










Review

# *Chlamydomonas reinhardtii*, an Algal Model in the Nitrogen Cycle

Carmen M. Bellido-Pedraza <sup>1</sup>, Victoria Calatrava <sup>1,†</sup>, Emanuel Sanz-Luque <sup>1</sup>,  
Manuel Tejada-Jiménez <sup>1</sup>, Ángel Llamas <sup>1</sup>, Maxence Plouviez <sup>2</sup>, Benoit Guieysse <sup>2</sup>,  
Emilio Fernández <sup>1,\*</sup> and Aurora Galván <sup>1,\*</sup>

<sup>1</sup> Departamento de Bioquímica y Biología Molecular, Campus de Rabanales y Campus Internacional de Excelencia Agroalimentario (CeIA3), Edif. Severo Ochoa, Universidad de Córdoba, 14071 Córdoba, Spain; b22bepec@uco.es (C.M.B.-P.); b82capom@uco.es (V.C.); q92salue@uco.es (E.S.-L.); q62tejim@uco.es (M.T.-J.); bb2llaza@uco.es (Á.L.)

<sup>2</sup> School of Food and Advanced Technology, Massey University, Private Bag, 11222 Palmerston North, New Zealand; M.Plouviez@massey.ac.nz (M.P.); B.J.Guieysse@massey.ac.nz (B.G.)

\* Correspondence: bb1feree@uco.es (E.F.); bb1gacea@uco.es (A.G.)

† Current address: Department of Plant Biology, Carnegie Institution for Science, Stanford, CA 94305, USA.

Received: 22 June 2020; Accepted: 13 July 2020; Published: 16 July 2020



**Abstract:** Nitrogen (N) is an essential constituent of all living organisms and the main limiting macronutrient. Even when dinitrogen gas is the most abundant form of N, it can only be used by fixing bacteria but is inaccessible to most organisms, algae among them. Algae preferentially use ammonium (NH<sub>4</sub><sup>+</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) for growth, and the reactions for their conversion into amino acids (N assimilation) constitute an important part of the nitrogen cycle by primary producers. Recently, it was claimed that algae are also involved in denitrification, because of the production of nitric oxide (NO), a signal molecule, which is also a substrate of NO reductases to produce nitrous oxide (N<sub>2</sub>O), a potent greenhouse gas. This review is focused on the microalga *Chlamydomonas reinhardtii* as an algal model and its participation in different reactions of the N cycle. Emphasis will be paid to new actors, such as putative genes involved in NO and N<sub>2</sub>O production and their occurrence in other algae genomes. Furthermore, algae/bacteria mutualism will be considered in terms of expanding the N cycle to ammonification and N fixation, which are based on the exchange of carbon and nitrogen between the two organisms.

**Keywords:** algae; *Chlamydomonas*; nitrogen cycle; nitric oxide; nitrous oxide

## 1. Introduction

Nitrogen (N) is an essential macronutrient that supports life in all living beings. This nutrient is an elemental constituent of biomolecules, such as nucleic acids, proteins, chlorophylls, cofactors, and signal molecules, among others.

In nature, living organisms can use different N reservoirs, and the biotic and abiotic conversions among different N forms constitute the biogeochemical N cycling. These N reservoirs and N flux in terrestrial and marine ecosystems have been recently reviewed [1]. Ammonium (NH<sub>4</sub><sup>+</sup>) bound into rocks and sediments is the largest N reservoir (1.8 × 10<sup>10</sup> Tg nitrogen). However, this NH<sub>4</sub><sup>+</sup> has a minimal impact on the annual N cycling because it is accessible only upon erosion [1]. The largest available N reservoir for organisms is dinitrogen gas (N<sub>2</sub>) with 3.9 × 10<sup>9</sup> Tg, which represents 79% of the atmospheric air. However, due to the reduced reactivity of the triple bonded N<sub>2</sub>, only a small group of organisms having the dinitrogenase enzyme complex can use it as N source [1–3]. The global and usable N-reservoirs are organic nitrogen (9 × 10<sup>5</sup> Tg), nitrate (NO<sub>3</sub><sup>-</sup>) (6 × 10<sup>5</sup> Tg), and nitrous

oxide ( $\text{N}_2\text{O}$ ) (2000 Tg) [1–4]. The ammonia ( $\text{NH}_3$ ) reservoir estimated in the marine ecosystem is 340–3600 Tg, but it is unknown in the terrestrial ecosystem [1]. Other global inorganic N sources such as nitrite and nitric oxide are considered minor reservoirs.

N compounds exhibit different oxidation states ranging from  $-3$  (organic N and  $\text{NH}_3/\text{NH}_4^+$ ) to  $+5$  ( $\text{NO}_3^-$ ), with intermediate values in compounds such as hydrazine ( $\text{N}_2\text{H}_4$ ) ( $-2$ ), hydroxylamine ( $\text{NH}_2\text{OH}$ ) ( $-1$ ),  $\text{N}_2$  ( $0$ ), nitrous oxide ( $\text{N}_2\text{O}$ ) ( $+1$ ),  $\text{NO}$  ( $+2$ ), nitrite,  $\text{NO}_2^-$  ( $+3$ ), and nitrogen dioxide ( $\text{NO}_2$ ) ( $+4$ ). Bacteria can catalyze many redox reactions involving the above compounds, which can be used to provide nitrogen for growth and redox energy to fuel cells. Bacterial species have been classically classified according to the reactions that they mediate within the N cycle (nitrification, denitrification, fixation, ammonification, anammox, assimilatory, and dissimilatory processes). Notwithstanding, DNA genome sequencing has revealed the set of genes present in each microorganism and highlighted the enormous metabolic flexibility of some of the organisms studied, making it almost impossible to classify them using only the classical divisions [1,2]. Moreover, it is expected that further research will unravel new N-transforming reactions and enzymes that will emphasize the metabolic flexibility of many of these organisms [1].

Prokaryotic organisms exhibit an enormous versatility to metabolize most of the N compounds, and they are the main players for most of the transformations in the N cycle. In addition, enzymes required for  $\text{N}_2$  fixation, such as nitrogenase, are exclusively found in prokaryotes or bacteria involved in symbiosis/mutualism with other eukaryotic organisms. For instance,  $\text{N}_2$ -fixing bacteria from the order Rhizobiales establish symbiotic interactions with legume plants, whose crops provide almost 20% of food protein in the world [5]. Other examples are found in marine ecosystems, where a symbiotic interaction between the haptophyte algae and the most widespread  $\text{N}_2$ -fixing cyanobacterium '*Candidatus Atelocyanobacterium thalassa*' (UCYN-A) has been described [6,7]. In the lab, the  $\text{N}_2$ -fixing bacterium *Azotobacter chroococcum* provides N to the alga *Chlamydomonas* and promotes its growth, whereas *Chlamydomonas* fixes  $\text{CO}_2$  and provides organic carbon to the bacterium. This symbiotic interaction has reported benefits for the industry by increasing lipid production in the microalga [8].

Anthropomorphic activities have affected the natural equilibrium of the N cycle for more than a century. The industrial  $\text{N}_2$ -fixation into ammonia (Haber-Bosch process) and the use of this ammonia as fertilizers has dramatically improved crop productivity but, at the same time, it has increased the reactive N load by more than 120%, which includes all biologically active, photochemically reactive, and radiatively active N compounds in the atmosphere and biosphere [1,2,9,10]. This increase in the N load comprises a higher global amount of reduced inorganic forms of N ( $\text{NH}_3$  and  $\text{NH}_4^+$ ), oxidized inorganic forms (as  $\text{NO}_x$ ,  $\text{HNO}_3$ ,  $\text{N}_2\text{O}$ , and  $\text{NO}_3^-$ ), and organic compounds (such as urea, amines, and proteins), in detriment to the unreactive  $\text{N}_2$  gas. One of the main unwanted effects of this imbalance in the N cycle is the transfer of reactive N from terrestrial to coastal systems and ground waters, causing algal blooms and the eutrophication of these ecological niches [9,10]. In addition, it is estimated that 3–5% of the N used in fertilizers is converted into  $\text{N}_2\text{O}$ , a greenhouse gas that is 310 times more potent than  $\text{CO}_2$  and considered the dominant ozone-depleting pollutant emitted in the 21st century [1,11–13]. The principal substrate for  $\text{N}_2\text{O}$  synthesis is  $\text{NO}$ , which is mainly reduced by bacterial  $\text{NO}$  reductases but also can be reduced by some eukaryotic enzymes that carry out nitrate dissimilatory reactions.

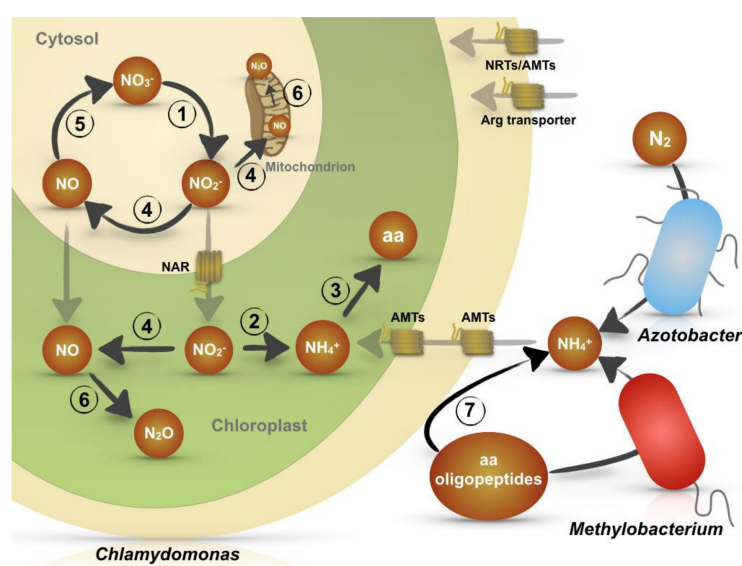
Nitrogen is a macronutrient for plants, algae, and bacterial growth. Nitrate is the most oxidized form of nitrogen and highly abundant in aquatic habitats. The nitrate assimilation pathway ( $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NH}_4^+ \rightarrow$  amino acids) is quantitatively the most important redox process, supporting almost 20% of marine algal growth [1,4]. Dissimilatory nitrate reduction pathways such as dissimilatory nitrate reduction to ammonium (DNRA), denitrification to  $\text{N}_2\text{O}$ , and total denitrification to  $\text{N}_2$  are also processes mediated by eukaryotes in aquatic systems. DNRA is described in diatoms as an adaptation and survival process to darkness and anoxia, where the intracellular  $\text{NO}_3^-$  is used as an electron acceptor instead of  $\text{O}_2$  [14,15]. On the other hand, a complete denitrification process ( $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$ ) has been described in some species belonging to the foraminifera

group, which represent the only eukaryotic organisms able to perform complete denitrification to  $N_2$  [16–18]. Other foraminifer species perform only partial nitrate denitrification to  $N_2O$  [17–19]. The genes involved in the foraminifer denitrification pathway have been recently identified [20]; however, the genes encoding the enzymes responsible for the first and last steps of this pathway, the dissimilatory nitrate reductases and nitrous oxide reductase, are missing in most of the foraminifer genomes but found in the associated bacteria. Partial nitrate denitrification to  $N_2O$  has also been well documented in fungi [21–25] and recently described in the green microalga *Chlamydomonas reinhardtii*, where a NO reductase (CYP55) [26] and flavodiiron proteins (FLVs) [27] catalyze the reduction of NO to  $N_2O$  in dark and light conditions, respectively. These two proteins constitute the molecular evidence that supports that microalgae contribute to  $N_2O$  emissions, something known for decades [28]. In this review, we analyze the metabolic flexibility of microalgae to carry out different steps of the N cycle with special attention to the denitrification pathway.

## 2. The Role of *Chlamydomonas reinhardtii* in the N Cycle

Traditionally the genus *Chlamydomonas* corresponds to unicellular, biflagellate, green microalgae and encompasses more than 500 species. However, current phylogenetic studies have reevaluated the taxonomy of *Chlamydomonas* into three species: *C. reinhardtii*, *C. incerta*, and *C. schloesseri* [29,30]. Today, all the unequivocally identified *C. reinhardtii* cells have been isolated from soil habitats suggesting a preference for rich nutrient environments. *Chlamydomonas* spp., other than *C. reinhardtii*, have been isolated from very different environments such as acid mine drainage, temperate marine, Antarctic ice, and even air [29]. *C. reinhardtii*, named as *Chlamydomonas* throughout, has been developed as a powerful model organism useful to study essential biological processes but also biotechnological applications [31].

Nitrogen assimilation has been widely studied in *Chlamydomonas* and the pathways for the assimilation of different forms of nitrogen, inorganic (nitrate, nitrite, and ammonium) as well as organic (urea, amino acids, and purines), have been identified [32–34]. However, besides the classical N assimilation pathways that have been described in axenic *Chlamydomonas* cultures, recent advances on nitrogen-transforming reactions have revealed that this microalga can also carry out both an ammonification process by mutualistic interactions with bacteria and a denitrification process that releases  $N_2O$  to the environment (Figure 1, Table 1).



**Figure 1.** Nitrogen-transforming reactions by *Chlamydomonas reinhardtii*.  $NO_3^-$  assimilation (steps 1,2,3);  $NO_3^-/NO_3^-$  cycle, (steps 1,4,5);  $NO_3^-$  dissimilation (steps 4,6) and ammonification from amino acids and peptides (step 7). Transporters for ammonium (AMT), nitrate (NRT), and arginine are indicated. Mutualism with bacteria for N/C exchange is also shown.

**Table 1.** Enzymes for nitrogen-transformation reactions in *Chlamydomonas*.

Step	Enzyme	Gene ID	Regulation	Localization	Ref.	
1	$\text{NO}_3^- + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$	NR	Cre09.g410950	$\text{NO}_3^-$	Cytosol	[33]
2	$\text{NO}_2^- + 6\text{e}^- + 8\text{H}^+ \rightarrow \text{NH}_4^+ + 2\text{H}_2\text{O}$	NIR	Cre09.g410950	$\text{NO}_3^-$		[33]
3	$\text{NH}_4^+ + 2\text{e}^- + \alpha\text{-ketoglutarate} + \text{ATP} \rightarrow \text{Glutamate} + \text{ADP} + \text{Pi}$	GS/GOGAT				[33]
4	$\text{NO}_2^- + \text{e}^- + 2\text{H}^+ \rightarrow \text{NO} + \text{H}_2\text{O}$	NR/NOFNIR	Cre09.g395950/Cre09.g389089	$\text{NO}_3^-$	Cytosol	[35]
		NirK	Cre08.g360550	Dark	Mitochondria	[36]
5	$\text{NO} + 2\text{H}_2\text{O} \rightarrow \text{NO}_3^- + 3\text{e}^- + 4\text{H}^+$	NR/THB1	Cre09.g410950/Cre14.g615400	$\text{NO}_3^-$	Cytosol	[37]
		CYP55	Cre01.g007950	$\text{NO}_3^-/\text{Dark}$	Mitochondria *	[38]
		FLVA	Cre12.g531900	-	Chloroplast	[27]
		FLVB	Cre16.g691800	-	Chloroplast	[27]
6	$2\text{NO} + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$	HCP1	Cre09.g391650	$\text{NO}_3^-/\text{Dark}$	Chloroplast	[39]
		HCP2	Cre09.g393543	$\text{NO}_3^-$	Mitochondria *	[38]
		HCP3	Cre09.g393506	$\text{NO}_3^-/\text{Dark}$	Chloroplast	[39]
		HCP4	Cre09.g391450	$\text{NO}_3^-/\text{Dark/anaerobiosis}$	Chloroplast	[40]
7	$\text{L-Amino acid} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{NH}_4^+ + \alpha\text{-keto acid} + \text{H}_2\text{O}_2$	LAO1	Cre12.g551353	Nitrogen starvation	Periplasm	[35]

Steps 1 to 7 correspond to those in Figure 1. Gene identification (Phytozome). \* Protein identified in the mitoproteome. NR (Nitrate Reductase), NIR (Nitrite Reductase), GS (Glutamine Synthetase), GOGAT (Glutamine Oxoglutarate Aminotransferase), NOFNIR (NO Forming Nitrite Reductase), NirK (Copper-Containing Nitrite Reductase), THB1 (Truncated Hemoglobin 1), CYP55 (Cytochrome P450), FLV (Flavodiiron Proteins), HCP (Hybrid Cluster Protein) and LAO1 (L-Amino Acid Oxidase).

### 2.1. Ammonification and Mutualism with Bacteria for N Scavenging from Amino Acids and Peptides

*Chlamydomonas* cells scavenge nitrogen from amino acids in three ways: transport, deamination/ammonification, and mutualistic interaction with bacteria [41]. Arginine is the only amino acid that is efficiently transported into the cell from the surrounding media and then used as carbon and nitrogen sources [42]. L-leucine and L-cysteine is also transported, but by passive diffusion [43]. However, most of the proteinogenic amino acids, including arginine, but also di- and tri-peptides, can be extracellularly deaminated, producing  $\text{NH}_4^+$  and the corresponding keto acids. This ammonification process is mediated by the periplasmic L-amino acid oxidase (LAO1) (step 7, Figure 1, and Table 1), which is induced upon inorganic N deprivation [41,44–47]. Interestingly, only the  $\text{NH}_4^+$  generated by the LAO1 enzyme is transported inside the cell by high-affinity ammonium transporters (AMT) and used by the alga, but the carbon skeletons remain in the medium [47]. It has been hypothesized that such carbon skeletons could be used for establishing mutualistic interactions with specific bacteria in the surrounding medium. These interactions depend on whether the released keto acid can meet the particular carbon requirements of a specific bacterium [41]. Besides ammonium and the keto acid, the ammonification process mediated by LAO1 also generates  $\text{H}_2\text{O}_2$ , which has bactericidal properties [48]. The eco-physiological role of  $\text{H}_2\text{O}_2$ , if any, is not clear but its production could repel other microorganisms to avoid competition for the organic N and, simultaneously, promote bacterial cell death making more N available. Alternatively,  $\text{H}_2\text{O}_2$  synthesis could also serve as a mechanism to select specific  $\text{H}_2\text{O}_2$ -tolerant bacterial species in order to restrict the mutualistic interactions. The role of  $\text{H}_2\text{O}_2$  in algal-bacterial mutualistic interaction has been previously reported [49].

*Chlamydomonas* LAO1 homologs have been found in other algal species, including Rhodophyta, Alveolata, Heterokonta, Haptophyta, and Dinophyta species, but not in other chlorophytes [41].

Despite the promiscuous LAO1 activity to deaminate amino acids and oligopeptides, Calatrava and collaborators [50] have reported that this enzyme cannot use proline and some oligopeptides as substrate. Interestingly, when proline is the only N source in the media, *Chlamydomonas* can establish a strict and specific mutualism with *Methylobacterium* species [50]. Under this situation, the bacteria mineralize the amino acid (or peptide) releasing ammonium that can be incorporated by the alga. In return, *Chlamydomonas*, which fixes  $\text{CO}_2$  to satisfy its carbon demand, produces C-compounds as glycerol that can be secreted and used as a C source for the bacterium.

Another step in which *Chlamydomonas* could participate in the N cycle is the use of  $\text{N}_2$ , as described from co-cultures with the nitrogen-fixing anaerobic bacteria *Azotobacter chroococcum*. In this situation, the bacteria supply the nitrogen source and  $\text{CO}_2$  to the alga by nitrogen fixation, meanwhile, the alga supplies carbohydrates and  $\text{O}_2$  via photosynthesis to the bacteria [8].

## 2.2. Nitrate Denitrification

The nitrate flux toward its assimilation is the primary nitrogen pathway that occurs in *Chlamydomonas* as well as in eukaryotic organisms containing an assimilatory nitrate reductase (NR). The nitrate assimilation appears to occur via a relatively simple linear pathway involving (1) nitrate uptake by specific nitrate transporters (i.e., NRT2.1/NAR2), (2) cytosolic nitrate reduction by a typical eukaryotic nitrate reductase (NR), (3) nitrite transport into the chloroplast by specific transporters (i.e., NAR1.1), (4) nitrite reduction to ammonium by a typical plant-like ferredoxin-dependent nitrite reductase (NIR), and finally (5) the ammonium incorporation into carbon skeletons by the GS/GOGAT cycle that synthesizes glutamate [33,34].

Besides its assimilatory role, the NR is a moonlighting protein that interacts with different partners to carry out alternative reactions. In photosynthetic organisms, NR is considered one of the principal nitric oxide (NO) sources [35] and also participates, as shown in *Chlamydomonas*, in the NO oxygenation to convert this signaling molecule back to nitrate. These reactions, in which NR is central and acts with different partners, can be called the  $\text{NO}_3^-/\text{NO}_3^-$  cycle (steps 1, 4, and 5 in Figure 1). Moreover, the intracellular NO can also be the substrate for NO reductases producing  $\text{N}_2\text{O}$  in mitochondria and chloroplasts [1]. Thus, NR seems to be a cross-point among three different pathways: nitrate assimilation, a partial nitrate-denitrification to NO and the contaminant gas  $\text{N}_2\text{O}$ , and the NO oxygenation to nitrate.

### 2.2.1. The $\text{NO}_3^-/\text{NO}_3^-$ Cycle

The eukaryotic NR is a homo-dimeric protein that contains three prosthetic groups in each subunit (FAD, Heme  $b_{557}$ , and molybdenum cofactor –Moco-). Nitrate reduction to nitrite is a redox reaction that occurs across a mini electron transport chain (mini-ETC) from NAD(P)H to nitrate through three prosthetic groups ( $\text{NAD(P)H} \rightarrow \text{FAD} \rightarrow \text{heme} \rightarrow \text{Moco} \rightarrow \text{NO}_3^-$ ). However, as mentioned above, NR can bind to other protein partners and detour electrons at different levels of this mini-ETC. When the partner protein is the molybdoprotein NOFNIR (NO forming nitrite reductase), electrons flow until the heme cofactor, where they reduce the NOFNIR that converts nitrite to NO [35,51]. When the partner is the truncated hemoglobin 1 (THB1), electrons go out of the mini-ETC at the FAD cofactor level and reduce the THB1 protein, which transforms NO into nitrate [33]. These three reactions constitute the named  $\text{NO}_3^-/\text{NO}_3^-$  cycle.

Nitric oxide is a signal molecule that regulates a high number of physiological processes in mammals, plants, and algae [52]. In *Chlamydomonas*, NO regulates N assimilation at the transcriptional and posttranslational level. NO is a negative regulator for the expression and activities of the high-affinity nitrate/nitrite transporters, the NR, and the high-affinity ammonium transporters [33,53]. As a signal molecule, NO is expected to have a temporal effect, and therefore, its synthesis and degradation have to be tightly controlled.

This  $\text{NO}_3^-/\text{NO}_3^-$  cycle could work for regulating nitrate assimilation without N loss. When the conditions are optimal, nitrate goes toward its assimilation up to glutamate. If the conditions are not optimal (i.e.,  $\alpha$ -ketoglutarate is deficient) and nitrite accumulates, NO is synthesized and stops the nitrate uptake and reduction. However, although NO is a gas and can diffuse out of the cells, the NR-THB complex could convert the NO into nitrate, avoiding the N loss.

### 2.2.2. NO Synthesis and Reduction: A Prokaryote Pathway

In plants and plant-like organisms, NO is produced by different mechanisms that use different substrates (i.e.,  $\text{NO}_2^-$  or arginine) in different intracellular compartments such as cytosol [35,54,55], mitochondria [26,56], chloroplast [57,58], and peroxisomes [59]. The oxidation of arginine to NO and citrulline by the NO synthase (NOS) is a well-known reaction in mammals, bacteria, and some algae [60,61]. NOS-like reactions have been described in land plants (peroxisomes) [62] and *Chlamydomonas* [63], but extensive genome analyses have ruled out the existence of NOS in



photosynthetic organisms except for some microalgae [60,61]. However, NO production from  $\text{NO}_2^-$  is well documented to occur in the cytosol, by NR [54,55] or NR/NOFNIR [35], and also in mitochondria [26,56] and chloroplast by the electron transport chains [57].

Besides NO conversion into nitrate, NO can also be reduced by NO reductases to  $\text{N}_2\text{O}$ . This denitrification process implies the loss of nitrogen to the atmosphere as a potent greenhouse gas. This pathway ( $\text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O}$ ) has been well defined in both prokaryotes [1] and eukaryotes (fungi). In fungi, denitrification involves NIRK (copper-containing nitrite reductase) ( $\text{NO}_2^- \rightarrow \text{NO}$ ) and the cytochrome P450, CYP55 ( $\text{NO} \rightarrow \text{N}_2\text{O}$ ), both acquired from bacteria [22]. For fungal CYP55, a horizontal gene transfer from bacteria is suggested, but for NIRK, a proto-mitochondrial origin has been proposed.

The *Chlamydomonas* genome contains two genes encoding for both proteins, NIRK and CYP55 (Table 1). CYP55 appears to be responsible for  $\text{N}_2\text{O}$  production in the dark [26,27]. Accordingly, CYP55 is located in mitochondria [38] and its transcript is accumulated in the dark [64] and in the presence of nitrate [38]. The dark NO reduction attributed to CYP55 activity has also been shown to be stronger when cells are grown in the presence of nitrate [27]. Although found in the mitochondrial proteome, CYP55 is also predicted to be targeted to the chloroplast [27]. Thus, it is possible that the protein is targeted to both organelles. In this case, CYP55 could be able to use electron donors from photosynthetic origin. Like CYP55, NIRK is also up-regulated in the dark [64] and in nitrate-containing media (Calatrava, unpublished data). In silico analyses strongly suggest that NIRK could also be localized in the mitochondria (Table 1), pointing out that *Chlamydomonas* seems to have a complete and operative  $\text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O}$  pathway in the mitochondria.

The second type of protein shown to reduce NO to  $\text{N}_2\text{O}$ , in *Chlamydomonas*, corresponds to FLVs (flavodiiron proteins) [27]. Flavodiiron proteins belong to a singular family of oxygen and/or nitric oxide reductases widespread in prokaryote and eukaryote organisms. This protein family has been classified into eight classes (A to H) according to the presence of modular domains [65,66]. Class A FDPs are the most dominant class in the family and only contain the two core domains (Fe-Fe and FMN) [65,66]. Some examples for this class A show a preference for  $\text{O}_2$  as revealed for the protozoal and archaeal enzyme [67–70], or they may have a dual activity toward NO and  $\text{O}_2$  as in *M. thermoacetica* or *D. gigas* [71,72]. Class B FDPs contain an additional C-terminal rubredoxin domain and, because of that, were named flavorubredoxins (FIRD) or NORV (from the encoding gene in *E. coli*) [65]. In *E. coli*, FLVB/FIRD/NORV was shown to have significant preference for NO over  $\text{O}_2$  and confers resistance to NO under anaerobic conditions [73,74]. Class C FDPs have a C-terminal flavin-binding domain, predicted to have a flavin reductases activity accepting electrons from NAD(P)H [65,66]. This class C is present in cyanobacteria, the protozoan *Paulinella chromatophora*, green algae, mosses, lycophytes, and gymnosperms, but absent in angiosperms [65]. *Chlamydomonas* FDPs correspond to class C (also called Flv), which forms a distinct cluster that falls into two groups. One of these groups (FlvA) has the ligand binding diiron site strictly conserved, while in the other (FlvB) the ligand binding sites are not conserved. The *Chlamydomonas* genome contains two genes, *FLVA* and *FLVB*, encoding both groups of flavodiiron proteins. Moreover, the *Chlamydomonas* FLVs could work as a heterodimer as shown by different insertional mutants of the *FLVB* gene that result in the loss of expression of both *FLVB* and *FLVA* proteins [71]. The study of these mutants revealed that FLVs participate in a Mehler-like reduction of  $\text{O}_2$ , providing an alternative valve for electrons during the dark to light transition in the chloroplast. Thus, FLVs are critical for the algal growth under fluctuating light regimes but not in continuous light [75]. These mutants were also useful to prove the FLVs role as a NO reductase, being the major enzyme responsible for NO reduction to  $\text{N}_2\text{O}$  under conditions of light in the presence of  $\text{O}_2$  in *Chlamydomonas*. In addition, in vivo experiments suggest that *Chlamydomonas* FLVs shows a higher affinity for NO than for  $\text{O}_2$  [27]. Therefore, *Chlamydomonas* has inherited two types of  $\text{N}_2\text{O}$ -producing proteins, FLVs in the chloroplast and CYP55 in the mitochondria.

Besides CYP55 and FLVs, the *Chlamydomonas* genome also contains four genes encoding for hybrid cluster proteins (HCPs) [39], which are metalloproteins characterized by the presence of an

iron-sulfur-oxygen cluster considered to be unique in biology [25]. The analysis of these protein sequences and their phylogenetic analyses support that the HCP family has emerged from a single gene of an alpha-proteobacterium with subsequent duplications in *Chlamydomonas* [39]. Like for CYP55 and NIRK, both nitrate and darkness contribute to the strong up-regulation of the HCPs in *Chlamydomonas* growing under oxic conditions [39]. In dark anoxia, the HCP4 transcript shows the stronger induction [76], and the studies performed in mutants obtained by gene silencing suggest a role in regulating fermentative and nitrogen metabolism pathways [77]. Concerning their cellular localization, HCP1, 3, and 4 are predicted to be in the chloroplast [39], but HCP2 is located in the mitochondria, as confirmed in mito-proteome analysis [38]. In bacteria, the function(s) of HCP is debated. Initially, they were considered hydroxylamine reductases, as they can use this substrate to produce ammonium in vitro, but the low affinity for hydroxylamine has questioned this physiological role [78]. Later, Wang and collaborators demonstrated that *E. Coli* HCP is a high-affinity nitric oxide (NO) reductase and proposed it as the major enzyme for reducing NO to N<sub>2</sub>O under physiologically relevant conditions [25]. The in vivo high affinity NOR activity is also demonstrated in *Methylobacter tundripaludum*, where NO mediates a response to hypoxia, quorum sensing, the secondary metabolite tundrenone, and methanol dehydrogenase [79]. What makes the history of HCP attractive is that it can function as a protein S-nitrosylase with a role in the NO sensing pathway. Thus, HCP is auto-S-nitrosylated and afterward results in the assembly of an HCP interactome, including enzymes that generate NO, synthesize SNO-proteins, and propagate SNO-based signaling mediating the NO-mediated stress responses [80]. The function(s) of HCP in *Chlamydomonas* as NOR and/or protein-S-nitrosylase has to be solved as well as its relationship with nitrate metabolism. Seth and collaborators propose that HCPs oxidize NO to NO<sup>+</sup>, which is the form required for S-nitrosylation [80]. However, the redox potentials of both HCP and HCR (HCP reductase, also required for HCP function) do not fit with an oxidase activity and Lis et al. have proposed that HCPs might exhibit reductase activity and perform the following two-electron reduction reaction,  $\text{NO}_3^- + 4\text{H}^+ + 2\text{e}^- \rightarrow \text{NO}^+ + 2\text{H}_2\text{O}$  [39].

### 3. N<sub>2</sub>O Production and Enzymes in Other Microalgae

For decades, N<sub>2</sub>O emission has been detected from lakes, coasts, and oceans, ecosystems where microalgae are mainly present [81], although the contribution of microalgae to these N<sub>2</sub>O emissions has been mostly neglected and attributed to the prokaryotic organisms present in these environments [28,82–84]. However, the correlation between algal bloom events, triggered by the excess of N from anthropogenic origins, and N<sub>2</sub>O emissions suggests that these microorganisms may be partly responsible for this production [1,28,83]. Importantly, many microalgae are currently cultivated at a large scale for commercial and biotechnological purposes. Therefore, the potential contribution to N<sub>2</sub>O emissions of these microalgae should be considered [26,28]. Microalgae cultures, supplied with NO<sub>3</sub><sup>-</sup> as the sole N source, have been estimated to emit N<sub>2</sub>O in amounts 2.5 to 14 times more than from natural vegetation [85,86]. Although the emission of N<sub>2</sub>O by microalgae depends on many environmental factors, the major ones seem to be the species of microalga and the N source [26,27,87]. While N<sub>2</sub>O is produced on nitrate or nitrite, its production is prevented on ammonium [28].

The existence of a full NO<sub>2</sub><sup>-</sup> → N<sub>2</sub>O dissimilation pathway in *Chlamydomonas* made interesting the analysis of other microalgae genomes. Public algal databases were searched for homologous proteins to the *Chlamydomonas* NIRK, possibly involved in NO synthesis and CYP55, FLVs, HCP as potential NO reductases. Those microalgae containing at least one of these proteins were selected. A total of 33 microalgae were considered, 23 Chlorophyta and 10 non-Chlorophyta species. Considering there are around one hundred microalgae genomes available, it can be assumed that almost 33% of microalgae contain at least one of these proteins. Phylogenetic trees of these proteins were constructed to provide a broader picture of the distribution of these proteins in the tree of life consistent with previous reports for FLV [65] (Figure S1), HCP [39] (Figure S2), CYP55 [88] (Figure S3), and NIRK [21] (Figure S4). These data were correlated to the production of N<sub>2</sub>O by the microalgae (Table 2) and discussed.

**Table 2.** Microalgal production of N<sub>2</sub>O and putative NIRK proteins, involved in NO formation, and putative HCP, CYP55, and FLV, involved in NO reduction to N<sub>2</sub>O.

Microalgae	NIRK	HCP	CYP55	FLVA	FLVB	N <sub>2</sub> O/Ref.
1-7 <i>Chlamydomonas reinhardtii</i>	PNW79625.1	XP_001694756.1 XP_001694571.1 XP_001694671.1 XP_001694454.1 GAX74118.1	XP_001700272.1	XP_001692916.1	PNW71243.1	+ [26,27]
<i>Chlamydomonas eustigma</i> <i>Chlorella variabilis</i> <i>Chlorella sorokiniana</i>	PRW57688.1	XP_005851548.1 PRW58172.1 PRW60486.1 PRW58278.1	XP_005848845.1 PRW58571.1 (A) PRW58572.1 (B)	GAX73888.1 XP_005849742.1 PRW59370.1	GAX73438.1 XP_005849474.1 PRW58882.1	+ [27]
<i>Scenedesmus obliquus</i> UTEX 393	SZX67391.1	ID:15469 ID:3042	ID:435	SZX62872.1 SZX76545.1	SZX63966.1 SZX639665.1	+ [89]
<i>Scenedesmus obliquus</i> EN0004	ID:579459	ID:571596 ID:618262 NSK 004142	ID:324854	ID:321778/332768 ID:342393	XP002948938.1	+ [89]
<i>Nannochloropsis salina</i> <i>Nannochloropsis oceanica</i> CCMP1779		599317				+ [90,91]
<i>Nannochloropsis gaditana</i> <i>Gonium pectorale</i>		XP_005853562.1 KXZ41847.1 KXZ44628.1	KXZ42279.1	KXZ51271.1	KXZ42718.1	- [27]
<i>Coccomyxa subelipsoidea</i> <i>Tetraabaena socialis</i> <i>Raphidocelis subcapitata</i>		PNH09786.1 GBF99305.1 GBF96263.1	GBF95475.1	GBF88523.1	GBF98453.1	+ [27]
<i>Volvox carteri</i> <i>Yamagishiella unicocca</i> <i>Micractinium conductrix</i>		XP_002952683.1 ARO50069.1 PSC73563.1 PSC72373.1 PSC68490.1		XP_002947563.1	XP_002948938.1	
<i>Micromonas pusilla</i> <i>Micromonas commoda</i>				XP_003057013.1 XP_002502419.1	XP_003057495.1 XP_002502038.1	
<i>Monoraphidium neglectum</i> SAG 48.87		XP013900867.1	ID:2893 ID:2894			
<i>Bathycoccus prasinus</i> <i>Bathycoccus lucimarinus</i> <i>Ostreococcus tauri</i> <i>Klebsormidium nitens</i>				XP007514084.1 XP001416100.1 XP003075211.2 GAQ79906.1	XP_007514099.1 XP001416098.1 XP003075215.2 GAQ89021.1	
<i>Galdieria sulphuraria</i>	XP_005707361.1					- [27]
<i>Porphyridium purpureum</i>						- [27]
<i>Phaeodactylum tricorutum</i>		ID:12416				- [27]
<i>Thalassiosira pseudonana</i>		ID:32716	ID:263399 <sup>a</sup>			- [27]
<i>Guillardia theta</i> CCMP2712		ID:152637				
<i>Andalucia godoyi</i>	KAF0852161.1					
<i>Phaeocystis antarctica</i> CCMP1374	ID:684					
<i>Emiliana huxleyi</i>	XP 005783766.1					

Reciprocal BLAST was performed using the *Chlamydomonas* homolog of each protein as a query. Green background highlights microalgal species that belong to the Chlorophyta phylum. For those algal species that did not show in a positive hit using the current available genome sequences, the gene coding for the corresponding queried protein was considered to be absent. Protein accession numbers correspond to those indicated in Figures S1–S4, from NCBI or Phycosm (protein references obtained from Phycosm are indicated as follows “ID: (ref number)”. “a” indicates partial sequence. N<sub>2</sub>O/Ref., positive or negative (+/–) N<sub>2</sub>O production by the microalgae and empty when it is unknown.

From the 33 selected microalgae, only three Chlorophyta species (*Chlamydomonas reinhardtii*, two subspecies of *Scenedesmus obliquus*, and *Chlorella sorokiniana*) contained a full set NIRK, CYP55, FLVA, FLVB, and HCP. In other words, they contain a prokaryote NO synthetase and redundant potential NO reductases. This represents almost 9% of the microalgae in Table 2. In addition, these three microalgae have been reported to produce N<sub>2</sub>O [26,27,89].

For the rest of microalgae in Table 2, an incomplete NO<sub>2</sub><sup>−</sup> → N<sub>2</sub>O pathway or the potential to produce NO (NIRK) or reduce NO to N<sub>2</sub>O through FLVs, CYP55, or HCP reductases was observed. The molecular evidence for these NO reductases is restricted to a few microalgae and comes from a recent study by Burlacot and collaborators [27]. Both CYP55 and FLVs were responsible for producing N<sub>2</sub>O under two different conditions, CYP55 in the dark and FLVs in the light. Therefore, microalga strains lacking CYP55 and/or FLV also are unable to produce N<sub>2</sub>O under its particular condition. However, whether HCP is or is not a NO reductase is unsolved in microalgae.



As previously reported, FLVs are present only in Chlorophyta and in approximately 74% of the selected chlorophytes in Table 2. These species contain both FLVA and FLVB, suggesting that these proteins may function as a heterodimer [75]. The phylogenetic tree suggests that FLVs from chlorophytes and early-diverged land plants are related to cyanobacteria (Figure S1), most likely derived from the endosymbiotic gene-transfer (EGT) of both genes from the chloroplast to the nuclear genome in the green lineage, after the divergence of the red algae and glaucophytes. Otherwise, both genes may have been transferred to the nucleus of the ancestral archaeplastidan and subsequently lost in red algae and glaucophytes.

As previously reported, HCP genes, although not present land plants, are highly represented in Chlorophyta; most of the chlorophytes harbor more than one copy of this gene [39]. In *Chlamydomonas reinhardtii*, the four HCP gene copies are phylogenetically related and located in the same chromosome, which suggests that these genes were recently duplicated [39]. In Table 2 it is shown that 69% of the chlorophytes harbor HCP. Moreover, putative HCP orthologs are also found in nonchlorophytes, including three *Nannochloropsis* spp. (Eustigmatales), the *Guillardia theta* and the Stramenopiles *Phaeodactylum tricornerutum* and *Thalassiosira pseudonana* (Table 2, Figure S2). Supported by further phylogenetic analyses, an endosymbiotic origin of the microalgal HCP genes from mitochondria has been proposed [39].

Although less abundant, CYP55 is in almost 39% of the Chlorophyta in Table 2 and Figure S3. It was also found only in one of other non-chlorophytes (*Thalassiosira pseudonana*), but it corresponds to a truncated sequence and it was not included in the phylogenetic tree. The most closely related to microalgal CYP55 are fungal members, which are hypothesized to have been acquired from Actinobacteria [22]. In fungi, CYP55, known as p450nor (cytochrome p450 NO reductase), is considered as a biomarker for N<sub>2</sub>O production, since it is detected in all fungal isolates producing N<sub>2</sub>O from NO<sub>2</sub><sup>-</sup> [24]. Because Actinobacteria are not considered canonical denitrifying bacteria, the prevalent hypothesis for the origin and evolution of CYP55/p450nor is its acquisition from Actinobacteria and next evolution to a new role in denitrification [22]. Recently, a new hypothesis suggests that fungal p450nor has a role in secondary metabolism rather than denitrification and that N<sub>2</sub>O is a minor bioproduct of the secondary metabolism [88].

The NO-forming NIRK, which shares a common bacterial origin in both fungi and microalgae [21], is poorly represented in chlorophytes (3 out of 33) (Table 2, Figure S4), but it is present in the genome of the red alga *Galdieria sulpharia*, the haptophyte *Emiliana huxleyi*, and in Excavata. However, the NO reductase activity by this protein in these species is still unknown.

#### 4. Concluding Remarks and Future Perspectives

N homeostasis in ecosystems relies on the balance between N fixation and N release to the atmosphere in gaseous forms (mainly N<sub>2</sub> and, to a lower extent, N<sub>2</sub>O and NO). In-depth studies of the reactions mediated by isolated organisms, as well as those carried out by consortiums, will help us to understand how organisms keep the equilibrium of the biogeochemical N cycle and will allow us to forecast putative imbalances.

*Chlamydomonas reinhardtii* performs a high number of N-transforming reactions. Quantitatively and preferentially are those devoted to inorganic nitrogen (NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>) assimilation for biomass production. The ammonification/deamination of amino acids and peptides constitutes a poor N supply but leads to bacterial mutualistic interactions for C/N exchange. NO<sub>3</sub><sup>-</sup> offers additional N-transforming reactions, such as the NO<sub>3</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> cycle, and dissimilation through a typical prokaryotic pathway NO<sub>2</sub><sup>-</sup> → NO → N<sub>2</sub>O by means of apparently redundant NO reductases.

Algae, playing essential roles in aquatic ecosystems in carbon and N cycles, were claimed for a long time to produce the potent greenhouse gas N<sub>2</sub>O. This point was recently further confirmed by molecular evidence with CYP55 and FLVs in *Chlamydomonas reinhardtii*. This review opens new perspectives for future investigations of the full or partial dissimilation pathway (NO<sub>2</sub><sup>-</sup> → NO → N<sub>2</sub>O) by algae, where NIRK, CYP55, FLVs, and HCPs may be involved. In fact, some green microalgae

seem to share the dissimilating full pathway  $\text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O}$  with *Chlamydomonas*. However, partial pathways seem to be prevalent in microalgae.

Understanding this  $\text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O}$  pathway operating in microalgae as complete or partial routes will be important to understand the production and regulation of either a signal molecule as is NO or the loss of nitrogen as the contaminant gas  $\text{N}_2\text{O}$ .

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2223-7747/9/7/903/s1>, Figure S1. Evolutionary relationships of putative FLV proteins, Figure S2. Evolutionary relationships of putative HCP proteins, Figure S3. Evolutionary relationships of putative CYP55 proteins, Figure S4. Evolutionary relationships of putative NIRK proteins.

**Author Contributions:** Conceptualization, A.G., E.F. and C.M.B.-P.; writing—Original draft preparation, A.G.; writing—Review and editing C.M.B.-P., V.C., E.S.-L., M.T.-J., Á.L., M.P., B.G., A.G. and E.F.; funding acquisition, A.G. and E.F. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was funded by MINECO (Grant BFU2015-70649-P), the European FEDER program, the Plan Propio de la Universidad de Córdoba, and the U.E.INTERREG POCTEP-055\_ALGARED\_PLUS5\_E.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## References

1. Kuypers, M.M.M.; Marchant, H.K.; Kartal, B. The microbial nitrogen-cycling network. *Nat. Rev. Microbiol.* **2018**, *16*, 263–276. [[CrossRef](#)] [[PubMed](#)]
2. Stein, L.Y.; Klotz, M.G. The nitrogen cycle. *Curr. Biol.* **2016**, *26*, R94–R98. [[CrossRef](#)]
3. Zerkle, A.L.; Mikhail, S. The geobiological nitrogen cycle: From microbes to the mantle. *Geobiology* **2017**, *15*, 343–352. [[CrossRef](#)] [[PubMed](#)]
4. Gruber, N.; Galloway, J.N. An Earth-system perspective of the global nitrogen cycle. *Nature* **2008**, *451*, 293–296. [[CrossRef](#)] [[PubMed](#)]
5. Burris, R.H.; Roberts, G.P. Biological nitrogen fixation. *Annu. Rev. Nutr.* **1993**, *13*, 317–335. [[CrossRef](#)] [[PubMed](#)]
6. Harding, K.; Turk-Kubo, K.A.; Sipler, R.E.; Mills, M.M.; Bronk, D.A.; Zehr, J.P. Symbiotic unicellular cyanobacteria fix nitrogen in the Arctic Ocean. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 13371–13375. [[CrossRef](#)]
7. Muñoz-Marin, M.D.C.; Shilova, I.N.; Shi, T.; Farnelid, H.; Cabello, A.M.; Zehr, J.P. The transcriptional cycle is suited to daytime  $\text{N}_2$  fixation in the unicellular cyanobacterium *Candidatus Atelocyanobacterium thalassa* (UCYN-A). *mBio* **2019**, *10*. [[CrossRef](#)]
8. Xu, L.; Cheng, X.; Wang, Q. Enhanced lipid production in *Chlamydomonas reinhardtii* by co-culturing with *Azotobacter chroococcum*. *Front. Plant Sci.* **2018**, *9*, 741. [[CrossRef](#)]
9. Erisman, J.Z.; Sutton, M.A.; Galloway, J.; Klimont, Z.; Winiwarter, W. How a century of ammonia synthesis changed the world. *Nat. Geosci.* **2008**, *1*, 636–639. [[CrossRef](#)]
10. Galloway, J.N.; Townsend, A.R.; Erisman, J.W.; Bekunda, M.; Cai, Z.; Freney, J.R.; Martinelli, L.A.; Seitzinger, S.P.; Sutton, M.A. Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. *Science* **2008**, *320*, 889–892. [[CrossRef](#)]
11. Ravishankara, A.R.; Daniel, J.S.; Portmann, R.W. Nitrous oxide ( $\text{N}_2\text{O}$ ): The dominant ozone-depleting substance emitted in the 21st century. *Science* **2009**, *326*, 123–125. [[CrossRef](#)] [[PubMed](#)]
12. Crutzen, P.J.; Moiser, A.R.; Smith, K.A.; Winiwarter, W.  $\text{N}_2\text{O}$  release from agro-biofuel production negates global warming reduction by replacing fossil fuels. *Atmos. Chem. Phys.* **2008**, *8*, 389–395. [[CrossRef](#)]
13. Davidson, A. The contribution of manure and fertilizer nitrogen to atmospheric nitrous oxide since 1860. *Nat. Geosci.* **2009**, *2*, 659–662. [[CrossRef](#)]
14. Kamp, A.; Stief, P.; Knappe, J.; de Beer, D. Response of the ubiquitous pelagic diatom *Thalassiosira weissflogii* to darkness and anoxia. *PLoS ONE* **2013**, *8*, e82605. [[CrossRef](#)] [[PubMed](#)]
15. Kamp, A.; de Beer, D.; Nitsch, J.L.; Lavik, G.; Stief, P. Diatoms respire nitrate to survive dark and anoxic conditions. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 5649–5654. [[CrossRef](#)] [[PubMed](#)]
16. Risgaard-Petersen, N.; Langezaal, A.M.; Ingvarsdén, S.; Schmid, M.C.; Jetten, M.S.; Op den Camp, H.J.; Derksen, J.W.; Pina-Ochoa, E.; Eriksson, S.P.; Nielsen, L.P.; et al. Evidence for complete denitrification in a benthic foraminifer. *Nature* **2006**, *443*, 93–96. [[CrossRef](#)]

17. Piña-Ochoa, E.; Hogslund, S.; Geslin, E.; Cedhagen, T.; Revsbech, N.P.; Nielsen, L.P.; Schweizer, M.; Jorissen, F.; Rysgaard, S.; Risgaard-Petersen, N. Widespread occurrence of nitrate storage and denitrification among Foraminifera and *Gromiida*. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1148–1153. [[CrossRef](#)]
18. Bernhard, J.M.; Casciotti, K.L.; McIlvin, M.R.; Beaudoin, D.J.; Visscher, P.T.; Edgcomb, V.P. Potential importance of physiologically diverse benthic foraminifera in sedimentary nitrate storage and respiration. *J. Geophys. Res.* **2012**, *117*, 1–14. [[CrossRef](#)]
19. Kamp, A.; Hogslund, S.; Risgaard-Petersen, N.; Stief, P. Nitrate storage and dissimilatory nitrate reduction by eukaryotic microbes. *Front. Microbiol.* **2015**, *6*, 1492. [[CrossRef](#)]
20. Woehle, C.; Roy, A.S.; Glock, N.; Wein, T.; Weissenbach, J.; Rosenstiel, P.; Hiebenthal, C.; Michels, J.; Schonfeld, J.; Dagan, T. A Novel eukaryotic denitrification pathway in foraminifera. *Curr. Biol.* **2018**, *28*, 2536–2543. [[CrossRef](#)]
21. Kim, S.W.; Fushinobu, S.; Zhou, S.; Wakagi, T.; Shoun, H. Eukaryotic *nirK* genes encoding copper-containing nitrite reductase: Originating from the protomitochondrion? *Appl. Environ. Microbiol.* **2009**, *75*, 2652–2658. [[CrossRef](#)] [[PubMed](#)]
22. Shoun, H.; Fushinobu, S.; Jiang, L.; Kim, S.W.; Wakagi, T. Fungal denitrification and nitric oxide reductase cytochrome P450<sub>nor</sub>. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2012**, *367*, 1186–1194. [[CrossRef](#)]
23. Maeda, K.; Spor, A.; Edel-Hermann, V.; Heraud, C.; Breuil, M.C.; Bizouard, F.; Toyoda, S.; Yoshida, N.; Steinberg, C.; Philippot, L. N<sub>2</sub>O production, a widespread trait in fungi. *Sci. Rep.* **2015**, *5*, 9697. [[CrossRef](#)]
24. Higgins, S.A.; Welsh, A.; Orellana, L.H.; Konstantinidis, K.T.; Chee-Sanford, J.C.; Sanford, R.A.; Schadt, C.W.; Löffler, F.E. Detection and diversity of fungal nitric oxide reductase genes (*p450<sub>nor</sub>*) in agricultural soils. *Appl. Environ. Microbiol.* **2016**, *82*, 2919–2928. [[CrossRef](#)]
25. Wang, J.; Vine, C.E.; Balasiny, B.K.; Rizk, J.; Bradley, C.L.; Tinajero-Trejo, M.; Poole, R.K.; Bergaust, L.L.; Bakken, L.R.; Cole, J.A. The roles of the hybrid cluster protein, Hcp and its reductase, Hcr, in high affinity nitric oxide reduction that protects anaerobic cultures of *Escherichia coli* against nitrosative stress. *Mol. Microbiol.* **2016**, *100*, 877–892. [[CrossRef](#)] [[PubMed](#)]
26. Plouviez, M.; Wheeler, D.; Shilton, A.; Packer, M.A.; McLenachan, P.A.; Sanz-Luque, E.; Ocana-Calahorra, F.; Fernandez, E.; Guieysse, B. The biosynthesis of nitrous oxide in the green alga *Chlamydomonas reinhardtii*. *Plant J.* **2017**, *91*, 45–56. [[CrossRef](#)]
27. Burlacot, A.; Richaud, P.; Gosset, A.; Li-Beisson, Y.; Peltier, G. Algal photosynthesis converts nitric oxide into nitrous oxide. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 2704–2709. [[CrossRef](#)] [[PubMed](#)]
28. Plouviez, M.; Shilton, A.; Packer, M.A.; Guieysse, B. Nitrous oxide emissions from microalgae: Potential pathways and significance. *J. Appl. Phycol.* **2019**, *31*, 1–8. [[CrossRef](#)]
29. Sasso, S.; Stibor, H.; Mittag, M.; Grossman, A.R. From molecular manipulation of domesticated *Chlamydomonas reinhardtii* to survival in nature. *eLife* **2018**, *7*. [[CrossRef](#)]
30. Proschold, T.; Marin, B.; Schlosser, U.G.; Melkonian, M. Molecular phylogeny and taxonomic revision of *Chlamydomonas* (Chlorophyta). I. Emendation of *Chlamydomonas Ehrenberg* and *Chloromonas Gobi*, and description of *Oogamochlamys* gen. nov. and *Lobochlamys* gen. nov. *Protist* **2001**, *152*, 265–300. [[CrossRef](#)]
31. Salome, P.A.; Merchant, S.S. A series of fortunate events: Introducing *Chlamydomonas* as a reference organism. *Plant Cell* **2019**, *31*, 1682–1707. [[CrossRef](#)] [[PubMed](#)]
32. Fernandez, E.; Galvan, A. Nitrate assimilation in *Chlamydomonas*. *Eukaryot. Cell* **2008**, *7*, 555–559. [[CrossRef](#)] [[PubMed](#)]
33. Sanz-Luque, E.; Chamizo-Ampudia, A.; Llamas, A.; Galvan, A.; Fernandez, E. Understanding nitrate assimilation and its regulation in microalgae. *Front. Plant. Sci.* **2015**, *6*, 899. [[CrossRef](#)]
34. Fernandez, E.; Galvan, A. Inorganic nitrogen assimilation in *Chlamydomonas*. *J. Exp. Bot.* **2007**, *58*, 2279–2287. [[CrossRef](#)] [[PubMed](#)]
35. Chamizo-Ampudia, A.; Sanz-Luque, E.; Llamas, A.; Ocana-Calahorra, F.; Mariscal, V.; Carreras, A.; Barroso, J.B.; Galvan, A.; Fernandez, E. A dual system formed by the ARC and NR molybdoenzymes mediates nitrite-dependent NO production in *Chlamydomonas*. *Plant Cell. Environ.* **2016**, *39*, 2097–2107. [[CrossRef](#)]
36. Düner, M.; Lambertz, J.; Mugge, C.; Hemschemeier, A. The soluble guanylate cyclase CYG12 is required for the acclimation to hypoxia and trophic regimes in *Chlamydomonas reinhardtii*. *Plant J.* **2018**, *93*, 311–337. [[CrossRef](#)]

37. Sanz-Luque, E.; Ocaña-Calahorra, F.; de Montaigu, A.; Chamizo-Ampudia, A.; Llamas, A.; Galvan, A.; Fernandez, E. THB1, a truncated hemoglobin, modulates nitric oxide levels and nitrate reductase activity. *Plant J.* **2015**, *81*, 467–479. [[CrossRef](#)]
38. Gerin, S.; Mathy, G.; Blomme, A.; Franck, F.; Sluse, F.E. Plasticity of the mitoproteome to nitrogen sources (nitrate and ammonium) in *Chlamydomonas reinhardtii*: The logic of *Aox1* gene localization. *Biochim. Biophys. Acta* **2010**, *1797*, 994–1003. [[CrossRef](#)]
39. van Lis, R.; Brugiere, S.; Baffert, C.; Coute, Y.; Nitschke, W.; Atteia, A. Hybrid cluster proteins in a photosynthetic microalga. *FEBS J.* **2020**, *287*, 721–735.
40. Atteia, A.; Adrait, A.; Brugiere, S.; Tardif, M.; van Lis, R.; Deusch, O.; Dagan, T.; Kuhn, L.; Gontero, B.; Martin, W.; et al. A proteomic survey of *Chlamydomonas reinhardtii* mitochondria sheds new light on the metabolic plasticity of the organelle and on the nature of the alpha-proteobacterial mitochondrial ancestor. *Mol. Biol. Evol.* **2009**, *26*, 1533–1548. [[CrossRef](#)]
41. Calatrava, V.; Hom, E.F.Y.; Llamas, A.; Fernandez, E.; Galvan, A. Nitrogen scavenging from amino acids and peptides in the model alga *Chlamydomonas reinhardtii*. The role of extracellular L-amino oxidase. *Algal Res.* **2019**, *28*, 101395. [[CrossRef](#)]
42. Kirk, D.L.; Kirk, M.M. Carrier-mediated uptake of arginine and urea by *Chlamydomonas reinhardtii*. *Plant Physiol.* **1978**, *61*, 556–560. [[CrossRef](#)] [[PubMed](#)]
43. Zuo, Z.; Qingqing, R.; Chen, K.; Yang, L.; Cheng, Z.; Peng, K.; Zhu, Y.; Bai, Y.; Wang, Y. Study of amino acids as nitrogen source in *Chlamydomonas reinhardtii*. *Phycol. Res.* **2012**, *60*, 161–168. [[CrossRef](#)]
44. Vallon, O.; Bulte, L.; Kuras, R.; Olive, J.; Wollman, F.A. Extensive accumulation of an extracellular L-amino-acid oxidase during gametogenesis of *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* **1993**, *215*, 351–360. [[CrossRef](#)] [[PubMed](#)]
45. Schroda, M.; Vallon, O. Chaperones and Proteases. In *The Chlamydomonas Sourcebook*, 2nd ed.; Harris, E.H., Stern, D.B., Witman, G.B., Eds.; Academic Press: Oxford, UK, 2009; pp. 671–729.
46. Schmollinger, S.; Muhlhaus, T.; Boyle, N.R.; Blaby, I.K.; Casero, D.; Mettler, T.; Moseley, J.L.; Kropat, J.; Sommer, F.; Strenkert, D.; et al. Nitrogen-sparing mechanisms in *Chlamydomonas* affect the transcriptome, the proteome, and photosynthetic metabolism. *Plant Cell* **2014**, *26*, 1410–1435. [[CrossRef](#)]
47. Muñoz-Blanco, J.; Hidalgo-Martinez, J.; Cardenas, J. Extracellular deamination of L-amino acids by *Chlamydomonas reinhardtii* cells. *Planta* **1990**, *182*, 194–198. [[CrossRef](#)] [[PubMed](#)]
48. Kasai, K.; Ishikawa, T.; Nakamura, T.; Miura, T. Antibacterial properties of L-amino acid oxidase: Mechanisms of action and perspectives for therapeutic applications. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 7847–7857. [[CrossRef](#)] [[PubMed](#)]
49. Hunken, M.; Harder, J.; Kirst, G.O. Epiphytic bacteria on the Antarctic ice diatom *Amphiprora kufferathii* Manguin cleave hydrogen peroxide produced during algal photosynthesis. *Plant Biol.* **2008**, *10*, 519–526. [[CrossRef](#)]
50. Calatrava, V.; Hom, E.F.Y.; Llamas, A.; Fernandez, E.; Galvan, A. OK, thanks! A new mutualism between *Chlamydomonas* and methylobacteria facilitates growth on amino acids and peptides. *FEMS Microbiol. Lett.* **2018**, *365*. [[CrossRef](#)]
51. Chamizo-Ampudia, A.; Sanz-Luque, E.; Llamas, A.; Galvan, A.; Fernandez, E. Nitrate reductase regulates plant nitric oxide homeostasis. *Trends Plant. Sci.* **2017**, *22*, 163–174. [[CrossRef](#)]
52. Leon, J.; Costa-Broseta, A. Present knowledge and controversies, deficiencies, and misconceptions on nitric oxide synthesis, sensing, and signaling in plants. *Plant Cell Environ.* **2020**, *43*, 1–15. [[CrossRef](#)] [[PubMed](#)]
53. de Montaigu, A.; Sanz-Luque, E.; Macias, M.I.; Galvan, A.; Fernandez, E. Transcriptional regulation of *CDP1* and *CYG56* is required for proper NH<sub>4</sub><sup>+</sup> sensing in *Chlamydomonas*. *J. Exp. Bot.* **2011**, *62*, 1425–1437. [[CrossRef](#)] [[PubMed](#)]
54. Rockel, P.; Strube, F.; Rockel, A.; Wildt, J.; Kaiser, W.M. Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. *J. Exp. Bot.* **2002**, *53*, 103–110. [[CrossRef](#)] [[PubMed](#)]
55. Yamasaki, H.; Sakihama, Y. Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: In vitro evidence for the NR-dependent formation of active nitrogen species. *FEBS Lett.* **2000**, *468*, 89–92. [[CrossRef](#)]
56. Gupta, K.J.; Stoimenova, M.; Kaiser, W.M. In higher plants, only root mitochondria, but not leaf mitochondria reduce nitrite to NO, in vitro and in situ. *J. Exp. Bot.* **2005**, *56*, 2601–2609. [[CrossRef](#)]



57. Jasid, S.; Simontacchi, M.; Bartoli, C.G.; Puntarulo, S. Chloroplasts as a nitric oxide cellular source. Effect of reactive nitrogen species on chloroplastic lipids and proteins. *Plant Physiol.* **2006**, *142*, 1246–1255. [[CrossRef](#)]
58. Tewari, R.K.; Prommer, J.; Watanabe, M. Endogenous nitric oxide generation in protoplast chloroplasts. *Plant Cell Rep.* **2013**, *32*, 31–44. [[CrossRef](#)]
59. Corpas, F.J.; Del Rio, L.A.; Palma, J.M. Plant peroxisomes at the crossroad of NO and H<sub>2</sub>O<sub>2</sub> metabolism. *J. Integr. Plant Biol.* **2019**, *61*, 803–816. [[PubMed](#)]
60. Foresi, N.; Mayta, M.L.; Lodeyro, A.F.; Scuffi, D.; Correa-Aragunde, N.; Garcia-Mata, C.; Casalongue, C.; Carrillo, N.; Lamattina, L. Expression of the tetrahydrofolate-dependent nitric oxide synthase from the green alga *Ostreococcus tauri* increases tolerance to abiotic stresses and influences stomatal development in *Arabidopsis*. *Plant J.* **2015**, *82*, 806–821. [[CrossRef](#)]
61. Jeandroz, S.; Wipf, D.; Stuehr, D.J.; Lamattina, L.; Melkonian, M.; Tian, Z.; Zhu, Y.; Carpenter, E.J.; Wong, G.K.; Wendehenne, D. Occurrence, structure, and evolution of nitric oxide synthase-like proteins in the plant kingdom. *Sci. Signal.* **2016**, *9*, re2. [[CrossRef](#)]
62. del Rio, L.A.; Corpas, F.J.; Barroso, J.B. Nitric oxide and nitric oxide synthase activity in plants. *Phytochemistry* **2004**, *65*, 783–792. [[CrossRef](#)] [[PubMed](#)]
63. Gonzalez-Ballester, D.; Sanz-Luque, E.; Galvan, A.; Fernandez, E.; de Montaigu, A. Arginine is a component of the ammonium-CYG56 signalling cascade that represses genes of the nitrogen assimilation pathway in *Chlamydomonas reinhardtii*. *PLoS ONE* **2018**, *13*, e0196167. [[CrossRef](#)] [[PubMed](#)]
64. Hemschemeier, A.; Duner, M.; Casero, D.; Merchant, S.S.; Winkler, M.; Happe, T. Hypoxic survival requires a 2-on-2 hemoglobin in a process involving nitric oxide. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 10854–10859. [[CrossRef](#)] [[PubMed](#)]
65. Folgosa, F.; Martins, M.C.; Teixeira, M. The multidomain flavodiiron protein from *Clostridium difficile* 630 is an NADH:oxygen oxidoreductase. *Sci. Rep.* **2018**, *8*, 10164. [[CrossRef](#)]
66. Romao, C.V.; Vicente, J.B.; Borges, P.T.; Frazao, C.; Teixeira, M. The dual function of flavodiiron proteins: Oxygen and/or nitric oxide reductases. *J. Biol. Inorg. Chem.* **2016**, *21*, 39–52. [[CrossRef](#)]
67. Seedorf, H.; Dreisbach, A.; Hedderich, R.; Shima, S.; Thauer, R.K. F<sub>420</sub>H<sub>2</sub> oxidase (FprA) from *Methanobrevibacter arboriphilus*, a coenzyme F<sub>420</sub>-dependent enzyme involved in O<sub>2</sub> detoxification. *Arch. Microbiol.* **2004**, *182*, 126–137. [[CrossRef](#)]
68. Smutna, T.; Goncalves, V.L.; Saraiva, L.M.; Tachezy, J.; Teixeira, M.; Hrdy, I. Flavodiiron protein from *Trichomonas vaginalis* hydrogenosomes: The terminal oxygen reductase. *Eukaryot. Cell* **2009**, *8*, 47–55. [[CrossRef](#)]
69. Di Matteo, A.; Scandurra, F.M.; Testa, F.; Forte, E.; Sarti, P.; Brunori, M.; Giuffre, A. The O<sub>2</sub>-scavenging flavodiiron protein in the human parasite *Giardia intestinalis*. *J. Biol. Chem.* **2008**, *283*, 4061–4068. [[CrossRef](#)]
70. Vicente, J.B.; Gomes, C.M.; Wasserfallen, A.; Teixeira, M. Module fusion in an A-type flavoprotein from the cyanobacterium *Synechocystis* condenses a multiple-component pathway in a single polypeptide chain. *Biochem. Biophys. Res. Commun.* **2002**, *294*, 82–87. [[CrossRef](#)]
71. Silaghi-Dumitrescu, R.; Coulter, E.D.; Das, A.; Ljungdahl, L.G.; Jameson, G.N.; Huynh, B.H.; Kurtz, D.M., Jr. A flavodiiron protein and high molecular weight rubredoxin from *Moorella thermoacetica* with nitric oxide reductase activity. *Biochemistry* **2003**, *42*, 2806–2815. [[CrossRef](#)]
72. Rodrigues, R.; Vicente, J.B.; Felix, R.; Oliveira, S.; Teixeira, M.; Rodrigues-Pousada, C. Desulfovibrio gigas flavodiiron protein affords protection against nitrosative stress in vivo. *J. Bacteriol.* **2006**, *188*, 2745–2751. [[CrossRef](#)] [[PubMed](#)]
73. Justino, M.C.; Vicente, J.B.; Teixeira, M.; Saraiva, L.M. New genes implicated in the protection of anaerobically grown *Escherichia coli* against nitric oxide. *J. Biol. Chem.* **2005**, *280*, 2636–2643. [[CrossRef](#)]
74. Gardner, A.M.; Helmick, R.A.; Gardner, P.R. Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in *Escherichia coli*. *J. Biol. Chem.* **2002**, *277*, 8172–8177. [[CrossRef](#)] [[PubMed](#)]
75. Chauv, F.; Burlacot, A.; Mekhalfi, M.; Auroy, P.; Blangy, S.; Richaud, P.; Peltier, G. Flavodiiron proteins promote fast and transient O<sub>2</sub> photoreduction in *Chlamydomonas*. *Plant Physiol.* **2017**, *174*, 1825–1836. [[CrossRef](#)] [[PubMed](#)]
76. Mus, F.; Dubini, A.; Seibert, M.; Posewitz, M.C.; Grossman, A.R. Anaerobic acclimation in *Chlamydomonas reinhardtii*: Anoxic gene expression, hydrogenase induction, and metabolic pathways. *J. Biol. Chem.* **2007**, *282*, 25475–25486. [[CrossRef](#)] [[PubMed](#)]
77. Olson, A.C.; Carter, C.J. The involvement of hybrid cluster protein 4, HCP4, in anaerobic metabolism in *Chlamydomonas reinhardtii*. *PLoS ONE* **2016**, *11*, e0149816. [[CrossRef](#)]



78. Overeijnder, M.L.; Hagen, W.R.; Hagedoorn, P.L. A thermostable hybrid cluster protein from *Pyrococcus furiosus*: Effects of the loss of a three helix bundle subdomain. *J. Biol. Inorg. Chem.* **2009**, *14*, 703–710. [[CrossRef](#)]
79. Yu, Z.; Pesesky, M.; Zhang, L.; Huang, J.; Winkler, M.; Chistoserdova, L. A Complex interplay between nitric oxide, quorum sensing, and the unique secondary metabolite tundenone constitutes the hypoxia response in *Methylobacter*. *mSystems* **2020**, *5*. [[CrossRef](#)]
80. Seth, D.; Hess, D.T.; Hausladen, A.; Wang, L.; Wang, Y.J.; Stamler, J.S. A Multiplex enzymatic machinery for cellular protein s-nitrosylation. *Mol. Cell* **2018**, *69*, 451–464. [[CrossRef](#)]
81. Bange, H.B.; Arevalo-Martinez, D.L.; Paz, M.; Farias, L.; Kaiser, J.; Kock, A.; Law, C.S.; Rees, A.P.; Rehder, G.; Tortell, P.D.; et al. A harmonized nitrous oxide (N<sub>2</sub>O) ocean observation network for the 21st century. *Front. Mar. Sci.* **2019**, *6*. [[CrossRef](#)]
82. Cohen, Y.; Gordon, L.G. Nitrous oxide in the oxygen minimum of the eastern tropical North Pacific: Evidence for its consumption during denitrification and possible mechanisms for its production. *Deep Sea Res.* **1978**, *25*, 509–524. [[CrossRef](#)]
83. Smith, C.J.; DeLaune, R.D.; Patrick, W.H. Nitrous oxide emission from Gulf Coast wetlands. *Geochim. Cosmochim.* **1983**, *47*, 1805–1814. [[CrossRef](#)]
84. Mengis, M.; Gächter, R.; Wehrli, B. Sources and sinks of nitrous oxide (N<sub>2</sub>O) in deep lakes. *Biogeochemistry* **1997**, *38*, 281–301. [[CrossRef](#)]
85. Guieysse, B.; Plouviez, M.; Coilhac, M.; Cazali, L. Nitrous Oxide (N<sub>2</sub>O) production in axenic *Chlorella vulgaris* microalgae cultures: Evidence, putative pathways, and potential environmental impacts. *Biogeosciences* **2013**, *10*, 6737–6746. [[CrossRef](#)]
86. Smeets, E.M.; Bouwman, L.F.; Stehfest, E.; Vuuren, D.P.; Postuma, A. Contribution of N<sub>2</sub>O to the greenhouse gas balance of first-generation biofuels. *Glob. Chang. Biol.* **2009**, *15*, 1–23. [[CrossRef](#)]
87. Bauer, S.K.; Grotz, L.S.; Connelly, E.B.; Colosi, L.M. Reevaluation of the global warming impacts of algae-derived biofuels to account for possible contributions of nitrous oxide. *Bioresour. Technol.* **2016**, *218*, 196–201. [[CrossRef](#)]
88. Higgins, S.A.; Schadt, C.W.; Matheny, P.B.; Löffler, F.E. Phylogenomics reveal the dynamic evolution of fungal nitric oxide reductases and their relationship to secondary metabolism. *Genome Biol. Evol.* **2018**, *10*, 2474–2489. [[CrossRef](#)]
89. Weather, P.J. N<sub>2</sub>O Evolution by green algae. *Appl. Environ. Microbiol.* **1984**, *48*, 1251–1253. [[CrossRef](#)]
90. Florez-Leiva, L.; Tarifeño, E.; Cornejo, M.; Kiene, R.; Farias, L. High production of nitrous oxide (N<sub>2</sub>O), methane (CH<sub>4</sub>) and dimethylsulphoniopropionate (DMSP) in a massive marine phytoplankton culture. *Biogeosci. Discuss.* **2010**, *7*, 6705–6723. [[CrossRef](#)]
91. Fagerstone, K.D.; Quinn, J.C.; Bradley, T.H.; De Long, S.K.; Marchese, A.J. Quantitative measurement of direct nitrous oxide emissions from microalgae cultivation. *Environ. Sci. Technol.* **2011**, *45*, 9449–9456. [[CrossRef](#)] [[PubMed](#)]

