Physiological responses of the freshwater N_2-fixing cyanobacterium *Raphidiopsis raciborskii* to Fe and N availabilities

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Originality-Significance Statement

In this study, the cylindrospermopsin-producing CS-506 strain and non-toxic CS-509 strain of the N_2-fixing cyanobacterium *Raphidiopsis raciborskii* (previously known as *Cylindrospermopsis raciborskii*) were employed to investigate the physiological responses of CS-506 and CS-509 strains under different conditions of Fe and N availability. Results herein show that *R. raciborskii* acclimated under Fe-limited conditions acquires Fe at higher rates than those under Fe replete conditions principally via unchelated Fe(II) formed as a result of photoreduction of complexed Fe(III). Our study also reveals that the CS-506 strain exhibited higher N_2-fixing and Fe uptake rates than CS-509 under N-free and Fe-limited conditions. Overall, the findings of this study highlight that Fe availability is of significance for the ecological advantage of CS-506 (the toxic strain) over CS-509 (the non-toxic strain) in N-deficient freshwater bodies with this information of potential importance in the management and control of harmful *R. raciborskii*-dominated blooms.

Summary

The cyanobacterium *Raciborskii raciborskii* is of environmental and social concern in view of its toxicity, bloom-forming characteristics and increasingly widespread occurrence. However, while availability of macro- and micro-nutrients such as N and Fe are critically important for the growth and metabolism of this organism, the physiological response of toxic and non-toxic strains of *R. raciborskii* to varying Fe and N availabilities remains unclear. By determining physiological parameters as a function of Fe and N availability, we demonstrate that *R. raciborskii* growth and N_2-fixing activity are facilitated at higher Fe availability under N_2-limited conditions with faster growth of the CS-506 (cylindrospermopsin-producing) strain compared to that of CS-509 (the non-toxic) strain. Radiolabeled Fe uptake assays indicated that *R. raciborskii* acclimated under Fe-limited conditions acquires Fe at significantly higher rates than under Fe replete conditions, principally via unchelated Fe(II) generated as a result of photoreduction of complexed Fe(III). While N_2-fixation of both strains occurred during both day and night, the CS-506 strain overall exhibited higher N_2-fixing and Fe uptake rates than the CS-509 strain under N-deficient and Fe-limited conditions. The findings of this study highlight that Fe availability is of significance for the ecological advantage of CS-506 over CS-509 in N-deficient freshwaters.

Introduction

The bloom-forming freshwater filamentous cyanobacterium, *Raphidiopsis raciborskii* (previously named *Cylindrospermopsis raciborskii* (Aguilera et al., 2018)), is of increasing global environmental and social concern because of its adverse effects on the quality of drinking water reservoirs and lakes as a result of the potential generation of toxic metabolites such as cylindrospermopsin (CYN) and saxitoxins (Hoff-Risseti et al., 2013; Lei et al., 2014; Botana, 2016; Martin et al., 2018). In recent decades, this diazotrophic cyanobacterium has spread from its native tropical region to subtropical and temperate zones (Burford et al., 2016; Burford et al., 2018). The rapid expansion of *R. raciborskii* may be due to several possibly synergistic factors including global climate change (Botana, 2016), strong physiological adaptability of this microorganism to low light and temperature (Antunes et al., 2015; Kehoe et al., 2015) and flexible acquisition of nutrients, particularly nitrogen (N) (Willis et al., 2016a). Although the N_2-fixation rate of *R. raciborskii* is lower than ammonium and nitrate uptake rates and accounts for less than 10% of total N assimilation by this organism, N_2-fixation by *R. raciborskii* plays a vital role in regulating the physiological adaptation of this organism in N-fluctuating environments. For example, N_2-fixation provides an essential N source to support the relatively low growth and cell homeostasis of *R. raciborskii* under prolonged N-limited conditions (Willis et al., 2016a). These facultative characteristics also provide competitive growth advantages for *R. raciborskii* under conditions where combined N concentrations fluctuate (Moisander et al., 2012; Burford et al., 2016).

N_2-fixation by most diazotrophs is mediated by nitrogenase with a cofactor containing iron (Fe) and other transition metals (Mo and V) (Yang et al., 2017). This metalloenzyme is composed of two component proteins, namely dinitrogenase (an Fe-X protein where X represents Fe, Mo, or V) and dinitrogenase reductase (an Fe protein) (Yang et al., 2017). Nitrogenase synthesis requires a large amount of Fe (25-39 atoms of Fe per nitrogenase molecule (Postgate, 1998) with 236 μmol of Fe bound to nitrogenase per mol of cellular carbon (Whittaker et al., 2011)). Numerous studies indicate that the Fe requirements for growth of phototrophic diazotrophs are higher than those for non-diazotrophs (Dutkiewicz et al., 2012; Dutkiewicz et al., 2014; Schoffman et al., 2016) with N_2-fixation commonly limited by the low Fe availability in oceanic and lake waters (Berman-Frank et al., 2007; Whittaker et al., 2011; Norman et al., 2014; Snow et al., 2015). For example, N_2-fixation rates of the marine diazotrophic cyanobacterium *Trichodesmium* increased from 0.07 to 0.5 mmol-N·mol-C^{-1}·h^{-1} on Fe supplementation from 4 nM to 4 μM (Berman-Frank et al., 2007). In addition to the positive correlation of N_2-fixation with dissolved Fe concentration (Moore et al., 2009), simulated results of a global ecosystem model revealed that the diazotroph biogeography is controlled by the Fe: N ratio in marine systems (Ward et al., 2013). Furthermore, in contrast to the very few studies regarding the physiological response of diazotrophic cyanobacteria to varying levels of Fe availability (Berman-Frank et al., 2007; Jacq et al., 2014), numerous studies of the impact of N and P availabilities on the growth of diazotrophic cyanobacteria including *R. raciborskii* have been

described (Kenesi et al., 2009; Muhid et al., 2013; Amaral et al., 2014; Chislock et al., 2014; Willis et al., 2016a; Yang et al., 2018).

Given the high Fe requirement for diazotrophic cells, efficient Fe uptake under N-deficient conditions is predicted to be important to the ecology of *R. raciborskii*. Despite the importance to diazotrophs, most comprehensive studies of Fe uptake in marine and freshwater systems have been undertaken on non-diazotrophic cells (Morel et al., 2008; Fujii et al., 2011b; Fujii et al., 2011a; Dang et al., 2012; Fujii et al., 2014b). Previous laboratory-based studies have revealed that Fe bioavailability is principally determined by the concentration of dissolved unchelated Fe (rather than total Fe) buffered by organic ligands for both eukaryotic and prokaryotic cells (Morel et al., 2008; Fujii et al., 2011b). Because dissolved ferric iron (Fe[III]) is typically tightly complexed by organic ligands (Fujii et al., 2008; Fujii et al., 2014a) and particulate Fe(III) oxyhydroxides display extremely low solubility at circumnetural pH (Liu and Millero, 2002), the reductive dissociation of chelated Fe(III) as a result of photochemical or biological processes to form more soluble unchelated ferrous forms (Fe[II]′) is considered to be a critical step in phytoplankton Fe uptake (Morel et al., 2008). The prevalence of unchelated Fe uptake by non-diazotrophic freshwater cyanobacteria including *Microcystis* (Fujii et al., 2014b) and *Synechocystis* (Jiang et al., 2015) as well as the lack of siderophore-associated genes in a number of prokaryotic phytoplankton (Hopkinson and Barbeau, 2012) is indicative of the importance of unchelated Fe uptake by phytoplankton. The limited number of studies reported in the literature have also shown that the facilitated production of unchelated Fe on cell surfaces is an important

adaptation strategy for diazotrophic cyanobacteria *Trichodesmium* and *Anabaena* with regard to acquisition of Fe (Berman-Frank et al., 2007; Wirtz et al., 2010; Benavides et al., 2013). However, there is a paucity of information regarding the mechanism of Fe uptake by freshwater diazotrophic cyanobacteria, particularly for bloom-forming N_2-fixers such as *R. raciborskii*.

Given that N_2-fixing primary producers are the main entry point of N into oligotrophic aquatic systems, an understanding of the physiological response of diazotrophs to Fe- and N-availability is critical not only to the nutritional status of these organisms but also to elemental cycles in aqueous systems in general. In addition, *R. raciborskii* can be divided into cylindrospermopsin-producing (toxic) and non-cylindrospermopsin (non-toxic) strains, which co-exist in *R. raciborskii*-blooms in Asia and Australia (Sinha et al., 2014; Burford et al., 2018). Nonetheless, there has been no study, to our knowledge, comparing the Fe uptake ability of toxic versus non-toxic *R. raciborskii* strains, and the subsequent influence on N_2-fixation, though the importance of cyanotoxin in regulating the cellular physiology such as oxidative stress induced by Fe limitation has been suggested (Alexova et al., 2011). As such, considerable scope exists to better understand the physiological response of toxic and non-toxic *R. raciborskii* strains to Fe availability, particularly under N-free conditions. In this study, we investigate the physiological characteristics and nitrogenase activity of toxic and non-toxic strains of *R. raciborskii* over a range of conditions of N and Fe availability. In addition, a radiolabelled iron (^{55}Fe) uptake assay was performed to investigate the Fe uptake mechanism of *R. raciborskii* in response to varying Fe and N availabilities.

Results and discussion

R. raciborskii growth

The effect of availability of N and Fe on the growth characteristics of *R. raciborskii* was examined in this study. For the control (Fig. 1), where combined nitrogen and 1000 nM Fe were supplied, both non-toxic (CS-509) and cylindrospermopsin-producing (CS-506) strains grew faster during the exponential phase and achieved the highest cell yield at stationary phase. Both strains generally showed comparable growth patterns and cell yields in the identical growth period, implying that there would non-discernible difference in CS-506 and CS-509 populations under nutrient-sufficient conditions. Previously, the CS-506 strain was observed to grow slightly faster and exhibit longer exponential growth phase than the CS-509 strain in the full JM (Fe-rich) medium with approximately 6-fold higher Fe concentration than that used in our control experiment (Pierangelini et al., 2014), suggesting a differing effect of Fe sufficiency on the growth of these two strains. In contrast, for CS-506 and CS-509 cultures in the absence of combined N, cellular growth of both strains was significantly lower ($p< 0.05$) with a gradual diminution in final yields with decreasing Fe concentrations, particularly for the CS-506 strain. The calculated specific growth rates of *R. raciborskii* were 0.058, 0.020, 0.016 and 0.012 d^{-1} for CS-506 incubated in JM*(+N 1000), JM*(-N 1000), JM*(-N 200), and JM*(-N 50), respectively, and 0.058, 0.014,0.013 and 0.013 d^{-1} for CS-509 incubated in JM*(+N 1000), JM*(-N 1000), JM*(-N 200), and JM*(-N 50), respectively. The growth rates of both strains supplemented with

combined N were significantly higher ($p< 0.05$) than those under N-free conditions. When cultures were supplied with identical levels of Fe under N-free conditions, the CS-506 exhibited significantly higher growth rates ($p< 0.05$) than CS-509. The growth rates of the CS-506 and CS-509 strains in this study were lower than previously reported under either N-free or fully nitrate-replete conditions (Saker et al., 1999; Pierangelini et al., 2014; Willis et al., 2015). This discrepancy is most likely a result of the lower Fe concentrations in this study (0.05-1 µM) than that (6.1 µM) present in the N-replete or N-deficient JM medium previously used. Our results suggest that the presence of nutrients including combined N and high concentration of Fe are beneficial to the growth of *R. raciborskii*.

Regarding the growth of *R. raciborskii*, the CS-506 strain exhibited a statistically significant ($p< 0.05$) higher cell yield than CS-509 in the incubation period of 6-16 d in the JM*(-N, 1000) treatment, implying a higher tolerance of CS-506 cells to N-deficiency in the presence of 1,000 nM Fe. This result is possibly a consequence of enhanced N_2-fixation of the CS-506 strain under Fe-rich conditions. On the other hand, as illustrated in Fig. 1, the cell density ratios of CS-506 relative to CS-509 within the 16-day incubation in JM*(-N, 200) and JM*(-N, 50) media were 1.00±0.01 and 1.01±0.01, respectively, implying similar growth of these two stains incubated in JM*(-N, 200) and JM*(-N, 50) media. While it has been previously reported that N_2-fixation by another CYN producer (CS-505) did not clearly result in a dominance of this toxic strain in N-free JM* media (Willis et al., 2016a), these *R. raciborskii* growth experiments suggest that the relative growth of the toxic CS-505 and CS-506 strains is optimal with limited N and replete Fe.

Fig. 1

Physiological characteristics

Since microorganisms can respond to the external nutrient conditions by adjusting their metabolic and physiological properties, physiological characteristics such as average trichome length, average heterocyst number per trichome, and Chl *a* content were examined under the different N and Fe conditions. As shown in Table S1, the highest values of average trichome length, vegetative cell number per trichome and overall Chl *a* content were observed in CS-506 and CS-509 strains cultured in the JM*(+N, 1000) medium with gradual diminution in average trichome length and average heterocyst number per trichome with decreasing Fe concentration under N-free conditions. In contrast, the lower Chl *a* content of both strains under N-free conditions was stable for different Fe availabilities (50-1000 nM). In contrast to the similar overall Chl *a* content of both strains under N-free conditions, the overall Chl *a* content of the CS-509 strain in JM*(+N, 1000) medium was slightly higher than that for the CS-506 strain. Moreover, the straight trichome of CS-509 in the medium with 200 and 1000 nM Fe was generally longer than the coiled trichome of CS-506. These results are consistent with the higher trichome length and Chl *a* content of the CS-509 strain compared to those for the CS-506 strain under non-diazotrophic conditions (Pierangelini et al., 2014). Similarly, the toxic CS-505 strain had shorter trichomes than non-toxic CS-510 under N-deficient conditions (Willis et al., 2015). Generally, the average trichome length per trichome and overall Chl *a* content of both strains under N-free conditions were 20%-90% and 53%-59% lower than those for JM*(+N, 1000),

respectively. These results on shorter trichome length and lower overall Chl *a* content are likely consistent with the growth characteristics seen in the previous section and can be associated with the adverse effect of N-deficiency on *R. raciborskii* growth. Indeed, shorter *R. raciborskii* trichomes were expected in the N-free media, since strains with long trichomes have lower surface area to volume ratios and consequently are at a disadvantage with regard to supplying N (in the form of compounds such as glutamine) to neighboring vegetative cells under N-deficient conditions (Bothe et al., 2010; Plominsky et al., 2015; Willis et al., 2015; Burford et al., 2018).

Vegetative cells in the middle of trichomes provide carbon in the form of low-molecular-weight sugars (such as disaccharides) to their adjacent cells. Furthermore, the production of both heterocysts and nitrogenase is energetically expensive (Burford et al., 2018). Therefore, it would be reasonable to conclude that the elevated number of vegetative cells and heterocysts per trichome with increasing Fe availability (as shown in Table S1 and Fig. S2) has a benefit for N_2-fixation of the toxic *R. raciborskii* strain. Given the relatively stable overall Chl *a* content of both strains under N-free conditions, the elevated vegetative cell number per trichome with increasing Fe availability resulted in a reverse trend in the cell number-normalized Chl *a* content (Table S1). Interestingly, the phenomenon of chlorosis was observed only for the non-toxic cultures (Fig. S3) under N-free conditions. Generally, chlorosis is an important indicator showing damage to the cyanobacterial photosystem caused by abiotic stresses. Therefore, analogous to other cyanobacteria such as *Microcystis aeruginosa* (Alexova et al., 2011), the bleaching resistance of the toxic CS-506 examined here under N-free and Fe-limited conditions is most likely to be a

strain-specific characteristic of *R. raciborskii* CYN-producers incubated in the medium without external nitrogen for 16 d.

Fig. 2

Nitrogenase activity

Effects of Fe availability on diel nitrogenase activity were examined by measuring the ethylene (C_2H_4) production in the acetylene reduction assay and its normalized values by Chl *a* content with results shown in Fig. S4 and Table 1 respectively. Similar temporal trends of overall C_2H_4 yields increasing gradually with incubation time are evident for CS-506 and CS-509 strains with significantly ($p< 0.05$) higher cumulative C_2H_4 production over 24 h for CS-506 cells than those for CS-509 cultures in the JM*(-N, 1000) and JM*(-N, 200) media (Fig. S4) with this result consistent with the high Fe requirement for N_2-fixation (Whittaker et al., 2011). Interestingly, the C_2H_4 yields (15.4-51.6 nmol at the termination of the experiment) for CS-506 and CS-509 strains treated with JM*(+N, 1000) revealed that both strains retained constitutive nitrogenase activity if heterocysts are present, even in the presence of nitrate throughout the incubation period (24 h), with this result mirroring previous studies in which nitrogenase activity was observed to be present in *R. raciborskii* incubated with nitrate or ammonium for 24-120 h (Sprober et al., 2003; Stucken, 2010). These observations are consistent with the fact that nitrogenase activity by *R. raciborskii* in N-replete media only ceases once the heterocysts are no longer present with their disappearance occurring after 10 days or more (Willis et al., 2016a). This basal N_2-fixation for both strains supplied with nitrate is in accordance with the relative

lower average heterocyst number per trichome in these strains (Table S1). Similarly, heterocysts were also previously observed in CS-506 and CS-505 cultures with nitrate, ammonium and urea, particularly within 100 h incubation periods (Saker et al., 1999) as well as the *R. raciborskii* strain ACT 9502 in media with nitrate or ammonium for 1.5 d periods (Kenesi et al., 2009). Furthermore, *R. raciborskii* strains from lakes Dora and Griffin in Florida were reported to express the N_2-fixation gene, *nifH*, in the presence of nitrate (Moisander et al., 2008).These characteristics may result from insufficient inactivation of *R. raciborskii* nitrogenase by 9.4×10^{-4} M nitrate, resulting in low but significant levels of N_2-fixation in this medium.

Table 1

The nitrogenase activity of both strains were then normalized by their Chl *a* contents to eliminate the effects of *R. raciborskii* growth on nitrogenase activity and to enable evaluation of the nitrogenase activity of each *R. raciboriskii* cell. As shown in Table 1, the Chl *a* content-normalized nitrogenase activity of the CS-509 strain under N-free conditions fluctuated steadily within the first 8 h, followed by a 0.39-2.56-fold increase at the termination of the experiment (24 h). In contrast, the normalized nitrogenase activity of the CS-506 strain under N-free conditions generally increased within the first 4-h, followed by near constancy until the end of the experiment. Results of diel C_2H_4 yield (Fig. S4) and normalized nitrogenase activity (Table 1 and Fig. S5) imply that CS-506 and CS-509 strains perform N_2-fixation not only during the light period (day time) but also during the dark period (night time). Moreover, these results also suggest that, in response to depletion of combined nitrogen, N_2-fixation occurs simultaneously

with photosynthesis during daytime (Willis et al., 2016a), and also occurs, in the absence of photosynthesis, during night.

With regard to the response to Fe availability, the average heterocyst number per trichome and Chl *a*-normalized nitrogenase activity measured at 8 h were observed to positively correlate with Fe concentration for CS-506 and CS-509 strains under N-free conditions ($p< 0.01$, Fig. 2) with this result suggesting that the N_2-fixing ability of both strains was enhanced by Fe availability. The slopes of linear regressions of the plots of overall and Chl *a*-normalized nitrogenase activity and average heterocyst number per trichome versus Fe concentrations for the CS-506 strain was 1.8-8.3 times greater than the values for the CS-509 strain (Figs. 2 and S2), indicating that the growth of heterocysts for CS-506 is likely more sensitive to Fe availability in order to achieve a high N_2-fixing potential. While N_2-fixation rate of CS-506 was higher than CS-509 in JM*(-N, 200) and JM*(-N, 50) media (Table 1), the average heterocyst number per trichome was lower for CS-506 compared to CS-509 (Fig. S2). These results suggest that the heterocysts of CS-506 are capable of fixing atmospheric N_2 at higher efficiency per heterocyst (Fig. S5) under the conditions examined with this result highlighting the strain variation in trichome N_2-fixation efficiency. As illustrated in Fig. S5, temporal variation of nitrogenase activity per heterocyst of both strains also suggests that heterocyst N_2-fixation rates differed during the 24-h incubation. The unstable N_2-fixation rate per heterocyst of CS-505 was also reported by Willis *et al*. (2016a) over a larger timescale (20 d). Therefore, the observed larger N_2-fixation rate for CS-506 compared with CS-509 under diazotrophic conditions is a combined

result of its faster heterocyst growth at high Fe availability and higher efficiency of heterocyst N_2-fixation. Moreover, together with slightly higher cell growth rates (Fig. 1), the higher N_2-fixing flexibility of the CS-506 strain with varying Fe availability may account for its ecological advantage over CS-509 cells N-free conditions.

At the termination of the incubation, compared to the CS-509 strain with the largest overall C_2H_4 yield in the JM*(-N, 1000), the optimal C_2H_4 yield of the CS-506 strain was observed in JM*(-N, 1000) and JM*(-N, 200) media (Fig. S4). This observation suggests that *R. raciborskii* could undertake high rate N_2-fixation in waters with moderate limitation to high availability of Fe, particularly for the CS-506 strain, with this result emphasizing its superiority over CS-509 strain in moderately Fe-limited conditions.

Fe uptake by R. raciborskii

Effect of light on cellular Fe uptake

Five hour short term ^{55}Fe uptake assays (using the CS-509 strain) were performed to elucidate the time-course of intercellular ^{55}Fe accumulation under dark and light conditions. A conspicuous impact of light on the ^{55}Fe accumulation is apparent as shown in Fig. 3. For example, cellular ^{55}Fe accumulation by Fe-replete and Fe-limited cells exhibited a strong linear dependence on time (R= 0.97-0.98, p <0.05) for incubation times of up to 5 h under light and N-free conditions with ^{55}Fe uptake rates of 9.0 and 19.1 amol·cell^{-1}·h^{-1} for Fe-replete and Fe-limited cells, respectively. In marked contrast to the effect of light, cellular ^{55}Fe assimilation in the dark (<0.57 amol·cell^{-1}·h^{-1}) was comparable to that observed for the blank setting where

addition of cells was omitted, indicating that *R. raciborskii* cells acquire ^{55}Fe primarily in the light phase. The substantially higher ^{55}Fe uptake rate of *R. raciborskii* cells acclimated under Fe-limited conditions (approximately 8-fold higher than that for Fe-replete conditions) indicates that Fe nutritional status is of importance in modulating the subsequent Fe accumulation by *R. raciborskii*.

In a manner similar to that observed here, the ^{55}Fe uptake rate by *M. aeruginosa* cultured in media containing 200 nM Fe buffered by 26 μM EDTA was found to be two orders of magnitude greater in the light compared to that under darkness (Fujii et al., 2011b). Photoreduction of organically complexed Fe(III) to bioassimilable Fe(II) has been demonstrated to be a crucial process for Fe acquisition by freshwater cyanobacteria including *Microcystis* (Fujii et al., 2011b; Fujii et al., 2014b), *Anabaena* (Wirtz et al., 2010) and *Synechocystis* (Jiang et al., 2015). Such light-induced ^{55}Fe uptake is most likely governed by the rate of Fe(II)′ formation via a ligand to metal charge transfer (LMCT) process (Barbeau et al., 2001; Fujii et al., 2015). Given that N_2-fixation by the *R. raciborskii* strains occurs in both light and dark phases, it is likely that Fe uptake and concurrent or subsequent nitrogenase activation of *R. raciborskii* occurs, for the most part, during the daylight hours in natural waters. The ^{55}Fe uptake rates of *R. raciborskii* in the presence of light determined in this study are 3.4-27.2 folds higher than those (0.33-2.65 amol·cell^{-1}·h^{-1}) for *M. aeruginosa* (Fujii et al., 2011a; Fujii et al., 2014b). This comparison indicates that the diazotrophic *R. raciborskii* possesses a significantly higher Fe uptake rate per unit cell than the non-diazotrophic cyanobacterium *M. aeruginosa*, which is in accord with their

relatively higher Fe requirements for the maintenance of metabolism and growth (Dutkiewicz et al., 2012; Dutkiewicz et al., 2014; Schoffman et al., 2016). These results suggest a superior competency in iron uptake of *R. raciborskii*, at least for the CS-506 and CS-509 strains, over the most common bloom-forming non-diazotrophic cyanobacteria (*e.g. M. aeruginosa*), in N-deficient but Fe-rich waters, with this superior competency likely partially accounting for *R. raciborskii*-dominated bloom-formation.

Fig. 3.

Cellular Fe uptake kinetic response to Fe limitation

As depicted in Fig. 4, ^{55}Fe uptake rates by CS-506 acclimated under Fe-replete and Fe-limited conditions are reasonably described by the Michaelis-Menten model as a function of Fe(II)' concentration (R= 0.96-0.97, p< 0.05). Despite the similar K_s values for Fe-replete and Fe-limited cultures (5.0×10^{-14} and 5.8×10^{-14} M, respectively), the calculated ρ^{max} value for CS-506 cells acclimated under Fe-replete conditions was approximately one fourth of that under Fe-limited conditions (62.4 and 241.8 amol·cell^{-1}·h^{-1}, respectively). These results suggest that *R. raciborskii* cells are most likely to respond to Fe limitation by regulating their Fe assimilation capacity (rather than affinity) to meet the Fe requirements under conditions of low Fe availability. This is consistent with the documented result that Fe-starved *M. aeruginosa* and *Chlorophyta* cells exhibit a higher Fe uptake capacity than Fe-replete cells (Weger et al., 2002; Alexova et al., 2011).

Fig. 4.

The enhanced Fe uptake capacity and rates of *R. raciborskii* cells on reducing Fe availability could be a result of one or more of the following:

(1) The basal metabolic performance of *R. raciborskii* is not obviously impeded by the Fe-limited media with the amount of Fe present apparently sufficient to maintain the basal requirements for *R. raciborskii* growth. This assumption is supported by the observations in Fig. 1 and Table S1 that CS-506 cell density increased by approximate 15% from lag to stationary phases and Chl *a* content under Fe-limited conditions accounted for 97% of that for Fe-replete cultures.

(2) Pre-incubation in Fe-limited media results in an increase in the number of cellular Fe transporters and/or plasma membrane ferric chelate reductase activity (Weger et al., 2002). In accord with the higher ^{55}Fe uptake rates in Fig. 3, Fe uptake capacities of the CS-506 stain are dramatically larger (by ~3 - ~800 folds) than those for *M. aeruginosa* cultured in the similar synthetic media (0.27-3.0 amol·cell^{-1}·h^{-1}) (Fujii et al., 2010; Dang et al., 2012). Moreover, the higher Fe uptake ability of *R. raciborskii* over the non-diazotroph *M. aeruginosa* may well contribute to the apparent dominant ecological advantage of diazotrophic phytoplankton over non-diazotrophic organisms under low nitrogen and high Fe conditions.

(3) Fe-limitation induces a decrease in the cell size of *R. raciborskii* with this change in size advantageous to energy and nutrient provision and ultimately favors Fe uptake under N-free conditions (Bothe et al., 2010; Plominsky et al., 2015; Burford et al., 2018). A profound decrease

in the cell volume of *Crocosphaera watsonii* was also identified under Fe-limited conditions compared with the size of cells grown under Fe-replete conditions (Jacq et al., 2014).

Effect of N availability on cellular Fe uptake

The ^{55}Fe uptake by *R. raciborskii* incubated in the JM* media either with or without 9.4×10^{-4} M nitrate was compared in order to investigate the influence of N-free conditions on cellular Fe requirements. It can be seen from Figs. 5(A) and S6 that the ^{55}Fe uptake rate of the *R. raciborskii* cells in the absence of nitrate is approximately 3-fold that for the cells incubated with nitrate ($p< 0.05$), consistent with the notion that cellular Fe acquisition is important in N_2 fixation due to the high requirement of Fe in nitrogenase (Postgate, 1998; Whittaker et al., 2011). This result is also consistent with a previous report that Fe requirements of *Trichodesmium* increased by up to 5-fold during the N_2-fixing process (Kustka et al., 2003). Additionally, the CS-506 strain showed relatively higher ^{55}Fe uptake compared to that of the CS-509 strain Fig. 5(B) under identical conditions where both Fe-replete and Fe-limited cells (2.5 and 3.8 folds, respectively) were used for the ^{55}Fe uptake experiment. These results highlight the advantageous potential of CS-506 strain for Fe acquisition and N_2-fixation under unfavorable conditions.

Relevance to natural environments

The Fe concentrations examined in this study (50-1,000 nM) are typical of natural freshwaters (Kikuchi et al., 2017). While high concentrations of different nutrients generally coexist in natural waters where blooms prevail, low levels of N can also be associated with high

Fe availability in some cases. For example, the eutrophic Dendre stone pit lake in Belgium contains low concentrations of N but higher Fe at a depth of approximately 15 m with concentrations comparable to the media used in this study (Roland et al., 2017). Similar results (below detection to 8.1×10^{-5} M and 5.4×10^{-7} to 1.8×10^{-3} M for nitrate and Fe, respectively) have also been observed in another surface water (Zobrist et al., 2009). Given that the incubation conditions employed in this study are typical of *Raphidiopsis*-blooming natural waters, the occurrence of *Raphidiopsis* species (particularly in toxic form) appear likely under low combined N but slightly enriched Fe conditions (Huisman et al., 2018).

Fig. 5.

Environmental implications and conclusions

The findings in this study provide insights into the physiological behavior and Fe uptake rate of a toxic and a non-toxic strains of *R. raciborskii* in response to Fe and N availability. A significant inhibitory effect of N-deficiency and a stimulatory effect of Fe supplementation under N-free conditions were observed on *R. raciborskii* growth. For both CS-506 (toxic) and CS-509 (non-toxic) strains, Fe uptake and C-fixation occur simultaneously with N_2-fixation under light and dark conditions in response to N-depletion. Overall, compared with the CS-509, the CS-506 was found to have a higher N_2-fixing potential and growth rate in waters with a broad range of Fe availabilities and to acquire Fe more efficiently via photochemical Fe(II)′ production. The observed different physiological responses of the CS-506 and CS-509 strains to varying Fe and nitrogen availability, therefore, imply different adaptation strategies of these strains in a

changing environment. The dominance of toxic and non-toxic *R. raciborskii* strains is recognized to be dependent on environmental conditions such as nutrient availabilities (Burford et al., 2014).

The ability of *R. raciborskii* to undertake N_2-fixation is, for example, considered to underpin the dominance of this organism under fluctuating nitrogen availability (Moisander et al., 2012; Yang et al., 2018). In addition to nitrogen availability, the presence of high phosphate concentration promoted a strain shift from less to more toxic strains within a short timescale (*e.g.* 5 d) in phytoplankton populations dominated by *R. raciborskii*, possibly as a result of the cellular stoichiometric changes of N: P ratios (Burford et al., 2014; Burford et al., 2018). Pierangelini *et al*. (2014) indicated that CS-506 had higher light requirement with faster growth than CS-509 under light-saturating conditions. Similarly, a previous study of the toxic and non-toxic strains of another well-known bloom forming cyanobacteria *Microcystis* revealed that altered environmental conditions led to a change in ecotype dominance, whereby toxic or non-toxic strains dominated the community depending on the levels of light and dissolved CO_2 (Van de Waal et al., 2011; Sandrini et al., 2016). Such strain-specific adaptation to different light and nutrient regimes may suggest that *R. raciborskii* has developed an adaption strategy to new freshwaters whereby there is dominance of fast growing strains (*e.g*., CS-506) at the beginning of invasion, followed by the appearance of strains with tolerance to low light (*e.g*., CS-509) over longer timescales (Pierangelini et al., 2014). These findings on the different physiological responses of *R. raciborskii* strains to environmental changes suggest that CYN production may ultimately improve the competitive dominance of *R. raciborskii* in mixed cyanobacterial

populations, particularly under N_2-fixation conditions, although the physiological roles of CYN remain unclear.

In addition to different CYN-producing characteristics, CS-506 (coiled) has a different morphology to CS-509 (straight). The coiled and straight morphotypes of *R. raciborskii* frequently coexist in *R. raciborskii*-blooming waters (Saker et al., 1999; Willis et al., 2016b; Willis et al., 2018) and likely respond differently to environmental changes. For example, the coiled *R. raciborskii* strains were more prone to dominate under conditions of high turbulence and high temperature than straight morphotypes (Saker et al., 1999; McGregor and Fabbro, 2000). Although the irreversible transformation from coiled to straight has been observed in a Chinese *R. raciborskii* strain (CHAB 151) grown in BG11 medium (Yang et al., 2018), to the best of our knowledge, there is no morphological transformation reported in Australian *R. raciborskii* strains including CS-506 and CS-509.

The diversity of genotypes and phenotypes in a bloom is, in all likelihood, beneficial in the physiological and ecological adaptation of bloom-forming cyanobacteria to environmental change. Indeed, high phenotypic and genetic variabilities of *R. raciborskii* are likely to underlie its flexible strategies and superior adaption in a wide ranges of ecological niches as well as contributing to the robust and opportunistic nature of *R. raciborskii* blooms (Burford et al., 2016; Willis et al., 2018). The results of this study suggest that the coiled and toxic nature together with higher Fe uptake and N_2-fixation rates under nitrogen-deficient conditions may render CS-506 an advantage as a result of its faster growth at the early stage of a *R. raciborskii* bloom. As the first

evidence of the physiological superiority of toxic CS-506 over non-toxic CS-509 under diazotrophic conditions, the findings of this study provide new insights into possible approaches to the management and control of *R. raciborskii* blooms in freshwater bodies, particularly those with low concentrations of nitrogen but moderate to high levels of Fe.

Experimental Procedures

Strain characteristics

In this study, the cylindrospermopsin-producing CS-506 strain and non-toxic CS-509 strain of the N_2-fixing cyanobacterium *Raphidiopsis raciborskii* (previously known as *Cylindrospermopsis raciborskii*) with different cell morphology and toxigenicity were obtained from the Australian National Algae Culture Collection, Hobart, Australia (ANACC, 2018). The coiled CS-506 stain was originally isolated from Solomon Dam in Queensland, Australia in 1996, while the CS-509 strain with straight trichrome morphology was originally isolated from Lake Julius in Queensland, Australia in 1995 (ANACC, 2018). It has previously been noted that *R. raciborskii* exhibits large physiological variation and genetic plasticity (Sinha et al., 2014; Willis et al., 2016b; Willis et al., 2018).

The previous genomic study for three *R. raciborskii* stains, CS-505 (straight, toxic), CS-506 (coiled, toxic) and CS-509 (straight, non-toxic), indicated that these strains shared high overall genetic identity (93.6-98.5%), had similar genome sizes and near identical G+C contents (Sinha

et al., 2014). At least 2,767 genes were shared between the CS-506 and CS-509 strains, including the taxonomically important *rpoc1, ssuRNA, lsuRNA, cpcA, cpcB, nifB* and *nifH* (Sinha et al., 2014). However, a number of genes were exclusive to the straight morphotype strains and the toxic strains, as well as each individual strain (Sinha et al., 2014).

The CS-506 and CS-509 strains contained at least 176 and 101 strain-specific (or non-homologous) genes, respectively, most of which were associated with DNA repair and modification, nutrient uptake and transport, or adaptive measures such as osmoregulation. Sinha et al. (2014), however, concluded that the cylindrospermopsin (CYN) biosynthesis (*cyr*) gene cluster was the only significant genetic difference between CS-506 and CS-509 strains. They did not identify any other distally encoded genes or gene clusters related to CYN production. The fact that the additional genomic differences between toxic and non-toxic strains were primarily associated with stress and adaptation genes suggests that CYN production may be linked to these physiological processes. Relevant to our current study, genes for N_2-fixation (*nifH*), Fe acquisition and transport were identified in both CS-506 and CS-509 strains.

In addition to previously reported morphological characteristics of the CS-506 and CS-509 strains (Saker et al., 1999; Pierangelini et al., 2014), some of their morphological parameters were examined in this study with results tabulated in Table S1.

Culture conditions

All chemicals used are described in SI1 of the Supporting Information. Both strains were maintained in the slightly modified Jaworski's Medium (JM* medium) detailed in SI2 and Table S2 at 27 °C under a 14:10 h light: dark cycle at light intensity of 157 μmol-photons·m^{-2}·s^{-1} for long-term cell maintenance culturing. In the JM* medium, $NaNO_3$ was used as the sole source of combined nitrogen by replacing $Ca(NO_3)_2 \cdot 4H_2O$ with $CaCl_2 \cdot 2H_2O$ and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ with $Na_2MoO_4 \cdot 2H_2O$ at equivalent Ca and Mo concentrations (Willis et al., 2015; Willis et al., 2016a). For N-free conditions in the combined N-free media, the JM* medium was further modified by replacing $NaNO_3$ with NaCl at the identical molar concentration.

To examine the effects of Fe and N supplements on the physiological characteristics of *R. raciborskii*, $NaNO_3$ concentrations were fixed at either 9.4×10^{-4} M for the medium containing combined N or 0 M for N-free medium, whereas Fe(III) concentrations were varied up to 1000 nM. The JM* media with different N and Fe concentrations are referred to as "JM*(+N/−N, Fe concentration in nM). For experiments in which physiological characteristics were examined, triplicate batches of each *R. raciborskii* strain were cultured under the incubation conditions aforementioned with the following media: JM*(-N, 1000) for the Fe-replete N-free condition, JM*(-N, 200) for the moderately Fe-limited N-free condition, JM*(-N, 50) for the Fe-starved N-free condition, and JM*(+N, 1000) for the N-sufficient condition.

Strains were harvested during the exponential growth phase (except for Chl *a* measurement where the culture in early stationary growth phase was employed). For the long-term cell stock culture, cells were incubated in either JM*(+N, 1000) or JM*(-N, 1000). The cultures were

regularly subcultured into fresh media at an initial cell concentration of ~10^5 cell·mL^{-1}. The subculturing was performed approximately two weeks after the commencement of incubation when cultures reached stationary growth phase. Cultures were randomly placed in the temperature- and light-controlled incubator (Thermoline Scientific) and agitated daily in order to both minimize shading from the light source and facilitate homogeneity.

Physiological characterization

Physiological parameters including cell density, average trichome length per trichome, average heterocyst number per trichome, average vegetative cell number per trichome, chlorophyll *a* (Chl *a*) content, and nitrogenase activity were determined with the methods described in SI3 to elucidate the physiological response of *R. raciborskii* strains to different Fe and N availabilities. In order to examine the diel nitrogenase activity, illumination was employed in the first 14 h, followed by a 10-h dark phase, and cultures were sampled at 1, 4, 8, and 24 h after initial acetylene (C_2H_4) injection.

Short-term ^{55}Fe uptake assay

CS-506 and/or CS-509 strains were incubated in the JM* media with different N and Fe availabilities (JM*(+N, 50), JM*(-N, 1000), JM*(-N, 200), and JM*(-N, 50)) and harvested during the exponential growth phase (~3×10^6 cell·mL^{-1}) in the light cycle by centrifugation (8,000 *g* for 10 min). The collected pellets were washed twice using either the Fe-free JM* media (JM*(+N, 0) or JM*(-N, 0)), resuspended into the same JM* media and then distributed (100 µL)

in a 96-well microplate at cell densities of ~2-4×10^6 cell·mL^{-1}. Subsequently, ^{55}Fe uptake assays were initiated by adding 6 µL of pre-equilibrated ^{55}Fe-EDTA solutions at final concentrations of 200 nM for ^{55}Fe and 3.5-200 µM for EDTA. The JM*(-N, 50) and JM*(+N, 50) media were also used to compare Fe uptake rates of the CS-506 strain under contrasting N-availabilities. *Cylindrospermopsis* cells were then incubated for 3 h in the absence and presence of illumination (157 µmol-photons·m^{-2}·s^{-1}). Details regarding preparation of the ^{55}Fe-EDTA solution are provided in SI1.

After incubation, cell cultures were transferred to a multiscreen 96-well microplate equipped with 1.2 µm membrane filters (MultiScreen, Millipore) in the bottom of each well and then vacuum filtered. The filtered cells were then washed gently three times with 200 µL of the EDTA/oxalate solution and subsequently rinsed for 10 min with 2 mM $NaHCO_3$ (200 µL) to remove Fe oxyhydroxides adsorbed to the cell surface. Finally, filtered cells were placed in glass scintillation vials with 5 mL of scintillation cocktail (Beckman ReadyScint). The ^{55}Fe activity was measured in a Packard TriCarb Liquid Scintillation Counter by converting sample scintillation counts (disintegrations per minute) to Fe mole amounts with concurrent counts of ^{55}Fe-ligand solutions (5-50 µL) in 5 mL scintillation cocktail. Process blanks were determined by performing the same procedures without *R. raciborskii* cells. The determined ^{55}Fe uptake rate and Fe mass balance analysis indicated that extracellular Fe concentrations available for uptake changed insignificantly during the short-term Fe uptake assays.

Data analysis

Dissolved unchelated Fe(II) (Fe(II)') arising from the photoreductive dissociation of Fe(III)-EDTA has been shown to be the major Fe substrate in the EDTA-buffered culture medium at pH 8 (Fujii et al., 2011b; Dang et al., 2012; Fujii et al., 2016). Here, the steady-state concentration of Fe(II)' was calculated using the same kinetic model and assuming that Fe(II)' is the major form of Fe available for uptake by *R. raciborskii* (SI4 and Table S2 in the Supplementary Content, respectively). Fe uptake parameters of *R. raciborskii* were estimated using the Michaelis-Menten model (Eq.(1)) via nonlinear regression analysis:

$$\rho_{\mathrm{Fe(II)}} = \frac{\rho^{\max} \times [\mathrm{Fe(II)}]_{ss}}{K_s + [\mathrm{Fe(II)}]_{ss}} \qquad (1)$$

where $\rho_{\mathrm{Fe(II)}}$ (amol·cell^{-1}·h^{-1}) is the Fe uptake rate of *R. raciborskii* at the given concentration of substrate (Fe(II)' in this study); $[\mathrm{Fe(II)}']_{ss}$ represents the steady-state substrate concentration in the media; $\rho^{\max}$ (amol·cell^{-1}·h^{-1}) is the maximum Fe uptake rate at saturated substrate concentration; K_s (M) is the half saturation constant (also called Michaelis-Menten rate constant) which is equal to the substrate concentration at $\rho_{\mathrm{Fe(II)'}} = \rho^{\max}/2$. Nonlinear regression analysis was performed using Sigmaplot 12.5 Software (Systat Software Inc.).

Student's *t*-test was implemented using Origin 9.0 (OriginLab, USA) to analyze the significance in difference among compared groups at $p \leq 0.05$. The covariance analysis was performed with SPSS software 19.0 (IBM SPSS Statistics, USA) to compare the difference of slopes of linear regression lines (Fe uptake rates) in Fe-replete and Fe-limited media under N-free conditions.

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References

Aguilera, A., Gomez, E.B., Kastovsky, J., Echenique, R.O., and Salerno, G.L. (2018) The polyphasic analysis of two native Raphidiopsis isolates supports the unification of the genera *Raphidiopsis* and *Cylindrospermopsis* (Nostocales, Cyanobacteria). *Phycologia* **57**: 130-146.

Alexova, R., Fujii, M., Birch, D., Cheng, J., Waite, T.D., Ferrari, B.C., and Neilan, B.A. (2011) Iron uptake and toxin synthesis in the bloom-forming *Microcystis aeruginosa* under iron limitation. *Environ Microbiol* **13**: 1064-1077.

Amaral, V., Bonilla, S., and Aubriot, L. (2014) Growth optimization of the invasive cyanobacterium *Cylindrospermopsis raciborskii* in response to phosphate fluctuations. *Eur J Phycol* **49**: 134-141.

ANACC (2018). Australian National Algae Culture Collection (ANACC) Strain Database for microalgae strains. URL https://anacc-db-cdc.it.csiro.au/fmi/webd/CMARC%20Database. Accessed on 7 November, 2018.

Antunes, J.T., Leao, P.N., and Vasconcelos, V.M. (2015) *Cylindrospermopsis raciborskii*: review of the distribution, phylogeography, and ecophysiology of a global invasive species. *Front Microbiol* **6**: 10.3389/fmicb.2015.00473.

Barbeau, K., Rue, E.L., Bruland, K.W., and Butler, A. (2001) Photochemical cycling of iron in the surface ocean mediated by microbial iron(III)-binding ligands. *Nature* **413**: 409-413.

Benavides, M., Aristegui, J., Agawin, N.S.R., Cancio, J.L., and Hernandez-Leon, S. (2013) Enhancement of nitrogen fixation rates by unicellular diazotrophs vs. *Trichodesmium* after a dust deposition event in the Canary Islands. *Limnol Oceanogr* **58**: 267-275.

Berman-Frank, I., Quigg, A., Finkel, Z.V., Irwin, A.J., and Haramaty, L. (2007) Nitrogen-fixation strategies and Fe requirements in cyanobacteria. *Limnol Oceanogr* **52**: 2260-2269.

Botana, L.M. (2016) Toxicological perspective on climate change: Aquatic toxins. *Chem Res Toxicol* **29**: 619-625.

Bothe, H., Schmitz, O., Yates, M.G., and Newton, W.E. (2010) Nitrogen Fixation and Hydrogen Metabolism in Cyanobacteria. *Microbiol Mol Biol Rev* **74**: 529-551.

Burford, M.A., Willis, A., Chuang, A., Man, X., and Orr, P. (2018) Recent insights into physiological responses to nutrients by the cylindrospermopsin producing cyanobacterium, *Cylindrospermopsis raciborskii*. *J Oceanol Limnol* **36**: 1032-1039.

Burford, M.A., Davis, T.W., Orr, P.T., Sinha, R., Willis, A., and Neilan, B.A. (2014) Nutrient-related changes in the toxicity of field blooms of the cyanobacterium, *Cylindrospermopsis raciborskii*. *FEMS Microbiol Ecol* **89**: 135-148.

Burford, M.A., Beardall, J., Willis, A., Orr, P.T., Magalhaes, V.F., Rangel, L.M. et al. (2016) Understanding the winning strategies used by the bloom-forming cyanobacterium *Cylindrospermopsis raciborskii*. *Harmful Algae* **54**: 44-53.

Chislock, M.F., Sharp, K.L., and Wilson, A.E. (2014) *Cylindrospermopsis raciborskii* dominates under very low and high nitrogen-to-phosphorus ratios. *Water Res* **49**: 207-214.

Dang, T.C., Fujii, M., Rose, A.L., Bligh, M., and Waite, T.D. (2012) Characteristics of the freshwater cyanobacterium Microcystis aeruginosa grown in iron-limited continuous culture. *Appl Environ Microbiol* **78**: 1574-1583.

Dutkiewicz, S., Ward, B.A., Monteiro, F., and Follows, M.J. (2012) Interconnection of nitrogen fixers and iron in the Pacific Ocean: Theory and numerical simulations. *Glob Biogeochem Cycle* **26**.

Dutkiewicz, S., Ward, B.A., Scott, J.R., and Follows, M.J. (2014) Understanding predicted shifts in diazotroph biogeography using resource competition theory. *Biogeosciences* **11**: 5445-5461.

Fujii, M., Rose, A.L., and Waite, T.D. (2011a) Iron uptake by toxic and nontoxic strains of *Microcystis aeruginosa*. *Appl Environ Microbiol* **77**: 7068-7071.

Fujii, M., Yeung, A.C.Y., and Waite, T.D. (2015) Competitive effects of calcium and magnesium ions on the photochemical transformation and associated cellular uptake of iron by the freshwater cyanobacterial phytoplankton *Microcystis aeruginosa*. *Environ Sci Technol* **49**: 9133-9142.

Fujii, M., Rose, A.L., Waite, T.D., and Omura, T. (2008) Effect of divalent cations on the kinetics of Fe(III) complexation by organic ligands in natural waters. *Geochim Cosmochim Acta* **72**: 1335-1349.

Fujii, M., Rose, A.L., Omura, T., and Waite, T.D. (2010) Effect of Fe(II) and Fe(III) Transformation Kinetics on Iron Acquisition by a Toxic Strain of *Microcystis aeruginosa*. *Environ Sci Technol* **44**: 1980-1986.

Fujii, M., Imaoka, A., Yoshimura, C., and Waite, T.D. (2014a) Effects of molecular composition of natural organic matter on ferric iron complexation at circumneutral pH. *Environ Sci Technol* **48**: 4414-4424.

Fujii, M., Dang, T.C., Bligh, M.W., and Waite, T.D. (2016) Cellular characteristics and growth behavior of iron-limited *Microcystis aeruginosa* in nutrient-depleted and nutrient-replete chemostat systems. *Limnol Oceanogr* **61**: 2151-2164.

Fujii, M., Dang, T.C., Rose, A.L., Omura, T., and Waite, T.D. (2011b) Effect of light on iron uptake by the freshwater cyanobacterium *Microcystis aeruginosa*. *Environ Sci Technol* **45**: 1391-1398.

Fujii, M., Dang, T.C., Bligh, M.W., Rose, A.L., and Waite, T.D. (2014b) Effect of natural organic matter on iron uptake by the freshwater cyanobacterium *Microcystis aeruginosa*. *Environ Sci Technol* **48**: 365-374.

Hoff-Risseti, C., Dorr, F.A., Schaker, P.D.C., Pinto, E., Werner, V.R., and Fiore, M.F. (2013) Cylindrospermopsin and Saxitoxin Synthetase Genes in *Cylindrospermopsis raciborskii* Strains from Brazilian Freshwater. *Plos One* **8,Doi.org/10.1371/journal.pone.0074238**.

Hopkinson, B.M., and Barbeau, K.A. (2012) Iron transporters in marine prokaryotic genomes and metagenomes. *Environ Microbiol* **14**: 114-128.

Huisman, J., Codd, G.A., Paerl, H.W., Ibelings, B.W., Verspagen, J.M.H., and Visser, P.M. (2018) Cyanobacterial blooms. *Nat Rev Microbiol* **16**: 471-483.

Jacq, V., Ridame, C., L'Helguen, S., Kaczmar, F., and Saliot, A. (2014) Response of the unicellular diazotrophic cyanobacterium *Crocosphaera watsonii* to iron limitation. *Plos One* **9,Doi.org/10.1371/journal.pone.0086749**: e86749.

Jiang, H.B., Lou, W.J., Ke, W.T., Song, W.Y., Price, N.M., and Qiu, B.S. (2015) New insights into iron acquisition by cyanobacteria: an essential role for ExbB-ExbD complex in inorganic iron uptake. *ISME J* **9**: 297-309.

Kehoe, M., O'Brien, K.R., Grinham, A., and Burford, M.A. (2015) Primary production of lake phytoplankton, dominated by the cyanobacterium *Cylindrospermopsis raciborskii*, in response to irradiance and temperature. *Inland Waters* **5**: 93-100.

Kenesi, G., Shafik, H., Kovacs, A., Herodek, S., and Presing, M. (2009) Effect of nitrogen forms on growth, cell composition and N_2 fixation of *Cylindrospermopsis raciborskii* in phosphorus-limited chemostat cultures. *Hydrobiologia* **623**: 191-202.

Kikuchi, T., Fujii, M., Terao, K., Jiwei, R., Lee, Y.P., and Yoshimura, C. (2017) Correlations between aromaticity of dissolved organic matter and trace metal concentrations in natural and effluent waters: A case study in the Sagami River Basin, Japan. *Sci Total Environ* **576**: 36-45.

Kustka, A.B., Sanudo-Wilhelmy, S.A., Carpenter, E.J., Capone, D., Burns, J., and Sunda, W.G. (2003) Iron requirements for dinitrogen- and ammonium-supported growth in cultures of *Trichodesmium* (IMS 101): Comparison with nitrogen fixation rates and iron: carbon ratios of field populations. *Limnol Oceanogr* **48**: 1869-1884.

Lei, L., Peng, L., Huang, X., and Han, B.-P. (2014) Occurrence and dominance of *Cylindrospermopsis raciborskii* and dissolved cylindrospermopsin in urban reservoirs used for drinking water supply, South China. *Environ Monit Assess* **186**: 3079-3090.

Liu, X.W., and Millero, F.J. (2002) The solubility of iron in seawater. *Mar Chem* **77**: 43-54.

Martin, R.M., Moniruzzaman, M., Mucci, N.C., Willis, A., Woodhouse, J.N., Xian, Y. et al. (2018) Cylindrospermopsis raciborskii Virus and host: genomic characterization and ecological relevance. *Environ Microbiol* **0**: doi:10.1111/1462-2920.14425.

McGregor, G.B., and Fabbro, L.D. (2000) Dominance of *Cylindrospermopsis raciborskii* (Nostocales, Cyanoprokaryota) in Queensland tropical and subtropical reservoirs: Implications for monitoring and management. *Lakes Reserv* **5**: 195-205.

Moisander, P.H., Paerl, H.W., and Zehr, J.P. (2008) Effects of inorganic nitrogen on taxa-specific cyanobacterial growth and *nif*H expression in a subtropical estuary. *Limnol Oceanogr* **53**: 2519-2532.

Moisander, P.H., Cheshire, L.A., Braddy, J., Calandrino, E.S., Hoffman, M., Piehler, M.F., and Paerl, H.W. (2012) Facultative diazotrophy increases *Cylindrospermopsis raciborskii* competitiveness under fluctuating nitrogen availability. *FEMS Microbiol Ecol* **79**: 800-811.

Moore, C.M., Mills, M.M., Achterberg, E.P., Geider, R.J., LaRoche, J., Lucas, M.I. et al. (2009) Large-scale distribution of Atlantic nitrogen fixation controlled by iron availability. *Nat Geosci* **2**: 867-871.

Morel, F.M.M., Kustka, A.B., and Shaked, Y. (2008) The role of unchelated Fe in the iron nutrition of phytoplankton. *Limnol Oceanogr* **53**: 400-404.

Muhid, P., Davis, T.W., Bunn, S.E., and Burford, M.A. (2013) Effects of inorganic nutrients in recycled water on freshwater phytoplankton biomass and composition. *Water Res* **47**: 384-394.

Norman, L., Cabanes, D.J.E., Blanco-Ameijeiras, S., Moisset, S.A.M., and Hassler, C.S. (2014) Iron Biogeochemistry in Aquatic Systems: From Source to Bioavailability. *Chimia* **68**: 764-771.

Pierangelini, M., Stojkovic, S., Orr, P.T., and Beardall, J. (2014) Photosynthetic characteristics of two *Cylindrospermopsis raciborskii* strains differing in their toxicity. *J Phycol* **50**: 292-302.

Plominsky, A.M., Delherbe, N., Mandakovic, D., Riquelme, B., Gonzalez, K., Bergman, B. et al. (2015) Intercellular transfer along the trichomes of the invasive terminal heterocyst forming cyanobacterium *Cylindrospermopsis raciborskii* CS-505. *FEMS Microbiol Lett* **362, Doi.org/10.1093/femsle/fnu009**.

Postgate, J. (1998) *Nitrogen Fixation*. Cambridge: Cambridge University Press.

Roland, F.A.E., Darchambeau, F., Morana, C., Bouillon, S., and Borges, A.V. (2017) Emission and oxidation of methane in a meromictic, eutrophic and temperate lake (Dendre, Belgium). *Chemosphere* **168**: 756-764.

Saker, M.L., Neilan, B.A., and Griffiths, D.J. (1999) Two morphological forms of *Cylindrospermopsis raciborskii* (Cyanobacterta) isolated from Solomon Dam, Palm Island, Queensland. *J Phycol* **35**: 599-606.

Sandrini, G., Ji, X., Verspagen, J.M.H., Tann, R.P., Slot, P.C., Luimstra, V.M. et al. (2016) Rapid adaptation of harmful cyanobacteria to rising CO_2. *Proc Natl Acad Sci USA* **113**: 9315-9320.

Schoffman, H., Lis, H., Shaked, Y., and Keren, N. (2016) Iron-nutrient interactions within phytoplankton. *Frontiers in Plant Science* **7: 10.3389/fpls.2016.01223**.

Sinha, R., Pearson, L.A., Davis, T.W., Muenchhoff, J., Pratama, R., Jex, A. et al. (2014) Comparative genomics of *Cylindrospermopsis raciborskii* strains with differential toxicities. *BMC Genomics* **15**: 10.1186/1471-2164-1115-1183.

Snow, J.T., Schlosser, C., Woodward, E.M.S., Mills, M.M., Achterberg, E.P., Mahaffey, C. et al. (2015) Environmental controls on the biogeography of diazotrophy and *Trichodesmium* in the Atlantic Ocean. *Glob Biogeochem Cycle* **29**: 865-884.

Sprober, P., Shafik, H.M., Presing, M., Kovacs, A.W., and Herodek, S. (2003) Nitrogen uptake and fixation in the cyanobacterium *Cylindrospermopsis raciborskii* under different nitrogen conditions. *Hydrobiologia* **506**: 169-174.

Stucken, K. (2010) Physiogenomics of *Cylindrospermopsis raciborskii* and R*aphidiopsis brookii* (Cyanobacteria) with emphasis on evolution, nitrogen control and toxin biosynthesis. In *Faculty of Biology and Chemistry*. Bremen: University Bremen.

Van de Waal, D.B., Verspagen, J.M.H., Finke, J.F., Vournazou, V., Immers, A.K., Kardinaal, W.E.A. et al. (2011) Reversal in competitive dominance of a toxic versus non-toxic cyanobacterium in response to rising CO_2. *ISME J* **5**: 1438-1450.

Ward, B.A., Dutkiewicz, S., Moore, C.M., and Follows, M.J. (2013) Iron, phosphorus, and nitrogen supply ratios define the biogeography of nitrogen fixation. *Limnol Oceanogr* **58**: 2059-2075.

Weger, H.G., Middlemiss, J.K., and Petterson, C.D. (2002) Ferric chelate reductase activity as affected by the iron-limited growth rate in four species of unicellular green algae (*Chlorophyta*). *J Phycol* **38**: 513-519.

Whittaker, S., Bidle, K.D., Kustka, A.B., and Falkowski, P.G. (2011) Quantification of nitrogenase in *Trichodesmium* IMS 101: implications for iron limitation of nitrogen fixation in the ocean. *Environ Microbiol Rep* **3**: 54-58.

Willis, A., Chuang, A.W., and Burford, M.A. (2016a) Nitrogen fixation by the diazotroph *Cylindrospermopsis raciborskii* (Cyanophyceae). *J Phycol* **52**: 854-862.

Willis, A., Chuang, A.W., Woodhouse, J.N., Neilan, B.A., and Burford, M.A. (2016b) Intraspecific variation in growth, morphology and toxin quotas for the cyanobacterium, *Cylindrospermopsis raciborskii*. *Toxicon* **119**: 307-310.

Willis, A., Adams, M.P., Chuang, A.W., Orr, P.T., O'Brien, K.R., and Burford, M.A. (2015) Constitutive toxin production under various nitrogen and phosphorus regimes of three ecotypes of *Cylindrospermopsis raciborskii* ((Woloszyriska) Seenayya et Subba Raju). *Harmful Algae* **47**: 27-34.

Willis, A., Woodhouse, J.N., Ongley, S.E., Jex, A.R., Burford, M.A., and Neilan, B.A. (2018) Genome variation in nine co-occurring toxic *Cylindrospermopsis raciborskii* strains. *Harmful Algae* **73**: 157-166.

Wirtz, N.L., Treble, R.G., and Weger, H.G. (2010) Siderophore-independent iron uptake by iron-limited cells of the cyanobacterium *Anabaena flos-aquae*. *J Phycol* **46**: 947-957.

Yang, J.G., Xie, X.Q., Yang, M.X., Dixon, R., and Wang, Y.P. (2017) Modular electron-transport chains from eukaryotic organelles function to support nitrogenase activity. *Proc Natl Acad Sci USA* **114**: E2460-E2465.

Yang, Y., Chen, Y., Cai, F., Liu, X., Wang, Y., and Li, R. (2018) Toxicity-associated changes in the invasive cyanobacterium *Cylindrospermopsis raciborskii* in response to nitrogen fluctuations. *Environ Pollut* **237**: 1041-1049.

Zobrist, J., Sima, M., Dogaru, D., Senila, M., Yang, H., Popescu, C. et al. (2009) Environmental and socioeconomic assessment of impacts by mining activities-a case study in the Certej River catchment, Western Carpathians, Romania. *Environ Sci Pollut Res* **16**: 14-26.

Legends and captions for Tables and Figures

Table 1 Temporal nitrogenase activity of CS-506 and CS-509 strains normalized by the Chl *a* content under different treatments.

Fig. 1. Growth curves of *R. raciborskii* CS-506 and CS-509 strains treated with different concentrations of Fe and N. All measurements are mean ± SD with $n = 3$ with mean results used for the calculation of overall specific growth rate.

Fig. 2. Relationship between the Chl *a*-normalized (A) and overall (B) nitrogenase activity and Fe availability for CS-506 and CS-509 strains under N-free condition. Symbol and error bars indicate the mean value and standard deviation from triplicate measurements. The solid line represents linear regression line for the experimentally determined data.

Fig. 3. Effects of light and Fe limitation on ^{55}Fe uptake by CS-509 strain. CS-509 cells acclimated in the Fe-replete ((JM*(-N, 1000)) and Fe-limited (JM*(-N, 200)) media were used for the ^{55}Fe uptake assay using the ^{55}Fe-EDTA bearing JM* medium (200 nM ^{55}Fe and 20 µM EDTA) under light and dark conditions. Symbol and error bars indicate the mean value and standard deviation from triplicate measurements. The solid line represents linear regression line for the experimentally determined data.

Fig. 4. Effects of Fe pre-supplementation on ^{55}Fe uptake by CYN^{+} strain. CS-506 cells acclimated in the Fe-replete ((JM*(-N, 1000)) and Fe-limited (JM*(-N, 200)) media employed for the ^{55}Fe uptake experiment in the ^{55}Fe-EDTA bearing JM* medium (200 nM ^{55}Fe and 3.5-200 µM EDTA) under light condition. Symbol and error bars indicate the mean value and standard deviation from triplicate measurements. The solid line represents non-linear regression line for the experimentally determined data.

Fig. 5. (A) ^{55}Fe uptake rates of CS-506 strain acclimated in JM* media supplemented with 50 nM Fe and no nitrate (JM*(-N, 50)) or with 50 nM Fe and 9.4×10^{-4} M nitrate (JM*(+N, 50)) and (A) ^{55}Fe uptake rates of *R. raciborskii* CS-506 and CS-509 strains acclimated in Fe-replete ((JM*(-N, 1000)) and Fe-limit (JM*(-N, 200)) conditions. The ^{55}Fe uptake experiment was conducted using these cells in the ^{55}Fe-EDTA bearing JM* medium (200 nM ^{55}Fe and 20 µM EDTA) under light condition. The asterisks above the columns indicate significant differences between the treatments ($p < 0.05$, $n = 3$). The symbol "NS" represents "not significant".

Table 1 Temporal nitrogenase activity of CS-506 and CS-509 strains normalized by the Chl *a* content under different treatments.

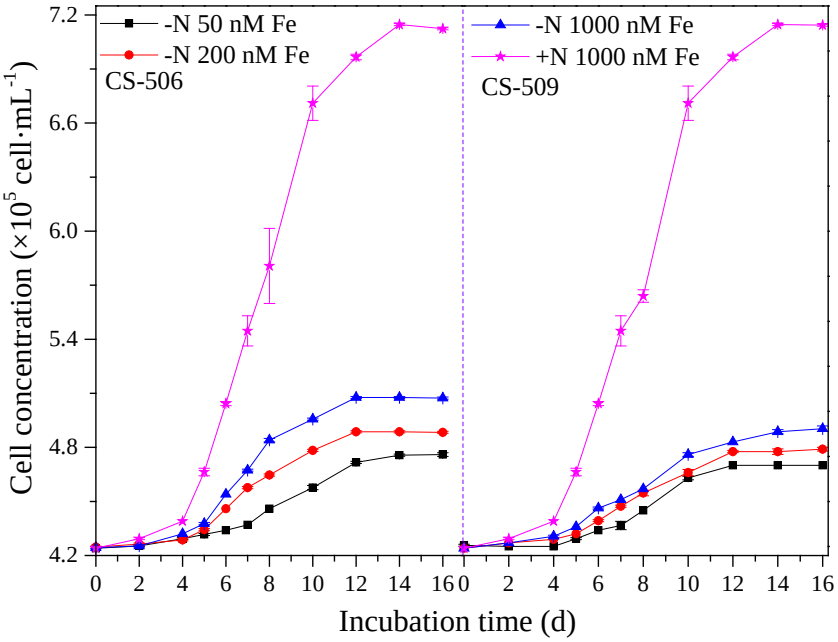
Time (h)	Nitrogenase activity for CS-506 Strain (nmol-C_2H_4·mL·μg-Chl a^{-1}·h^{-1})				Nitrogenase activity for CS-509 Strain (nmol-C_2H_4·mL·μg-Chl a^{-1}·h^{-1})			
	JM*(+N, 1000)[a]	JM*(-N, 1000)[b]	JM*(-N, 200)[c]	JM*(-N, 50)[d]	JM*(+N, 1000)[a]	JM*(-N, 1000)[b]	JM*(-N, 200)[c]	JM*(-N, 50)[d]
2	1.18 ± 0.26	5.46 ± 0.25	1.65 ± 0.12	3.77 ± 0.11	2.48 ± 0.26	1.84 ± 0.19	1.58 ± 0.10	1.40 ± 0.10
4	2.88 ± 0.29	11.25 ± 2.13	0.75 ± 0.06	2.93 ± 0.26	1.94 ± 0.13	1.91 ± 0.08	1.79 ± 0.21	1.58 ± 0.15
8	0.81 ± 0.26	11.90 ± 2.26	8.39 ± 1.26	9.30 ± 1.16	2.50 ± 0.20	1.85 ± 0.20	1.69 ± 0.28	1.37 ± 0.26
24	0.87 ± 0.31	12.20 ± 1.46	8.45 ± 1.37	8.66 ± 1.04	1.10 ± 0.13	3.51 ± 0.32	4.06 ± 0.65	1.91 ± 0.16

[a] JM*(+N, 1000) represents the JM* medium in the presence of 9.4×10^{-4} M nitrate and 1000 nM Fe(III);

[b] JM*(-N, 1000) represents the JM* medium in the presence of 0 M nitrate and 1000 nM Fe(III);

[c] JM*(-N, 200) represents the JM* medium in the presence of 0 M nitrate and 200 nM Fe(III);

[d] JM*(-N, 50) represents the JM* medium in the presence of 0 M nitrate and 50 nM Fe(III).



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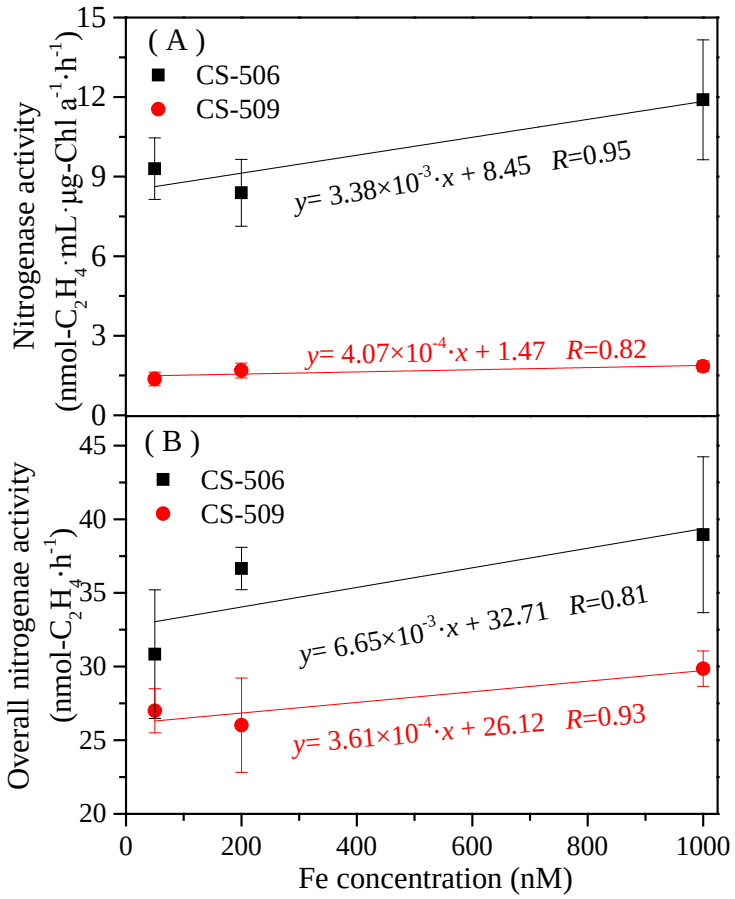

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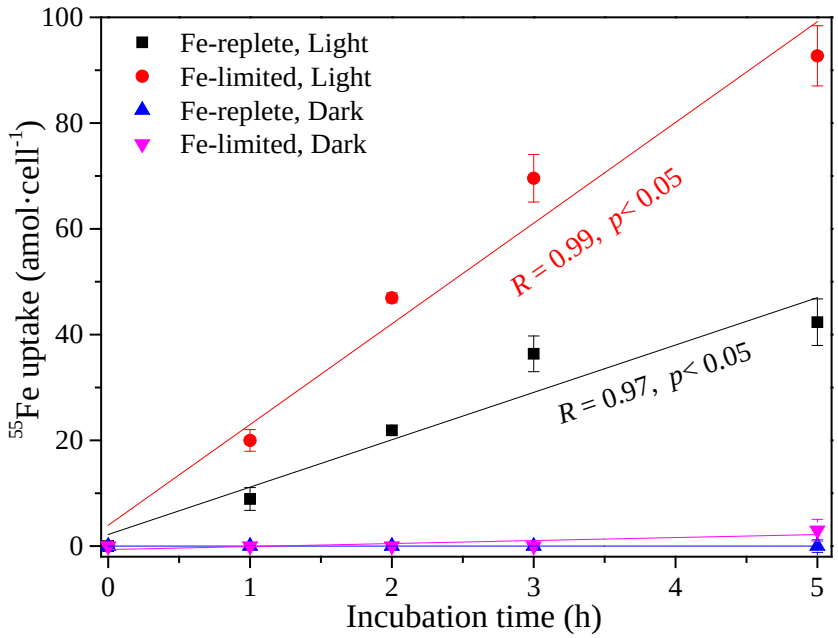

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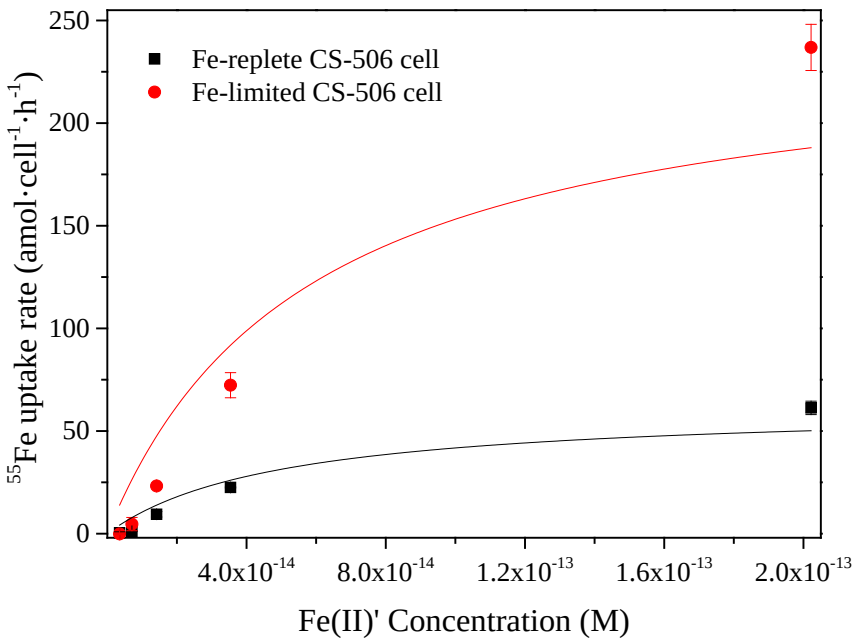

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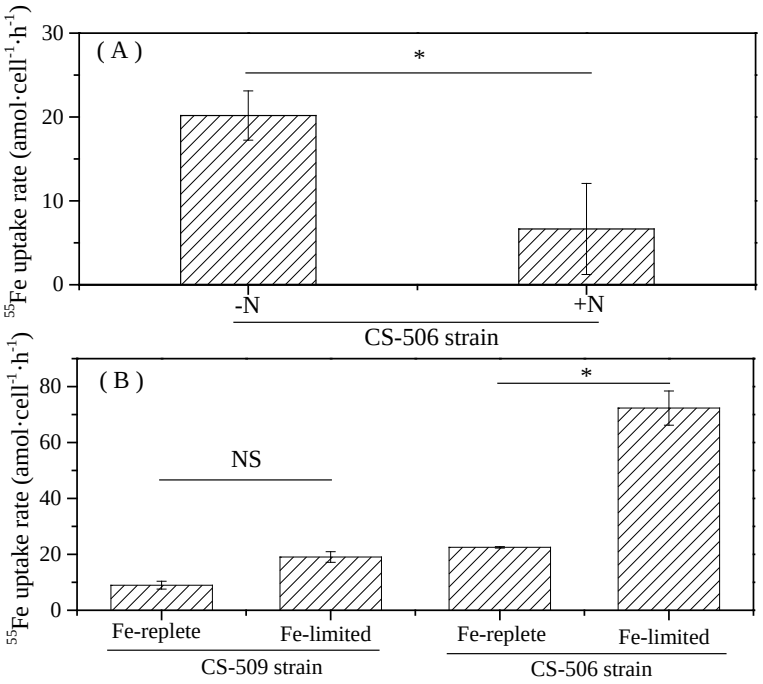

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