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 NH₄⁺ and dark Cᵢ-assimilation under anaplerosis (Giordano, Norici, Forssen, Eriksson, & Raven, 2003; Vanlerberghe, Schuller, Smith, & Turpin, 1990) as well as photosynthetic CO₂-assimilation (Turpin, Vanlerberghe, Amory, & Guy, 1991). In Synechococcus sp., the use of NH₄⁺ over NO₃⁻ stimulated photosynthesis and growth under a light limitation (Ruan & Giordano, 2017).

Most phytoplankton species are able to use NH₄⁺ and NO₃⁻ as the nitrogen source (Raven & Giordano, 2016). Several studies...
observed a higher photosynthetic or growth rate of macro- and microalgae for a certain N\textsubscript{2}-source; reflected, for example, by different affinities to NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{-} 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\textsubscript{4}\textsuperscript{+} is considered the preferred N\textsubscript{2}-source, and negatively signals NO\textsubscript{3}\textsuperscript{-} 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 (Kolza et al., 2014). In contrast to NO\textsubscript{3}\textsuperscript{-}, NH\textsubscript{4}\textsuperscript{+} is directly incorporated into amino acids by condensation with glutamate to form glutamine catalyzed by glutamine synthetase (Miflin & Lea, 1980; Sanzu-Luque, Chamizo-Ampudia, Llamas, Galvan, & Fernandez, 2015). The demand to acquire CO\textsubscript{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\textsubscript{3}\textsuperscript{-} than NH\textsubscript{4}\textsuperscript{+} (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\textsubscript{4}\textsuperscript{+} (compared to NO\textsubscript{3}\textsuperscript{-}) 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\textsubscript{2}-source on several parameters describing the inorganic carbon acquisition (C\textsubscript{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, & Pröschild, 2005) and replete N\textsubscript{2} concentrations (i.e. 0.22 mM), 91% of which is in the form of NH\textsubscript{4}\textsuperscript{+} (Bissinger et al., 2000).

We hypothesize that the acquisition of NH\textsubscript{4}\textsuperscript{+} compared to NO\textsubscript{3}\textsuperscript{-} allows a higher rate of nitrogen assimilation and that the reduced metabolic energy requirement of NO\textsubscript{3}\textsuperscript{-} use enables energy allocation to other cellular processes, such as photosynthesis and growth. Thus, C\textsubscript{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\textsubscript{3}\textsuperscript{-}-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\textsubscript{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\textsubscript{i}-uptake in Chlorella emersonii (Beardall, Roberts, & Raven, 2005). The concentration of CO\textsubscript{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\textsubscript{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\textsubscript{2}-source on different physiological parameters might depend on other factors, such as light (Ruan & Giordano, 2017) and CO\textsubscript{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\textsubscript{2}-source. Therefore, as both C\textsubscript{i} (Tittel, Bissinger, Gaedke, & Kamjunke, 2005) and P\textsubscript{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\textsubscript{i}- and CO\textsubscript{2}-supply/limitation under two different N\textsubscript{2}-sources, in a full-factorial design (eight different treatments).

We expect the highest photosynthetic and C\textsubscript{i}-assimilation rates at NH\textsubscript{4}\textsuperscript{+}, P\textsubscript{i} and CO\textsubscript{2}-replete conditions, and the lowest at NO\textsubscript{3}\textsuperscript{-}, P\textsubscript{i}- and CO\textsubscript{2}-limiting conditions. The influence of provided N\textsubscript{2}-sources on the ecophysiology of C. acidophila was examined by measuring a wide range of physiological parameters such as C\textsubscript{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\textsuperscript{-2} s\textsuperscript{-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\textsubscript{2}. We varied the N\textsubscript{2}-source (NO\textsubscript{3}\textsuperscript{-} or NH\textsubscript{4}\textsuperscript{+}), P\textsubscript{i} concentration (P\textsubscript{i}-limited: 1 µM and P\textsubscript{i}-replete: 100 µM) and C\textsubscript{i}-supply (low CO\textsubscript{2}- air, high CO\textsubscript{2}: 4.5%) in a full-factorial design. Nitrogen was intended not to become a limiting nutrient, and therefore, NO\textsubscript{3}\textsuperscript{-} was provided in excess of 2 mM and NH\textsubscript{4}\textsuperscript{+} in excess of 1 mM. We added less NH\textsubscript{4}\textsuperscript{+} than NO\textsubscript{3}\textsuperscript{-} to prevent ammonium from causing an uncoupling of the photosynthetic H\textsuperscript{+} gradient (Krause & Behrend, 1986). All cultures were aerated with normal air or with CO\textsubscript{2} enriched air from a gas cylinder (4.5% CO\textsubscript{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\textsubscript{2} in the flasks were 800 µM at high CO\textsubscript{2}, 14 µM at low CO\textsubscript{2}, P\textsubscript{i}-limiting, and 3 µM at low CO\textsubscript{2}, P\textsubscript{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.
Reutlingen, Germany). 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<sub>i</sub>, N<sub>i</sub>, P<sub>i</sub>) 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<sub>t</sub>, 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<sup>2</sup> s<sup>−1</sup>). Subsequently, the response to an addition of P<sub>i</sub> (final concentration in cuvette: 10 µM KH<sub>2</sub>PO<sub>4</sub>, N<sub>i</sub> (100 µM N<sub>i</sub> as (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> or NaNO<sub>3</sub>) or C<sub>i</sub> (100 µM NaHCO<sub>3</sub>), and combinations of the nutrients, was recorded until the fluorescence signal remained constant again (F<sub>d</sub>). All spike solutions were prepared in acidified water of pH 2.5 (with the C<sub>i</sub> solution diluted 1 min before addition of CO<sub>2</sub> after conversion of most of the HCO<sub>3</sub>), 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<sub>i</sub> concentration was above 4 µM, based on results in Roberts, Shelly, and Beardall (2008), and N<sub>i</sub> concentrations for maximal NIFT responses and a final CO<sub>2</sub> 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<sub>i</sub> and C<sub>i</sub>, a rapid decrease in the fluorescence was observed in P<sub>i</sub>- or CO<sub>2</sub>-limited cultures. We calculated the ΔF<sub>d</sub> (difference between lowest fluorescence after decrease, F<sub>p</sub>, and steady-state fluorescence, F<sub>t</sub>) (Shelly et al., 2010) as a percentage of F<sub>t</sub> for these two nutrient additions (Figure 1a). The response of N<sub>i</sub>-limited cells to NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>−</sup> might be different, as described by Beardall, Young, and Roberts (2001), because we found a small rise of fluorescence (F<sub>d</sub>, highest fluorescence as response to nutrient) followed by a drop to F<sub>d</sub> after NH<sub>4</sub><sup>+</sup> addition, and a strong rise of fluorescence to F<sub>p</sub> with a recovery to initial values after NO<sub>3</sub><sup>−</sup> injection (F<sub>p</sub>). Therefore, for these two nutrients, we calculated ΔF<sub>p</sub> 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<sub>i</sub> and C<sub>i</sub>, C<sub>i</sub> and NH<sub>4</sub><sup>+</sup>, or all three nutrients) to the culture, at first a decrease (F<sub>d</sub>), then an increase (F<sub>p</sub>), and finally a second decrease of the fluorescence was observed (second F<sub>d</sub>). We calculated the response by forming the sum of ΔF<sub>p</sub> and ΔF<sub>d</sub> (Figure 1c). Such a response to two potentially limiting nutrients has been shown before for C. acidoiphila growing under P<sub>i</sub>- and C<sub>i</sub>-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<sub>i</sub>: 1 mM; N<sub>i</sub> (NO<sub>3</sub><sup>−</sup> or NH<sub>4</sub><sup>+</sup> depending on culture conditions) and C<sub>i</sub>: 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<sub>2</sub>EDTA (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 MgCl₂ (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) content, pellets were resuspended in 500 µl of SDS (1%) in NaOH according to Jeffrey and Humphrey (1975). The remaining pellet 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 KNO₃, 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 H₂O) directly before use, after which, 100 µl of the coloring mixture was added to 100 µl supernatant in an 96-well microplate. Absorption was measured at 570 nm (infinite F200PRO, TECAN, Männedorf, Switzerland).

2.5 | Ci-accumulation factor

We measured the Ci-accumulation factor (CCF) to check for the effect of N₂-source on Ci-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 Ci and then placed on a silicon oil layer on the top of a killing fluid (Spijkerman, 2005). The accumulation of Ci 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 NaH¹⁴CO₃ with a specific activity of about 1,739 GBq/mmol (PerkinElmer, Germany), for the algae while illuminating with 200 µmol PAR m⁻² s⁻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 Ci-accumulation from the difference between total and acid stable fractions. After 15- to 20-min incubation with 37 kBq ³H-H₂O (specific activity: 37 kBq · mmol⁻¹ Hartmann-analytic), cell volume was measured according to Beardall (1981). The CCF of the accumulated Ci over the 10 s was calculated in two ways: one based on cell volume determined with ³H-H₂O, 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 | ¹⁴C-fixation rates

To check for the effect of N₂-source on the rate of Ci-acquisition, we measured the primary productivity by Ci-fixation rate. Each culture was sampled and measured as quickly as possible to avoid changes in the CO₂ 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 NaH¹⁴CO₃ (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 Ci 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 ¹⁴C (PO¹⁴C), 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 ¹⁴C (T¹⁴C). 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 ¹⁴C fixation rate was calculated following equation 1; with DIC (the concentration of dissolved Ci) 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 ¹²C and ¹⁴C by phytoplankton (Steemann, 1952).

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¹⁴C \text{ fixation rate} = \frac{\text{PO}^{14}\text{Ci}}{\text{max filter incubation time}} \times \text{activity(spec.)}^{-1} \times (\frac{\text{DIC}}{\text{PO}^{14}\text{Ci}}) \times 1.06
\]

where

- PO¹⁴Ci is the particulate organic ¹⁴C
- activity(spec.) is the activity of the sample
- DIC is the dissolved inorganic carbon concentration
- PO¹⁴Ci is the particulate organic ¹⁴C
- max filter incubation time is the maximum filter incubation time
- 1.06 is a factor for the isotope-discrimination between ¹²C and ¹⁴C by phytoplankton (Steemann, 1952).
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$_4$-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$_2$O 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$_4$-source on the efficiency for C$_i$-acquisition, we measured C$_i$-uptake algal 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), by photosynthesis, the maximal uptake rate V$_{max}$, and the affinity V$_{max}$/K$_{0.5}$(C) 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) 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 (WTG binder, Tutlingen, 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$_2$S$_2$O$_8$ 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$_4$-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$_4$-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$_4$-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
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 Pi-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 ΔFpd of around 10% if C was added to low CO2-grown cells, implying a slight CO2 limitation in all these cultures (Figure 2a, c). When NO3− was the available nitrogen source, under low CO2, the responses of cells to nutrient additions were slightly, but consistently, stronger than of cells grown with NH4+ (ΔFpd, Figure 2a, c). In contrast, the response of NH4+ grown cells was often stronger than NO3−-grown cells under high CO2 conditions (Figure 2b, d). Against expectations, we found a clear response to all added nutrients (i.e., C, N, and P) in the NH4+/Pi-replete/high CO2 treatment (Figure 2d). The responses of nutrient combinations (sum of ΔFpd and ΔFpd) 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 NO3−. In contrast to this expectation, C. acidophila had NR activity and the N-source affected the activity of NR in all combinations of factors (Figure 3, three-way ANOVA, N × P × CO2: F1,16 = 110.3, p < 0.001. Supporting information Table S4). In detail, by comparing the responses of NR between N-sources within each CO2 - P combination (Figure 3, contrast analyses, for details see methods),
a higher activity was observed in treatments with NO\textsuperscript{−}\textsubscript{3} instead of NH\textsuperscript{+}\textsubscript{4} (contrast analyses, \p < 0.05), except under \Pi\textsubscript{t}-replete and high CO\textsubscript{2} conditions. Unexpectedly, in the latter case, a higher activity was detected when NH\textsuperscript{+}\textsubscript{4} was provided compared to NO\textsuperscript{−}\textsubscript{3} (contrast analysis, \p < 0.05), which might be due to the N\textsubscript{t}-limitation. The strongest positive effect of NO\textsuperscript{−}\textsubscript{3} on the NR activity was observed under low CO\textsubscript{2} and \Pi\textsubscript{t}-replete conditions, with an eightfold increase compared with the NH\textsubscript{4}\textsuperscript{+} treatment (contrast analysis, \p < 0.001). We found an interaction between N\textsubscript{t} and both \Pi\textsubscript{t} and CO\textsubscript{2} (three-way ANOVA, \N \times \Pi: \F = 9.4, \N \times \CO\textsubscript{2}: \F = 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\textsuperscript{−}\textsubscript{3} compared to NH\textsuperscript{+}\textsubscript{4}. This might be visible in different steady-state levels of amino acids in cells grown under different N\textsubscript{t}-sources. Glutamate generally accumulated in cells grown with NO\textsuperscript{−}\textsubscript{3}, but surprisingly, NO\textsuperscript{−}\textsubscript{3} also led to an increase in threonine, alanine, and glycine under \Pi\textsubscript{t}-limited conditions, and on tyrosine under high CO\textsubscript{2} conditions (Table 1, Supporting Information Table S7). The positive influence of NO\textsuperscript{−}\textsubscript{3} on the cellular content of valine was only observed under high CO\textsubscript{2} conditions and limiting \Pi\textsubscript{t}-supply (Table 1, Supporting Information Table S7). Additionally to our main interest concerning the influence of N\textsubscript{t}-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 \Pi\textsubscript{t}-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\textsubscript{2} (Supporting information Table S7).

### TABLE 1

| Amino acid | Low CO\textsubscript{2} | High CO\textsubscript{2} |
|------------|-----------------|-----------------|
|            | \Pi\textsubscript{t}-limited | \Pi\textsubscript{t}-replete | \Pi\textsubscript{t}-limited | \Pi\textsubscript{t}-replete |
| NO\textsubscript{3} | NH\textsubscript{4}\textsuperscript{+} | NO\textsubscript{3} | NH\textsubscript{4}\textsuperscript{+} |

#### Influence of N\textsubscript{t}-source

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

#### Notes

Mean and standard deviation in parentheses. Different letters in superscript indicate significant differences in cellular amino acid contents between the two N\textsubscript{t}-sources within each \Pi\textsubscript{t} \ CO\textsubscript{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 Ni-source, but the effects of the Ni-source clearly changed with the supply of Pi and CO2 (three-way ANOVA, N_i × P_i × CO2: 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 CO2 conditions (contrast analysis, p < 0.001, Figure 4), but under high CO2, NO_3^−-grown cells had a higher chl a content than NH_4^+ grown cells (contrast analysis, p < 0.001, Figure 4).

3.5 | 14C-fixation rates

The C_i fixation rate was measured by 14C-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 CO2 supply was observed (three-way ANOVA, N_i × P_i × CO2: 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 14C-fixation rates only under low CO2 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 effectiveness 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 CO2 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 CO2 conditions affected the half-saturation constant K_{0.5} (CO2) (three-way ANOVA, N_i × P_i × CO2: F_{1,16} = 5.1, p < 0.05, Table 2 and Supporting information Table S5). Within P_i-replete-high CO2-conditions, the K_{0.5} (CO2) 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 CO2/P_i-replete treatment, some of the interactions became statistically significant.

A higher affinity for CO2 (V_{max}/K_{0.5}) was detected in low CO2 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 CO2 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 CO2 supply (contrast analysis, p < 0.05). Interactions were found between N_i and both P_i and CO2 (three-way ANOVA, N_i × P_i × CO2: F_{1,15} = 25.41, p < 0.001, N_i × CO2: F_{1,15} = 12.84, p < 0.01, Supporting information Table S5). The interaction between N_i and CO2 is represented by different intensities of an increased alpha. Thus, alpha was 32% higher in NO_3^−-conditions relative to NH_4^+ under low CO2 and only 6% higher under high CO2. We observed the lowest values for alpha when P_i was limited, and CO2 was also low. Interestingly, the presence of

\[ \frac{\text{pg chl a/cell}}{\text{ng chl a/g chl}} \times 10^{-2} \]
only one of these nutrients in surplus enhanced the photosynthetic efficiency by about 50%, but phosphorus seemed to have a slightly stronger effect than CO₂.

### 3.7 | Carbon-concentrating factor

The CCF is a factor indicating the presence of CCMs, which extent might be larger when a N₄-source is used that requires less metabolic energy to accumulate. Therefore, we expected a greater CCF in NH₄⁺-grown cells compared to NO₃⁻-grown cells, and yet we found no influence of the N₄-source, Pᵢ, and CO₂ on the CCF and Cᵢ-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 Cᵢ-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-H₂O, 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 (N₄ × Pᵢ × CO₂: F₁,16 = 7.0, Figure 7, Supporting information Table S4). An influence of the N₄-source on cell density was only observed in cultures grown under Pᵢ-replete and high CO₂-conditions (Figure 7, contrast analysis, p < 0.001). Under these conditions, the cell density was about 75% higher when NO₃⁻ was provided, but as the NH₄⁺ cultures were N₄-limited, N₄-source had no effect on the cell density.

### TABLE 2  Mean and standard deviation (mentioned in parentheses) of CO₂ uptake kinetics as measured by oxygen evolution (n = 3), Vₘₐₓ (mmol O₂ (g chl a h)⁻¹), K₅₀ (CO₂) (µM) and Vₘₐₓ/K₅₀

| Parameter | Low CO₂                                     | High CO₂                                     |
|-----------|---------------------------------------------|----------------------------------------------|
|           | Pᵢ-limited                                  | Pᵢ-replete                                  |
|           | NO₃⁻                                    | NH₄⁺                               | NO₃⁻                                    | NH₄⁺                                |
| Vₘₐₓ      | 48 (9)                                      | 56 (6)                                      | 73 (6)                                   | 64 (4)                              |
| K₅₀ (µM)  | 1.3 (0.6)                                   | 1.4 (0.2)                                   | 2.1 (0.4)                                | 2.0 (0.4)                           |
| Vₘₐₓ/K₅₀  | 44 (27)                                     | 41 (4)                                      | 36 (5)                                   | 33 (5)                              |

Notes. Different letters in superscript indicate significant differences in kinetic parameters between the two N₄-sources within each P × CO₂ combination (three-way ANOVA followed by contrast analyses with R package emmeans).

### 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: Pᵢ-limitation, +P: Pᵢ-replete, -C: low CO₂, +C: high CO₂

### 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: Pᵢ-limitation, +P: Pᵢ-replete, -C: low CO₂, +C: high CO₂
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 P$_i$-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 Spijkerkman 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 α 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 NO₃⁻-grown cells under low CO₂, and in NH₄⁺-grown cells under high CO₂ conditions. In contrast to results in enrichment experiments, we detected an unexpected (and unintended) N limitation via NIFTs under a high supply of both P and CO₂. 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 source on the studied physiological and metabolic traits of C. acidophila was very diverse and often influenced by C₇ and/or P limitation. Nitrate seemed to be the preferred N-source for photosynthesis and growth and led to more pronounced P-limitations under low CO₂ conditions, emphasizing the enhanced energy requirement to assimilate this N-source. Our results suggest that the CO₂-user, C. acidophila, was little influenced in its C₇-acquisition by N-source, and that the CO₂-limitation seemed to be stronger under NO₃⁻-use. Possibly the use of NH₄⁺ provides an additional proton stress for the cells that compensates for the metabolic advantages of this N-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-source (i.e., NH₄⁺) of their natural environment. Our species synthesized NR in the presence of NO₃⁻ similar to neutrophiles (Chen et al., 2012; Li, Fingrut, & Maxwell, 2009). Local adaptation did not influence the N-source preference of this acidophile, whereas the green alga Closterium aciculare (Coese, 1991), and most isolates from the acidotolerant C. pitschmannii (Pollio et al., 2005), were restricted to ammonium for their N-uptake. Such preferences are of interest for biofuel production, as lipid production in Tetraselmis sp. was higher when cells were grown with NO₃⁻ than with NH₄⁺. In general, effects of the N-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**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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