



Article

# Regulation of the Emissions of the Greenhouse Gas Nitrous Oxide by the Soybean Endosymbiont *Bradyrhizobium diazoefficiens*

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**Abstract:** The greenhouse gas nitrous oxide (N<sub>2</sub>O) has strong potential to drive climate change. Soils are a major source of N<sub>2</sub>O, with microbial nitrification and denitrification being the primary processes involved in such emissions. The soybean endosymbiont *Bradyrhizobium diazoefficiens* is a model microorganism to study denitrification, a process that depends on a set of reductases, encoded by the *napEDABC*, *nirK*, *norCBQD*, and *nosRZDYFLX* genes, which sequentially reduce nitrate (NO<sub>3</sub><sup>-</sup>) to nitrite (NO<sub>2</sub><sup>-</sup>), nitric oxide (NO), N<sub>2</sub>O, and dinitrogen (N<sub>2</sub>). In this bacterium, the regulatory network and environmental cues governing the expression of denitrification genes rely on the FixK<sub>2</sub> and NnrR transcriptional regulators. To understand the role of FixK<sub>2</sub> and NnrR proteins in N<sub>2</sub>O turnover, we monitored real-time kinetics of NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, NO, N<sub>2</sub>O, N<sub>2</sub>, and oxygen (O<sub>2</sub>) in a *fixK<sub>2</sub>* and *nnrR* mutant using a robotized incubation system. We confirmed that FixK<sub>2</sub> and NnrR are regulatory determinants essential for NO<sub>3</sub><sup>-</sup> respiration and N<sub>2</sub>O reduction. Furthermore, we demonstrated that N<sub>2</sub>O reduction by *B. diazoefficiens* is independent of canonical inducers of denitrification, such as the nitrogen oxide NO<sub>3</sub><sup>-</sup>, and it is negatively affected by acidic and alkaline conditions. These findings advance the understanding of how specific environmental conditions and two single regulators modulate N<sub>2</sub>O turnover in *B. diazoefficiens*.

**Keywords:** denitrification; dinitrogen; gene expression; nitric oxide; nitrous oxide reductase



**Citation:** Bueno, E.; Mania, D.; Mesa, S.; Bedmar, E.J.; Frostegård, Å.; Bakken, L.R.; Delgado, M.J. Regulation of the Emissions of the Greenhouse Gas Nitrous Oxide by the Soybean Endosymbiont *Bradyrhizobium diazoefficiens*. *Int. J. Mol. Sci.* **2022**, *23*, 1486. <https://doi.org/10.3390/ijms23031486>

Academic Editors: Rosa María Martínez-Espinosa and Carmen Lucía Pire-Galiana

Received: 22 December 2021

Accepted: 20 January 2022

Published: 27 January 2022

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## 1. Introduction

Under shortage of oxygen, bacteria can adapt and thrive by two ATP-generating mechanisms: (i) induction of dedicated high-affinity terminal oxidases that permit bacteria to respire oxygen at very low concentrations or (ii) making use of inorganic terminal electron acceptors such as nitrate (NO<sub>3</sub><sup>-</sup>), which can be reduced through the denitrification pathway to dinitrogen (N<sub>2</sub>) or through dissimilatory nitrate reduction to ammonium (DNRA). Although such anaerobic respiration generates less ATP per mol electron than aerobic respiration, it allows bacteria to grow and persist in the absence of oxygen (O<sub>2</sub>) [1]. Denitrification has been defined as the sequential reduction of NO<sub>3</sub><sup>-</sup> or nitrite (NO<sub>2</sub><sup>-</sup>) to nitric oxide (NO), nitrous oxide (N<sub>2</sub>O), and N<sub>2</sub> [2]. This process is catalyzed by the periplasmic (Nap) or membrane-bound (Nar) nitrate reductase, nitrite reductases (NirK/NirS), nitric oxide reductases (cNor, qNor, or Cu<sub>A</sub>Nor), and nitrous oxide reductase (N<sub>2</sub>OR) encoded by *nap/nar*, *nirK/nirS*, *nor*, and *nos* genes, respectively [2–4]. In addition to denitrification, multiple pathways for N<sub>2</sub>O generation have been reported, including nitrification, nitrifier

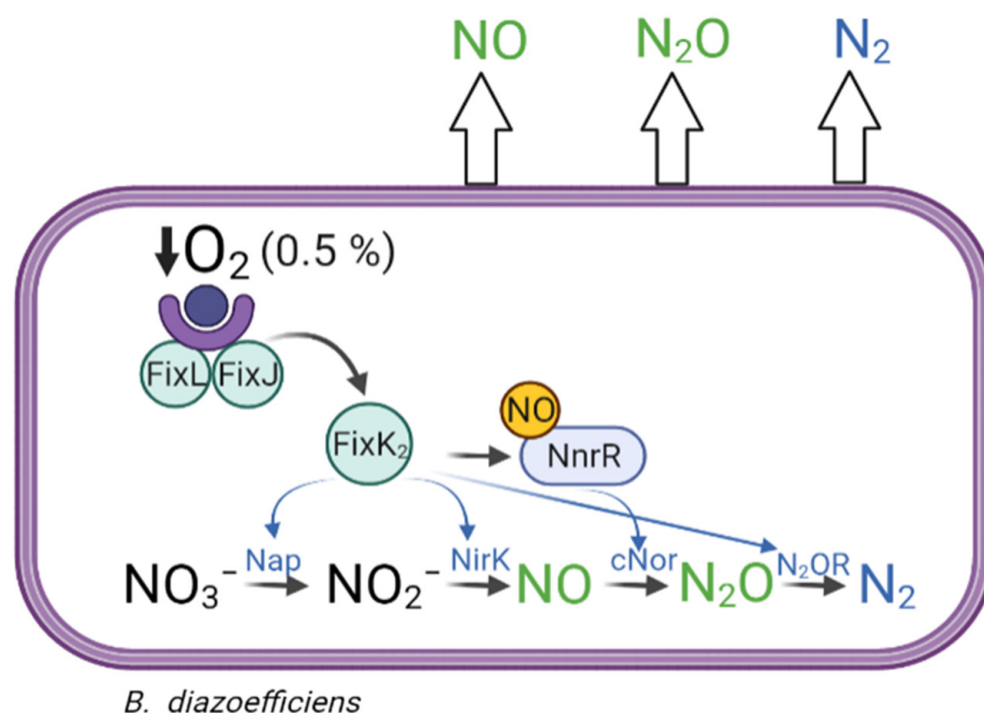
denitrification, nitrite oxidation, ammonia oxidation, heterotrophic denitrification, anaerobic ammonium oxidation (anammox), and DNRA [3,5]. N<sub>2</sub>OR is the only known enzyme catalyzing the reduction of N<sub>2</sub>O to N<sub>2</sub> [6]. Accordingly, expression and activity of N<sub>2</sub>OR are considered natural targets to mitigate N<sub>2</sub>O emissions from agricultural soils [7].

Given the impact of N<sub>2</sub>O as a powerful greenhouse gas in global warming and in depletion of the ozone layer [5,7], understanding its dynamics of production/reduction in soils and aquatic environments has become a priority. In fact, the application of synthetic nitrogen (N) fertilizers to agricultural soils, as well as local oxygen concentrations, water content, carbon availability, and pH, greatly affect N<sub>2</sub>O emissions from soils and aquatic ecosystems [7–9].

Many legumes establish symbiotic associations with soil bacteria, collectively termed “rhizobia”, which fix nitrogen in so-called root nodules on legume roots and on the stems of some aquatic legumes. Following invasion of the plant cells via a complex signaling pathway between bacteria and plant, rhizobia stop dividing and undergo differentiation into nitrogen-fixing bacteroids, at which point the nitrogenase complex reduces atmospheric N<sub>2</sub> into biologically useful forms in a process called “Biological N<sub>2</sub> Fixation”. Consequently, cultivation of legumes can reduce the need for environmentally polluting synthetic nitrogen fertilizers, thus decreasing N<sub>2</sub>O emissions as well as protecting ground water from toxicity while improving soil fertility. However, legume crops can also contribute to N<sub>2</sub>O emissions in several ways: (i) by biologically fixed N<sub>2</sub> being converted to NO and N<sub>2</sub>O through nitrification and denitrification [10]; (ii) by providing N-rich residues for decomposition [11], and (iii) directly by some rhizobia that can denitrify under free-living conditions or in symbiotic association with legumes [12,13]. In this context, one strategy to reduce N<sub>2</sub>O emissions from legume crops is to use as inoculants rhizobia strains with high N<sub>2</sub>OR activity. In fact, it has been shown that N<sub>2</sub>O emissions from soybean crops can be reduced by inoculating legumes with strains of the soybean endosymbiont *Bradyrhizobium diazoefficiens* that overexpress N<sub>2</sub>OR [14,15].

*B. diazoefficiens* is considered a model bacterium to study denitrification in rhizobia, since it is the only rhizobial species that, in addition to fixing N<sub>2</sub>, has the ability to grow under anoxic conditions by reducing NO<sub>3</sub><sup>−</sup> through the complete denitrification pathway, a process widely studied in this bacterium both in free-living conditions and in symbiosis with soybeans [12,13,16]. *B. diazoefficiens* possesses the complete set of *napEDABC*, *nirK*, *norCBQD*, and *nosRZDFYLX* denitrification genes [12], which encode the periplasmic nitrate reductase (Nap), copper-containing nitrite reductase (NirK), nitric oxide reductase type c (cNor), and nitrous oxide reductase (N<sub>2</sub>OR), respectively (Figure 1). Like many other denitrifiers, expression of denitrification genes in *B. diazoefficiens* requires both oxygen limitation and the presence of NO<sub>3</sub><sup>−</sup> or a nitrogen oxide (NO<sub>x</sub>) derived from its reduction. The response to low oxygen (<0.5% O<sub>2</sub> in the gas phase, i.e., <5–10 μM O<sub>2</sub>) is mediated by the FixLJ-FixK<sub>2</sub>-NnrR regulatory cascade [3], in which the response regulator FixJ in its active phosphorylated form induces the expression of several genes, including *fixK<sub>2</sub>*, which encodes the transcriptional regulator FixK<sub>2</sub> (Figure 1). This protein induces the expression of more than 300 genes, including genes associated with microoxic metabolism (*fixNOQP*), denitrification genes (*napEDABC*, *nirK*, *norCBQD*, and *nosRZDFYLX*), and regulatory genes (*rpoN<sub>1</sub>*, *fixK<sub>1</sub>*, and *nnrR*) [12,17,18]. It has also been demonstrated that expression of *napEDABC*, *nirK*, and *nosRZDFYLX* genes requires microoxic conditions and directly depends on FixK<sub>2</sub>, while expression of *norCBQD* genes relies on NO, being the transcriptional regulator NnrR the candidate that directly interacts with *norCBQD* promoter [19,20] (Figure 1).

Although much is known about the role of FixK<sub>2</sub> and NnrR in the regulation of denitrification in *B. diazoefficiens*, this knowledge needs to be extended to include relevant physiological conditions that this bacterium is expected to meet in nature. In the present work, we investigated the dynamics of N<sub>2</sub>O balance by FixK<sub>2</sub> and NnrR as well as the influence of specific environmental conditions such as the presence of nitrogen oxides, O<sub>2</sub> concentration, pH, and the redox state of C-sources.



**Figure 1.** Schematic representation of the denitrification process and its regulation in *Bradyrhizobium diazoefficiens*. *B. diazoefficiens* can reduce nitrate ( $\text{NO}_3^-$ ) to nitrite ( $\text{NO}_2^-$ ), nitric oxide (NO), nitrous oxide ( $\text{N}_2\text{O}$ ), and dinitrogen ( $\text{N}_2$ ) by the periplasmic nitrate reductase (Nap), copper-containing nitrite reductase (NirK), nitric oxide reductase type *c* (cNor), and nitrous oxide reductase ( $\text{N}_2\text{OR}$ ) enzymes, respectively. In *B. diazoefficiens*, expression of denitrification enzymes is tightly regulated by the FixLJ, FixK<sub>2</sub>, and NnrR regulatory proteins (see Introduction for further details). However, despite the coordinated activation of each reductase, environmental unfriendly gases such as NO and  $\text{N}_2\text{O}$  can leak from denitrification and be released to the atmosphere.

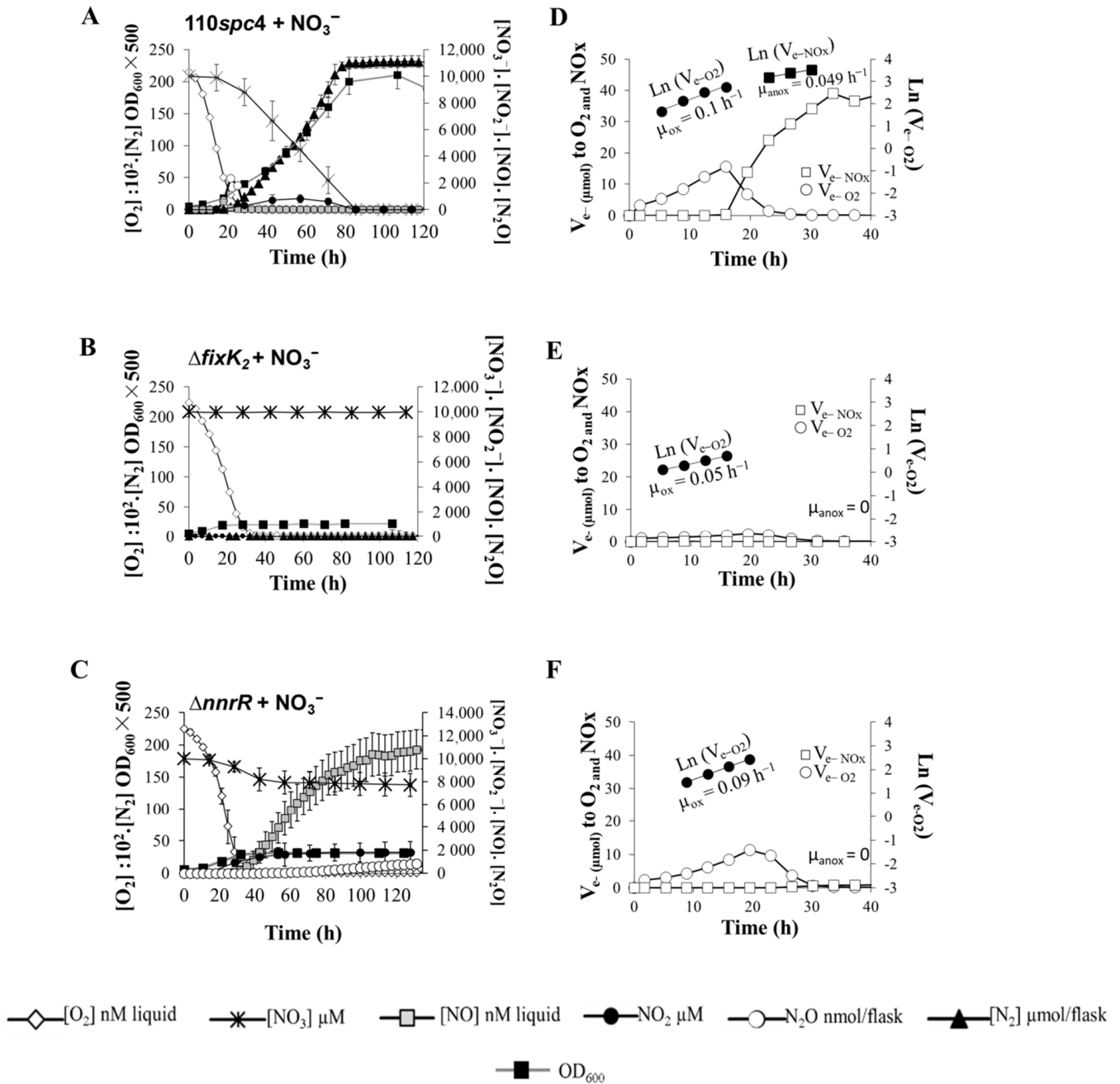
## 2. Results

### 2.1. $\text{N}_2\text{O}$ Emissions by *B. diazoefficiens* 110spc4 Depend on the FixK<sub>2</sub> and NnrR Regulatory Proteins

*B. diazoefficiens* strains were raised oxicly under vigorous stirring, and aliquots were inoculated into culture vials to an initial  $\text{OD}_{600}$  of 0.01 ( $8 \times 10^6$  cells  $\text{mL}^{-1}$ ). Next, 2%  $\text{O}_2$  and 10 mM  $\text{NO}_3^-$  were added as oxic and anoxic electron acceptors, respectively. Figures 2 and S1 show the  $\text{O}_2$ ,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , NO,  $\text{N}_2\text{O}$ , and  $\text{N}_2$  concentrations throughout the 120 h incubation of *B. diazoefficiens* 110spc4 (wild type) (Figures 2A and S1A) and *fixK<sub>2</sub>* (Figure 2B) and *nnrR* (Figures 2C and S1B) mutant strains. *B. diazoefficiens* wild type consumed  $\text{O}_2$  within 28 h, and bacterial  $\text{OD}_{600}$  increased following  $\text{O}_2$  depletion.

Rates of  $\text{O}_2$  consumption for each time increment between two samplings were taken to calculate electron ( $\text{e}^-$ ) flow rates to oxygen ( $V_{\text{e}-\text{O}_2}$ ). As shown in Figure 2D,  $V_{\text{e}-\text{O}_2}$  increased exponentially in the wild type during the first 16 h and declined gradually in response to diminishing  $\text{O}_2$  concentrations. The increase in electron flow can be taken as an indirect measure of growth ( $\mu_{\text{ox}}$ ) [21]. Thus, the apparent  $\mu_{\text{ox}}$  estimated by linear regression of  $\ln(V_{\text{e}-\text{O}_2})$  against time was  $0.10 (\pm 0.03) \text{ h}^{-1}$  (Figure 2D, Table 1A). Final  $\text{OD}_{600}$  resulting from the consumption of 2%  $\text{O}_2$  was  $0.080 (\pm 0.005)$  ( $6.40 \times 10^7$  cells  $\text{mL}^{-1}$ , Table 1B), equivalent to a yield of  $13.3 (\pm 1.1)$  cells  $\text{pmol}^{-1} \text{ e}^-$  to  $\text{O}_2$  (Table 1A). Remarkably, in contrast to the fast depletion of  $\text{O}_2$  observed in the parental strain, the capacity to consume  $\text{O}_2$  in the *fixK<sub>2</sub>* and *nnrR* mutant strains was slightly reduced (Figure 2A–C). In the case of the *fixK<sub>2</sub>* mutant,  $V_{\text{e}-\text{O}_2}$  increased exponentially throughout the first 19 h and then declined gradually (Figure 2E). As shown in Table 1A, the apparent  $\mu_{\text{ox}}$  was  $0.055 (\pm 0.008)$  (Figure 2E, Table 1A). The final  $\text{OD}_{600}$  from  $\text{O}_2$  respiration was  $0.044 (\pm 0.003)$  (Table 1B), resulting in a yield of  $6.6 (\pm 0.3)$  cells  $\text{pmol}^{-1} \text{ e}^-$  to  $\text{O}_2$  (Table 1A). In the *nnrR* mutant,

electron flow to  $O_2$  increased exponentially throughout the first 19 h with an apparent  $\mu_{ox}$  of  $0.090 (\pm 0.004)$  and then decreased slowly (Figure 2F). The final  $OD_{600}$  during oxic phase was  $0.079 (\pm 0.001)$  (Table 1B), with a subsequent yield of  $13.1 (\pm 0.6)$  cells  $pmol^{-1} e^-$  to  $O_2$  (Table 1A).



**Figure 2.** Denitrification phenotypes of the parental strain *B. diazoefficiens* 110spc4 (A) and the two mutant strains  $\Delta fixK_2$  (B) and  $\Delta nnrR$  (C). (A–C) measurement of  $O_2$  and  $NO_3^-$  respiration, concentrations of denitrifying intermediaries ( $NO_2^-$ , NO,  $N_2O$ ,  $N_2$ ), and bacterial growth ( $OD_{600}$ ) yielded from such dynamics. (D–F) electron flow rates to  $O_2$  and nitrogen oxides ( $NO_x$ ). Cells were incubated with 2%  $O_2$  and 10 mM  $NO_3^-$  as oxic and anoxic respiratory substrates, respectively.  $O_2$ ,  $NO_x$  concentrations, and bacterial growth were monitored by automatic sampling from headspace and liquid phase. See Figure S1 to visualize individual gases' dynamics from (A,C). Data are the means and standard deviations of at least three different cultures.

Initiation of denitrification in the parental strain, hallmarked by the reduction of  $\text{NO}_3^-$  and transient emissions of NO and  $\text{N}_2\text{O}$  (Figures 2A and S1A), was observed at  $\text{O}_2$  concentrations of  $\leq 5$  ( $\pm 0.3$ )  $\mu\text{M}$   $\text{O}_2$  (Figures 2A and S1A and Table 1B) after 17 h of incubation. Rapidly,  $\text{N}_2$  production was detected as individual final product from  $\text{NO}_3^-$  denitrification, with 100% of  $\text{NO}_3^-$  being converted to  $\text{N}_2$  within 80 h of growth.  $\text{NO}_2^-$  accumulated for a longer period than NO and  $\text{N}_2\text{O}$ ; however, its concentration was maintained at low levels until it was totally reduced to its depletion (Figure S1A).

**Table 1.** Summary of growth parameters from  $\text{O}_2$  (oxic growth phase) and  $\text{NO}_3^-$  respiration (anoxic growth phase) in the *B. diazoefficiens* 110spc4 parental and *fixK2* and *nnrR* mutant strains (A) and other parameters observed through anoxic  $\text{NO}_3^-$  respiration (B).

A					
Genotype	Oxic Growth Phase		Anoxic Growth Phase		
	$\mu_{\text{ox}}$ ( $\text{h}^{-1}$ )	Yield <sub>ox</sub> (cell $\text{pmol}^{-1} \text{e}^-$ )	$\mu_{\text{anox}}$ ( $\text{h}^{-1}$ )	Yield <sub>anox</sub> (cell $\text{pmol}^{-1} \text{e}^-$ )	
110spc4	0.10 ( $\pm 0.03$ ) a	13.3 ( $\pm 1.1$ ) a	0.049 ( $\pm 0.004$ )	5.1 ( $\pm 0.8$ )	
$\Delta\text{fixK}_2$	0.055 ( $\pm 0.008$ ) b	6.6 ( $\pm 0.3$ ) b	0.00	-	
$\Delta\text{nnrR}$	0.090 ( $\pm 0.004$ ) a	13.1 ( $\pm 0.6$ ) a	0.00	-	
B					
Genotype	[ $\text{O}_2$ ] at Onset of $\text{NO}_3^-$ Reduction ( $\mu\text{M}$ $\text{O}_2$ )	Max [NO] in Liquid (nM)	Fraction of $\text{NO}_3^-$ Reduced to $\text{N}_2$ (%)	Final OD <sub>600</sub> (oxic)	Final OD <sub>600</sub> (anoxic)
110spc4	5 ( $\pm 0.3$ ) a	600 ( $\pm 400$ ) a	100	0.080 ( $\pm 0.005$ ) a	0.40 ( $\pm 0.05$ ) a
$\Delta\text{fixK}_2$	-	-	-	0.044 ( $\pm 0.003$ ) b	0.042 ( $\pm 0.002$ ) b
$\Delta\text{nnrR}$	3.3 ( $\pm 2.3$ ) b	10797 ( $\pm 1700$ ) b	-	0.079 ( $\pm 0.001$ ) a	0.061 ( $\pm 0.004$ ) b

All the experimental vials contained an initial  $\text{O}_2$  concentration of 2% at headspace and 10 mM  $\text{NO}_3^-$  in the growth medium. Data are means with standard error (in parenthesis) from at least three independent cultures. Values in a column followed by the same lower-case letter are not significantly different according to One-Way ANOVA and the Tukey HSD test at  $p \leq 0.05$ . Apparent oxic growth ( $\mu_{\text{ox}}$ ,  $\text{h}^{-1}$ ) and anoxic growth ( $\mu_{\text{anox}}$ ,  $\text{h}^{-1}$ ) rates based on  $\text{O}_2$  consumption during the oxic phase or reduction of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , or  $\text{N}_2\text{O}$  during the anoxic phase. Yield (cells per mole electron) based on increase in OD vs. cumulated consumption of  $\text{O}_2$  or reduction of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , or  $\text{N}_2\text{O}$ . -, not detected.

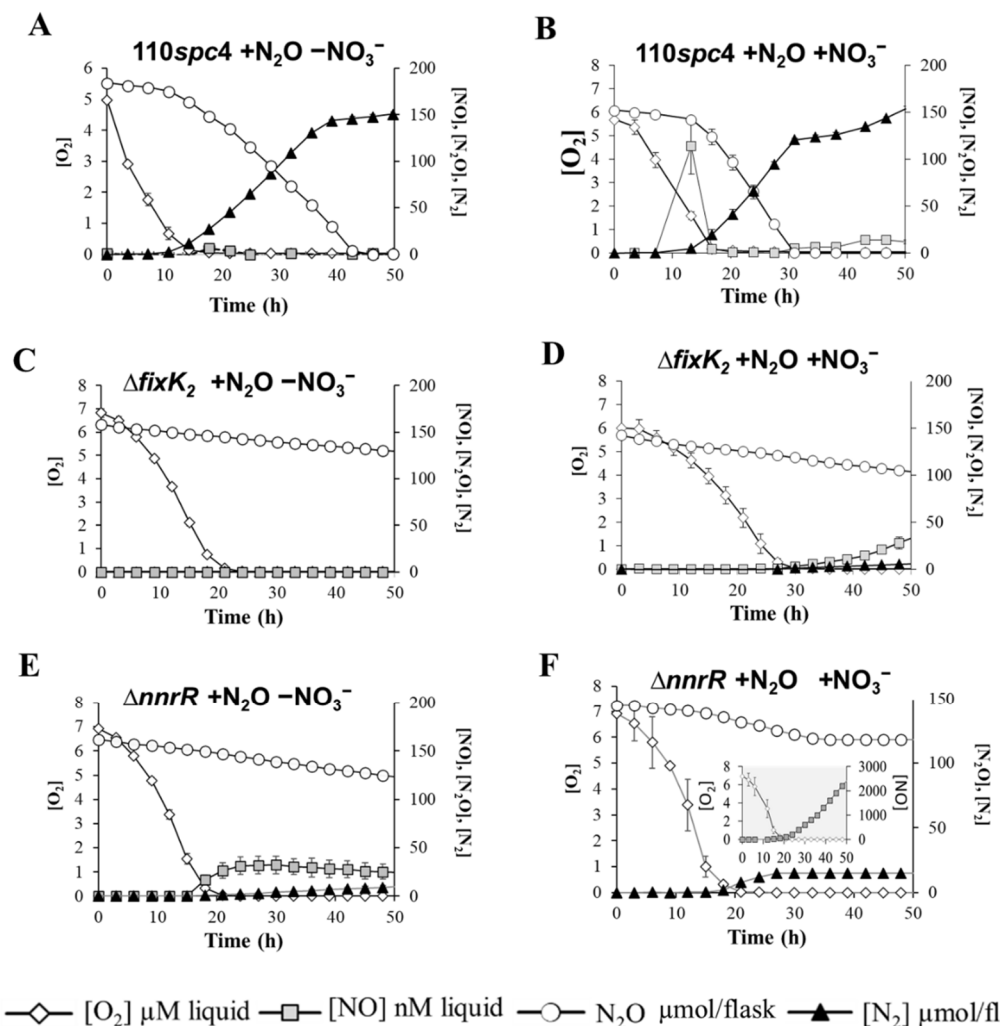
As shown in Figure 2A, growth of *B. diazoefficiens* increased proportionally with  $\text{NO}_3^-$  respiration. Interestingly, the parental strain was able to derive electrons to  $\text{NO}_3^-$  reduction during the oxic phase before  $\text{O}_2$  was depleted, thus securing the transition from aerobic to anaerobic respiration and avoiding anaerobic entrapment (Figure 2D). Electron flow to  $\text{NO}_3^-$  increased exponentially during the anoxic phase, with an estimated growth rate ( $\mu_{\text{anox}}$ ) of 0.049 ( $\pm 0.004$ )  $\text{h}^{-1}$  (Figure 2D; Table 1A). The final OD<sub>600</sub> was 0.40 ( $\pm 0.05$ ) (Table 1B) and cell yield resulting from  $\text{NO}_3^-$  respiration (5.1 ( $\pm 0.8$ ) cells  $\text{pmol}^{-1} \text{e}^-$  to  $\text{NO}_3^-$ ) (Table 1A) was around 2.6-fold lower than that observed during oxic respiration.

In contrast to the competent transition from aerobic to anaerobic  $\text{NO}_3^-$  respiration by the parental strain, the *fixK2* mutant strain was unable to shift to anaerobic respiration (Figure 2B), and following the oxygen depletion, the electron flow dropped drastically to zero (Figure 2E). Remarkably,  $\Delta\text{nnrR}$  was able to initiate denitrification at  $\text{O}_2$  concentrations of  $\leq 3.3$   $\mu\text{M}$  ( $\pm 2.3$ ) after 31 h incubation but was unable to consume NO derived from  $\text{NO}_2^-$  reduction, and consequently, NO accumulated in the headspace of the incubation medium (Figures 2C and S1B and Table 1B). This accumulation of NO probably inhibited  $\text{NO}_3^-$  reduction and concomitant growth.

## 2.2. $\text{N}_2\text{O}$ Reduction by *B. diazoefficiens* 110spc4 Relies on the *FixK2* and *NnrR* Regulatory Proteins in a Nitrogen-Oxides-Independent Manner

Transient detection of  $\text{N}_2\text{O}$  in *B. diazoefficiens* wild type and inhibition of the denitrification process in  $\Delta\text{fixK}_2$  and  $\Delta\text{nnrR}$  strains precluded comparison of their  $\text{N}_2\text{O}$  reduction

capacities. Thus, to specifically assess the capacity of *B. diazoefficiens* wild type and *fixK2* and *nnrR* mutant strains to consume  $N_2O$ , we undertook a complementary approach. We supplied *B. diazoefficiens* bacterial cells with artificial  $N_2O$  and analyzed their capacity to consume it.  $N_2O$  reduction and subsequent  $N_2$  production were monitored in vials containing 5%  $N_2O$  injected into the headspace. In addition, to study the impact that the presence of nitrogen oxides ( $NO_x$ ) might exert on  $N_2O$  reduction, we also examined *B. diazoefficiens*' capacity to consume  $N_2O$  in the absence (Figure 3A,C,E) and in the presence (Figure 3B,D,F) of  $NO_3^-$ . In addition to  $N_2O$ , 0.5%  $O_2$  was also added to the headspace as aerobic respiratory substrate due to the incapacity of *B. diazoefficiens* to initiate growth in the total absence of  $O_2$  (data not shown). Regardless of the presence of  $NO_3^-$ , externally supplied  $N_2O$  was rapidly reduced to  $N_2$  by the parental strain until its complete depletion (Figure 3A,B). The final  $OD_{600}$  (Table 2B) and yield (Table 2A) of *B. diazoefficiens* parental cells were also monitored upon  $N_2O$  consumption, and we found that both growth parameters were significantly enhanced when the bacterium was simultaneously incubated with both alternative electron acceptors,  $N_2O$  and  $NO_3^-$  (Table 2A,B).



**Figure 3.** Impact of *FixK2* and *NnrR* inactivation on  $N_2O$  consumption. Measurement of  $O_2$  and  $N_2O$  respiration and concentrations of  $NO$  and  $N_2$ . *B. diazoefficiens* *110spc4* parental strain (A,B) and *fixK2* (C,D) and *nnrR* (E,F) mutant strains were incubated in vials containing 0.5%  $O_2$  and 5%  $N_2O$  as oxic respiratory and anoxic respiratory substrates, respectively. In addition, a second set of vials were also supplemented with 10 mM  $NO_3^-$  (B,D,F) as anoxic respiratory substrate. The gradual decline in  $N_2O$  concentration in (C,D,E) corresponds to dilution of headspace gases due to sampling. Data are the means and standard deviations of at least three different cultures.

In the absence of  $\text{NO}_3^-$ ,  $\text{N}_2\text{O}$  reduction was initiated at  $\text{O}_2$  concentrations of  $\leq 0.66$  ( $\pm 0.05$ )  $\mu\text{M}$  in the parental strain (Figure 3A; Table 2B). Under these conditions, electron flow to  $\text{N}_2\text{O}$  increased with an apparent growth rate ( $\mu_{\text{N}_2\text{O}}$ ) of  $0.028$  ( $\pm 0.002$ )  $\text{h}^{-1}$  estimated by linear regression of  $\ln(V_{e-\text{N}_2\text{O}})$  against time (Figure S2A, Table 2A). Electron flow rates to  $\text{N}_2\text{O}$  remained unnoticeable during the first 5 h of oxic respiration; however, they increased exponentially after 8 h when electron flow to  $\text{O}_2$  was high. Similar to that which was previously observed during anaerobic  $\text{NO}_3^-$  respiration, this premature induction of the  $\text{N}_2\text{OR}$  in the presence of  $\text{O}_2$  might be a mechanism to elude anoxia entrapment during the transition from oxic to anoxic conditions.

**Table 2.** Summary of growth parameters from  $\text{N}_2\text{O}$  consumption in the *B. diazoefficiens* 110spc4 parental and *fixK2* and *nmrR* mutant strains (A) and other parameters observed through the incubations, depending on the presence or absence of  $\text{NO}_3^-$  (B).

A						
Anoxic $\text{N}_2\text{O}$ Respiration $-\text{NO}_3^-$			Anoxic $\text{N}_2\text{O}$ Respiration $+\text{NO}_3^-$			
Genotype	$\mu_{\text{N}_2\text{O}}$ ( $\text{h}^{-1}$ )	Yield (cell $\text{pmol}^{-1} \text{e}^-$ )	$\text{N}_2\text{OR}$ ( $\mu\text{mol N}_2 \text{h}^{-1}$ )	$\mu_{\text{N}_2\text{O}}$ ( $\text{h}^{-1}$ )	Yield (cell $\text{pmol}^{-1} \text{e}^-$ )	$\text{N}_2\text{OR}$ ( $\mu\text{mol N}_2 \text{h}^{-1}$ )
110spc4	0.028 ( $\pm 0.002$ )	29 ( $\pm 8$ )	4.6 ( $\pm 0.5$ )	0.046 ( $\pm 0.003$ )	48 ( $\pm 13$ )	8.4 ( $\pm 0.8$ )
$\Delta\text{fixK}_2$	-	-	-	-	-	-
$\Delta\text{nmrR}$	-	-	-	-	-	-
B						
$-\text{NO}_3^-$						
Genotype	$[\text{O}_2]$ at Onset of $\text{N}_2\text{O}$ Reduction ( $\mu\text{M O}_2$ )	Max [NO] in Liquid (nM)		% $\text{N}_2\text{O}$ Reduced to $\text{N}_2$		Final $\text{OD}_{600}$
110spc4	0.66 ( $\pm 0.05$ )	-		100 a		0.13 ( $\pm 0.03$ ) a
$\Delta\text{fixK}_2$	-	-		-		0.06 ( $\pm 0.01$ ) b
$\Delta\text{nmrR}$	-	32.2 ( $\pm 8.8$ )		8 ( $\pm 0.5$ ) b		0.05 ( $\pm 0.02$ ) b
$+\text{NO}_3^-$						
Genotype	$[\text{O}_2]$ at Onset of $\text{N}_2\text{O}$ Reduction ( $\mu\text{M O}_2$ )	Max [NO] in Liquid (nM)		% $\text{N}_2\text{O}$ Reduced to $\text{N}_2$		Final $\text{OD}_{600}$
110spc4	0.15 ( $\pm 0.05$ ) a	113 ( $\pm 30$ ) a		100 a		0.2 ( $\pm 0.05$ ) a
$\Delta\text{fixK}_2$	-	35 ( $\pm 6.9$ ) b		-		0.05 ( $\pm 0.01$ ) b
$\Delta\text{nmrR}$	0.8 ( $\pm 0.3$ ) b	2250 ( $\pm 85$ ) c		12.5 ( $\pm 2.1$ ) b		0.07 ( $\pm 0.01$ ) b

All the experimental vials contained an initial  $\text{O}_2$  concentration of 0.5% at headspace, 5%  $\text{N}_2\text{O}$ , and 10 mM  $\text{NO}_3^-$  in the growth medium when indicated. Values in a column followed by the same lower-case letter are not significantly different according to One-Way ANOVA and the Tukey HSD test at  $p \leq 0.05$ . Data are means with standard error (in parenthesis) from at least three independent cultures. Apparent anoxic growth ( $\mu_{\text{N}_2\text{O}}$ ,  $\text{h}^{-1}$ ) rates based on  $\text{N}_2\text{O}$  reduction. Yield (cells per mole electron) based on increase in OD vs. cumulated consumption of  $\text{N}_2\text{O}$  without or with  $\text{NO}_3^-$ . -, not detected.

When  $\text{NO}_3^-$  was present, initiation of denitrification, hallmarked by a transient emission of NO ( $113 \text{ nM} \pm 30$ ), took place after 7 h incubation under nearly anaerobic conditions ( $\text{O}_2$  concentrations of  $1.5$  ( $\pm 0.15$ )  $\mu\text{M}$ ) (Table 2B and Figure 3B, respectively), and it preceded induction of  $\text{N}_2\text{O}$  consumption. In fact,  $\text{N}_2\text{O}$  reduction was initiated at lower  $\text{O}_2$  concentrations of  $\leq 0.15$  ( $\pm 0.05$ )  $\mu\text{M}$  (Figure 3B; Table 2B), indicating the *B. diazoefficiens'* preference for  $\text{NO}_3^-$  as terminal electron acceptor. Estimated anaerobic growth rate supported by  $\text{N}_2\text{O}$  in the presence of  $\text{NO}_3^-$  was  $\mu_{\text{N}_2\text{O}} = 0.046$  ( $\pm 0.003$ )  $\text{h}^{-1}$  (Figure S2B, Table 2A). Equivalently to that which was observed in the absence of  $\text{NO}_3^-$ , electron flow to  $\text{N}_2\text{O}$  reduction occurred during active  $\text{O}_2$  respiration after 5 h incubation in the presence of  $\text{NO}_3^-$  (Figure S2B).

Strikingly, *B. diazoefficiens* strains lacking the regulatory transcriptional factors *FixK2* or *NnrR* were severely impaired in  $\text{N}_2\text{O}$  consumption capacity and growth (Figure 3C–F, Table 2A,B). Despite  $\Delta\text{fixK}_2$  being unable to reduce  $\text{N}_2\text{O}$  and grow either in the absence or the presence of  $\text{NO}_3^-$ , traces of NO gas were detected after 40 h incubation with  $\text{NO}_3^-$  (Figure 3D), but such residual respiratory activity was not coupled to growth. A mutant strain defective in the *nmrR* gene was significantly defective in its capacity to reduce  $\text{N}_2\text{O}$

when incubated without  $\text{NO}_3^-$  (only  $8 (\pm 0.5)\%$  of  $\text{N}_2\text{O}$  was reduced to  $\text{N}_2$ ), likely due to its incapacity to detoxify  $\text{NO}$ , which permanently accumulated in the medium up to  $32.2 (\pm 8.8)$  nM (Figure 3E, Table 2B). The presence of  $\text{NO}_3^-$  slightly induced  $\text{N}_2\text{O}$  reduction by the *nmrR* mutant ( $12.5 (\pm 2.1)\%$  of  $\text{N}_2\text{O}$  was reduced to  $\text{N}_2$ ) at  $\text{O}_2$  concentrations of  $0.8 (\pm 0.3)$   $\mu\text{M}$  after 13 h incubation (Figure 3F, Table 2B). However, under these conditions,  $\text{NO}_3^-$  in the medium was further reduced to  $\text{NO}$ , which was accumulated after 20 h incubation reaching levels up to  $\sim 2$   $\mu\text{M}$  after 50 h incubation (Figure 3F, insert, Table 2B).

Our results explain that *B. diazoefficiens* can co-respire  $\text{NO}_3^-$  and  $\text{N}_2\text{O}$  and that activation of the  $\text{N}_2\text{O}$  reductase relies on the FixK<sub>2</sub> and NnrR regulatory proteins, independently of the presence of nitrogen oxides. Lastly, we also found that  $\text{N}_2\text{O}$  reductase activity in *B. diazoefficiens* is highly sensitive to accumulation of endogenous  $\text{NO}$  derived from  $\text{NO}_3^-$  respiration, further supporting the importance of coordinated activation of denitrifying reductases by the FixK<sub>2</sub> and NnrR regulators.

### 2.3. Acidic and Alkaline pHs Impair $\text{N}_2\text{O}$ Reduction by *B. diazoefficiens* 110spc4

To further elucidate how environmental cues prevailing in *B. diazoefficiens* niches might modulate  $\text{N}_2\text{O}$  reduction, we monitored the expression of *nosRZDFYLX* genes and the capacity of *B. diazoefficiens* to reduce  $\text{N}_2\text{O}$  in the presence of C-substrates commonly encountered in a plant's rhizosphere [22], such as succinate, which generates 2 mol e<sup>-</sup> per C-mol oxidized, and butyrate, which generates 5 mol e<sup>-</sup> per C-mol oxidized. Interestingly, such C-sources did not affect expression of the *nos* operon (Figure S3A). Next, we analyzed  $\text{N}_2\text{O}$  consumption by *B. diazoefficiens* determined as changes in  $\text{N}_2\text{O}$  concentration in the headspace of vials containing 0.5%  $\text{O}_2$  plus 5%  $\text{N}_2\text{O}$  inoculated with aerobically raised bacterial cells. Monitoring  $\text{O}_2$  uptake by *B. diazoefficiens* during the oxic phase also allowed us to evaluate any effect of C-source on bacterial metabolism/energetic that could subsequently alter  $\text{N}_2\text{O}$  respiration. Despite  $\text{N}_2\text{O}$  consumption was delayed around 20 h in the presence of butyrate compared to succinate (Figure 4A,B), such impairment could be attributed to a general metabolic defect, as oxygen consumption during the first hours of growth also was attenuated in that C-source. Further metabolic analyses are required to shed light on this respiratory inhibition induced by reduced C-sources.

To understand if local changes in soil pH might affect  $\text{N}_2\text{O}$  emissions from *B. diazoefficiens*, we also examined  $\text{N}_2\text{OR}$  gene expression and  $\text{N}_2\text{O}$  consumption in cells incubated at different pHs. As shown in Figure S3B, *nos* expression levels after 20 and 30 h incubation were not affected by different pH levels. Interestingly, while  $\text{O}_2$  consumption was similar at different pH levels,  $\text{N}_2\text{O}$  reduction was strongly diminished at pH 6 and 8 (Figure 4C–F). These findings imply that, in addition to the impact of FixK<sub>2</sub> and NnrR regulatory proteins on  $\text{N}_2\text{O}$  reduction, relevant environmental factors such as pH importantly influence dynamics of  $\text{N}_2\text{O}$  reduction by *B. diazoefficiens*.

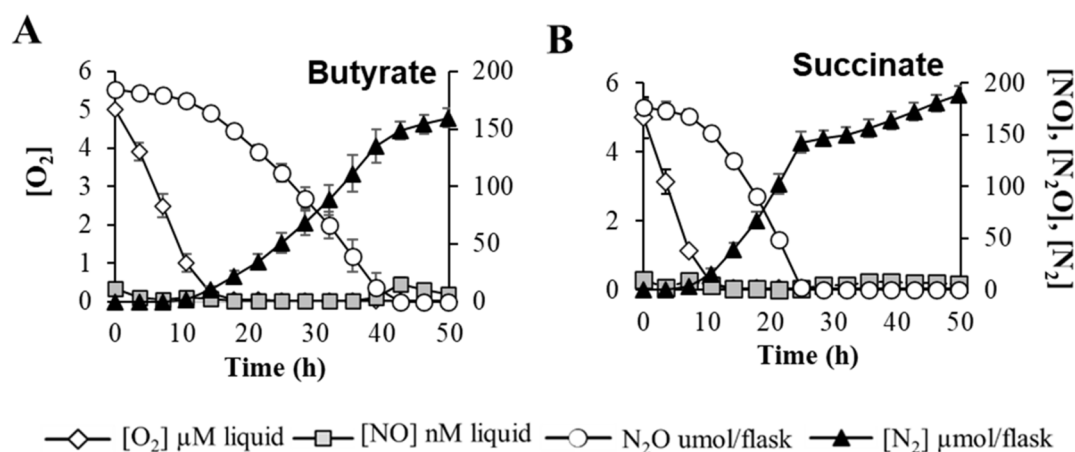
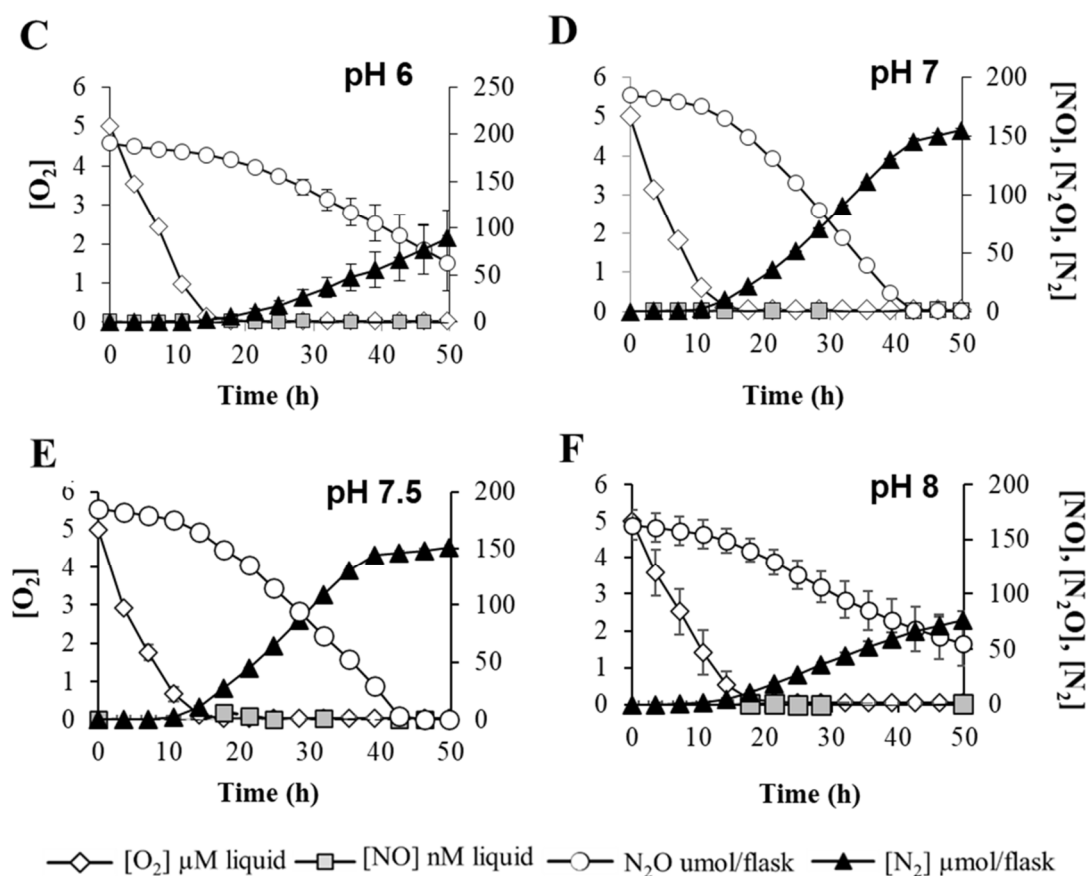


Figure 4. Cont.





**Figure 4.** Impact of C-source and pH on N<sub>2</sub>O consumption. Measurement of O<sub>2</sub> and N<sub>2</sub>O respiration and concentrations of NO and N<sub>2</sub>. *B. diazoefficiens* 110spc4 parental strain was incubated in vials containing 0.5% O<sub>2</sub>, 5% N<sub>2</sub>O, and 10 mM NO<sub>3</sub><sup>−</sup> as substrates for aerobic and anaerobic respiration, respectively. C-sources (A,B) and pH (C–F) of the growth medium were modified as shown on the graphs (see Material and Methods for further details). O<sub>2</sub> and NO<sub>x</sub> concentrations were monitored by automatic sampling from headspace phase. Data are the means and standard deviations of at least three different cultures.

### 3. Discussion

Given the damaging effect of N<sub>2</sub>O on climate, strategies to mitigate N<sub>2</sub>O emissions arising from intensive agricultural practices must be developed. These strategies include: (i) management of soil chemistry and microbiology to ensure that bacterial denitrification proceeds to completion, forming N<sub>2</sub>; (ii) promotion of sustainable agriculture, i.e., obtaining higher output from the same cultivated area of land; (iii) a better understanding of the environmental and regulatory factors that contribute to the generation and consumption of biological N<sub>2</sub>O; and (iv) reducing the dependence on fertilizers by using engineered crops that fix dinitrogen themselves or, alternatively, through application of nitrogen-fixing bacteria to legume crops. Despite the latter being one of the most promising alternatives to reduce N<sub>2</sub>O emissions, denitrification within endosymbiotic and free-living rhizobia released from nodules also contributes to the emission of N<sub>2</sub>O [10,13,16,23,24]; therefore, a better knowledge of the environmental and cellular factors controlling rhizobial denitrification is required.

Environmental cues (oxygen tensions and nitrogen oxides) and regulatory proteins (FixK<sub>2</sub> and NnrR) governing denitrification in *B. diazoefficiens* are well-known [19,20,25] (Figure 1). In this work, we have validated, under physiological conditions, the importance of the FixK<sub>2</sub> and NnrR transcription factors in real-time N<sub>2</sub>O dynamics using a robotized incubation system. Hence, we were able to simultaneously monitor changes in O<sub>2</sub>, NO<sub>3</sub><sup>−</sup>,

$\text{NO}_2^-$ ,  $\text{NO}$ ,  $\text{N}_2\text{O}$ , and  $\text{N}_2$  concentration during the transition from aerobic to anaerobic respiration in *B. diazoefficiens* wild type and *fixK<sub>2</sub>* and *nnrR* regulatory mutants. In addition, we also performed precise estimations of growth parameters (i.e.,  $\mu$ , yield) and defined accurately the  $\text{O}_2$  concentrations in which each step of the denitrification process is triggered. Therefore, we were able to determine that the denitrification process in *B. diazoefficiens* occurs at  $\text{O}_2$  concentrations of  $\leq 5 (\pm 0.3) \mu\text{M}$ . This concomitant induction of the denitrifying machinery with oxic respiration ensures a smooth and efficient transition from aerobic to anaerobic respiration, avoiding depression of electron flow when  $\text{O}_2$  is scarce (Figure 2A,D). A similar scenario was previously observed in the plant pathogen *Agrobacterium tumefaciens* [26]. In contrast to the early induction of the denitrification process found in these plant-interacting bacteria, *Paracoccus denitrificans* initiates transcription of nitrite reductase very late, resulting in entrapment of the majority of cells in anoxia [27].

We have also demonstrated, for the first time, that *B. diazoefficiens* 110*spc4* is an efficient denitrifier, as it is able to transform 100% of  $\text{NO}_3^-$  to  $\text{N}_2$  (Figures 2A and S1A). Interestingly, emission of  $\text{N}_2\text{O}$  was detected at an early peak in  $\text{O}_2$  concentration of  $\leq 5 \mu\text{M}$  (Figures 2A and S1A and Table 2) during the transition from aerobic to anaerobic respiration, but the bacteria rapidly reduced its concentration, keeping it under very low levels ( $\sim 2$  ppm in headspace; 40 nM in the liquid). Collectively, these results reveal that denitrification in *B. diazoefficiens* 110*spc4* emits marginal amounts of  $\text{N}_2\text{O}$ , implying, as demonstrated by Mania et al., (2020) [28] and by Gao et al., (2021) [29], that bradyrhizobia can constitute a strong sink of the  $\text{N}_2\text{O}$  released by neighboring organisms in the soil. Such denitrifying activity depends on coordinated activities of FixK<sub>2</sub> and NnrR regulatory proteins. The tight control on emission of  $\text{N}_2\text{O}$  and other denitrifying gases has been previously described in diverse bacterial species [26–29].

Although  $\text{NO}$  is a key signal molecule for the regulation of many processes, at high concentrations it exerts toxicity at different cellular levels [30–32]. Consequently, bacteria employ dedicated regulatory systems to keep  $\text{NO}$  at very low concentrations. Strikingly, we found that  $\text{NO}$  levels in *B. diazoefficiens* cultures reached very high concentrations ( $\sim 600$  nM) (Table 1B). Similarly, *A. tumefaciens* also accumulates large amounts of  $\text{NO}$ ; however, those  $\text{NO}$  concentrations were not detrimental for this closely related rhizobium [26]. Conversely, *P. denitrificans*, *Pseudomonas aerofaciens*, and strains from the genus *Thauera* present a relatively tight control of  $\text{NO}$  production, maintaining  $\text{NO}$  concentration lower than 10–50 nM [21,27,33]. Although the reason for these differences in control of and tolerance to  $\text{NO}$  concentrations is unknown, it might arise from differential selective pressures exhibited by their ecological niches. Hence, while *P. denitrificans*, *P. aerofaciens*, and *Thauera* genus comprise bacteria that exist under free-living conditions, *A. tumefaciens* and *B. diazoefficiens* are bacteria that can interact with plants establishing pathogenic and symbiotic relationship, respectively. During its interaction with plants, *A. tumefaciens* might face diverse host defense systems such as  $\text{NO}$  production. Thus, a high tolerance to  $\text{NO}$  might confer a certain fitness advantage in respect to other soil competitors.  $\text{NO}$  is also known to be produced by plants in early stages during its interaction with nitrogen-fixing bacteria, as well as within the mature nodule [34–37]. In this context, symbiotic bacteria might require higher tolerance to  $\text{NO}$  to establish a productive symbiotic interaction with the plant.

In contrast to the efficient denitrifying capacity of *B. diazoefficiens* wild type, we found that the *fixK<sub>2</sub>* mutant was unable to initiate  $\text{NO}_3^-$  reduction. On the contrary, *nnrR* mutant cells were able to initiate the reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  and to  $\text{NO}$ ; however, they were entrapped into anoxia due to accumulation of toxic concentrations of  $\text{NO}$  (Figure 2B,C). This disparate response of *fixK<sub>2</sub>* and *nnrR* mutants confirms previous results in vitro, where we demonstrated that FixK<sub>2</sub> directly controls the expression of *napEDABC*, *nirK*, and *nosRZDFYLYX* genes in response to microoxic conditions and NnrR is the regulator that directly interacts with *norCBQD* promoter in response to  $\text{NO}$  [19,20]. Similar denitrification phenotypes were observed in *P. denitrificans* mutants deficient in the  $\text{O}_2$  and  $\text{NO}$  sensors FnrP and NNR, respectively [38].

Since  $\text{NO}_3^-$  reduction in  $\Delta\text{fixK}_2$  and  $\Delta\text{nnrR}$  was abrogated, we could not value their capacity to produce or consume  $\text{N}_2\text{O}$  resulting from  $\text{NO}_3^-$  reduction. To achieve this goal, we incubated the cells in the presence of  $\text{N}_2\text{O}$ , and we analyzed  $\text{N}_2\text{O}$  and  $\text{N}_2$  fluxes. In the parental strain *B. diazoefficiens* 110*spc4*,  $\text{N}_2\text{O}$  reduction was initiated at  $\text{O}_2$  concentrations of 0.15 ( $\pm 0.05$ ) and 0.66 ( $\pm 0.05$ )  $\mu\text{M}$  in the presence and in the absence of  $\text{NO}_3^-$ , respectively. In contrast to the low  $\text{O}_2$  concentration required to trigger  $\text{N}_2\text{O}$  consumption in *B. diazoefficiens*, in other rhizobia species such as *Ensifer meliloti* strain 1021,  $\text{N}_2\text{O}$  consumption was initiated at  $\text{O}_2$  concentrations of 8  $\mu\text{M}$  [39], indicating that *B. diazoefficiens* presents a  $\text{N}_2\text{OR}$  more sensitive to  $\text{O}_2$  than other closely related rhizobial species.

In addition to microoxia, the nitrogen oxide  $\text{NO}_3^-$  and its reduction products  $\text{NO}_2^-$  or  $\text{NO}$  are considered essential inducers of denitrification in *B. diazoefficiens* [3,20]. Remarkably, we demonstrated in this work that  $\text{N}_2\text{O}$  reduction in this bacterium was triggered in the absence of  $\text{NO}_3^-$ . Supporting our observations, it has been previously reported that microoxia is the main signal of expression of *B. diazoefficiens* *nosRZDYFLX* genes and  $\text{N}_2\text{OR}$  activity [19]. This independence from  $\text{NO}_3^-$  was also reported in *E. meliloti* [39] and *P. denitrificans* [33].

When  $\text{N}_2\text{O}$  was externally supplied, the parental strain reduced 100% of  $\text{N}_2\text{O}$  to  $\text{N}_2$ . In contrast, the  $\text{N}_2\text{O}$ -reducing capacity of the *fixK<sub>2</sub>* mutant was totally abolished in a medium without or with  $\text{NO}_3^-$ . However, *nnrR* mutant cells were able to reduce some  $\text{N}_2\text{O}$  to  $\text{N}_2$  in the absence or in the presence of  $\text{NO}_3^-$  (8 ( $\pm 0.5$ )% and 12.5 ( $\pm 2.1$ )%, respectively) (Table 2B). These results confirm previous reports that propose *FixK<sub>2</sub>* but not *NnrR* as the main transcriptional activator of the *nosRZDYFLX* genes [19]. In contrast to the disparate contribution of *FixK<sub>2</sub>* and *NnrR* observed in our studies, it has been proposed that the homologous regulators of *P. denitrificans* *FnrP* and *NNR* contribute equally to  $\text{N}_2\text{OR}$  induction [38,40,41]. Interestingly, cultures from  $\Delta\text{nnrR}$ , with or without nitrate, showed a weak  $\text{N}_2\text{OR}$  activity. In contrast to the transient accumulation of  $\text{NO}$  detected in cultures from the WT strain with  $\text{NO}_3^-$  (Figure 3B), the  $\Delta\text{nnrR}$  mutant seems to be unable to detoxify  $\text{NO}$ , which remains permanently in the medium throughout the incubation (Figure 3F, insert). This long-lasting accumulation of  $\text{NO}$  was also observed when the medium was not supplemented with nitrate (Figure 3E). This  $\text{NO}$  may arise from traces of nitrate present in this medium (~50–100  $\mu\text{M}$ , data not shown). The permanent accumulation of  $\text{NO}$  (32 nM) in  $\Delta\text{nnrR}$  cells incubated without nitrate or when they were incubated with nitrate (~2  $\mu\text{M}$ ) might impair  $\text{N}_2\text{O}$  reduction of the *B. diazoefficiens*  $\Delta\text{nnrR}$  mutant.

An optimal management of soils is crucial to induce  $\text{N}_2\text{OR}$  activity. In this context, it has been reported that maintaining soil pH at high ranges promotes  $\text{N}_2\text{OR}$  activity. This strategy is based on the reported sensitivity of the  $\text{N}_2\text{O}$  reductase activity to low pH in denitrifying bacteria [33,39], in bacterial communities extracted from soils and in intact soils [42]. Carbon availability also has an important role in  $\text{N}_2\text{O}$  emissions from soils [43]. However, how specific forms of reductants might affect expression and activity of  $\text{N}_2\text{OR}$  is largely unexplored. To study ecologically relevant environmental factors that could influence *B. diazoefficiens*  $\text{N}_2\text{OR}$  expression and activity, we analyzed the expression of a *nosR-lacZ* transcriptional fusion as well as  $\text{N}_2\text{OR}$  activity by monitoring  $\text{N}_2\text{O}$  consumption, in the presence of reduced or oxidized C-sources such as butyrate or succinate and at different pH values. Despite the fact that expression of the *nos* genes was not affected by any of the conditions tested,  $\text{N}_2\text{OR}$  activity was significantly attenuated when *B. diazoefficiens* cells were incubated under acidic and alkaline pHs (i.e., pH 6 and pH 8). Moreover,  $\text{N}_2\text{OR}$  activity was also negatively affected when cells were incubated with reduced C-sources. However, reduced C-sources also affected oxygen consumption, which may indicate a general defect in bacterial metabolism when using such a C-source.

Confirming these observations, low pH had little effect on the transcription of the *nosZ* gene in *P. denitrificans* [33]. Instead, the enzymatic rate of  $\text{N}_2\text{O}$  reduction was significantly attenuated at low pH levels, suggesting that environmental pH may have a direct posttranslational effect on the assembly and/or activity of the  $\text{N}_2\text{OR}$  holoenzyme. Consistent with these findings, pH did not affect gene expression of *Marinobacter hydrocarbonoclasticus*  $\text{N}_2\text{OR}$

genes; however, the amount of N<sub>2</sub>O reductase isolated from cells grown at pH 6.5 was lower than that at pH 7.5 and 8.5, pointing to a post-transcriptional regulation [44]. Indeed, biochemical studies of the *M. hydrocarbonoclasticus* N<sub>2</sub>OR revealed that redox properties of its catalytic site are significantly altered by changes in pH values ranging from 6.5 to 8.5 [44]. Similarly, as observed in *B. diazoefficiens*, an inhibitory effect of reduced carbon sources such as butyrate or low pH on N<sub>2</sub>OR activity was already observed in *E. meliloti* [39]. In contrast to *E. meliloti* [39] and *M. hydrocarbonoclasticus* [44], in our work, *B. diazoefficiens* N<sub>2</sub>OR was also inhibited at a high pH, buttressing the importance of controlling soils pH regarding N<sub>2</sub>O emissions. Such sensitivity of *B. diazoefficiens* N<sub>2</sub>OR to high pH is currently under investigation.

Altogether, these observations expand the knowledge of the regulatory and environmental factors that control N<sub>2</sub>O emissions by bacterial species associated with legumes. This information should be taken into consideration when developing new programs to manage N<sub>2</sub>O emissions from legume crops.

## 4. Materials and Methods

### 4.1. Bacterial Strains and Growth Conditions

*Bradyrhizobium diazoefficiens* 110spc4 [45] and  $\Delta fixK_2::\Omega$  and  $\Delta nnrR::aphII$  mutant strains [20] were used in this work. To analyze expression of the *nosRZDYFLX* genes, a *B. diazoefficiens* strain (110spc4-BG0306) containing a chromosomally integrated transcriptional fusion within the *nosRZDYFLX* genes promoter and the *lacZ* reporter gene was used [19]. *B. diazoefficiens* strains were firstly grown aerobically in 120 mL serum vials each containing a magnetic stirring bar and 50 mL of Peptone-Salts-Yeast extract (PSY) complete medium [46] at 30 °C. To analyze anaerobic growth from *B. diazoefficiens*, aliquots from aerobic cultures raised under vigorous stirring to avoid anoxic microzones by cells aggregation were transferred to vials with minimal defined Bergersen's medium [47]. Oxygen from vials was removed by 6 cycles of air evacuation for 360 s and helium (He) filling for 40 s. Influence of pH on N<sub>2</sub>O consumption was analyzed by cultivating *B. diazoefficiens* under N<sub>2</sub>O respiring conditions in minimally defined medium buffered with 50 mM phosphate buffer at pH 6, 7, 7.5, and 8. In all the treatments, the headspace was filled with an initial concentration of O<sub>2</sub> of 0.5 or 2% (6 or 24 μM dissolved O<sub>2</sub> at 30 °C, respectively). To study the N<sub>2</sub>O consumption by the bacterium, vials were also supplemented with N<sub>2</sub>O 5% (1.2 mM). A concentration of 10 mM KNO<sub>3</sub><sup>-</sup> was also added to the cultures as alternative respiratory substrate as indicated in the text. When needed, antibiotics were used at the following concentrations (in μg/mL): kanamycin, 30; spectinomycin, 25; streptomycin, 25; tetracycline, 10.

### 4.2. Gas Measurements

After transferring aerobically grown bacteria into anaerobic vials, they were placed together with blanks and gas standards in a thermostatic water incubator at 30 °C. Cells dispersion and equal distribution of gases throughout the vial liquid and headspace was achieved by continuous stirring at 700 rpm. Emission of gases (O<sub>2</sub>, NO, N<sub>2</sub>O, and N<sub>2</sub>) resulting from bacterial aerobic and anaerobic metabolism were monitored by automatic gas sampling. Gas measurements were analyzed as described by Bueno et al., 2015, and Molstad et al., 2007 [39,48]. Briefly, the gas samples were drawn from each bacterial culture, and with each sampling an equal volume of He was pumped back into the vials to maintain gas pressure at 1 atm. Sampling and gases' measurements were performed as previously described in detail [39].

### 4.3. Determination of Bacterial Growth and NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> Concentrations

To measure bacterial growth and NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> concentrations, aliquots from the liquid phase of vials were withdrawn manually by using sterile syringes. Bacterial growth was determined by measuring cell density at 600 nm (OD<sub>600</sub>). Concentrations of NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> were determined as described by Bueno et al., 2015 [39].

#### 4.4. Kinetic Analysis from Aerobic and Anaerobic Respiration

Aerobic and anaerobic respiration kinetics were determined as described by Bueno et al., 2015 [39]. To determine O<sub>2</sub> and NO concentrations in the liquid, we considered the pressure of the gases, their solubilities, and their transport coefficients among headspace and liquid. O<sub>2</sub> dissolved in liquid was also calculated considering O<sub>2</sub> respiration rate during bacterial growth (see Molstad et al., 2007 for details). We analyzed N<sub>2</sub>O concentrations as  $\mu\text{mol N}_2\text{O vial}^{-1}$ , while N<sub>2</sub> was estimated as net production of N<sub>2</sub>. Growth rates ( $\mu_{\text{ox}}$ ) and reduction of NO<sub>x</sub> during the anoxic phase ( $\mu_{\text{anox}}$ ) were determined by regression [ $\ln(V_{e-})$  against time] for the phases with exponentially increasing rates. Determination of cells yield (cells  $\text{pmol}^{-1} \text{e}^{-}$ ) was estimated considering the number of biomass produced per pmol electron consumed by the transport electron chain to reduce O<sub>2</sub> to H<sub>2</sub>O in the oxic phase (Yield<sub>ox</sub>) or by the denitrifying machinery during the anoxic phase (Yield<sub>anox</sub>). V<sub>max</sub> tells us about the specific efficiency for O<sub>2</sub> and NO<sub>x</sub> respiration per cell. For further details regarding these calculations, see Molstad et al. (2007) [48] and Nadeem et al. (2013) [27].

#### 4.5. Determination $\beta$ -Galactosidase Activity

$\beta$ -galactosidase activity to investigate gene expression was analyzed as previously described [49]. In brief, 5 mL of cells incubated for 20 and 30 h under the conditions detailed in the text were collected, centrifuged, and resuspended in 500  $\mu\text{L}$  of growth medium. In total, 25  $\mu\text{L}$  of this culture was mixed with 20  $\mu\text{L}$  of freshly prepared SDS 0.1%, 25  $\mu\text{L}$  chloroform, and 100  $\mu\text{L}$  of Z-buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, and 50 mM  $\beta$ -mercaptoethanol). Next, 20  $\mu\text{L}$  of ONPG (4 mg/mL) was added to initiate the reaction. Reaction mix was incubated at room temperature before the reaction was terminated by addition of 75  $\mu\text{L}$  of 1 M Na<sub>2</sub>CO<sub>3</sub>. Supernatant was collected and absorbance at OD<sub>420</sub> and OD<sub>550</sub> used to determine  $\beta$ -galactosidase specific activity in Miller units.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/ijms23031486/s1>.

**Author Contributions:** Conceptualization, E.B., Å.F., L.R.B. and M.J.D.; methodology, E.B., D.M., Å.F., L.R.B. and M.J.D.; validation, E.B., Å.F., L.R.B. and M.J.D.; formal analysis, E.B., Å.F., L.R.B. and M.J.D.; investigation, E.B.; resources, Å.F., L.R.B., S.M. and M.J.D.; writing—original draft preparation, E.B., writing—review and editing, E.B., Å.F., L.R.B., E.J.B., S.M. and M.J.D.; visualization, E.B., Å.F., L.R.B. and M.J.D.; supervision, E.B., Å.F., L.R.B. and M.J.D.; project administration, E.B., Å.F., L.R.B. and M.J.D.; funding acquisition, E.B., Å.F., L.R.B., S.M. and M.J.D. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by MCIN/AEI/10.13039/501100011033, “ERDF A way of making Europe”, grant AGL2017-85676-R to María J Delgado, grants AGL2015-63651-P and PID2020-114330GB-100 to Socorro Mesa, and also Junta de Andalucía, grant P18-RT-1401 to María J Delgado and Socorro Mesa. EB was supported by a personal visiting researcher grant—IS-MOBIL (Oslo University, Norway) and the CSIC JAE-DOC Program co-financed by ESF.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Acknowledgments:** We acknowledge the continuous support from Junta de Andalucía. We also acknowledge the support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

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

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