valuable tool to study NO_2^- dissimilation to N_2O . We show that the NR–ARC complex strongly contributes to N_2O emissions in cells incubated in the presence of NO_2^- . Our results corroborate NR function in synthesizing NO and suggest that this cytosolic enzyme is the primary NO source for N_2O synthesis, carried out in the chloroplast and mitochondria. Furthermore, we show that excess NO_2^- and NR-dependent NO impacts CO_2 emissions under our experimental conditions, and we briefly discuss the impact on CO_2 fixation and release.

2. Results

2.1. Nitrite Reductase Mutants (*nii1*) Cannot Use NO_3^-/NO_2^- for Growth but Can Reduce Them to N_2O

Chlamydomonas nii1 mutants (G1, M3, and M4) cannot reduce NO_2^- to ammonium (NH_4^+) and, therefore, do not grow in media containing either NO_3^- or NO_2^- as the sole nitrogen (N) source (Figure 1a). The G1 strain is a deletion mutant that lacks the entire cluster of the NO_3^- assimilation genes. This cluster, located in chromosome 9, contains

the genes that encode NO_3^- and NO_2^- reductases (*NIA1* and *NIII1*, respectively) and the high-affinity $\text{NO}_3^-/\text{NO}_2^-$ transport components (*NRT2.1*, *NRT2.2*, and *NAR2*) [40,41]. By genetic crosses, either the *NIA1* and *NRT2.1-NAR2* sets of genes or only *NIA1* were transferred to the G1 strain, generating the M3 and M4 mutants, respectively (see [41] for more details). As previously mentioned, *Chlamydomonas* cells can reduce NO_2^- to NO [37] and NO to N_2O [26,27]; therefore, we used these mutants as model organisms to study this process in microalgae. First, we studied NO_2^- evolution in the M3 strain. NH_4^+ -grown cells were washed and transferred to fresh media containing 0.1 and 1 mM NO_3^- or NO_2^- , and NO_2^- concentration in the medium was determined at different time points (Figure 1b–d). Cells exposed to 0.1 mM NO_3^- showed a stoichiometric excretion of NO_2^- after 4 h (Figure 1b), as previously reported [41]. Subsequently, extracellular NO_2^- concentration slowly decreased, being completely exhausted from the medium after 24 h. Similar depletion rates and kinetics were observed when 0.1 mM NO_2^- was added instead, but a lag of 4–6 h was observed before the concentration started to decrease.

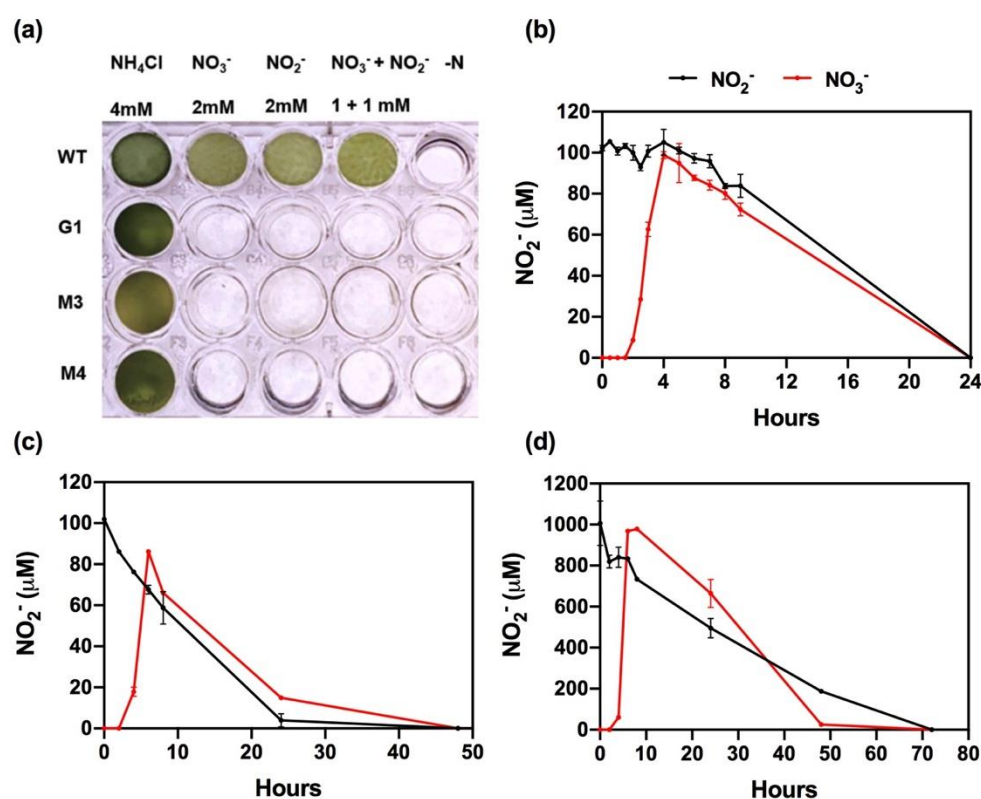


Figure 1. M3 strain cannot grow in NO_3^- or NO_2^- but metabolizes them. (a) Growth test of the *niii1* mutants (G1, M3, and M4) and the WT (6145c) strain on the indicated N sources. Plate wells were inoculated with 0.1×10^6 cells ml^{-1} and cultured for 7 days. (b–d) Extracellular NO_2^- quantification in cultures incubated in the presence of either NO_3^- (red line) or NO_2^- (black line). NH_4^+ -grown cells were washed and transferred to NO_3^- - or NO_2^- -containing media at 0.1 mM in non-sealed bottles (b), 0.1 mM in sealed bottles (c), or 1 mM in sealed bottles (d). Error bars represent $\pm\text{SD}$, $n \geq 3$.

The same experiment was performed in sealed bottles, in which N_2O emission would be retained and could be quantified. Under these conditions, similar rates of accumulation and depletion of NO_2^- were observed (Figure 1c). However, NO_2^- depletion was induced faster than in non-sealed cultures (2 h vs. 6–8 h); therefore, NO_2^- excretion after NO_3^- reduction was not stoichiometric and reached only a concentration of 86 μM . Furthermore, as observed in non-sealed bottles, NO_2^- was exhausted before 24 h. A similar pattern was observed when cells were exposed to 1 mM NO_3^- or NO_2^- , although total depletion required longer incubations (Figure 1d).

To monitor N_2O emissions in the headspace of the cultures, we used Cavity Ring-Down Spectroscopy (CRDS) (see Material and Methods), which allows continuous N_2O measurements. The M3 cultures produced N_2O in a NO_2^- concentration-dependent manner and from both NO_3^- and NO_2^- (Figure 2). When the cells were incubated with 0.1 mM NO_2^- , N_2O started to accumulate after 2–3 h with a rate of 3.3 ppm/h and plateaued after 21 h, reaching a final concentration of 62 ppm after 24 h (Figure 2a). In the presence of 10 mM NO_2^- , although N_2O accumulation was also detected after 2 h of induction, the gas was released at ~15-fold higher rate (51 ppm/h) than in 0.1 mM NO_2^- , and no saturation was observed after 24 h when N_2O concentration was 864 ppm (Figure 2b). When the cells were incubated with 10 mM NO_3^- (Figure 2c), N_2O release was delayed as expected due to the requirement to reduce NO_3^- to NO_2^- , but the production rate was boosted after 14 h (92 ppm/h), almost doubling that observed in the cells supplemented with NO_2^- . As expected, cells incubated in N-free media did not emit detectable amounts of N_2O (Supplementary Table S1).

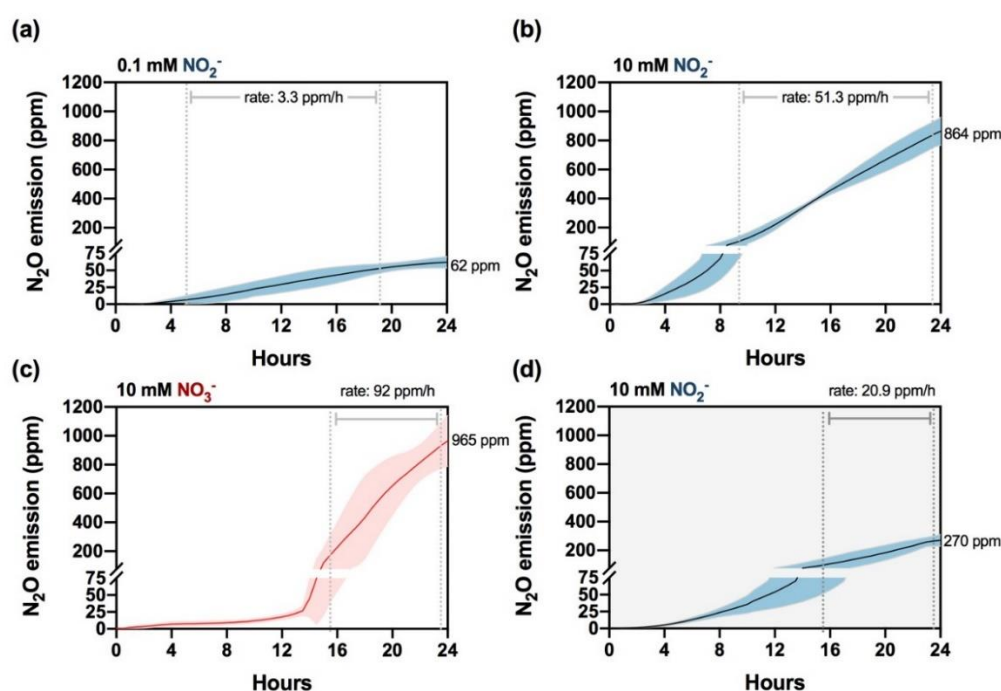


Figure 2. Kinetics of N_2O emission in the M3 strain in the presence of NO_3^- and NO_2^- . NH_4^+ -grown cells were washed and transferred to NO_2^- -containing media in sealed bottles at 0.1 mM (a) and 10 mM (b) in the light. Cells were also incubated in the presence of 10 mM NO_3^- under illumination (c) and 10 mM NO_2^- in the dark (d). Each data line represents an average of three biological replicates, and the colored area corresponds to \pm SD.

In *Chlamydomonas*, N_2O production may involve light-dependent and light-independent pathways [26,27]; therefore, we additionally studied N_2O production in cells incubated with NO_2^- in the dark. In this condition, total N_2O accumulation (270 ppm) and production rate (20 ppm/h) were both strongly reduced (Figure 2d), highlighting the importance of light in this process in the M3 strain.

In these experiments, the earliest N_2O emissions were achieved during incubation with 10 mM NO_2^- , a concentration previously used by Plouviez and collaborators [26]; therefore, we set this concentration for further studies. Moreover, the kinetics and high rates of N_2O production observed in the M3 strain led us to use this mutant as a model to study the role of other players involved in the reduction of NO_2^- to N_2O .

2.2. Nitrate Reductase Is the Primary NO Source Involved in N₂O Emissions from NO₂⁻ in the *niil* Mutants

The enzymes responsible for NO reduction to N₂O are located in the chloroplast (FLV) [27] and mitochondria (CYP55) [42] in *Chlamydomonas*. However, the NO sources that feed these reactions are not well understood. In plants and algae, the cytosolic NR seems to be the main enzyme involved in NO synthesis from NO₂⁻ [30,31,43], although other pathways for NO synthesis have been proposed in chloroplasts [44] and mitochondria [26]. Here, we set out to elucidate the possible role of the NR-ARC complex as a NO source for the synthesis of N₂O. First, N₂O emissions were compared in the *niil* mutants G1 (NR⁻) and M4 (NR⁺) (Figure 3a). The lack of NR led to a dramatic reduction in N₂O accumulation after 24 h in both light (31 ppm) and dark (77 ppm) conditions, while the M4 strain behaved similarly to the M3 mutant, reaching 904 ppm after 24 h in the light and 395 ppm in the dark (Figure 3a,b).

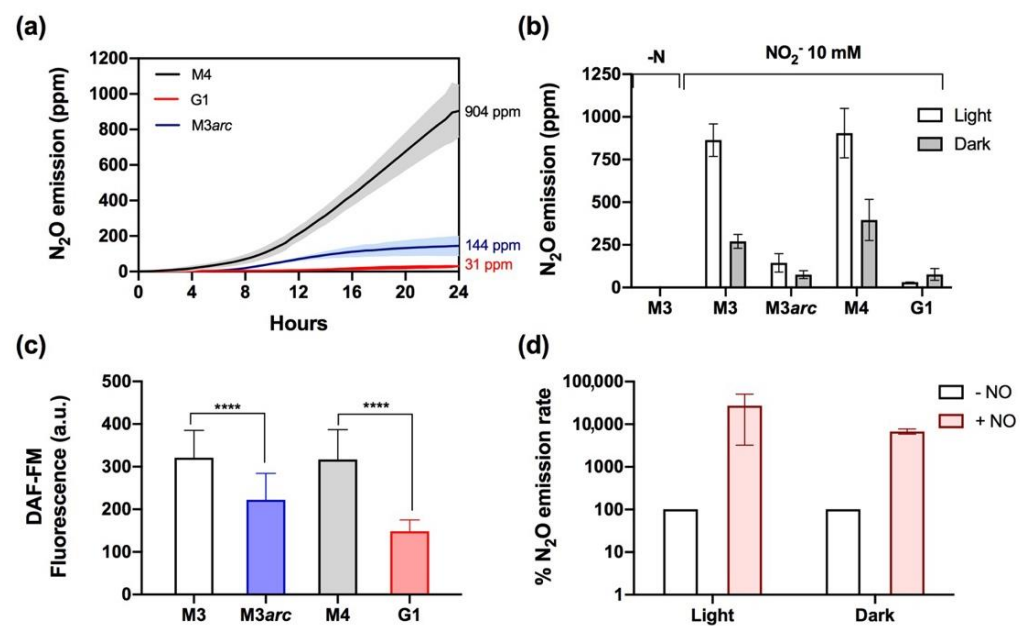


Figure 3. N₂O emission by the *niil* mutants mainly depends on the functionality of the NR-ARC complex to reduce NO₂⁻ to NO. (a) N₂O emissions by M3, G1, and M3arc strains in media containing 10 mM NO₂⁻ in the light. (b) Effect of light and dark on total N₂O emission in the *niil* mutants in the presence of 10 mM NO₂⁻ after 24 h. (c) DAF-FM fluorescence in the *niil* mutants after 24 h incubation in 10 mM NO₂⁻ in the light. (d) N₂O emission rates after adding 40 μM DEA-NONOate to G1 cultures in light and dark conditions. The initial rates (100%) correspond to 0.66 ppm/h (light) and 4.42 ppm/h (dark). Each data line in (a) represents an average of three biological replicates, and the colored area corresponds to ±SD. Error bars represent ±SD, $n \geq 3$. Student's *t* test was performed. **** $p \leq 0.0001$.

Secondly, to study the potential role of ARC in N₂O emission, we transferred the *arc* mutation to the M3 background by genetic crossing. This new strain (M3arc) showed a significant reduction in N₂O accumulation after 24 h in both light (~144 ppm) and dark (~69 ppm) conditions (Figure 3a,b), suggesting that the NR-ARC complex is responsible for the synthesis of most of the NO that sustains N₂O production. To confirm this idea, NO levels were measured in these four strains (M3, M3arc, M4, and G1) using the DAF-FM fluorescent probe in cells incubated for 24 h in 10 mM NO₂⁻ under illumination (Figure 3c). G1 and M3arc strains exhibited a pronounced reduction in fluorescence (50% and 30%, respectively) compared to their corresponding strain of reference, M4, and M3. Our results suggest that NR-ARC is the main player in NO synthesis to feed NO reductases, but also that other NR-ARC-independent pathways should be considered.

If NR is required for N₂O production as a key NO supplier, then the exogenous addition of NO should enhance N₂O production in the NR-lacking G1 strain. To test this hypothesis, G1 cells were incubated for 20 h with 10 mM NO₂⁻ in either light or dark conditions and then were exposed to NO donor (40 μM DEA-NONOate). In both conditions, an immediate burst of N₂O emission was observed. Before NO donor addition, N₂O was produced with a rate of 0.66 ppm/h and 4.42 ppm/h in light and dark, respectively; after NO donor supplementation, the rate increased up to 131 ppm/h in light and 150 ppm/h in the dark (Figure 3d). These results suggest that the low N₂O emissions observed in the G1 strain are due to a limitation in NO synthesis.

2.3. Nitrite Impacts CO₂ Emissions through a NR-Dependent Process in the *nii1* Mutants

NO is a signal molecule that inhibits a wide variety of processes in *Chlamydomonas*, including photosynthesis [45] and mitochondrial respiration [46]. Thus, taking advantage of the CRDS analyzer's functionality to quantify CO₂, we studied CO₂ evolution to understand how NO accumulation, and indirectly N₂O emissions, might impact central metabolism in the *nii1* mutants. Under mixotrophic conditions, CO₂ emissions are mainly a result of the flux balance between CO₂ fixation (photosynthesis and Calvin–Benson–Bassham cycle) and CO₂ release by the Tricarboxylic Acid Cycle (TCA) that is fed with acetate as an exogenous carbon source, although CO₂ emissions can also be impacted by other processes such as carbon mobilization from storage compounds (i.e., starch and lipids) and, to a lesser extent, photorespiration [47–49]. Therefore, we assayed how the different *nii1* mutants were affected in CO₂ evolution.

Total CO₂ accumulation in the headspace of the cultures was monitored after 24 h of induction in the presence of 10 mM NO₂⁻ in light and dark conditions. In the dark, when cells cannot fix carbon, CO₂ emissions were higher in G1 (9024 ppm) than in the M4 and M3 strains (3658 ppm and 2852 ppm) (Figure 4a and Supplementary Table S1). The same experiment, carried out under illumination, showed the opposite effect: lower CO₂ emission in the G1 mutant (1153 ppm) than in the M4 and M3 strains (5188 ppm and 6151 ppm). Similar results were obtained for M3arc and M3 strains in the dark (M3arc accumulated more CO₂, 9169 ppm, than M3, 2852 ppm) but not in the light, where they showed almost identical CO₂ accumulation (Figure 4a). We suggest that this different phenotype in the light might be a consequence of the slightly higher NO levels observed in M3arc compared to G1 (Figure 3c), as CO₂ emission patterns in light and darkness seem to be affected by NO. To test this hypothesis, the G1 cultures were supplied with a NO donor in dark and light conditions after 20 h induction in 10 mM NO₂⁻. The NO addition led to a three-fold increase in the CO₂ emission rate in the light but not in the dark, where only a slight reduction was observed (Figure 4b). To further confirm whether NO reduces CO₂ emission in the dark, the M3 strain was treated with NO donor in N-free medium in the dark, and after a short incubation time (75 min) (Supplementary Figure S1). Before NO donor addition, the CO₂ emission rate was 242 ppm/h, but after NO donor addition, the CO₂ emission rate decreased to 88 ppm/h. Accordingly, the N₂O emission rate increased from 0 to 8 ppm/h (Supplementary Figure S1).

CO₂ emission was also studied in M3 cells under N deprivation and different NO₂⁻ concentrations in the light (Supplementary Figure S2). In N-free medium, the atmospheric CO₂ was consumed, and almost no emission was detected after 24 h. However, CO₂ was released in the presence of NO₂⁻ in a concentration-dependent manner (4718 ppm and 6152 ppm in 0.1 mM and 10 mM NO₂⁻, respectively). These data highlight the regulatory role of NO₂⁻-derived NO in CO₂ emission levels (see Discussion Section).

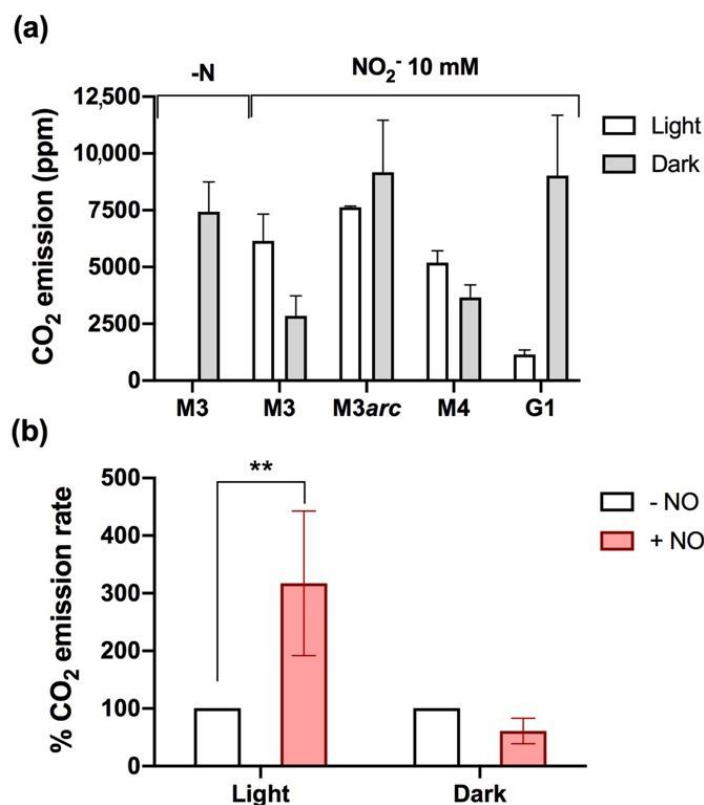


Figure 4. NR impacts CO₂ evolution in the *nii1* mutants. (a) CO₂ emission by the *nii1* mutants under light and dark incubation in 10 mM NO₂⁻ and N-free media after 24 h. (b) CO₂ emission rates after adding 40 μM DEA-NONOate to G1 cultures in light and dark conditions. The initial rates (100%) correspond to 44.28 ppm/h and 78.21 ppm/h in light and dark, respectively. Error bars represent ±SD, $n \geq 3$. Student's *t* test was performed. ** $p \leq 0.001$.

2.4. N₂O and CO₂ Emissions in the NO₃⁻/NO₂⁻ Assimilation Wild Type Strain 6145c and the *nit1nit2* Mutant CMJ030

To better understand how the NO₃⁻/NO₂⁻ assimilation pathway impacts N₂O and CO₂ emissions, we studied the accumulation of these gases in sealed cultures of the WT strain (6145c) and CMJ030, a mutant that cannot assimilate NO₃⁻ and exhibits a limited growth on NO₂⁻. By genetic crossing, we demonstrated that CMJ030 is a *nit1nit2* mutant (see Supplementary Figure S3) that lacks NR activity and also NIT2, which is the key transcriptional factor involved in the regulation of the NO₃⁻/NO₂⁻ assimilation pathway [36,40].

Both 6145c and CMJ030 strains accumulated much less N₂O than the M3 and M4 mutants; N₂O emission reached 18 ppm in 6145c and 4 ppm in CMJ030 after 24 h in the light (Figure 5a,b). After normalization using chlorophyll concentration (as 6145c cultures double their chlorophyll content after 24 h in NO₂⁻), N₂O production in 6145c was two-fold higher than in CMJ030 (Supplementary Table S1). In the dark (where no growth was observed), normalized emission increased ~five-fold (Supplementary Table S1), showing characteristic kinetics with two phases of production separated by another phase in which N₂O was not accumulated (Figure 5a,b). The lower N₂O emissions observed in the *nit1nit2* mutant further support that the NO₃⁻/NO₂⁻ assimilation pathway impacts N₂O synthesis in *Chlamydomonas*.

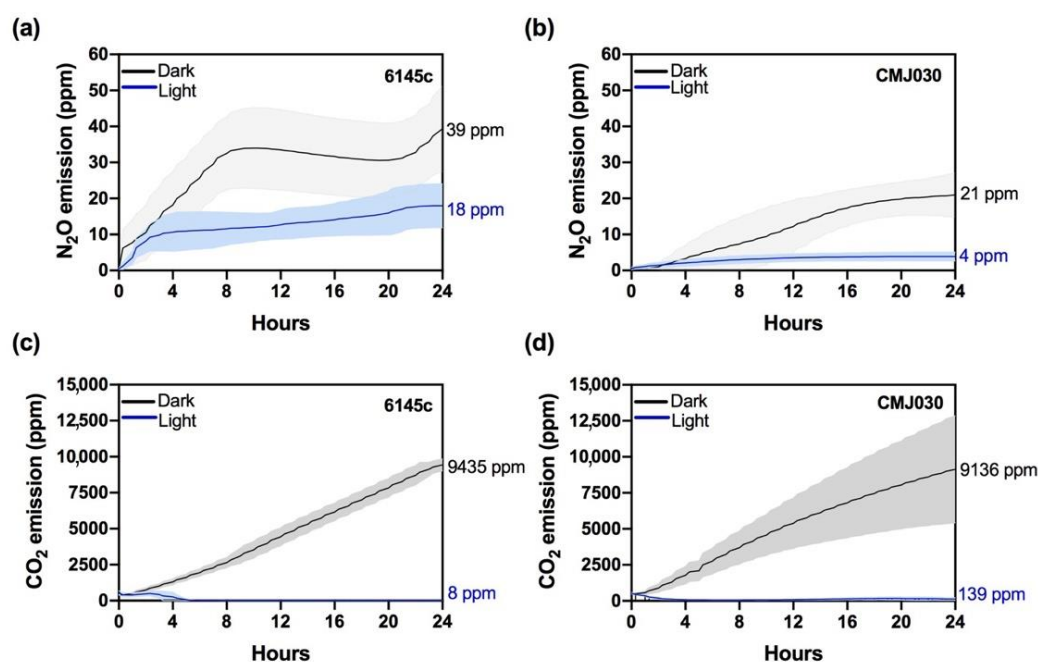


Figure 5. N₂O and CO₂ emissions in the wild-type strain 6145c and the *nit1nit2* mutant CMJ030. NH₄⁺-grown cells were washed and transferred to 10 mM NO₂⁻-containing media in sealed bottles in the light (blue line) or the dark (black line). N₂O emissions (a,b) and CO₂ (c,d) were quantified during 24 h. Each data line represents an average of three biological replicates, and the colored area corresponds to ±SD.

The low N₂O production detected in these strains seems to point out that NO is not highly accumulated. Consequently, both strains exhibited high CO₂ emissions in the dark and low CO₂ levels in light (Figure 5c,d, and Supplementary Table S1), suggesting that 10 mM NO₂⁻ is not enough to alter CO₂ evolution under our experimental conditions.

3. Discussion

Plants and algae can produce the potent greenhouse gas N₂O, which can be emitted at significant amounts into the atmosphere as a result of high inputs of NO₃⁻/NO₂⁻ [16,17,22]. Despite its potentially high environmental and ecological impact, the molecular mechanisms involved in N₂O production by photosynthetic organisms remain largely unknown, and genetic evidence supporting N₂O emissions has been only recently described in the model organism *Chlamydomonas reinhardtii* [26,27]. Recent works have documented the existence of two NO reductases, FLVs and CYP55, able to produce N₂O when the alga is supplied with NO. Most of these experiments were performed in a *Chlamydomonas nit1nit2* genetic background and demonstrated that N₂O production mostly relies on FLVs in the light and on CYP55 in the dark. Another approach by Plouviez and collaborators studied the N₂O production from NO₂⁻ by *Chlamydomonas* strains with different genetic backgrounds for NO₃⁻ assimilation. Their results showed that N₂O production by the WT strain, able to assimilate NO₃⁻, occurs from NO₂⁻ and mainly in the dark linked to CYP55. This result was supported later by Burlacot and collaborators showing that NO uptake and N₂O production in the dark were much higher when WT cells were grown with NO₃⁻ as the sole nitrogen source and reflecting the regulation of CYP55 by NO₃⁻ metabolism. Different processes have been proposed to synthesize NO from NO₂⁻, the intermediary step in N₂O production [17,35,44]. Plouviez et al., 2017 [26] suggest two phases in the *Chlamydomonas* N₂O emissions by WT in the dark, an early one involving NR (3.5 h) and a late phase involving the mitochondrial COX (24 h). Here, we present and discuss new data on the NO₂⁻-to-N₂O denitrification process in *Chlamydomonas nii1* mutants and how CO₂ emissions are affected in these strains.

When $\text{NO}_3^-/\text{NO}_2^-$ assimilation is interrupted because of the absence of NiR activity, two main conclusions are considered: (1) NR and ARC (NOFNiR) have a vast impact on N_2O emissions, and (2) this NR-dependent N_2O emission is significantly higher (4.5-fold) in the light than in the dark, a result in accord with Plouviez et al., 2017. Our results highlight that the NO synthesized by the cytosolic NR/ARC complex can diffuse to other organelles such as mitochondria and chloroplast, and this NO seems to regulate processes involved in CO_2 emissions (later discussed). Despite the importance of NR as the main NO source in the *nii1* mutants, the remaining NO and N_2O levels observed in G1 cultures point out alternative NO synthesis pathways such as that involving COX, as previously reported [26].

When $\text{NO}_3^-/\text{NO}_2^-$ assimilation is totally functional, N_2O emissions are tremendously diminished. This result reveals that N_2O emissions in *Chlamydomonas* seem to be mainly restricted to conditions in which $\text{NO}_3^-/\text{NO}_2^-$ cannot be properly assimilated and used for growth. This might support why the WT strain emits more N_2O in the dark, as cells need to acclimate to this condition and $\text{NO}_3^-/\text{NO}_2^-$ assimilation is less efficient. According to this, we could expect high N_2O emission in $\text{NO}_3^-/\text{NO}_2^-$ -rich environments depleted of other nutrients. Therefore, growth limitation in the presence of high $\text{NO}_3^-/\text{NO}_2^-$ concentrations should lead to high N_2O synthesis rates. Finally, the two phases of N_2O emission observed in dark-incubated WT cells could be attributed to NO generated by NR (first phase) and mitochondrial COX (second phase), as previously reported [17].

When $\text{NO}_3^-/\text{NO}_2^-$ assimilation is impaired (*nit1nit2* mutant), N_2O emissions are lower than in the WT. In this genetic background, neither NR nor the regulatory NIT2 proteins are functional, and NO_2^- assimilation is slow, allowing a limited growth in this N source [50]. This residual NO_2^- assimilation is enough to avoid NO_2^- dissimilation to N_2O . In addition, NIT2 also controls other steps in NO_3^- assimilation, including $\text{NO}_3^-/\text{NO}_2^-$ transporters [36,40] and NO metabolism-related proteins such as AOX1 [51], THB1 and THB2 [38,39], and probably CYP55, which increases in response to NO_3^- [29]. Moreover, a putative NO_3^- -dependent regulation of the N_2O production, mediated by NIT2, is also supported by the significant increase in the N_2O emission rate observed in M3 cells incubated in NO_3^- compared to those incubated in NO_2^- (Figure 2c).

CO_2 emissions are closely related to NO_2^- -dependent N_2O emissions. Our results show a relationship between N_2O and CO_2 emissions that will require further investigation to understand the metabolic adaptations of *Chlamydomonas* to heterotrophic and mixotrophic conditions in the presence of NO_3^- or NO_2^- . In both conditions, acetate is the main carbon source, but it is essential only in the dark to feed the TCA cycle and provide energy to the cells, releasing CO_2 [47,52,53].

This study shows that low N_2O emissions correlate with high CO_2 release in the dark and vice versa; high N_2O emissions correlate with less CO_2 release. The link between N_2O and CO_2 emissions appears to be the NO signal molecule, produced mainly by the NR/ARC complex in the *nii1* mutants. NO could inhibit acetate metabolism and CO_2 release, also supported by the slight inhibition of the CO_2 emission rate by NO donor.

In the light, we found the opposite correlation: low N_2O accumulation, due to low NO synthesis, leads to a reduced CO_2 emission and vice versa. Under illumination, NO inhibits photosynthesis [45], reducing CO_2 fixation. In fact, NO supply increased by three-fold the CO_2 emissions in the light, suggesting that CO_2 fixation is very sensitive to NO. Thus, CO_2 fixation would be more active in those strains/conditions in which low NO is synthesized (low N_2O emitted) and, therefore, lower CO_2 levels would be accumulated. The role of NO as a photosynthesis inhibitor has been described in plants and algae and has been considered a mechanism to avoid photo-damage in algae under nutrient deprivation [45,54,55]. Nitrogen- [56] or sulfur-starved [57] *Chlamydomonas* cells accumulate NO, which causes the degradation of the cytochrome b_6f complex and Rubisco by the FtsH and Clp proteases. More recently, transcriptomic analyses reported the molecular mechanisms underlying the acclimation of *Chlamydomonas* after NO supply [45]. Among

the regulated process, NO decreases photosynthesis, respiration, N availability, and induces NO scavenging (THB1, THB2, FLVB, and CYP55).

The contributions of plants and algae to the N₂O atmospheric budget are not being considered by the expert panels, even when increasing reports support their participation in this process, and the high input of nitrogen fertilizers is the primary cause [8,17,22]. Our data shed light on the mechanisms involved in the N₂O synthesis and highlight the *nit1* mutants as good models to study the molecular bases of the N₂O emission in photosynthetic organisms. Moreover, the NR role on N₂O emission raises an important link between NO₃⁻ assimilation and dissimilation, making of this enzyme a good candidate for future studies in order to acquire a better understanding on those environmental conditions that promote NO₃⁻ dissimilation over assimilation.

4. Materials and Methods

4.1. Strains and Growth Conditions

The strains used in this study are listed in Supplementary Table S1. G1 strain is a deletion mutant affected at the *NIT1* locus and lacking nitrite reductase (NiR), nitrate reductase (NR) and the high-affinity NO₃⁻/NO₂⁻ transporters (Δ (*NIII1*, *NIA1*, *NRT2.2*, *NRT2.1*, *NAR2*)). By genetic crosses, *NIA1* (the gene encoding NR) was transferred to G1 to generate the M4 strain (Δ (*NIII1*, *NIA1*, *NRT2.2*, *NRT2.1*, *NAR2*):*NIA1*). Similarly, *NIA1* plus the gene encoding the NO₃⁻/NO₂⁻ transporter *NRT2.1* were added to obtain the M3 strain (Δ (*NIII1*, *NIT1*, *NRT2.2*, *NRT2.1*, *NAR2*):*NIT1*, :(NRT2.1, NAR2)) [41]. Strain 6145c is a WT strain for NO₃⁻ assimilation and CMJ030 is a *nit1nit2* mutant. Finally, the M3arc strain (Δ (*NIII1*, *NIT1*, *NRT2.2*, *NRT2.1*, *NAR2*):*NIT1*, :(NRT2.1, NAR2), *arc*) was obtained in this work by crossing M3 (mt⁺) and LMJ.RY0402.255418 (mt⁻), where LMJ.RY0402.255418 is an insertional mutant where the *ARC* gene was interrupted with the paramomycin cassette [58] (obtained from the *Chlamydomonas* Library Project (CLiP), <https://www.chlamylibrary.org>).

All the cell cultures were performed in TAP medium (Tris, Acetate, Phosphate) [59] in a chamber (AlgaeTron AG 230, Photon System Instruments, Drásov, Czech Republic) at 25 °C, with continuous agitation (120 rpm) and illumination (light intensity 130 $\mu\text{mol photons}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$). When indicated, cell cultures were transferred to dark in the same chamber.

Cells were grown in TAP medium with NH₄⁺ as a nitrogen source (8 mM NH₄Cl) (pre-cultures). At the exponential phase of the culture, cells were harvested by centrifugation (2 min at 3000 \times g), washed twice with nitrogen-free TAP and transferred to new media containing the indicated nitrogen sources. The initial chlorophyll concentration was adjusted to 9–10 $\mu\text{g mL}^{-1}$.

For unsealed flask, Erlenmeyer flasks covered with foil paper were used. The same flasks were hermetically sealed with screw caps (sealed flasks), and a syringe was used to collect samples from the culture.

4.2. Chlorophyll, NO₂⁻, and Cell Counting Measurements

Samples of 1 mL were centrifuged at 15,000 \times g for 5 min, and the supernatant (cell-free medium) and the pellet were separately frozen at -20 °C. NO₂⁻ was quantified in the cell-free medium using the Griess reagents according to Snell and Snell (1949) [60]. For chlorophyll concentration, the pellet was resuspended in 1 mL ethanol and incubated for 3 min, at room temperature. Afterwards, the samples were centrifuged and the chlorophyll concentration in the supernatant was quantified as previously described [61]. Cell quantification of liquid cultures was determined using the Sysmex Microcellcounter F-500 cell counter.

4.3. NO Measurements

Cells cultures (25 mL) were induced in media with 10 mM NO₂⁻ during 24 h. Then, 2 μM of DAF-FM (4,5-Diaminofluorescein) was added and incubated for 1h. An amount of 200 μL of the culture was used for NO quantification in a fluorescence spectrophotometer

(Varioskan Lux, Thermo scientific, Waltham, MA USA) using OptiPlate Black Opaque 96-well Microplate (PerkinElmer, Waltham, MA USA). The excitation and emission wavelengths for the NO indicator were 485 and 515 nm, respectively. Data are represented as arbitrary fluorescence units.

4.4. Determination of N₂O and CO₂ Emissions

N₂O and CO₂ were simultaneously quantified by using a Cavity Ring-Down Spectroscopy (CRDS) analyzer (PICARRO G2508). For this purpose, we used 1 L bottles (DURAN™) that were hermetically sealed with screw caps (GL 45 with 2 or 3 connectors) both from DWK Life Sciences (Mainz, Germany). The bottles were set with 250 mL liquid culture medium and 750 mL headspace (gas phase). The CRDS analyzer was connected to the bottle through a combined inlet and outlet Teflon tubes (2.5 m in length). The outlet tube extracted the sample from the headspace of the bottle (0.3 L/min), and the inlet tube returned the sample into the gas phase of the cultures, passing the air through a 0.22 µm PVDF filter (Duaalex™-Plus; Merck, Darmstadt, Germany) to avoid culture contamination.

4.5. Genetic Crosses

Genetic crosses were performed according to [62] and the random spore plating method. Then, 100 segregants were analyzed, and several of them were chosen for further experiments.

4.6. Chemicals and Statistical Analysis

DEA-NONOate [2-(N,N-diethylamino)-diazonolate 2-oxide sodium salt] (D-184) and DAF-FM (4,5-Diaminofluorescein) (D224-1MG) were purchased from Merck (Darmstadt, Germany). For statistical analysis (Student's *t* test), PRISM software v8.4.3 (GraphPad Software, LLC, San Diego, CA, USA) was used.

Supplementary Materials: The supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms23169412/s1>.

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