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ORIGINAL RESEARCH

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Nitrate or ammonium: Influences of nitrogen source on the physiology of a green alga

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Abstract

In freshwaters, algal species are exposed to different inorganic nitrogen (N_i) sources whose incorporation varies in biochemical energy demand. We hypothesized that due to the lesser energy requirement of ammonium (NH_4^+)-use, in contrast to nitrate (NO_3^-)-use, more energy remains for other metabolic processes, especially under CO_2- and phosphorus (P_i) limiting conditions. Therefore, we tested differences in cell characteristics of the green alga *Chlamydomonas acidophila* grown on NH_4^+ or NO_3^- under covariation of CO_2 and P_i-supply in order to determine limitations, in a full-factorial design. As expected, results revealed higher carbon fixation rates for NH_4^+-grown cells compared to growth with NO_3^- under low CO_2 conditions. NO_3^--grown cells accumulated more of the nine analyzed amino acids, especially under P_i-limited conditions, compared to cells provided with NH_4^+. This is probably due to a slower protein synthesis in cells provided with NO_3^-. In contrast to our expectations, compared to NH_4^+-grown cells NO_3^--grown cells had higher photosynthetic efficiency under P_i-limitation. In conclusion, growth on the N_i-source NH_4^+ did not result in a clearly enhanced C_i-assimilation, as it was highly dependent on P_i and CO_2 conditions (replete or limited). Results are potentially connected to the fact that *C. acidophila* is able to use only CO_2 as its inorganic carbon (C_i) source.

KEYWORDS
amino acids, carbon uptake kinetics, CO_2 conditions, nitrogen, phosphorus limitation

1 | INTRODUCTION

Green algae (Chlorophyta), with around 6,500 recognized species (Guiry & Guiry, 2017), can be found in highly diverse habitats, such as in soils, streams, lakes, and even on stones, trees, and animals (Andersen, 1992). In these systems, algae might be exposed to different nutrient sources and limitations. Nitrogen is, next to phosphorus, an important nutrient that is more often (co-)limiting in freshwater systems than previously thought (Elser et al., 2007). The assimilation of nitrogen into amino acids and proteins requires energy and organic carbon skeletons (Huppe & Turpin, 1994). Therefore, it is quite obvious that interactions between photosynthesis-related processes and the acquisition of nitrogen occur, which is, for example, shown by a causal relationship between the assimilation of NH_4^+ and dark C_i-fixation under anaplerosis (Giordano, Norici, Forssen, Eriksson, & Raven, 2003; Vanlerberghe, Schuller, Smith, & Turpin, 1990) as well as photosynthetic CO_2-fixation (Turpin, Vanlerberghe, Amory, & Guy, 1991). In *Synechococcus* sp., the use of NH_4^+ over NO_3^- stimulated photosynthesis and growth under a light limitation (Ruan & Giordano, 2017).

Most phytoplankton species are able to use NH_4^+ and NO_3^- as the nitrogen source (Raven & Giordano, 2016). Several studies

observed a higher photosynthetic or growth rate of macro- and microalgae for a certain N_i-source; reflected, for example, by different affinities to NH_4^+ and NO_3^- uptake, or changes in a broad range of physiological parameters related to the growth response to different nitrogen sources (Ale, Mikkelsen, & Meyer, 2011; Beamud, Diaz, & Pedrozo, 2010; Giordano, 1997; Giordano & Bowes, 1997; Reay, Nedwell, Priddle, & Ellis-Evans, 1999). For *Chlamydomonas* species, NH_4^+ is considered the preferred N_i-source, and negatively signals NO_3^- assimilation (Fernandez & Galvan, 2007). These preferences might be a factor for competition, as the concentration of both nutrients varies through out the year in lakes (Kolzau et al., 2014). In contrast to NO_3^-, NH_4^+ is directly incorporated into amino acids by condensation with glutamate to form glutamine catalyzed by glutamine synthetase (Miflin & Lea, 1980; Sanz-Luque, Chamizo-Ampudia, Llamas, Galvan, & Fernandez, 2015). The demand to acquire CO_2 increases with increasing nitrogen assimilation. In order to satisfy this demand, the provision of carbon skeletons via photosynthesis is partly energized from mitochondrial respiration (Giordano et al., 2003; Weger, Birch, Elrifi, & Turpin, 1988). Thus, phosphoenolpyruvate carboxylase (PEPc) activity (Giordano et al., 2003) is also enhanced.

Some phytoplankton species seem to grow faster with NO_3^- than NH_4^+ (Dortch, 1990), potentially as an adaptation to the more available nitrogen source in their natural environment. Using ammonium, the algal cell avoids energy consuming steps of nitrogen reduction and the production of nitrate reductase (NR) and nitrite reductase (NiR) (Pritchard, Hurd, Beardall, & Hepburn, 2015; Raven, 1985). As no nitrification occurs in acidic lakes below pH 3 (Jeschke, Falagan, Knöller, Schultze, & Koschorreck, 2013), a high concentration of NH_4^+ (compared to NO_3^-) was observed in many of these lakes (Bissinger, Jander, & Tittel, 2000; Jeschke et al., 2013). Therefore, we suggest a preference for ammonium, and possibly a lack of NR activity, as an adaptation of acidophilic algae to their environment and decided to study the influence of N_i-source on several parameters describing the inorganic carbon acquisition (C_i-acquisition) of *Chlamydomonas acidophila* Negoro (SAG 2045). The strain was isolated from an acidic mining lake with a pH of about 2.7 (Gerloff-Elias, Spijkerman, & Pröschold, 2005) and replete N_i concentrations (i.e. 0.22 mM), 91% of which is in the form of NH_4^+ (Bissinger et al., 2000).

We hypothesize that the acquisition of NH_4^+ compared to NO_3^- allows a higher rate of nitrogen assimilation and that the reduced metabolic energy requirement of NO_3^- use enables energy allocation to other cellular processes, such as photosynthesis and growth. Thus, C_i-uptake might be increased and photosynthetic parameters optimized to enhance photosynthesis, which probably also affects the cellular amino acid content. Some amino acids such as glutamate might be accumulated in NO_3^--grown cells as the protein synthesis is slowed and consequently the realized cell number decreased. Phosphorus also plays an important role in the energy budget as limiting P_i-conditions reduce the total adenylate concentration (Gauthier & Turpin, 1994; Theodorou, Elrifi, Turpin, & Plaxton, 1991) and consequently decreased, for example, the maximal photosynthetic and growth rate of *C. acidophila* (Spijkerman, 2010), and decreased the affinity for C_i-uptake in *Chlorella emersonii* (Beardall, Roberts, & Raven, 2005). The concentration of CO_2 is the key factor for the activation of carbon-concentrating mechanisms (CCMs); therefore, effects due to energy consuming CCMs (Raven & Beardall, 2014) might be amplified under low CO_2. We realized that effects might be small as there are possibly no loss processes in the CCMs (Raven, Beardall, & Giordano, 2014).

It was previously shown that the influence of the N_i-source on different physiological parameters might depend on other factors, such as light (Ruan & Giordano, 2017) and CO_2 (Giordano, 1997), but to our knowledge there are no studies combining two factors involved in energy and carbon metabolism in comparison with the effect of a different N_i-source. Therefore, as both C_i (Tittel, Bissinger, Gaedke, & Kamjunke, 2005) and P_i (Spijkerman 2008) have been identified as potential (co-)limiting factors for *C. acidophila* in the acidic Lake 111 (Spijkerman, Stojkovic, Holland, Lachmann, & Beardall, 2016), we included both factors in our setup. Consequently, we studied interactions among P_i- and CO_2- supply/limitation under two different N_i-sources, in a full-factorial design (eight different treatments). We expect the highest photosynthetic and C_i-assimilation rates at NH_4^+, P_i- and CO_2-replete conditions, and the lowest at NO_3^-, P_i-, and CO_2-limiting conditions. The influence of provided N_i-sources on the ecophysiology of *C. acidophila* was examined by measuring a wide range of physiological parameters such as C_i-uptake kinetics, amino acid levels, and NR activity.

2 | MATERIALS AND METHODS

2.1 | Cultivation and number of cells

Three replicates of *C. acidophila* Negoro (SAG 2045) were cultivated semi-continuously by daily dilution at a low steady-state growth rate of 0.2/day in a climate chamber, to obtain stringent nutrient limiting conditions. Cultures were exposed to saturating light conditions (approximately 100 µmol photons m^{-2} s^{-1} as measured inside the culture flasks; Gerloff-Elias, Spijkerman, & Schubert, 2005) at 20 ± 1°C in a modified Woods Hole Medium (Nichols, 1973); without silicate, at pH 2.5, buffered with $FeCl_3$. We varied the N_i-source (NO_3^- or NH_4^+), P_i concentration (P_i-limited: 1 µM and P_i-replete: 100 µM) and C_i-supply (low CO_2: air, high CO_2: 4.5%) in a full-factorial design. Nitrogen was intended not to become a limiting nutrient, and therefore, NO_3^- was provided in excess of 2 mM and NH_4^+ in excess of 1 mM. We added less NH_4^+ than NO_3^- to prevent ammonium from causing an uncoupling of the photosynthetic H^+ gradient (Krause & Behrend, 1986). All cultures were aerated with normal air or with CO_2 enriched air from a gas cylinder (4.5% CO_2 in air (v/v), Air Liquide) and each comprised 600 ml of culture volume within a 1-l Erlenmeyer flask. Measured concentrations of CO_2 in the flasks were 800 µM at high CO_2, 14 µM at low CO_2, P_i-limiting, and 3 µM at low CO_2, P_i-replete conditions (following Spijkerman, Castro, & Gaedke, 2011). Algal growth was monitored by daily dilution and measurements of the optical density (OD) at 800 nm on a spectrophotometer

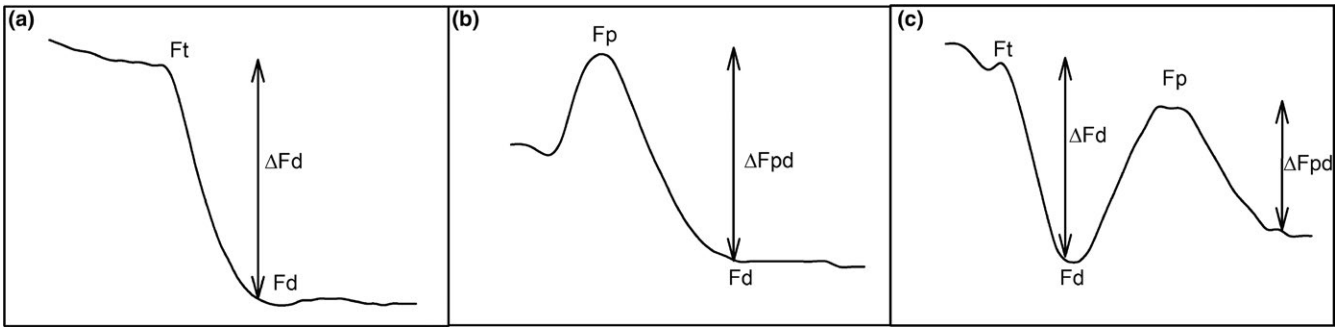

FIGURE 1 Possible shapes of nutrient-induced fluorescence transients (NIFT) in response to the in vivo fluorescence of chl *a* and parameters for calculating the response. The parameters and shapes of the response are described in the text

(UV-2401 PC; Shimadzu, Kyoto, Japan). Experiments were performed with algae in steady state, which means that the OD was stable for at least 20 days (three total exchanges of culture volume). The number of cells was measured by fixation with Lugol's iodine (1%) and counting on an automatic cell counter (CASY®1 TT, Schärfe System, Reutlingen, Germany).

2.2 | Nutrient-induced fluorescence transients

Nutrient-induced fluorescence transients (NIFTs) are a quick method to indicate nutrient depletion in algae (C_i, N_i, P_i) due to transient change in chlorophyll *a* (chl *a*) fluorescence after a nutrient spike (Shelly, Holland, & Beardall, 2010; Spijkerman et al., 2016). On a Phyto-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany), the fluorescence (F_t, chl *a* fluorescence in steady state and under actinic light) was recorded (Phytowin_v1.47) every three seconds without a saturating pulse for at least one minute (Gain: 8–18, actinic light: 120 µmol m^{-2} s^{-1}). Subsequently, the response to an addition of P_i (final concentration in cuvette: 10 µM KH_2PO_4), N_i (100 µM N_i as $(NH_4)_2SO_4$ or $NaNO_3$) or C_i (100 µM $NaHCO_3$), and combinations of the nutrients, was recorded until the fluorescence signal remained constant again (F_d). All spike solutions were prepared in acidified water of pH 2.5 (with the C_i solution diluted 1 min before addition of CO_2 after conversion of most of the HCO_3), and 20 µl was pipetted into a cuvette with 2 ml of diluted culture, to reach the previously denoted nutrient concentrations in the cuvette. Final P_i concentration was above 4 µM, based on results in Roberts, Shelly, and Beardall (2008), and N_i concentrations for maximal NIFT responses and a final CO_2 concentration of 100 µM were selected, according to Young and Beardall (2003) and Spijkerman et al. (2016), respectively. A fresh sample was taken for each measurement to avoid potential influences of previous additions of nutrients. After addition of P_i and C_i, a rapid decrease in the fluorescence was observed in P_i- or CO_2-limited cultures. We calculated the ΔF_d (difference between lowest fluorescence after decrease, F_d, and steady-state fluorescence, F_t (Shelly et al., 2010)) as a percentage of F_t for these two nutrient additions (Figure 1a). The response of N_i-limited cells to NH_4^+ and NO_3^- might be different, as described by Beardall, Young, and Roberts (2001), because we found a small rise of fluorescence (F_p, highest fluorescence as response to nutrient) followed by a drop to F_d after NH_4^+ addition, and a strong rise of fluorescence to F_p with a recovery to initial values after NO_3^- injection (F_t). Therefore, for these two nutrients, we calculated ΔF_{pd} as representing the difference between the highest fluorescence at the top of the peak and the lowest value after decreasing (Figure 1b). Under colimitation of three nutrients, a third response was observed: after adding nutrient combinations (P_i and C_i, C_i and NH_4^+, or all three nutrients) to the culture, at first a decrease (F_d), then an increase (F_p), and finally a second decrease of the fluorescence was observed (second F_d). We calculated the response by forming the sum of ΔF_d and ΔF_{pd} (Figure 1c). Such a response to two potentially limiting nutrients has been shown before for *C. acidophila* growing under P_i- and C_i-deplete conditions (Spijkerman et al., 2016).

2.3 | Traditional nutrient enrichments

We tested for the growth limiting nutrient of cultures by performing enrichment experiments. Eighty microliters of spike solutions was added (P_i: 1 mM; N_i (NO_3^- or NH_4^+ depending on culture conditions) and C_i: both 10 mM) to 8 ml of culture. Acidified water was added as a control. After three days of growth under the above-mentioned culture conditions, the optical densities were measured. The influence of nutrients was detected by calculating differences in biomass yield between enriched and control cultures.

2.4 | Chl *a*, protein content, and NR activity

During steady state, part of the culture, remaining after daily dilution, was centrifuged (2,000 ***g***, 5 min, 6°C), washed with demineralized water and quickly frozen at −80°C. For extraction and following analyses, these pellets of algae were resuspended in 0.7 ml extraction buffer (pH 8), which consisted of 50 mM HEPES (N-2-Hydroxyethyl piperazine-N-2-ethane sulfonic acid), 0.1% Triton-X-100, 10% glycerol and 1 mM Na_2EDTA (Ethylenediaminetetraacetic acid), DTT (Dichlorodiphenyltrichloroethane; 1 mM), and protease inhibitor (S8820, Sigma-Aldrich, 18 ng/ml). Glass beads (1.0 mm, BioSpec Products, Inc., Bartlesville, USA) were added to homogenize cells in a cooled bead beater (Precellys 24; PEQLAB, Erlangen, Germany) by shaking 6 times for 10 s at 5,000 rpm. After centrifugation (5 min, 1,800 ***g***; Biofuge Stratos; Heraeus, Hanau Germany), the supernatant

was enriched with 10 mM $MgCl_2$ (final concentration) and stored at −20°C for <1 week, until analysis.

The NR activity was standardized for protein, and this was measured by a method that determines chl *a* and protein in the same sample (Peterson, 1977). To measure chl *a* concentration in cells, cell extracts were well-mixed with precooled acetone (90%) in a bead beater (3 × 10 s at 5,000 rpm) and centrifuged (5 min at 18,000 ***g*** at 7°C). We measured absorption of the supernatant at 750, 664 and 647 nm in a spectrophotometer, and calculated concentrations of chl *a* according to Jeffrey and Humphrey (1975). The remaining pellet was used for protein determination. For the measurement of protein content, pellets were resuspended in 500 µl of SDS (1%) in NaOH (0.1 M), following Peterson (1977). Bovine serum albumin (BSA) was used as a standard. Two dyes were freshly prepared containing, A: CTC (0.1% CuSO4 × 5 H_2O, 0.2% NaKTartrate, 10% Na_2CO_3 (w/v)), 10% SDS, 0.8 M NaOH and H_2O mixed in equal amounts, and B: Folin reagent diluted in a fivefold amount of water. Firstly, to the resuspended pellet or standard 500 µl of dye A was added and directly mixed. After 10 min, 250 µl of dye B was pipetted in and mixed. Another 30 min of incubation was needed before 300 µl aliquots was filled in wells of a 96-well microplate and absorption was measured at 750 nm (infinite F200PRO, TECAN, Männedorf, Switzerland).

We modified the NR activity measurements following Chen, He, and Hu (2012), as follows. Fifty µl of cell extracts was mixed with reaction solution consisting of 50 mM HEPES (pH 7.5), 10 mM KNO_3, and 0.1 mM EDTA. The reaction was started by adding 5 µl NADH (40 mM), while extracts incubated in a 30°C water bath. The incubation time varied based on potential activities (15–30 min; which was linear and tested beforehand) and the enzyme reaction was stopped by adding 50 µl zinc acetate solution (1 M). Afterward, extracts were centrifuged for 3 min at 6,700 ***g*** (Minispin; Eppendorf; Hamburg, Germany) and nitrite content in the supernatant was determined with the help of two coloring solutions. These were mixed 1:1 (10 g/L sulfanilamide in 3 N HCl and 200 mg/L N-(1-Naphthyl) ethylenediamine dihydrochloride in H_2O) directly before use, after which, 100 µl of the coloring mixture was added to 100 µl supernatant in an 96-well microplate. The filled microplates were covered with a plastic film and incubated for 15 min under gentle shaking. Finally, absorption was measured in a multimode microplate reader at 540 nm. This absorption was converted to NR activity via a calibration curve and presented as nmol nitrite min^{-1} mg $protein^{-1}$.

2.5 | C_i-accumulation factor

We measured the C_i-accumulation factor (CCF) to check for the effect of N_i-source on C_i-accumulation. Concentrated cell suspension (after centrifugation at 1,500 *g* for 5 min and resuspension in the growth medium, and that to approached an OD of 2) was illuminated for one hour to deplete C_i and then placed on a silicon oil layer on the top of a killing fluid (Spijkerman, 2005). The accumulation of C_i was measured following Badger, Kaplan, and Berry (1980) and Spijkerman, Stojkovic, and Beardall (2014). Preliminary experiments showed that an effective oil mixture of 1:1 or 1:2 (v/v) of silicon oil "type 3," and "500" for gas chromatography (Merck, Darmstadt, Germany), sufficed. We provided between 50 and 70 µM $NaH^{14}CO_3$, with a specific activity of about 1,739 GBq/mmol (PerkinElmer, Germany), for the algae while illuminating with 200 µmol PAR $m^{-2\ -1}$ (incident irradiance), and the carbon uptake was stopped after 10 s by centrifugation (12,000 ***g***, 15 s). Centrifuge tubes were flash frozen in liquid nitrogen, and the algal pellets removed simply by cutting off the end of the tube. The pellets were resuspended in 400 µl NaOH (0.1 M), and then, we transferred 150 µl into the same amount of NaOH (0.1 M; total fraction) or HCl (0.5 M in methanol; acid stable fraction). Acid-labile carbon in the HCl samples evaporated under a fume head over night. 2.5 ml of Ultima Gold (PerkinElmer) was then added before counting in a liquid scintillation analyzer (Tri-Carb 2,810; PerkinElmer). We calculated the C_i-accumulation from the difference between total and acid stable fractions. After 15- to 20-min incubation with 37 kBq ^3H-H_2O (specific activity: 37 kBq · $mmol^{-1}$ Hartmann-analytic), cell volume was measured according to Beardall (1981). The CCF of the accumulated C_i over the 10 s was calculated in two ways: one based on cell volume determined with ^3H-H_2O, and one, based on the cell volume calculated with cell diameter analyzed with the automatic cell counter CASY®1 TT (Schärfe System, Reutlingen, Germany).

2.6 | ^{14}C-fixation rates

To check for the effect of N_i-source on the rate of C_i-acquisition, we measured the primary productivity by C_i-fixation rate. Each culture was sampled and measured as quickly as possible to avoid changes in the CO_2 equilibration. Three technical replicates were kept in light while the one control was placed in the dark. We added a final concentration of 2 µM $NaH^{14}CO_3$ (3,480 Bq/ml) from stock solution (1.74 GBq/mmol specific activity; PerkinElmer, Germany) and incubated the algae depending on their P-status ranging from 2 to 13 min. The fixation of C_i was stopped by rapid filtration over a 0.25-µm nucleopore filter (Whatman, Maidstone, UK) under a maximal pressure of 200 mbar and rinsing with demineralized water. For detection of fixed particulate organic ^{14}C ($PO^{14}C$), filters were dissolved in 0.5 ml Soluene (Perkin Elmer). Separately, 0.5 ml of each sample was taken to measure the total activity of ^{14}C ($T^{14}C$). We added 2.5 ml of scintillation fluid (Ultima Gold, PerkinElmer) to all samples before counting radioactive decay in a liquid scintillation analyzer (Tri-Carb 2810 TR, Perkin Elmer). The ^{14}C fixation rate was calculated following equation 1; with DIC (the concentration of dissolved C_i) measured by an injection of 4 ml culture into a liquid carbon analyzer (High TOC; Elementar Analysensysteme GmbH, Hanau, Germany), and 1.06, a factor for the isotope-discrimination between ^{12}C and ^{14}C by phytoplankton (Steemann, 1952).

$$^{14}\text{C fixation rate} = \frac{\left(\frac{PO^{14}C(\text{corr.})}{\text{ml(filter)}\times\text{incubation time}} \times \text{activity(spec.)}^{-1}\right)\times\left(\frac{\text{DIC}}{T^{14}C}\right)\times 1.06}{\text{chlorophyll } a \text{ concentration}}$$

2.7 | Extraction of algal samples and measurements of amino acids (HPLC)

The accumulation of amino acids in cells can indicate hampering of protein synthesis. The effect of N_i-sources on the steady-state levels of amino acids was therefore measured by extracting the amino acids from algal cells. Firstly, 10 ml of culture was quickly cooled down to −60°C in 20 ml quenching solution, consisting of 70% methanol (cooled by −70°C ethanol), flash frozen in liquid nitrogen, and stored at −80°C. Samples were dried and then extracted according to Mettler et al. (2014). Extracts were dissolved in 50 µl HCl (0.1 M). As the optimal pH for derivatization is between 8.2 and 10, we added 2 µl of 1 N NaOH and 8 µl H_2O to 10 µl samples, from which 10 µl were taken for derivatization by AccQ-Tag Ultra Reagent Powder (Waters Corporation, Milford, MA, USA), according to the manufacturer's instructions. Derivates were separated by liquid chromatography and detected at 260 nm using a 1,290 UHPLC system coupled to diode array detector (Agilent, USA), according to Rademacher et al. (2016).

2.8 | C_i-uptake kinetics and photosynthetic measurement

To assess the effect of N_i-source on the efficiency for C_i-acquisition, we measured C_i-uptake kinetics. Algal cultures were centrifuged (5 min at 1,500 g) and resuspended in fresh medium (pH 2.5) without the limiting nutrient, in order to concentrate cells. ODs differed among varying P_i and CO_2 (P_i-replete and low CO_2: 0.20 – 0.23, P_i-replete and high CO_2: 0.31 – 0.36, P_i-limited low CO_2: 0.31 – 0.42; P_i-limited, high CO_2: 0.44 – 0.67). Oxygen evolution rates were measured, following Lachmann, Maberly, and Spijkerman (2016b), with a Clark electrode at an incident saturating light intensity of 500 µmol m^{-2} s^{-1} for the dense samples in a light dispensation system (Illuminova, Uppsala, Sweden). The half-saturation constant for C_i $K_{0.5}(C_i)$ by photosynthesis, the maximal uptake rate V_{max}, and the affinity $V_{max}/K_{0.5}(C_i)$ were calculated by modeling the response of oxygen evolution rates to C_i concentrations and performing a linearization, according to Hofstee (1952). As measurements were taken under pH 2.5, all C_i quickly converts to CO_2 and therefore, hereafter, $K_{0.5}(C_i)$ is mentioned as $K_{0.5}(CO_2)$.

Additionally, photosynthetic electron transport rate was also measured via rapid light curves using a Phyto-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) and following Grzesiuk, Wacker, and Spijkerman (2016). We determined the electron transport rate (ETR) with PhytoWin (V2.13) under different light intensities, and calculated alpha, the slope at the beginning of the light curve. This parameter represents the relative photosynthetic efficiency on the basis of electrons, which might be related to the usage of different nitrogen sources.

2.9 | Chemical analyses

For determining the content of carbon and nitrogen (Supporting information Tables S2 and S3) in cells, a defined volume of algal cultures was filtered through precombusted (4 hr at 450°C) GF/F filters. Filters were dried at least 48 hr at 45°C (WTC binder, Tuttlingen, Germany), packed in tin cartridges (10x10 mm, HEKAtech GmbH, Wegeberg, Germany) and measured in a CHNS-O Elemental Analyzer (EA 3000; EuroVector SpA, Milan, Italy).

Particulate phosphorus content of cells (Supporting information Tables S2 and S3) was determined by filtering algal culture on polysulphone filters (0.45 µm; Pall Corporation, Port Washington, NY, USA). Subsequently, filters were oxidized by adding $K_2S_2O_8$ and autoclaved at 120°C and 120 kPa for 1 hr. The molybdate blue reduction method according to Murphy and Riley (1962) was used, and phosphorus concentrations were measured at 880 nm on a spectrophotometer (UV-2401 PC; Shimadzu, Kyoto, Japan), and compared to a similarly treated calibration curve.

For cellular chl *a* content, concentrations were determined by filtering culture samples on glass fiber, GF/F filters (Whatman, Buckinghamshire, UK). Chl *a* was extracted overnight with 60°C warm ethanol (90%). Measurements of extracts were conducted in a fluorometer (TD-700, Turner Designs, GAT Bremerhaven, Germany), following Welschmeyer (1994), and quantified using a calibration curve prepared from commercially obtained chl *a* (Sigma).

2.10 | Statistical analyses

Statistical analyses were performed with the software R version 3.4 (R Core Team, 2017). Influences of nutrients on tested variables were detected using three-way ANOVAs. To estimate the particular effects of the N_i-source, we calculated contrasts between the NO_3^- and NH_4^+ treatments within each CO_2 – P combination using the R package "emmeans" (Russell, 2018). As cutoff level of significance, $p > 0.05$ was used. This way, we test straightforwardly for the modulating effects of CO_2 and P_i availability on the potential of N_i-source utilization.

3 | RESULTS

The order of the results will follow the potential path of nitrogen through the metabolism of the alga. At first, the limitation(s) of cells is described to better understand the results. In short, we consider the NR activity as the first step of the nitrate metabolism and follow this by the synthesis of amino acids for both N_i-sources. Then, we examine the production of chlorophyll, whose content will influence parameters involved in photosynthesis. Finally, the cell density is presented, which reflects the total efficiency of all metabolic processes resulting in population productivity, as our experiments were performed in a fixed steady-state rate of growth.

3.1 | Detection of nutrient limitations

We compared two different methods for verifying the suggested factors (co-)limiting the differently treated cultures, as the identification of P_i and C_i limitations was essential for the basis of the

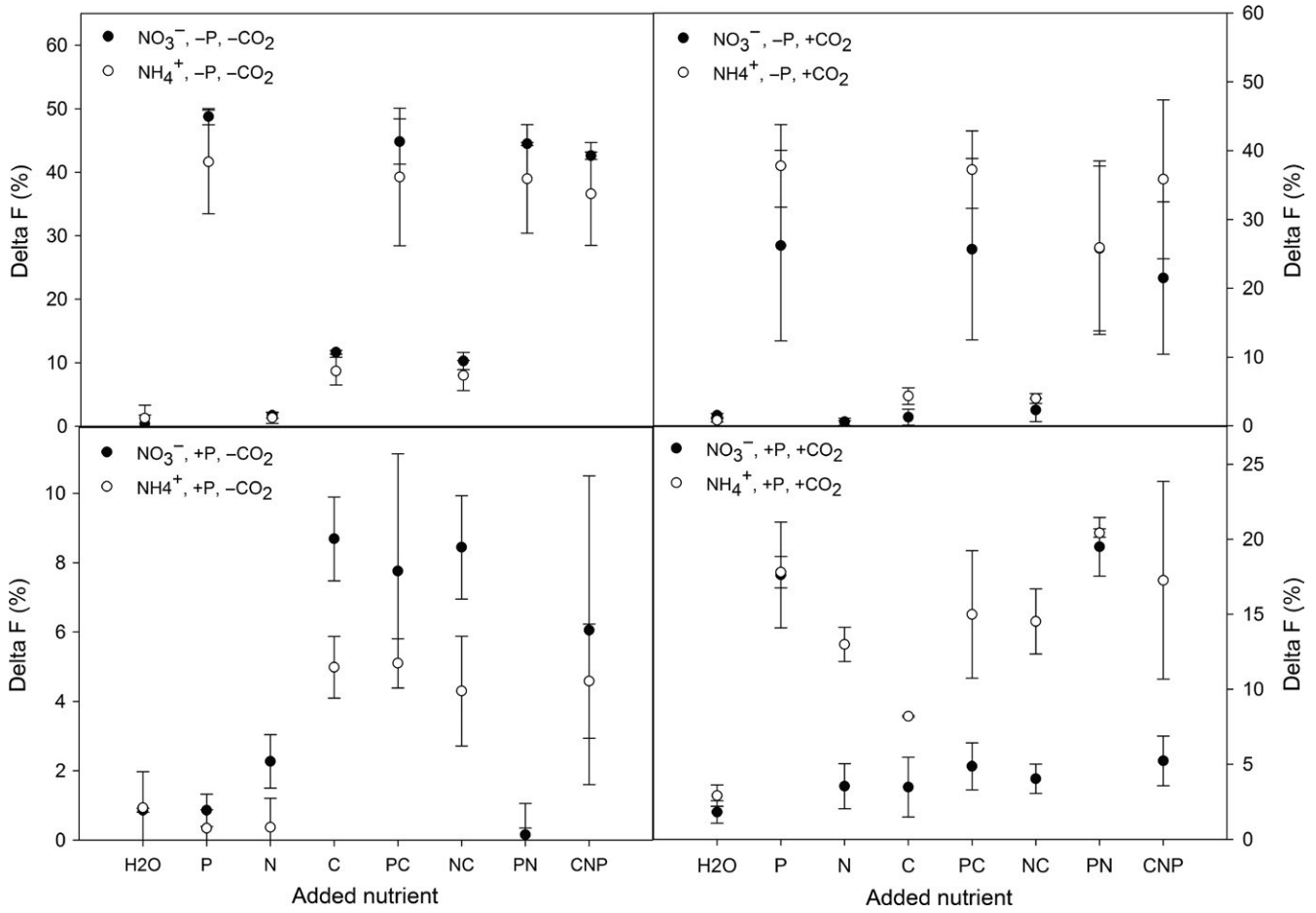

FIGURE 2 Nutrient-induced fluorescence transients (NIFT) response to added nutrients in all culture treatments calculated differently as described in methods; data points reflect mean and standard deviation of three replicates. In each panel, responses are compared between algae provided with NH_4^+ or NO_3^- under a certain nutrient condition; a: P_i-limited conditions under low CO_2, b: P_i-limited conditions under high CO_2, c: P_i-replete conditions under low CO_2, d: P_i-replete conditions under high CO_2. Please note the differences in scales on the y-axes between panel a, b and c, d

conducted experiment. Traditional enrichment experiments detect limitations via additions of nutrients, after some days of growth, and reveal the growth limiting nutrient *in* sensu Liebig (Sperfeld, Raubenheimer, & Wacker, 2016; von Liebig, 1841). In contrast, nutrient-induced fluorescence transients (NIFTs) can be used as a much more rapid method for detecting nutrient (co-)limitations affecting photosynthesis and consequently metabolic rates (i.e., Blackman limitation). In these, the direct photosynthetic response to nutrient additions is visible immediately and reflects the current nutrient status (Spijkerman et al., 2016). Both the traditional enrichment (Supporting information Table S1) and NIFT experiments (Figure 2) confirmed the desired P_i-limitation. Additionally, NIFT experiments revealed further effects of nutrients on photosynthesis and possible interactions, for which traditional enrichment experiments were not sensitive enough. We detected a ΔF_d of around 10% if C_i was added to low CO_2-grown cells, implying a slight CO_2 limitation in all these cultures (Figure 2a, c). When NO_3^- was the available nitrogen source, under low CO_2, the responses of cells to nutrient additions were slightly, but consistently, stronger than of cells grown with NH_4^+ (ΔF_{pd}, Figure 2a, c). In contrast, the response of NH_4^+- grown cells was often stronger than NO_3^--grown cells under high CO_2 conditions (Figure 2b, d). Against expectations, we found a clear response to all added nutrients (i.e., C, N, and P) in the NH_4^+/P_i-replete/highCO_2 treatment (Figure 2d). The responses of nutrient combinations (sum of ΔF_d and ΔF_{pd}) included fast evolving transients as depicted in Figure 1c, suggesting the presence of a co-limitation for all three nutrients. How these limitations relate to each other in a physiological sense could not be unraveled.

3.2 | NR activity

The activity of NR might be the first reaction to changing nitrogen conditions: and we hypothesized that the studied algae might lack NR activity as an adaptation to an acidic environment without NO_3^-. In contrast to this expectation, *C. acidophila* had NR activity and the N_i-source affected the activity of NR in all combinations of factors (Figure 3, three-way ANOVA, N × P × CO_2: $F_{1,16} = 110.3$, $p < 0.001$, Supporting information Table S4). In detail, by comparing the responses of NR between N_i-sources within each CO_2 – P combination (Figure 3, contrast analyses, for details see methods),

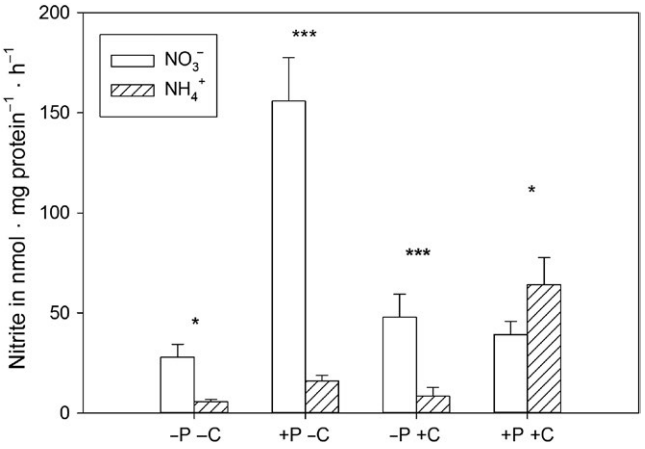

FIGURE 3 Mean and standard deviation of the activity of NR [nmol nitrite mg protein^{-1} hr^{-1}] (n = 3). Asterisks show significant differences between treatments differing in nitrogen sources (contrast analysis, * $p < 0.05$, ***$p < 0.001$), letters and signs of the x-axis describe treatments: -P: P_i-limitation, +P: P_i-replete, -C: low CO_2, +C: high CO_2

a higher activity was observed in treatments with NO_3^- instead of NH_4^+ (contrast analyses, $p < 0.05$), except under P_i-replete and high CO_2 conditions. Unexpectedly, in the latter case, a higher activity was detected when NH_4^+ was provided compared to NO_3^- (contrast analysis, $p < 0.05$), which might be due to the N_i-limitation. The strongest positive effect of NO_3^- on the NR activity was observed under low CO_2 and P_i-replete conditions, with an eightfold increase compared with the NH_4^+ treatment (contrast analysis, $p < 0.001$). We found an interaction between N_i and both P_i and CO_2 (three-way ANOVA, N × P: $F_{1,16} = 9.4$ $p < 0.01$, N × CO_2: $F_{1,16} = 72.8$, $p < 0.001$, Supporting information Table S4).

3.3 | Amino acids

The condensation of ammonia and glutamate to glutamine is the first step of fixing nitrogen within the algal cell. Since it needs additional steps of reduction, we expected a slower turnover from nitrogen to amino acids when cells were grown with the more oxidized NO_3^- compared to NH_4^+. This might be visible in different steady-state levels of amino acids in cells grown under different N_i-sources. Glutamate generally accumulated in cells grown with NO_3^-, but surprisingly, NO_3^- also led to an increase in threonine, alanine, and glycine under P_i-limited conditions, and on tyrosine under high CO_2 conditions (Table 1, Supporting Information Table S7). The positive influence of NO_3^- on the cellular content of valine was only observed under high CO_2 conditions and limiting P_i-supply (Table 1, Supporting Information Table S7). Additional to our main interest concerning the influence of N_i-source, the major differences in amino acid levels were found between high and low phosphorus supply. Six of the nine analyzed amino acids were increased under P_i-limited conditions (alanine, glycine, isoleucine, leucine, threonine, valine; Table 1 and Supporting information Table S7). Furthermore, the level of glycine was also affected by the variation of CO_2 (Supporting information Table S7).

TABLE 1 Cellular content of different amino acids (in pmol · 10^{-6} cells) in *Chlamydomonas acidophila*, grouped by the factors influencing them

	Low CO_2				High CO_2			
	P_i-limited		P_i-replete		P_i-limited		P_i-replete	
Amino acid	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+
Influence of N_i-source								
General influence								
Glutamate	639 (279)^a	99.1 (9.3)^b	361 (80)^a	134 (119)^b	300 (21)	98.5 (34.9)	364 (119)^a	65.4 (46.1)^b
Threonine	212 (16)^a	94.4 (36.1)^b	109 (36)	98.9 (36.3)	325 (23)^a	121 (79)^b	183 (53)^a	39.4 (19.2)^b
Influence under P_i-limitation								
Alanine	752 (125)^a	247 (60)^b	402 (53)	312 (84)	906 (76)^a	210 (100)^b	255 (85)	146 (84)
Glycine	1710 (393)^a	500 (118)^b	607 (140)	947 (310)	2,255 (5)^a	423 (192)^b	139 (65)	243 (40)
Influence under high CO_2								
Tyrosine	214 (33)	125 (70)	186 (48)	110 (45)	295 (38)^a	86.3 (40.8)^b	222 (41)^a	69.6 (66.1)^b
Influence under high CO_2 and P_i-limitation								
Valine	130 (109)	201 (94)	84.2 (20.8)	182 (53)	407 (148)^a	117 (67)^b	131 (53)	46.2 (29.4)
No Influence on N_i-source								
Isoleucine	155 (71)	129 (86)	84.2 (38.2)	165 (112)	207 (6)	186 (62)	66.2 (29.3)	33.6 (11.6)
Leucine	245 (117)	177 (156)	99.2 (44.4)	107 (89)	278 (11)	270 (76)	71.4 (20.7)	34.1 (27.4)
Phenylalanine	268 (151)	247 (220)	140 (56)	297 (360)	206 (30)	300 (249)	190 (71)	28.2 (17.1)

Notes. Mean and standard deviation in parentheses. Different letters in superscript indicate significant differences in cellular amino acid contents between the two N_i-sources within each P_i - CO_2 combination (three-way ANOVA followed by contrast analyses with R package emmeans).

3.4 | Chl *a* per cell

Chl *a* is produced from glutamate and plays an important role for the photosynthetic capacity of an algal cell. Since the different nitrogen sources affect glutamate synthesis differently, we also expected changes in chl *a* levels. Indeed, the chl *a* content per cell was significantly affected by the N_i-source, but the effects of the N_i-source clearly changed with the supply of P_i and CO_2 (three-way ANOVA, $N_i \times P_i \times CO_2$: $F_{1,16} = 156$, $p < 0.001$, Figure 4, Supporting information Table S4). The presence of NH_4^+, instead of NO_3^-, enhanced the chl *a* content per cell under P_i-replete and low CO_2 conditions (contrast analysis, $p < 0.001$, Figure 4), but under high CO_2, NO_3^--grown cells had a higher chl *a* content than NH_4^+-grown cells (contrast analysis, $p < 0.001$, Figure 4).

3.5 | ^{14}C-fixation rates

The C_i-fixation rate was measured by ^{14}C-labelled incorporation of C_i, and these rates might be decreased under nutrient and energy limiting conditions. An interaction of the N_i-source with the CO_2 supply was observed (three-way ANOVA, $N_i \times CO_2$: $F_{1,16} = 14.3$, $p < 0.01$, Figure 5, Supporting information Table S4), but no higher or other interaction with P_i was discerned (Supporting information Table S4). The N_i-source influenced the ^{14}C-fixation rates only under low CO_2 conditions; under these conditions, a higher rate was observed when NH_4^+ was used by algal cells instead of NO_3^- (contrast analysis, $p < 0.05$).

3.6 | C_i-uptake kinetics

Kinetic parameters of photosynthesis reflect the effectivity of C_i-acquisition: which we expected to be enhanced in NH_4^+-grown cells compared to cells grown in the presence of NO_3^-. The kinetic parameter V_{max} was lower under P_i-limiting than P_i-replete conditions (three-way ANOVA, P_i: $F_{1,16} = 15$, $p = 0.001$, Supporting information Table S5). Among N_i-treatments, only under high CO_2 and P_i-replete conditions was the V_{max} of NH_4^+-provided cells lower than the value of NO_3^--cells (contrast analysis, $p < 0.01$, Table 2), which was probably due to the nitrogen limitation in the NH_4^+ treatment. Therefore, we suggest that V_{max} was unaffected by N_i-source, but negatively affected by nitrogen limitation.

The N_i-source and the P_i-supply in concert with the CO_2 conditions affected the half-saturation constant $K_{0.5}$ (CO_2) (three-way ANOVA, $N_i \times P_i \times CO_2$: $F_{1,16} = 5.1$, $p < 0.05$, Table 2 and Supporting information Table S5). Within P_i-replete-high CO_2-conditions, the $K_{0.5}$ (CO_2) was lower when NH_4^+ was the N_i-source provided (contrast analysis, $p < 0.01$, Table 2), indicating the onset of a slight C_i-limitation, also observed in the NIFT measurements (Figure 2d). As a result of the slight C_i- and/or N_i-limitation present in the high CO_2/P_i-replete treatment, some of the interactions became statistically significant.

A higher affinity for CO_2 ($V_{max}/K_{0.5}$) was detected in low CO_2 conditions (Table 2, three-way-ANOVA, $F_{1,16} = 39$, $p < 0.001$). The P_i-supply and the species of N_i-source had no influence on the affinity (Supporting information Table S5).

The species of N_i played an important role under P_i-limited conditions in both CO_2 conditions, as the photosynthetic efficiency (alpha) was lower when NH_4^+ was the N_i-source provided (Figure 6, contrast analysis, $p < 0.05$). Under P_i-replete conditions, significant influences of the N_i-source were only detected under low CO_2 supply (contrast analysis, $p < 0.05$). Interactions were found between N_i and both P_i and CO_2 (three-way ANOVA, $N_i \times P_i$: $F_{1,15} = 25.41$, $p < 0.001$, $N_i \times CO_2$: $F_{1,15} = 12.84$, $p < 0.01$, Supporting information Table S5). The interaction between N_i and CO_2 is represented by different intensities of an increased alpha. Thus, alpha was 32% higher in NO_3^--conditions relative to NH_4^+ under low CO_2 and only 6% higher under high CO_2. We observed the lowest values for alpha when P_i was limited, and CO_2 was also low. Interestingly, the presence of

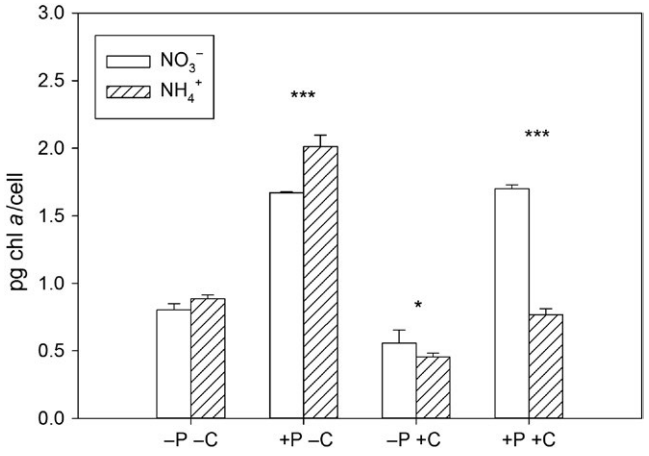

FIGURE 4 Mean and standard deviation of chl *a* content in *C. acidophila* ($n = 3$). Asterisks show differences between treatments differing in nitrogen sources (contrast analysis, $^*p < 0.05$, $^{***}p < 0.001$), letters and signs of the x-axis describe treatments: -P: P_i-limitation, +P: P_i-replete, -C: low CO_2, +C: high CO_2

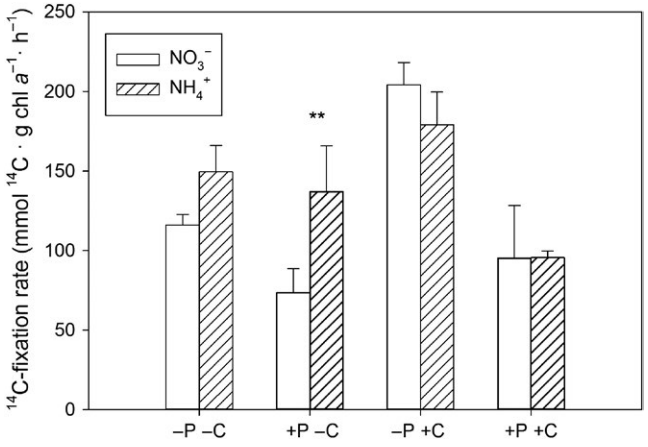

FIGURE 5 Mean and standard deviation of the ^{14}C-fixation rate [mmol ^{14}C g chl a^{-1} hr^{-1}] ($n = 3$). Asterisks show differences between treatments differing in nitrogen sources (contrast analysis,** $p < 0.01$), letters and signs of the x-axis describe treatments: -P: P_i-limitation, +P: P_i-replete, -C: low CO_2, +C: high CO_2

TABLE 2 Mean and standard deviation (mentioned in parentheses) of CO_2 uptake kinetics as measured by oxygen evolution (n = 3), V_{max} (mmol O_2 (g chl a h)$^{-1}$), $K_{0.5}$ (CO_2) (µM) and $V_{max}/K_{0.5}$

	Low CO_2				High CO_2			
	P_i-limited		P_i-replete		P_i-limited		P_i-replete	
Parameter	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+
V_{max}	48 (9)	56 (6)	73 (6)	64 (4)	42 (7)	33 (16)	64 (6)[a]	38 (14)[b]
$K_{0.5}$ (CO_2)	1.3 (0.6)	1.4 (0.2)	2.1 (0.4)	2.0 (0.4)	4.5 (1.0)	5.0 (0.8)	4.2 (0.9)[a]	2.1 (0.6)[b]
$V_{max}/K_{0.5}$	44 (27)	41 (4)	36 (5)	33 (5)	9 (1)	7 (4)	16 (2)	18 (5)

Notes. Different letters in superscript indicate significant differences in kinetic parameters between the two *N*-sources within each P - CO_2 combination (three-way ANOVA followed by contrast analyses with R package emmeans).

only one of these nutrients in surplus enhanced the photosynthetic efficiency by about 50%, but phosphorus seemed to have a slightly stronger effect than CO_2.

3.7 | Carbon-concentrating factor

The CCF is a factor indicating the presence of CCMs, which extent might be larger when a N_i-source is used that requires less metabolic energy to accumulate. Therefore, we expected a greater CCF in NH_4^+-grown cells compared to NO_3^--grown cells, and yet we found no influence of the N_i-source, P_i and CO_2 on the CCF and C_i-pool (three-way ANOVA, all factors $p > 0.22$ Supporting information Table S6). Values of the CCF varied around 29 ± 10 (mean ± *SD*), when using the traditional method via tritium-labeled water, to determine cell volume; or was 7.8 ± 4 (mean ± *SD*), when using the automatic cell counter for cell volume determination. The C_i-pool in the cells after 10 s was 2,535 ± 868 µM (mean ± *SD*). All values originated from three biological replicates per treatment, with N = 24; see Supporting information Table S6 for statistic details. CCF only varied depending on the method of calculation because the CASY estimated a larger cell volume. Therefore, the CCF calculated in the traditional way, by determining the cell volume with ^3H-H_2O, was three times higher than the CCF calculated with the measured cell volume in the automatic cell counter (CASY).

3.8 | Cell density

The cell density is assumed to reflect the sum of all measured parameters, as it is the result of the population productivity. Cultures were grown at the same steady-state growth rate of 0.2/d, which prevents the possibility to study growth rates for productivity. The three-way ANOVA indicated that all factors have an impact on the cell density ($N_i \times P_i \times CO_2$: $F_{1,16}$ = 7.0, Figure 7, Supporting information Table S4). An influence of the N_i-source on cell density was only observed in cultures grown under P_i-replete and high CO_2-conditions (Figure 7, contrast analysis, $p < 0.001$). Under these conditions, the cell density was about 75% higher when NO_3^- was provided, but as the NH_4^+-cultures were N_i-limited, N_i-source had no effect on the cell density.

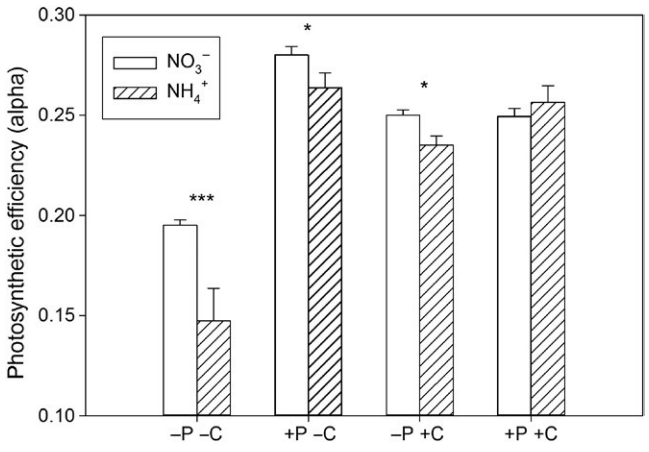

FIGURE 6 Mean and standard deviation of alpha which reflects the photosynthetic efficiency on the basis of electron transport rate (n = 3). Asterisks show differences between treatments varying in nitrogen sources (contrast analysis, * $p < 0.05$, *** $p < 0.001$), letters and signs of the x-axis describe treatments: -P: P_i-limitation, +P: P_i-replete, -C: low CO_2, +C: high CO_2

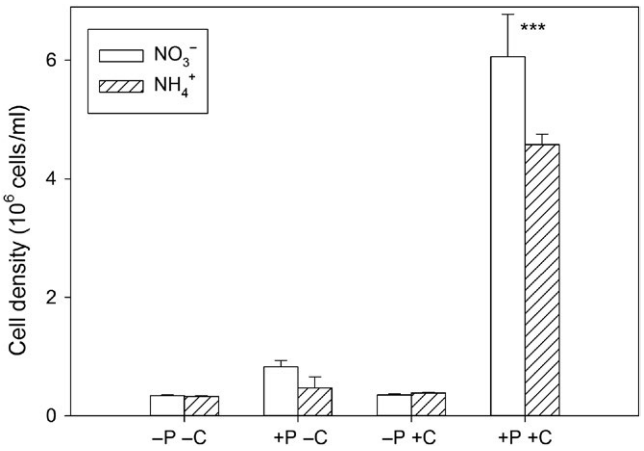

FIGURE 7 Mean and standard deviation of cell densities at the end of culturing (n = 3). Asterisks show differences between treatments differing in nitrogen sources (contrast analysis, *** $p < 0.001$), letters and signs of the x-axis describe treatments: -P: P_i-limitation, +P: P_i-replete, -C: low CO_2, +C: high CO_2

4 | DISCUSSION

4.1 | From nitrogen to amino acids

The relatively high glutamate content in cells grown with NO_3^- probably reflects an accumulation of this amino acid due to a lower turnover of glutamate into other amino acids or proteins. This may be in line with our hypothesis that NH_4^+ allows a faster turnover of nitrogen into amino acids and through the subsequent metabolic pathways (e.g., into chl *a* synthesis; see below). In addition, under low CO_2 conditions, NO_3^--grown cells had a higher total protein content than NH_4^+-cultures (51 vs. 38 pg/cell, respectively; results not shown). These observations are consistent with a faster stimulation of amino acid synthesis when NH_4^+ was added to nitrogen-starved cells of cyanobacteria, shown by Tapia, Ochoa de Alda, Llama, and Serra (1996). Opposing initial expectations, some other amino acids also reached higher concentrations in cells provided with NO_3^- instead of NH_4^+. This phenomenon implies an effect of the N_i-source on downstream processes also; when, for example, the assembling of proteins proceeds slower. This hypothesis is supported by the detection of some higher amino acid contents in P_i-limited *C. acidophila*, than in P_i-replete cells as a higher production of amino acids would be intuitively expected under P_i-deficient, but N_i-replete conditions. Furthermore, high CO_2 and P_i-limited conditions at the same time seemed to intensify the differences between different N_i-sources, as higher amounts of valine in NO_3^--cells were only seen under high CO_2 supply. This might be an effect of a stronger P_i-limitation when high concentrations of CO_2 are available as this was shown by a lower minimum cell quota for *C. acidophila* in a previous study (Spijkerman, Bissinger, Meister, & Gaedke, 2007).

4.2 | Adaptations to acidic environment—NR activity

Contrary to our hypothesis that an acidophilic alga might have a preference for ammonium as an N_i-source, and possibly lacks NR activity as an adaptation to their environment, which consists of 91% NH_4^+ (Bissinger et al., 2000), we found NR activity in *C. acidophila*. We predominantly detected NR in NO_3^--grown cells, with high activities when NO_3^- was provided under P_i-replete, low CO_2 conditions: which has been known since at least 1969 for phytoplankton (by Eppley, Coatsworth, & Solorzano, 1969, who varied P_i conditions under low CO_2 in cell cultures). The N_i-limitation in P_i-replete, high CO_2, NH_4^+-grown cells revealed that a N_i-limitation enhanced NR activity. The NR activity in this NH_4^+-treatment was even higher than that in NO_3^--grown cells (Figure 3). Earlier, Kessler and Osterheld (1970) had also detected NR activity after ammonium was exhausted in cultures of two *Chlorella* strains. Probably, when NH_4^+ is exhausted, genes expressing NR (nit2) are activated (Fernandez & Galvan, 2007). Modern approaches suggest a role of the signaling molecule nitric oxide that functions as a signaling molecule in *Chlamydomonas* (Calatrava et al., 2017).

Interestingly, the NR activity in NO_3^--grown cells under low CO_2 was higher in P_i-replete than in P_i-limited cells, supporting the assumption that an increased energy demand, in terms of ATP, for NO_3^--acquisition (Ruan & Giordano, 2017) was also present in *C. acidophila*.

4.3 | Photosynthetic parameters—differences to neutrophiles/bicarbonate users

The higher ^{14}C-fixation rates in NH_4^+-grown cells fits with the previously discussed faster turnover of nitrogen, expressed by a lower glutamate content in NH_4^+-grown cells. This effect was only observed under a low CO_2 condition, which is consistent with our hypothesis that effects from the N_i-source might be enhanced under low CO_2 due to energy consuming CCMs (Raven & Beardall, 2014) or a decreased cellular ATP content (Raven et al., 2014). This was, however, not reflected in an enhanced biomass production in NH_4^+-cultures, suggesting other metabolic costs.

We expected to find a more efficient photosynthesis when NH_4^+ was supplied instead of NO_3^-, but instead no effects were found. This, for example, is in contrast to a higher maximal photosynthetic rate in *Dunaliella salina* grown in NH_4^+ rather than NO_3^- (Giordano, 1997), and a more efficient CCM in the same species (Giordano & Bowes, 1997). In *C. acidophila*, the CCF was similar among N_i-treatments, and also, no other indicators for more efficient CCMs in NH_4^+-grown cells were found. Possibly, the different response originates from the large physiological differences in the CCM between the neutrophile, marine *Dunaliella salina* and our acidophile, freshwater *C. acidophila*. This difference is especially evident from the C_i-species supporting photosynthesis, as *D. salina* is able to use both bicarbonate and carbon dioxide, while *C. acidophila* is restricted to CO_2 (Lachmann, Maberly, and Spijkerman 2016a, 2016b). It has been proposed that HCO_3^--based CCMs requires more metabolic energy than CO_2^--based ones (Raven et al., 2014), which we confirm here. Consequently, *D. salina* might be more dependent on active HCO_3^--uptake mechanisms in its CCM, and consequently photosynthetic parameters are more profoundly affected by N_i-source. Possibly, the metabolic advantages of NH_4^+-grown cells for CO_2 acquisition and photosynthesis in *C. acidophila* were compensated by enhanced proton extrusion necessities.

4.4 | Cell density and chl *a*

An influence of the N_i-source on cell density was visible only in the treatment with the unexpected N_i-limitation, although the chl *a* content per cell was higher in NH_4^+-grown cells than in NO_3^--grown cells under low CO_2 and P_i-replete conditions. The latter was also shown for *D. salina* (Giordano, 1997). Because cell densities were independent of the N_i-source, other metabolic processes were enhanced in the NH_4^+-grown cells (possibly related to proton extrusion). At P_i-limited conditions, the chl *a* content per cell did decrease slightly in cells grown on NH_4^+ compared to NO_3^-, under high CO_2 conditions. Although we expected more pronounced differences due to a decreased energy budget in P_i-limited conditions (Lachmann et al., 2016a,b), the low chl *a* content might be close to a (minimum) threshold value, below which algae might face strong physiological

restrictions, or below which the synthesis of chl *a* is prioritized for energy allocation (Spijkerman et al., 2007).

4.5 | Methods to detect nutrient limitations

NIFT experiments revealed nutrient limitations and co-limitations that remained hidden in (traditional) enrichment experiments. Enrichment experiments are often used because they are easier to conduct, as no special, expensive equipment is needed; however, they might fail to show a limitation in growth rate when the increase in biomass due to nutrient additions is low, for example, because cultures reach another limitation quickly. It was shown that NIFT experiments are also more suitable for detecting moderate nutrient limitations and co-limitations, due to the rapid detection of the response (Spijkerman et al., 2016). We generally found a slight but consistently stronger response to additions of limiting nutrients in NO_3^--grown cells under low CO_2, and in NH_4^+-grown cells under high CO_2 conditions. In contrast to results in enrichment experiments, we detected an unexpected (and unintended) N_i-limitation via NIFTs under a high supply of both P_i and CO_2. Without this knowledge, we would have come to a false conclusion. Interestingly, the response to additions of more than one nutrient was much more complex when three nutrients were limiting (Figure 1c), suggesting complex physiological interactions among nutrient limitations (Koussoroplis, Pincebourde, & Wacker, 2017). It would be exciting to analyze this in further research, especially in relation to other physiological acclimations.

4.6 | Conclusion and ecological implications

In conclusion, the effect of N_i-source on the studied physiological and metabolic traits of *C. acidophila* was very diverse and often influenced by C_i- and/or P_i-limitation. Nitrate seemed to be the preferred N_i-source for photosynthesis and growth and led to more pronounced P_i-limitations under low CO_2 conditions, emphasizing the enhanced energy requirement to assimilate this N_i-source. Our results suggest that the CO_2-user, *C. acidophila*, was little influenced in its C_i-acquisition by N_i-source, and that the CO_2-limitation seemed to be stronger under NO_3^--use. Possibly the use of NH_4^+ provides an additional proton stress for the cells that compensates for the metabolic advantages of this N_i-source (Giordano & Raven, 2014) as a higher mitochondrial activity is required (Weger et al., 1988).

None of our results suggested that *C. acidophila* developed special physiological adaptations to the higher concentrated N_i-source (i.e., NH_4^+) of their natural environment. Our species synthesized NR in the presence of NO_3^-: similar to neutrophiles (Chen et al., 2012; Li, Fingrut, & Maxwell, 2009). Local adaptation did not influence the N_i-source preference of this acidophile, whereas the green alga *Closterium aciculare* (Coesel, 1991), and most isolates from the acidotolerant *C. pitschmannii* (Pollio et al., 2005), were restricted to ammonium for their N_i-uptake. Such preferences are of interest for biofuel production, as lipid production in *Tetraselmis* sp. was higher when cells were grown with NO_3^- than with NH_4^+. In general, effects of the N_i-source may be visible at the interactions among trophic levels. As *C. acidophila* is the most important photoautotroph, and the base of the food web in its lake of origin (Kamjunke, Gaedke, Tittel, Weithoff, & Bell, 2004), its physiological changes might have strong effects on its consumers and competitors.

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CONFLICT OF INTEREST

None Declared.

AUTHORS CONTRIBUTION

The idea and design of experiments were conceived by SCL and ES. Laboratory work was conducted by SCL, ES (kinetic), and TMA (amino acids). SCL analyzed the data and wrote the manuscript. AW contributed to statistics of the study. SCL received comments to the written form of the manuscript by all co-authors.

DATA ACCESSIBILITY

Chemical and physiological data are accessible at Dryad (https://doi.org/10.5061/dryad.2k16k4b).

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SUPPORTING INFORMATION

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