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Nitrous oxide (N_2O) synthesis by *Microcystis aeruginosa*

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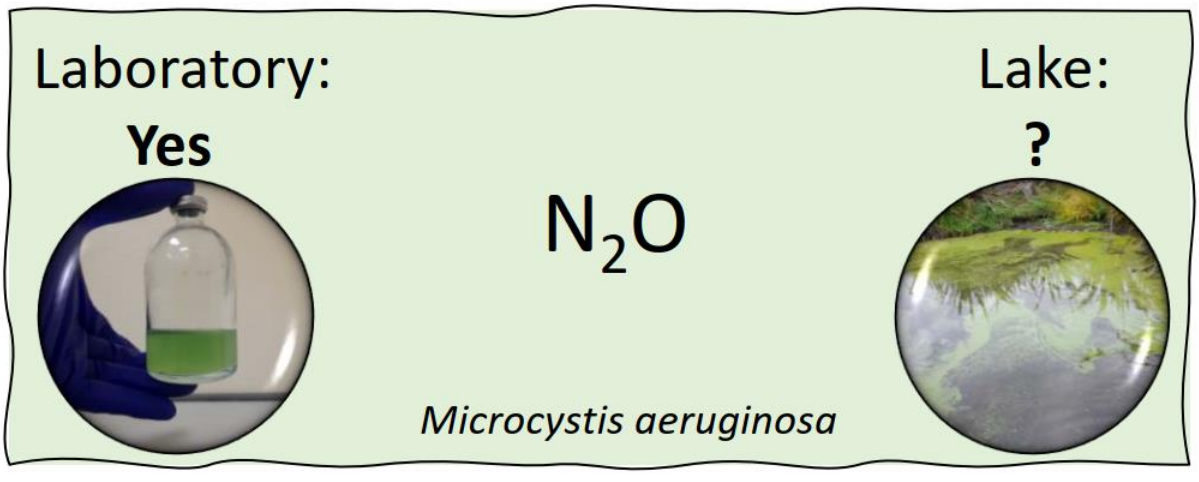
Abstract. Pure cultures of *Microcystis aeruginosa* synthesized nitrous oxide (N_2O) when supplied with nitrite (NO_2^-) in darkness (198.9 nmol·g-DW^{-1}·h^{-1} after 24 hours) and illumination (163.1 nmol·g-DW^{-1}·h^{-1} after 24 hours) whereas N_2O production was negligible in abiotic controls supplied with NO_2^- and in cultures deprived of exogenous nitrogen. N_2O production was also positively correlated to the initial NO_2^- and *M. aeruginosa* concentrations, but low to negligible when nitrate (NO_3^-) and ammonium (NH_4^+) were supplied as the sole exogenous N source instead of NO_2^-. A protein database search revealed *M. aeruginosa* possesses protein homologues to eukaryotic microalgae enzymes known to catalyse the successive reduction of NO_2^- into nitric oxide (NO) and N_2O. Our laboratory study is the first demonstration that *M. aeruginosa* possesses the ability to synthesize N_2O. As *M. aeruginosa* is a bloom-forming cyanobacterium found globally, further research (including field monitoring) is now needed to establish the significance of N_2O synthesis by *M. aeruginosa* under relevant conditions (especially in terms of N supply). Further work is also needed to confirm the biochemical pathway and potential function of this synthesis.

Graphical Abstract.



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1 Introduction

Emissions of the potent ozone depleting greenhouse gas nitrous oxide (N_2O) have been reported from various aquatic ecosystems characterized by a high level of photosynthetic activity and several authors have suggested that N_2O emissions from eutrophic lakes could be globally significant (Delsontro et al., 2018; Plouviez et al., 2019). Noteworthy, Delsontro et al. (2018) determined that N_2O emissions from lakes and impoundments could be expected to increase as a function of lake size and chlorophyll a (an indicator of the presence of primary producer such as microalgae). Because eutrophication is an increasing global issue (Delsontro et al., 2018; Kapsalis and Kalavrouziotis, 2021; Maure et al., 2021), N_2O emissions from these ecosystems could also be expected to increase. Several species of microalgae and cyanobacteria can indeed synthesize N_2O (Weathers, 1984; Weathers and Niedzielski, 1986; Bauer et al., 2016; Plouviez et al., 2019) and a biochemical pathway for this synthesis has been established in the model microalga *Chlamydomonas reinhardtii* (Plouviez et al., 2017b; Burlacot et al., 2020). Despite these critical advances, the true global significance of microalgal N_2O synthesis in microalgae-rich eutrophic aquatic bodies remains unknown (Plouviez et al., 2019; Burlacot et al., 2020; Plouviez and Guieysse, 2020). Microcystis species are cyanobacteria commonly found in eutrophic ecosystems (Xiao et al., 2018; Zhou et al., 2020; Hernandez-Zamora et al., 2021) but the ability of this genus to synthesize N_2O is currently unknown. We, therefore, investigated the ability of N_2O production by the most notorious bloom-forming cyanobacterium reported in freshwaters and model cyanobacterium *M. aeruginosa* (Qian et al., 2010; Kataoka et al., 2020; Zhou et al., 2020) under conditions known to induce or impact N_2O production in microalgae (Guieysse et al., 2013; Alcantara et al., 2015; Bauer et al., 2016; Plouviez et al., 2017b; Burlacot et al., 2020).

2 Results and discussion

2.1 N_2O synthesis bioassays

The ability of *M. aeruginosa* to synthesize N_2O was investigated using a protocol successfully used for the microalgae *C. vulgaris* and *C. reinhardtii* (Alcantara et al., 2015; Guieysse et al., 2013; Plouviez et al., 2017b). As can be seen in **Fig. 1**, N_2O was only significant in cultures supplied NO_2^- as there was no significant production in the absence of the cyanobacterium (abiotic control) or the absence of NO_2^- (negative control). Further assays showed a positive correlation between biomass concentration and N_2O production (**Fig. 2**), confirming the biological origin of N_2O synthesis.

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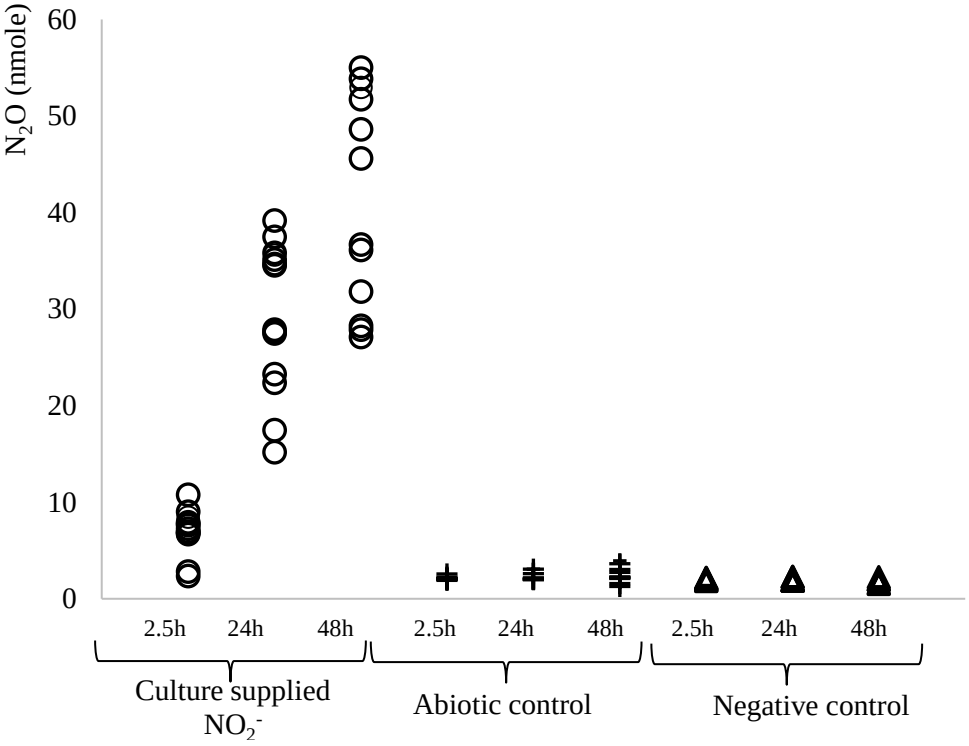

Figure 1. Total N_2O accumulation (nmole) from *M. aeruginosa* supplied with 10 mM NO_2^- under continuous illumination (○, n ≥ 12), abiotic control N-free media with 10 mM NO_2^- (+, n ≥ 10) and negative control: *M. aeruginosa* cultures incubated in N-free media (Δ, n ≥ 10).

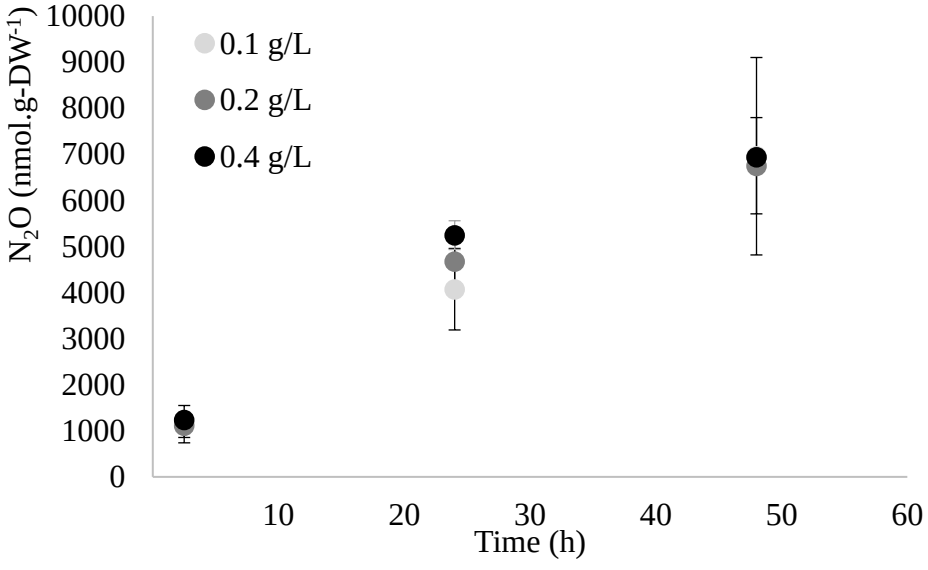

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Figure 2. N_2O (nmol·g-DW^{-1}) recorded from *M. aeruginosa* cultures with different biomass concentrations (0.1, 0.2 and 0.4 g-DW·L^{-1}; n ≥ 6, n ≥ 12, n = 4, respectively) in sealed flasks supplied light and 10 mM NO_2^-. N_2O synthesis was statistically different when comparing the rates between 2.5 and 24 h and between 24 and 48 h (p < 0.05, two samples t-test).

In comparison to cultures supplied with NO_2^-, low and negligible N_2O synthesis was recorded in cultures supplied with NO_3^- and NH_4^+, respectively (**Table. 1**). This showed that NO_2^- was the substrate to N_2O synthesis, as reported for other microalgae (Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse et al., 2013; Alcantara et al., 2015; Bauer et al., 2016; Plouviez et al., 2017b; Burlacot et al., 2020). This was also confirmed by the positive correlation between NO_2^- concentration and N_2O synthesis (**Fig. 3,** Vm = 185 nmol·g-DW^{-1}·h^{-1} and Km for NO_2^- = 2.22 mM).

Table 1. N_2O emissions in different conditions (n = number of replicates)

Light conditions	N source	N_2O production (nmol·g-DW^{-1}·h^{-1})	Standard error	n
Light	1 mM NO_2^-	59.5	13.7	18
	5 mM NO_2^-	131.5	21.5	16
	10 mM NO_2^-	163.1	31.5	23
	10 mM NO_3^-	3.9	1.4	6
	10 mM NH_4^+	0.07	0.7	4
	-	0.9	0.5	4
Dark	10 mM NO_2^-	198.9	30.5	5
	-	1.5	1.7	6

M. aeruginosa was able to synthesize N_2O in both darkness and illumination (**Table. 1**), respectively representing 0.07% and 0.06% of the amount of N supplied (g-N-N_2O produced/g-N supplied × 100). The N_2O produced under illumination was statistically lower than in darkness (p-value < 0.05, two samples t-test, n = 5 replicates from experiments performed on the same day). The negative impact of light was previously observed in *C. vulgaris* and *C. reinhardtii* tested under similar conditions (Guieysse et al., 2013; Alcantara et al., 2015; Plouviez et al., 2017b), although N_2O production was positively correlated with light supply in *C. vulgaris* grown outdoors (Plouviez et al., 2017a). The difference we observed during this study may be explained by light-dependent mechanisms impacting enzymatic activities and consequently intracellular NO_2^- accumulation (e.g. the rates of NO_2^- reduction into NH_4^+ and N_2O), as suggested by Plouviez et al, (2017a). While small, N_2O synthesis was statistically significant in *M. aeruginosa* fed NO_3^- as the sole exogenous N source (p-value < 0.05**,** two samples t-test when compared with the negative controls). As in *C. vulgaris* and *C. reinhardtii*, the intracellular reduction of NO_3^- into NO_2^- by the enzyme nitrate reductase (narB) is the first step of NO_3^- assimilation in *M. aeruginosa* (Ohashi et al., 2011; Zhou

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et al., 2020). Hence, intracellular NO_2^- production likely generated this substrate for N_2O synthesis during NO_3^- exogenous supply but competitive use of NO_2^- (for protein synthesis via NH_4^+ generation) could have competed with N_2O synthesis. Intracellular NO_2^- was not possible when NH_4^+ was supplied as the sole exogenous N source, explaining the absence of N_2O production (p-value = 0.91, two samples t-test when compared with the negative controls). In *M. aeruginosa*, NO_3^- uptake and the transcriptional regulation of nitrate reductase have been shown to be activated by light, NO_3^- and NO_2^- (Chen et al., 2009; Ohashi et al., 2011; Chen and Liu, 2015). While the transcriptional and post-translational regulation of nitrate reductase in *M. aeruginosa* still needs to be investigated in relation to N_2O synthesis and varying environmental parameters (e.g. light supply), it is possible that the pattern of N_2O synthesis during outdoor *M. aeruginosa* growth would be similar to that seen in *C. vulgaris*.

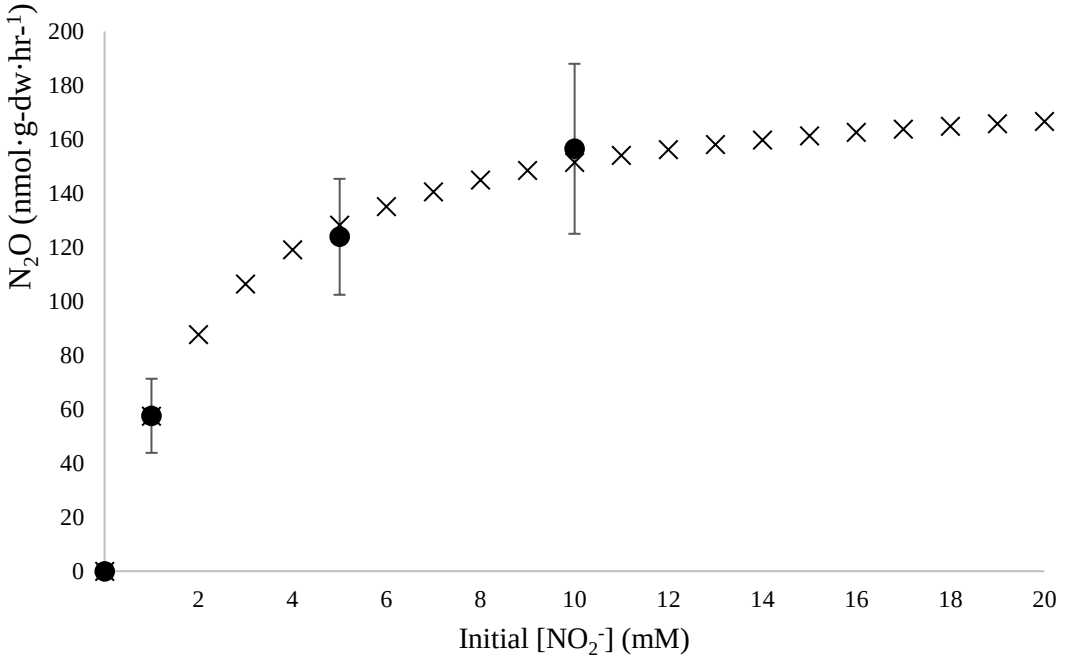

Figure 3. Impact of initial NO_2^- concentration (0, 1, 5 and 10 mM; n ≥ 7, n = 18, n = 16, n = 23, respectively) on the N_2O production in *M. aeruginosa* cultures incubated 24 hours in light (○). The observed kinetic followed a Michaelis-Menten kinetic (×): Vmax and Km were graphically estimated at 185 nmol·g-DW^{-1}·h^{-1} and 2.22 mM, respectively and used to simulate the Michaelis-Menten kinetic based on the equation described by (Johnson and Goody, 2011).

2.2 Putative pathways

In the eukaryotic microalga *C. reinhardtii*, cytoplasmic NO_2^- is sequentially reduced to nitric oxide (NO) and N_2O. The first step, NO_2^- reduction into NO, is catalysed by the dual enzyme nitrate reductase-NO forming nitrite reductase (NR-NoFNiR) or, potentially, the copper containing nitrite reductase (NirK). The second step, NO reduction into N_2O, can then be catalysed by cytochrome P450 (CYP55, (Plouviez et al., 2017b; Burlacot et al., 2020), Flavodiirons (FLVs, (Burlacot et al., 2020;

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Bellido-Pedraza et al., 2020), or potentially by the Hybrid Cluster proteins (HCPs, (Bellido-Pedraza et al., 2020) involved in nitrogen metabolism (Van Lis et al., 2020). Interestingly, NO_2^- reduction into NO by nitrate reductase (narB) has been demonstrated in *M. aeruginosa* (Tang et al., 2011; Song et al., 2017) and *M. aeruginosa* possesses homologs of the CYP55, FLVs, and HCPs found in *C. reinhardtii* (**Table. 2**). While the functions of these proteins need to be confirmed, their presence suggests N_2O synthesis in *M. aeruginosa* could involve similar NO_2^- and NO reduction pathways to those found in *C. reinhardtii*.

Table 2. Summary of Blastp results for proteins potentially involved in N_2O synthesis in *Chlamydomonas reinhardtii*. Accession numbers were retrieved from (Bellido-Pedraza et al., 2020) and used as query sequence for blastp (protein-protein BLAST) protein searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of *M. aeruginosa* (taxid:1126) protein sequences database.

Protein	*C. reinhardtii* accession number	e-value	*M. aeruginosa* accession number	% Similarity	*M. aeruginosa* protein
NirK	PNW79625.1	-		-	-
HCP	XP_001694756.1	3e-158	NCR75269.1	45.38	Hydroxylamine reductase
	XP_001694571.1	5e-160	WP_002787796.1	44.79	Hydroxylamine reductase
	XP_001694671.1	2e-157	NCR75269.1	45.03	Hydroxylamine reductase
	XP_001694454.1	2e-159	WP_002787796.1	45.96	Hydroxylamine reductase
CYP55	XP_001700272.1	3e-45	NCR09918.1	29.90	CYP55
FLV	XP_001692916.1	6e-138	WP_193956217.1	43.45	Diflavin flavoprotein
FLV	PNW71243.1	0	WP_110545956.1	52.18	Diflavin flavoprotein

2.3 Metabolic function

The metabolic function of N_2O synthesis in eukaryotic microalgae is currently unknown and it has been suggested that NO_2^- reduction into N_2O enables cells to expend excess energy or instead, is the fortuitous result of dual enzymatic activity (Guieysse et al., 2013; Plouviez et al., 2017b). The intermediate NO is a ubiquitous signalling molecule in algae (Astier et al., 2021). Interestingly, NO stimulates the allelopathic response of *M. aeruginosa* against competitors (Song et al., 2017) and promotes the growth of this cyanobacteria (Tang et al., 2011). While the link between NO and N_2O still needs to be determined, it is possible that the NO and N_2O biosynthetic pathways is/are involved in cell-to-cell communications in *M. aeruginosa* and more broadly, in microalgae. Further research is needed.

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2.4 Potential environmental implications

N_2O emissions from aquatic environments where microalgae abound have been repeatedly reported in the literature (Bauer et al., 2016; Plouviez et al., 2019; Zhang et al., 2022). Based on the data available, DelSontro et al. (2018) and Plouviez and Guieysse, (2020) estimated that global N_2O emissions from eutrophic lakes alone could represent 110 to 450 kt $N\text{-}N_2O \cdot yr^{-1}$, which represent 14-56% of the natural and anthropogenic N_2O emissions reported from inland and coastal waters (Tian et al., 2020). Importantly, Delsontro et al. (2018) predicted that N_2O emissions from lakes and impoundments would increase with lake size and chlorophyll a concentration. The N_2O synthesis rates reported during our study are in the same order of magnitude as the rate previously reported for members of the green microalgae, cyanobacteria, and diatoms (Bauer et al., 2016; Plouviez et al., 2019). Our findings therefore support past predictions of the global relevance of photosynthetic N_2O emissions from eutrophic aquatic bodies as Microcystis is globally found and often the dominant genus in these ecosystems (Qian et al., 2010; Kataoka et al., 2020; Zhou et al., 2020). Further research is now needed to quantify N_2O emissions from eutrophic aquatic ecosystems where *M. aeruginosa* abounds. This is especially timely considering that the frequency and geographic distribution of harmful algae blooms have increased due to anthropogenic activities (Paerl et al., 2018; Kataoka et al., 2020).

3 Conclusions

We herein present the first demonstration that *M. aeruginosa* synthesizes N_2O. *Microcystis aeruginosa* synthesized N_2O when supplied with NO_2^- in darkness (198.9 $nmol \cdot g\text{-}DW^{-1} \cdot h^{-1}$ after 24 hours) and illumination (163.1 $nmol \cdot g\text{-}DW^{-1} \cdot h^{-1}$ after 24 hours), and this production was positively correlated to the initial NO_2^- and *M. aeruginosa* concentrations. A protein database search also revealed *M. aeruginosa* possesses proteins homologues to eukaryotic microalgae known to catalyse the successive reduction of NO_2^- into NO and N_2O. As *M. aeruginosa* is globally distributed, further research (including field monitoring) is now needed to evaluate the significance of N_2O synthesis by these cyanobacteria under relevant conditions (especially in terms of N supply). Further studies are also needed to confirm the genes/proteins involved.

4 Appendix: Materials and Methods

4.1 Strain and culture maintenance

Microcystis aeruginosa UTEX 2385 was obtained from the culture collection of the University of Texas at Austin (https://utex.org/). Pure cultures were maintained on 100 mL low-phosphate minimal media (Plouviez et al., 2021) incubated at 25ºC (INFORS HT Multitron) under continuous illumination (20 $\mu mol \cdot cm^{-2} \cdot s^{-1}$) and agitation (150 rotation per minutes, rpm). Cultures thus incubated for more than a week were supplied with 100 µL of a solution of KH_2PO_4/K_2HPO_4 (0.4 M/0.6 M) to prevent P limitation. The purity of the cultures was verified via sequencing (**S1**).

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4.2 Cultivation and Bioassays

M. aeruginosa was cultivated on 400 mL low-phosphorus minimal media in 500 mL Duran bottles for 5 days. These cultures were incubated under fluorescent tubes (F15W/GRO sylvania gro-lux) providing illumination at 20 µmol·cm^{-2}·s^{-1} at the culture surface. Mixing was provided by bubbling filtered (0.22µm) air at 1.5 L·min^{-1}. On the day of the experiment, 15 mL aliquots were withdrawn from the cultures to measure the cell dry weight (DW) according to (Bechet et al., 2015). Then, 100-400 mL aliquots were centrifuged at 4400 rpm for 3 min. The supernatants were discarded, and the pellets were re-suspended in N-free medium to a final concentration of 0.2 g-DW L^{-1} as previously described (Guieysse et al., 2013). Twenty-five mL aliquots of these suspensions were transferred into 120 mL serum flasks supplied with 1 mL of $NaNO_2$, $NaNO_3$ or NH_4Cl stock solutions (250 mM) to reach a final concentration of 10 mM. Sterile abiotic controls were not inoculated but were supplied with 10 mM nitrite (NO_2^-) while negative controls were *M. aeruginosa* cultures incubated in N-free media. The flasks were immediately sealed with rubber septa and aluminium caps and incubated at 25°C under continuous agitation (150 rpm) under either constant illumination (20 µmol·cm^{-2}·s^{-1}) or darkness. A similar protocol was used to evaluate the impact of the initial cell (0.1 – 0.4 g-DW·L^{-1}), NO_2^- (1 – 10 mM) or nitrate (NO_3^-, 10 mM) or ammonium (NH_4^+, 10 mM) concentrations on N_2O synthesis. Unless otherwise stated, each condition was tested in triplicate flasks and repeated at least twice. All glassware and media were autoclaved prior to the experiments. An additional experiment confirmed the purity of the *M. aeruginosa* stock cultures and the cultures used during the bioassays (**S1**).

4.3 Analysis

Gas samples (5 mL) were withdrawn from the flask headspace using a syringe equipped with a needle. The headspace N_2O concentration in those samples was then quantified using gas chromatography (Shimadzu GC-2010, Shimadzu, Japan). Total N_2O was calculated as the sum of gaseous N_2O and dissolved N_2O as described by Guieysse et al. (2013). Briefly, Assuming the gas and the liquid phase N_2O concentrations were at equilibrium at the time of sampling, the total amount of N_2O produced in the flask was calculated by summing up the amounts of N_2O present in the gas and liquid phases. The amount of dissolved N_2O in the liquid phase was calculated using Henry's law at 25°C (Eq. 1):

$$n^t_{N_2O_{total}} = x^t_{N_2O} \cdot P^t \cdot \left(\frac{V_g}{R \cdot T} + H_{N_2O} \cdot V_l\right) \quad (1)$$

Where $n^t_{N_2O_{total}}$ is the total amount of N_2O produced in the Duran bottle at time t (moles N_2O); $x^t_{N_2O}$ is the molar fraction of N_2O in the gas phase at time t (mol N_2O·mol gas^{-1}); P^t is the pressure in the gas headspace at time t (typically 101325 Pa unless otherwise stated); V_g is the volume of gas in the flask (mL); R is the ideal gas constant (8.314 J·mol^{-1}·K^{-1}); T is the temperature inside the bottle (298.15 K); H_{N_2O} is the Henry law constant of N_2O at T (2.5·10^{-7} mol·L^{-1}·Pa^{-1}); and V_l is the volume of liquid in the serum flask (mL).

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Supplementary information

The purity of M. aeruginosa cultures was confirmed by PCR and sequencing. The methodology used and the results obtained are presented in the supplement S1.

Authors contribution

F.F. performed the investigation, data visualization and curation, and contributed to the writing - review & editing of the manuscript. M.P. was involved with the writing - original draft and contributed to conceptualization, methodology, and data curation and visualization with B.J. B.J. and J.P. were involved with the writing - review & editing of the manuscript before submission. Finally, B.J., J.P. and M.P. were all involved with the funding acquisition.

Competing interests

The authors declare that they have no conflict of interest.

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