# Limnospira fusiformis harbors dinitrogenase reductase (nifH)-like genes, but does not show N2 fixation activity

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# *Limnospira fusiformis* harbors dinitrogenase reductase (*nifH*)-like genes, but does not show $N_2$ fixation activity

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ABSTRACT

East African soda lakes (EASLs), some of them world-renowned for their large flocks of flamingos, range amongst the most productive aquatic ecosystems worldwide. The non-heterocytous filamentous cyanobacterium *Limnospira fusiformis* (formerly *Arthrospira fusiformis* or *Spirulina platensis*), forming almost unialgal blooms, is supposed to be a key driver in those ecosystems and is gaining increasing attention because of its nutritional value. Compared to phosphorus and carbon availability, these lakes show reduced nitrogen supply. We studied the possibility of molecular nitrogen ($N_2$) fixation in *Limnospira*, as contradictory statements have been published, and some closely related taxa were confirmed as $N_2$ fixers (diazotrophs). We cultivated nine isolates originating from various EASLs under nitrate-rich and nitrate-depleted conditions. We detected dinitrogenase reductase (*nifH*)-like genes in all strains; however, the genes grouped within *nifH* cluster IV that mostly contains nitrogenases not functioning in $N_2$ fixation. Accordingly, incubations with $^{15}N_2$ gas did not support $N_2$ fixation activity of the investigated strains. Under laboratory conditions, all strains faded during nitrate-depleted growth after approximately three weeks. Both phycocyanin and chlorophyll-a dropped to a threshold, and chlorophyll fluorescence indicated a severe problem with nitrogen supply. In summary, our data indicate that the investigated *Limnospira fusiformis* strains are not capable of $N_2$ fixation.

## 1. Introduction

*Limnospira fusiformis* (Voronikhin) Nowicka-Krawczyk, Mühlsteinová & Hauer is sometimes still referred to as its synonym *Arthrospira fusiformis* [1,2], or sold under the trademark *Spirulina platensis* [3]. It prospers in alkaline-saline lakes (ASL) in Africa and Eurasia [4–6], mostly at salinity between 20 and 60 [6–9]. ASL rank amongst the most productive aquatic ecosystems worldwide due to high inorganic carbon availability [10,11]. Phosphorus is also available in excess, but nitrogen (N) limitation may be a growth-limiting factor for many photoautotrophs [9,12–15]. N limitation is further enhanced by N losses via denitrification processes [16], high temperatures and anoxic conditions in near-bottom zones [17]. Factors like internal nutrient cycling can only partly describe the discrepancies between the high biomass and productivity and the low levels of inorganic N compounds observed for soda lakes [11]. According to Sorokin et al. [18], there is a gap of knowledge about the role of biological dinitrogen ($N_2$) fixation in ASL, which could considerably revise our understanding of the N-cycle under oxic conditions. Studies performed in Mono Lake, California [19], and in Russian ASL [14] suggest that benthic phototrophs are important for $N_2$ fixation at elevated salinity, but for the highly turbid and shallow ASL of the tropics this possibility is questionable. These circumstances, and the growing number of non-heterocytous cyanobacteria known to fix $N_2$, gave rise to the hypothesis that *L. fusiformis* might be capable of $N_2$ fixation.

$N_2$ fixation is a highly regulated process on both a transcriptional [20,21] and post-translational level [22]. The presence of alternative sources of N such as nitrate, ammonium, and urea, not only inhibits nitrogenase activity, but also downregulates its synthesis to prevent wasting energy and reductants [20,23]. Cyanobacterial nitrogenase consists of two protein components [24]: dinitrogenase with two subunits α and β (MoFe-protein, heterotetramer of NifD and NifK proteins),

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and dinitrogenase reductase (Fe-protein, homodimer of NifH protein) encoded by the *nifH* gene [25]. In general, about 16 different *nif* genes are known from cyanobacteria, eight of them are closely related to the core $N_2$ fixation-process [26]. However, the *nif* gene-cluster varies between different diazotrophs [27].

The nitrogenase complex is highly conserved in diazotrophs and it is assumed to have a common ancestor [23,28]. Since it is inactivated by oxygen, the genetic information was lost in many species during bacterial evolution as the atmosphere gradually became oxygenated [23,29,30]. Cyanobacteria as global players of $N_2$ fixation are oxygenic photoautotrophs, which poses a serious problem to the $N_2$ fixation within a non-compartmented prokaryotic cell [29]. To overcome this problem, some taxa of the orders Nostocales and Stigonematales developed heterocytes [31,32], which was long thought to be the only strategy for fixing $N_2$ in oxygenic photoautotrophs [33]. Today we know that also temporal separation of the two processes enable certain non-heterocytous taxa to fix $N_2$. Already Bergman et al. [33] reported 17 non-heterocytous genera, which are capable of $N_2$ fixation.

The number of non-heterocytous filamentous cyanobacteria that are capable of fixing $N_2$ is steadily increasing. Numerous studies have focused on the closely related marine filamentous, non-heterocytous genus *Trichodesmium,* which is able to fix $N_2$ also during the photoperiod [34–37]. *Plectonema boryanum* Gomont is another well-studied taxon, which has the ability to fix $N_2$ during the photoperiod under micro-oxic conditions [38,39]. Recently, Berrendero et al. [40] reported $N_2$ fixation of *Schizothrix* mats of a mountain river in Spain. It is therefore justified to study also *Limnospira* for the ability of $N_2$ fixation. Surprisingly, conflicting reports on this topic have been published. Whole-genome shotgun sequencing of '*Arthrospira platensis*' revealed that it carried the *nif* genes [41]. '*Arthrospira platensis*' is an invalid taxon, but from the Peruvian origin of the strain, we conclude that it probably belongs to *Limnospira maxima* (Setchell & N.L.Gardner) Nowicka-Krawczyk, Mühlsteinová & Hauer, indicating that *L. fusiformis* might be capable of $N_2$ fixation. Kumaresan et al. [42] mentioned in their transcriptome-analysis that *Arthrospira* and *Spirulina* are able to fix $N_2$, but they did not list the coding genes. In the closely related *Limnospira maxima* clone CS 328, *nifJ* and *nifS* genes were detected, while *nifH*, *-K* and —*D* were not mentioned [25]. Contrarily, Fujisawa et al. [43] did not detect nitrogenase genes in their nearly complete genome analysis, although other genes required for heterocyte maturation were conserved in the studied strain NIES-39, identified as '*Arthrospira platensis*'. As the strain was isolated from Lake Chad, it probably belongs to *L. fusiformis*.

The possibility of $N_2$ fixation in non-heterocytous taxa was not considered in the past. New findings of non-heterocytous $N_2$-fixing taxa that are closely related to *Limnospira*, together with genomic studies on this genus (in most cases published as synonyms *Arthrospira* and *Spirulina*), however suggest examining the potential of $N_2$ fixation in this taxon. We tested this hypothesis via (1) growth experiments under laboratory conditions with six strains of different origin, cultured under different N supply. (2) We examined nine strains for the existence of the *nifH* gene that is commonly used as marker gene to identify the potential of diazotrophy. (3) We studied *nifH* gene expression under nitrate- rich and N-depleted conditions in these strains, and (4) we incubated these nine strains in N-limited culture medium containing $^{15}N_2$ gas to test its incorporation into biomass.

This study has also practical benefits: *L. fusiformis* and *L. maxima* are commercially exploited because of their high protein content, nutritional value, and anti-inflammatory effects [44]. Even the European Space Agency is interested in scientific enterprises like the Micro-Ecological Life Support System Alternative (MELiSSA), a circular life support system for extended space missions to eventually produce food, drinking water, and oxygen from mission wastes [45–47].

## 2. Material and methods

### 2.1. Growth experiments

The study was conducted with *Limnospira* strains originating from different ASL of the East African Rift Valley (Table 1). Strains are maintained in the ASW culture collection (Algensammlung Wien) in Zarrouk medium at 25 °C and a light:dark cycle of 16 h:8 h (irradiance supply via fluorescent tubes at 15 μmol photons $m^{-2}$ $s^{-1}$). Detailed growth experiments were carried out with six strains of *L. fusiformis* in a climate chamber at constant temperature of 27 °C (Table 1). Batch cultures were grown in standard Zarrouk medium [48] as control group (+N) and under nitrate-limited conditions (—N) in Zarrouk medium without $NaNO_3$, but with addition of the equimolar NaCl. Before running the experiments, pre-cultures with standard Zarrouk medium (control) and 20 % $NaNO_3$ medium (for —N treatments) were prepared and grown for a few days for acclimation. To avoid N carryover, we pre-filtered cultures through a plankton net (mesh size 30 μm), rinsed them with new medium and re-suspended them before main experiments started. Cultures were microscopically checked for contamination every 4th day. Strains were cultivated in 5 L bubble column reactors illuminated via LED panels (50 μmol photons $m^{-2}$ $s^{-1}$; 12 h:12 h light:dark cycle) with 4 replicates for each treatment. Cultures were continuously bubbled with sterile-filtered air during daytime. At the onset of the dark period, air supply was stopped and the cultures were flushed with $N_2$ gas for around 5 min to establish micro-oxic conditions (~15 % dissolved $O_2$). All strains were cultivated for 12–13 days until final harvest.

Optical density (OD) as a proxy of biomass was monitored daily. A pilot study confirmed that OD is highly correlated with dry mass (DM; $r^2 = 0.938$, $n = 96$, data not shown). OD was measured photometrically (Hitachi U-200) at 750 nm against Zarrouk medium. Based on OD, growth rates (μ) and doubling times ($t_d$) were calculated: $\mu = (\ln OD_t - \ln OD_0)/t$ and $t_d = (\ln 2 - \ln 1)/\mu$ with t = time [$d^{-1}$]. Maximum dark fluorescence yields of PSII ($F_v/F_m$) were measured with a PAM-2500/US device (Walz company) after 10 min pre-darkening: $F_v = F_m - F_0$ ($F_0$ = minimum fluorescence after dark adaption, $F_m$ = maximum inducible fluorescence during a light flash, $F_v$ = variable fluorescence; [49]). $F_v/F_m$ serves as a proxy of the overall photosynthetic efficiency [50].

In addition to the monitoring variables, a set of parameters including photosynthetic pigments, nitrate-N and carbon/nitrogen (CN) contents, and organic dry mass (DM) were analyzed every 4th day during the experiment. For pigments (chlorophyll-a = chl-a, total carotenoids and phycobilins), a defined volume of the algal suspension was filtered onto GF/C filters (Ederol company) and frozen at −20 °C until further treatment. Frozen filters were then cut, resuspended in 90 % acetone (for chl-a and total carotenoids), or in 0.1 m phosphate buffer adjusted to pH = 6.8, respectively (for phycocyanin and phycoerythrin). Filters were homogenized via ultra-sonication (Branson Sonifier 250) and extracted at 4 °C for 12 h (chl-a and total carotenoids), or 24 h (phycobilins), respectively. After extraction, the suspension was centrifuged and the supernatant measured spectrophotometrically at 750 nm, 663 nm, 520 nm and 480 nm (for chl-a and total carotenoids) and 730 nm, 618 nm and 564 nm (for phycobilins). Chl-a content was calculated according to Lorenzen [51], total carotenoids according to Strickland and Parsons [52]. Phycoerythrin and phycocyanin were estimated according to Sampath-Wiley and Neefus [53]. For nitrate-N, DM and CN analyses, a defined volume of the algal suspension was filtered onto pre-combusted and pre-weighed GF/C filters. DM-filters were dried at 90 °C for 24 h and reweighed. For determination of nitrate-N, the filtrate was analyzed by means of ion chromatography (according to DIN EN ISO ÖNORM 10304). To remove all inorganic carbon, CN-filters were rinsed with 10 % HCl followed by rinsing with milliQ-water. CN-filters were then dried at 60 °C, packed in tin foil and analyzed in the elemental analyzer Vario MICRO Cube.

**Table 1**
Origin of strains, strain number in the ASW culture collection and experiments conducted. The asterisk indicates if the mentioned experiment was performed with the particular strain.

| Lake, country | Coordinates | Strain number | Growth experiments | Molecular analysis | $^{15}N_2$ incubation |
|---|---|---|---|---|---|
| Arenguade (Ethiopia) | 8.6957, 38.9765 | 01 101 | * | * | * |
| Abijata (Ethiopia) | 7.6179, 38.6000 | 01 107 | * | * | * |
| Chitu (Ethiopia) | 7.4053, 38.4206 | 01 104 | | * | * |
| Nakuru (Kenya) | −0.3508, 36.1000 | 01 100 | * | * | * |
| Simbi (Kenya) | −0.3672, 34.6289 | 01 105 | * | * | * |
| Sonachi (Kenya) | −0.7824, 36.2621 | 01 103 | | * | * |
| Oloidien (Kenya) | −0.8135, 36.2778 | 01 108 | * | * | * |
| Big Momella (Tanzania) | −3.2235, 36.9100 | 01 106 | * | * | * |
| L.B. Momella (Tanzania) | −3.2235, 36.9100 | 01 102 | | * | * |

### 2.2. $^{15}N_2$ incubations

Incubation experiments were performed with nine strains of *L. fusiformis* (Table 1) according to the method of Mohr et al. [54]. For $^{15}N_2$-medium preparation, N-depleted Zarrouk medium (pH 8.5) was degassed at ≤200 mbar and stirred for 30 min. After transferring it into a vial until overflow, 1 mL $^{15}N_2$ gas was added per 26 mL followed by shaking to ensure that all $^{15}N_2$ was equally dissolved. For incubations, we used 14 mL-vials filled with 12.6 mL of N-depleted medium, which was pre-inoculated with the respective clone. The remaining 1.4 mL were filled with $^{15}N_2$ -enriched medium. $^{15}N_2$ gas (98 at.%) used in incubations was purchased from CAMPRO Scientific (Berlin, Germany, lot # MBBB0968V) and tested for $^{15}N$-labelled ammonia gas contaminations using the hypobromite oxidation method [55,56]. As contamination with $^{15}N$-labelled ammonia gas (155.16 ppm) was detected, we subtracted this potential $^{15}N$ source from the measured $^{15}N$ enrichment for data analysis. We used triplicates of both, cultures grown in $^{15}N_2$-labelled and unlabelled medium, which were exposed to a light: dark cycle of 8:16 h at 24 °C for 5 d. The prolonged darkness was chosen to avoid any interferences of nitrogenase with oxygen (oxygen measurements proved a concentration below 1 mg $L^{-1}$ after 5 h darkness). We used the diazotrophs *Nostoc* sp., strain ASW 01020 and *Anabaena torulosa* ASW 01028 as positive controls. Harvesting was done by vacuum filtration followed by freeze-drying of the material. Finally, 0.3–0.8 mg of dried biomass were packed into stannic tons and the $^{15}N/^{14}N$ isotopic ratios were determined using an elemental analyzer (EA 110; CE Instruments, Milan, Italy) coupled to an isotope ratio mass spectrometer (DELTA Plus; Finnigan MAT, Bremen, Germany).

### 2.3. Molecular analysis

For detailed protocols see Supplemental Material. Briefly, sequencing was done with nine clones of *L. fusiformis* (Table 1). We used triplicates grown in 1 L SCHOTT-flasks on shakers with 130 rpm under conditions previously mentioned ((+N) and nitrate-limited conditions (—N). Cultures were harvested during the exponential growth phase by vacuum filtration onto glass fiber filters (Munktell MGC) and immediately frozen at −80 °C. DNA and RNA extractions were conducted according to the modified protocol of [57]. DNA digestion was performed with Turbo DNase (Thermo Scientific TM) and RNA purification using GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Scientific TM). cDNA strands were synthesised from pure RNA using Invitrogen's SuperScriptTM III (Thermo Scientific TM). PCR targeting *nifH* was conducted with DNA, RNA, and cDNA of each strain using the primer pair IGK3 (5'-GCIWTHTAYGGIAARGGIGGIATHGGIAA-3′) and DVV (5'-ATIGCRAAICCICCRCAIACIACRTC-3′) [58]. PCR amplification was carried out with the following thermocycler program: initial 4 min at 94 °C, following by 35 cycles of 45 s at 52 °C, 30 s at 72 °C, 30 s at 94 °C and a final step for 45 s at 52 °C, 72 °C for 10 min and a cooling down phase at 4 °C. For amplification of the 16S rRNA, the general bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5'-CGGTTACCTTGTTACGACTT-3′) were used, under the same PCR conditions. Clone libraries were created by using Invitrogen's TOPO TA cloning kit (Thermo Scientific TM) and both fragments, *nifH* and 16S rRNA, were sequenced by Sanger sequencing at Microsynth AG, Balgach, Switzerland.

For both, *nifH* and 16S rRNA, primer and vector sequences were removed using cutadapt (v1.15; [59]). *nifH* sequences were processed using NifMAP (v1.2; [60]). DNA sequences were translated to amino acids using FrameBot [61], aligned using mafft [62] and classified using blastp [63] and CART [64]. For 16S rRNA sequences, contig merging was done using BBMerge [65]. Alignment was done using sina [66] against the SILVA database (V138.1; [67]). Phylogenetic trees for both genes were calculated using IQ-TREE 2 [68].

### 2.4. Statistics

Statistical analyses were carried out in SPSS Statistics 21 (IBM) on a significance level of $p < 0.05$. A repeated measures ANOVA (rmANOVA) was conducted to show significant differences for cellular pigment content over time and between treatments. Levene's test was used to check homogeneity of variances and sphericity was tested via Mauchley's test of sphericity. A Mann-Whitney *U* test was conducted to show significant changes of N content between start and end of the experiments and significant differences of $t_d$ between treatments. Linear regression models were calculated to show relations of measured parameters of biomass and homogeneity of regression slopes was tested via ANCOVA to show significant differences between the regression slopes.

## 3. Results

### 3.1. Growth experiments

All cultures showed differences between the N-rich and N-limited treatments. Within a few days, +N treatments of all strains developed from pale green to very dense, dark-green cultures, whereas —N treatments turned into yellow-green to almost colorless suspensions (Fig. 1). Microscopical observations showed only healthy, greenish, long filaments in +N treatments and increasingly shorter and fragmented, yellowish-brownish filaments in —N treatments towards the end of the experiments (data not shown). Growth curves showed continuous growth of all +N treatments until end of the experiments, while —N treatments showed, at best, only slight growth throughout the experiments (Fig. S1). The doubling time ($t_d$) varied significantly between treatments of all strains (Mann Whitney U test; $p < 0.05$), with highest observed $t_d$'s in the range of 2.6 to 39.0 days for —N treatments and 1.5 to 8.2 days for +N treatments (Fig. 2). $F_v/F_m$ measurements of all strains revealed a comparable pattern within the respective treatment (Fig. 3). —N treatments showed a slight increase of dark yields during the first days of the experiments, followed by a strong decrease until the end of the experiments. In contrast, dark yields of +N treatments remained at the same level throughout the experiments with values between 0.4 and 0.6 or even slightly higher (Big Momella).

Direct biomass parameters particulate C and DM showed a highly

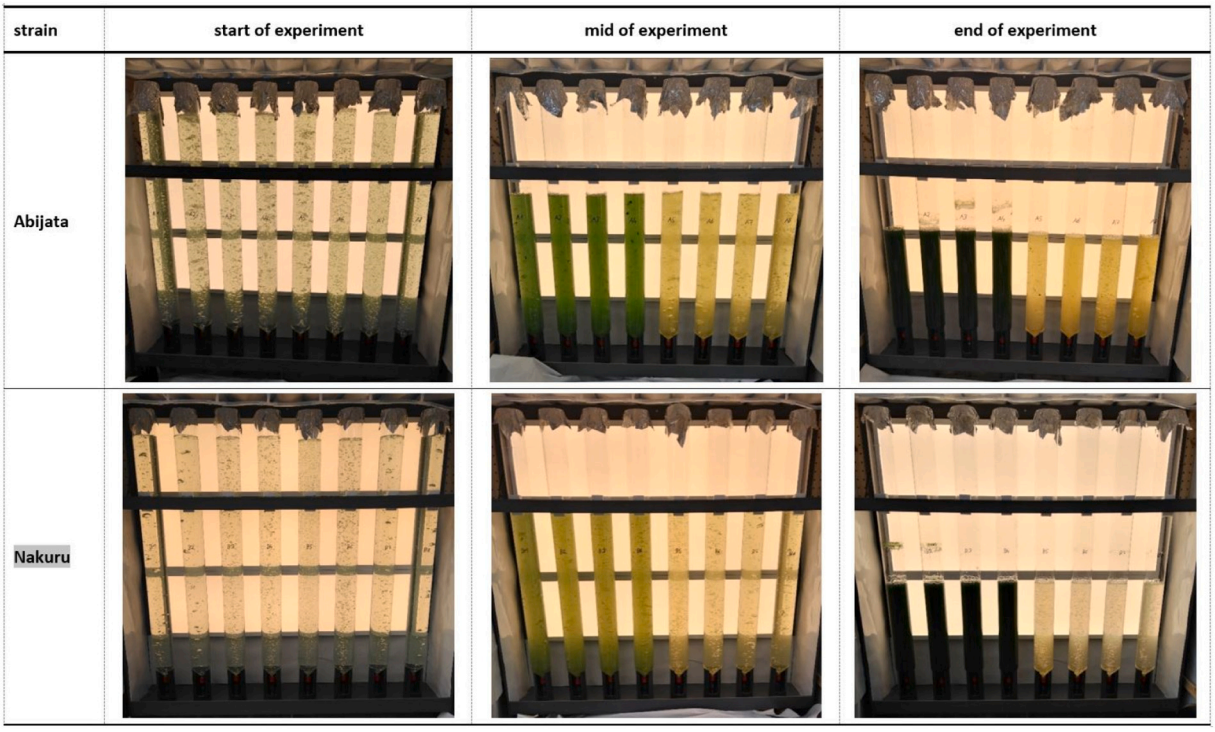


**Fig. 1.** Macroscopic development of the cultures for two selected strains (Abijata and Nakuru). Left four cultures were grown in N-rich media, while the right three cultures contained N-limited media.

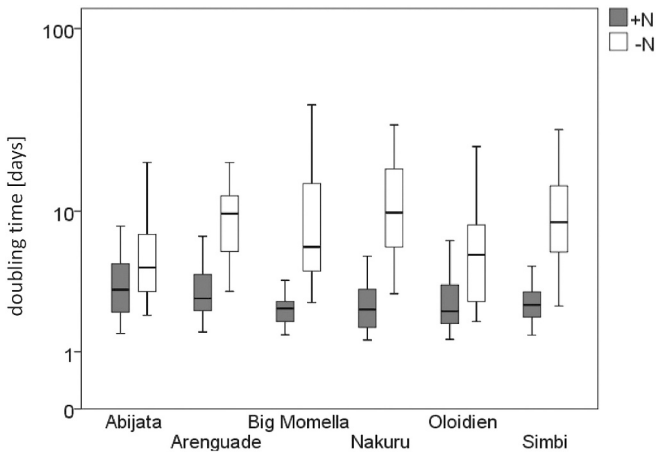


**Fig. 2.** Boxplot diagram of calculated doubling times (in days) for all examined strains. Median, 25- and 75 % percentiles, minimum and maximum are depicted, $n = 40$.

significant and strong relation with no significant differences between the treatments (Fig. 4). Based on DM, —N treatments had significantly lower chl-a concentrations; also particulate N turned out to be significantly lower in —N treatments (Fig. 4). A significant and sharp decrease of phycocyanin from day 1 towards the end of the experiments was observed in all —N treatments (Fig. 5). In contrary, in most of the +N treatments (except Big Momella and Simbi), the phycocyanin content first dropped from day 1 to day 5 and then increased to even higher contents at the end of the experiments. In general, phycocyanin contents of well-supplied +N cultures ranged from 7.5 mg $g^{-1}$ DM (Big Momella, day 9) to 83.7 mg $g^{-1}$ DM (Nakuru, day 13), values of —N cultures dropped below 3.1 mg $g^{-1}$ DM. Phycoerythrin contents of algal biomass were rather low with values of +N cultures ranging from 0.8 to 4.1 mg $g^{-1}$ DM. rmANOVA showed a significant variation of phycobilin contents between treatments of all examined strains, except phycoerythrin content of Nakuru with no significant difference between treatments (Table 2). The interaction of the main effects time x treatment was also significant in most cases, except for phycocyanin and phycoerythrin of Big Momella, and phycoerythrin content of Simbi, where both time and treatment led to a decrease of pigment content. In agreement with the phycobilins analysis, a decrease of chl-a and total carotenoids in the course of the experiments was observed in all —N treatments (Fig. 6). The decrease of chl-a and total carotenoids per unit DM over time in —N treatments was significant, while it significantly increased in all +N treatments except Abijata (Table 2). Rm-ANOVA showed significant differences of chl-a and total carotenoid content of all strains between treatments and significant change of pigment content over time for all treatments except +N treatment of Abijata (Table 2). The interaction between time x treatment was significant for all cases. Maximum chl-a content was recognized towards the end of the experiment in +N treatments. Chl-a ranged from 3.6 mg $g^{-1}$ DM (Big Momella) to 11.1 mg $g^{-1}$ DM (Nakuru). Total carotenoids showed maxima between 1.3 mg $g^{-1}$ DM (Big Momella) to 3.6 mg $g^{-1}$ DM (Oloidien). Minimum values of chl-a were observed in —N treatments at the end of the experiments and varied around 2 mg $g^{-1}$ DM (1.3–3.2 mg $g^{-1}$ DM); total carotenoid was around 1 mg $g^{-1}$ DM (0.6–1.2 mg $g^{-1}$ DM).

Total N of all treatments showed no increase over the course of the experiment with only one exception due to inaccuracy of nitrate analysis (Simbi, Table 3). For all +N treatments, a slight increase of N per unit DM was obtained with mean values ranging from 64 to 89 mg $g^{-1}$ DM at the end of the experiments, while N per unit DM values dropped significantly to around 20 mg $g^{-1}$ DM in all —N treatments (Table 3). The significant decrease of N content of algal biomass in —N treatments throughout the experiments is also reflected in higher C:N ratios (Table 3, Figs. 4 and 7). While the C:N ratios of algal biomass in +N treatments remain constant at around 4,3:1; C:N ratios of —N treatments are increasing to rates as high as 17:1 (Abijata, Fig. 7).

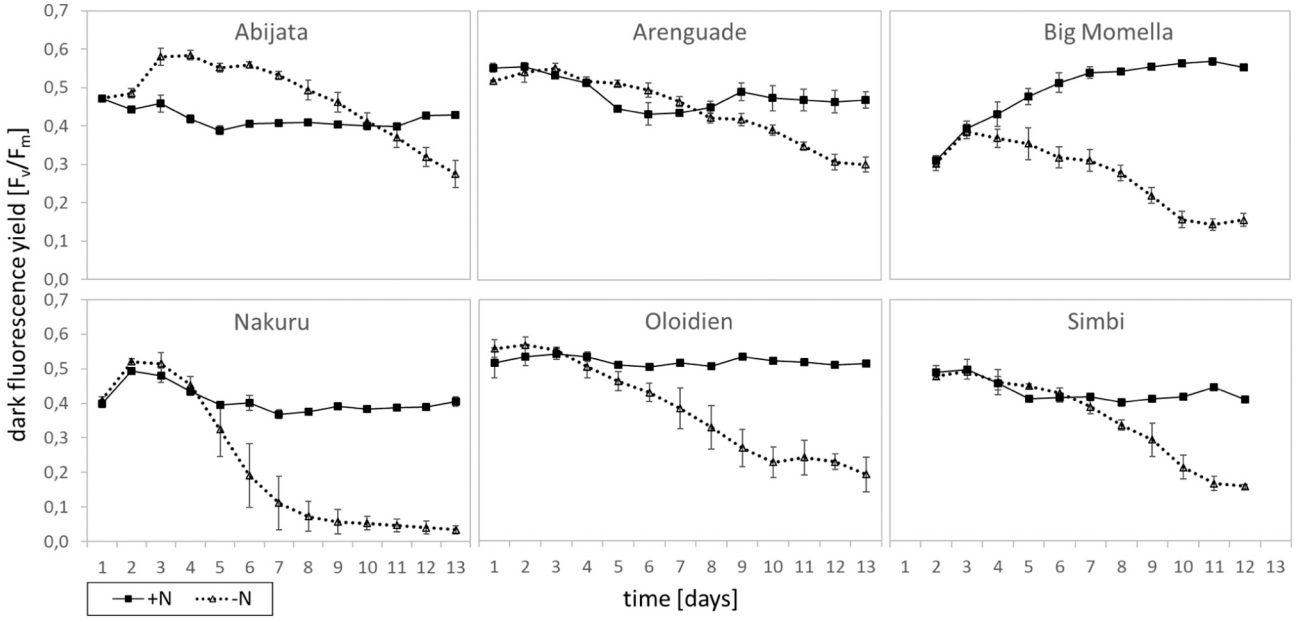


**Fig. 3.** Dark fluorescence yield of all examined strains measured every day during the experiments, error bars = SD, $n = 4$.

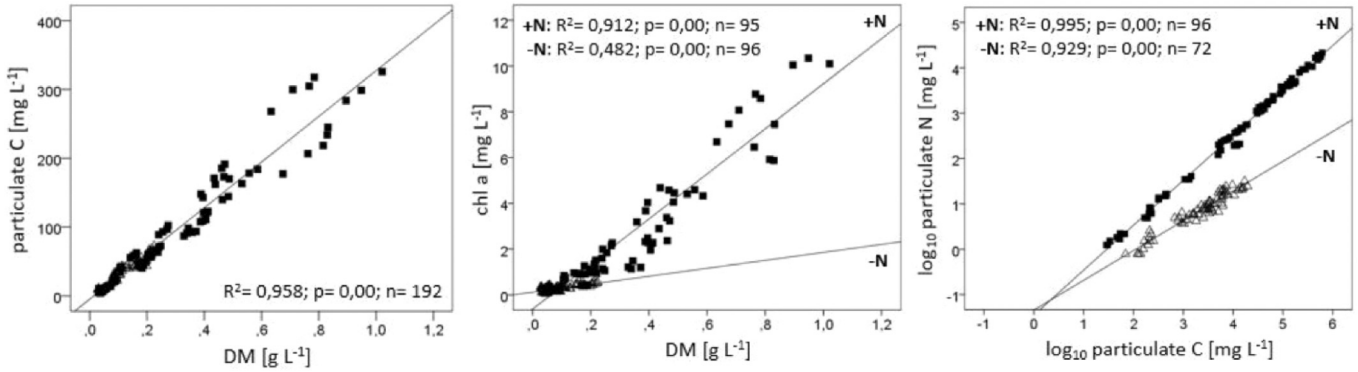


**Fig. 4.** Linear regressions of direct biomass parameters particulate C and DM, DM and chl a, and particulate C and particulate N. ANCOVA results showed significant ($p < 0.05$) differences of treatments between regression slopes of chl a/DM and particulate N/particulate C.

### 3.2. $^{15}N_2$ incubations

For all investigated *L. fusiformis* strains, no significant $^{15}N_2$ fixation activity could be detected (Fig. 8), in contrast to the diazotrophic strains *Nostoc* and *Anabaena*.

### 3.3. Molecular analysis

The 16S rRNA tree, comparing strains of this study with others obtained from the SILVA database, revealed a rather homogeneous cluster of all *L. fusiformis* strains (Fig. S2). All nine examined strains carried a *nifH* gene that, based on classification and regression trees, grouped with *nifH* cluster IV [64]. The *nifH* tree (Fig. 9) showed a very high similarity between the strains obtained in this study, but a small variation between the *nifH* gene sequences of *L. fusifomis* and that of other diazotrophs was recognized. Six of the strains (Arenguade, Sonachi, Simbi, Big Momella, Abijata, Nakuru) expressed the *nifH* gene under N-limited conditions, while *nifH* transcripts could be detected in four strains (Arenguade, Simbi, Big Momella, Abijata) under N-rich conditions. L.B. Momella, Chitu and L. Oloidien, did not express *nifH* independent of the treatment (Table 4, Fig. S3).

## 4. Discussion

Compiling data exactly matching *L. fusiformis* is challenging because of its puzzling nomenclature [2]. Moreover, information on the origin of isolates is often lacking, which hampers attempts for comparing and drawing conclusions on autecology and distribution patterns. Former molecular analyses provide a blurred picture with contradictory results. Fujisawa et al. [43] performed an almost complete genome sequencing of *L. fusiformis* (referred to as *Spirulina platensis* NIES-39) originating from Lake Chad. The authors assumed that this isolate is not diazotrophic, as they could not detect any *nif* genes. Although the authors detected *nif*-related genes together with heterocyte encoding ones, they did not comment on this inconsistency. The whole genome of "*Arthrospira platensis* C1 (*Arthrospira* sp. PCC 9438)" was sequenced by Cheevadhanarak et al. [69]. Strain PCC 9438 was isolated from a Lake near Mogadishu in Somalia and most probably belongs to *L. fusiformis* (Avigad Vonshak, pers. comm.). The authors compared its genome with other cyanobacteria and concluded that this strain is not diazotrophic, but without providing any details [69]. Sequencing of "*Arthrospira* sp. (PCC 8005)" by Janssen et al. [47] revealed that essential *nif* genes are lacking, but the authors did not provide any further information. Moreover, the origin of this strain is unknown, as the information was lost according to Pasteur Culture Collection. Contrary to the above-

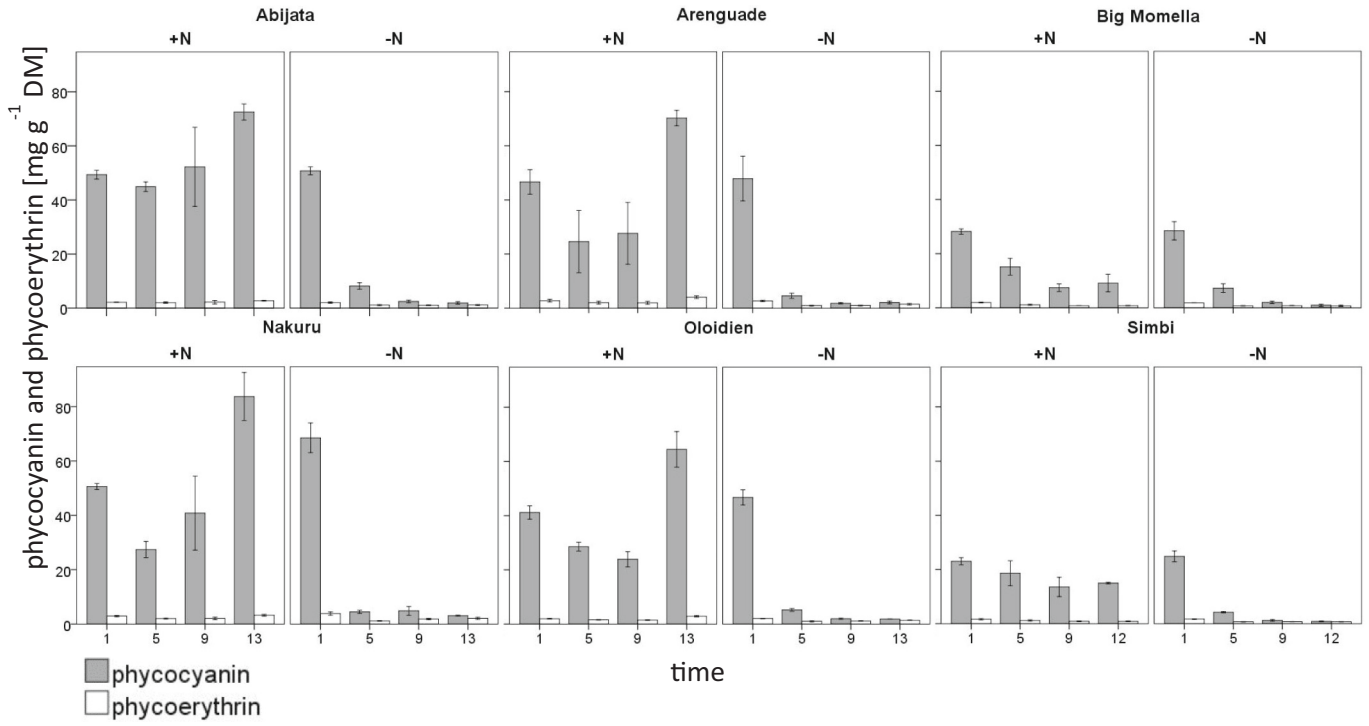


**Fig. 5.** Phycocyanin and phycoerythrin content of all examined strains measured 4 times during the experiment for +N treatments (left) and —N treatments (right), error bars = SD, n = 4.

**Table 2**
Rm-ANOVA of pigments per unit DM (significance level of $p < 0{,}05$). +… significant increase of pigment content over time, −… significant decrease of pigment content over time, =… no significant change in pigment content over time, *… general significant difference of pigment content between treatments.

| | Phycocyanin | | Phycoerythrin | | Chlorophyll *a* | | Carotenoids | |
|---|---|---|---|---|---|---|---|---|
| Abijata + N | + | * | = | * | = | * | = | * |
| Abijata -N | − | | − | | − | | − | |
| Arenguade + N | + | * | + | * | + | * | + | * |
| Arenguade -N | − | | − | | − | | − | |
| Big Momella + N | − | * | − | * | + | * | + | * |
| Big Momella -N | − | | − | | − | | − | |
| Nakuru + N | + | * | + | | + | * | + | * |
| Nakuru -N | − | | − | | − | | − | |
| Oloidien + N | + | * | + | * | + | * | + | * |
| Oloidien -N | − | | − | | − | | − | |
| Simbi + N | − | * | − | * | + | * | + | * |
| Simbi -N | − | | − | | − | | − | |

mentioned studies, other studies suggested diazotrophy of *Limnospira*: Lefort et al. [41] stated that "*Arthrospira platensis*" from Peru is capable of $N_2$ fixation, but did not provide further evidence. According to Kumaresan et al. [42], *Arthrospira* and *Spirulina* are able to fix $N_2$ based on transcriptome sequencing using Illumina sequencing and de novo assembly. Another study focusing on *Limnospira maxima* clone CS 32 mentioned the occurrence of *nifJ* and *nifS* genes in this taxon [25]. We were able to detect the *nifH* gene in all examined strains, and in some clones it was also expressed. However, these *nifH* genes grouped with *nifH* cluster IV (Table S1), which with few exceptions [70] is mostly comprised of nitrogenases not functional in $N_2$ fixation [71]. All the *nifH* genes detected in our study clustered together and are closely related to *nifH* genes from other diazotrophs, most notably '*Arthrospira platensis*' (Fig. 9). With this assay, however, we were not able to assess if genes for the entire enzyme complex is encoded/expressed, so we conducted additional laboratory experiments.

All +N treatments showed continuous growth and healthy filaments. On the contrary, —N treatments had only slight growth during the first days of the experiments followed by a decay towards the end of the growth experiments. Cell divisions during the first days were possible using internal storage products such as cyanophycin. Cyanophycin is a polypeptide consisting of the two amino acids aspartic acid and arginine [72,73], and a well-known N-storage protein occurring in Cyanobacteria [74,75]. It was previously observed in *L. fusiformis*, but only at temperatures below 17 °C and at times when cultures approached the stationary phase [76].

Much more important at provided growth conditions is phycocyanin located in phycobilisomes in its different forms [23], but also known as an internal N source [77]. Phycobilisomes contain up to 25 % of cellular N [78]. Phycocyanin and phycoerythrin content of —N treatments dropped strongly on day five of the experiments, indicating severe N-starvation and complete consumption of the entire N-storage pool. None of the strains were able to recover from N-starvation, which would have indicated the ability of $N_2$ fixation. Phycocyanin amounts of +N treatments varied highly between the different strains (7.5 to 83.7 mg $g^{-1}$ DM) and were at the lower end of the range of phycocyanin contents reported for *Limnospira* between 50 and 180 mg $g^{-1}$ DM [79–82]. It is quite striking that the studied strains showed such big variations in

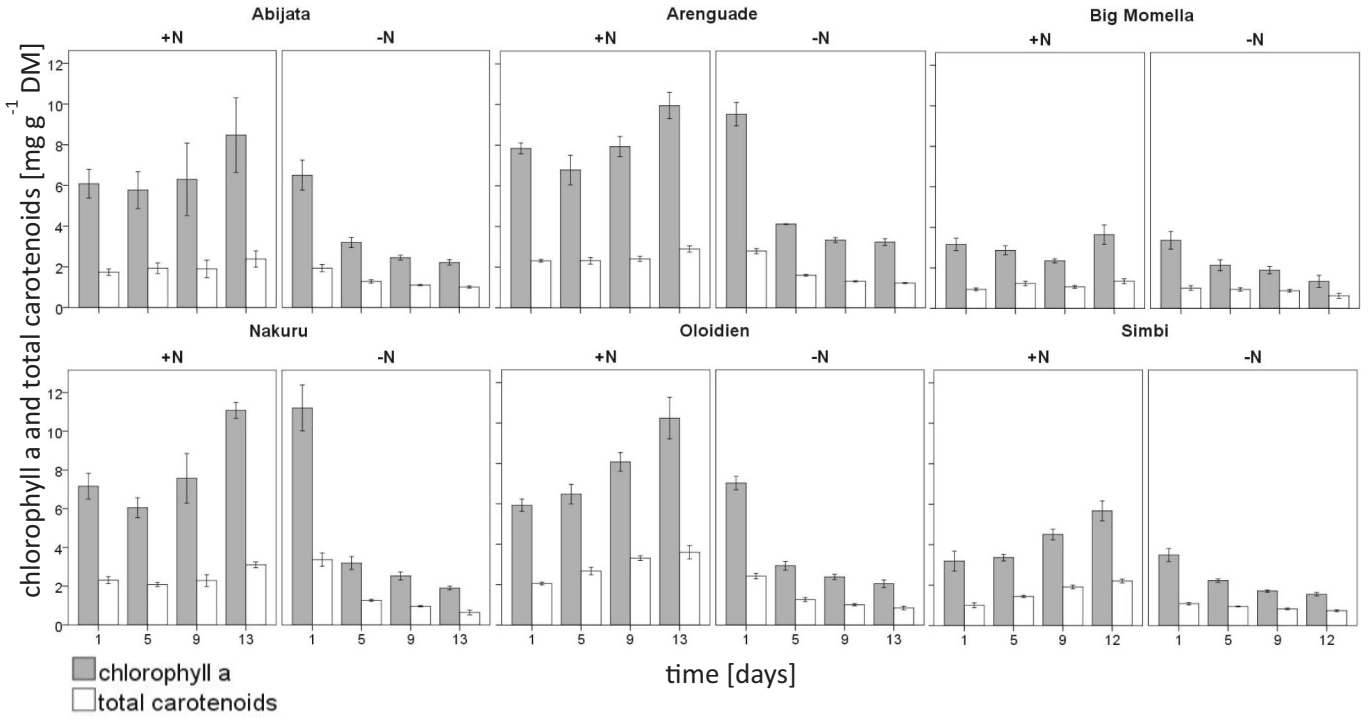


**Fig. 6.** Chlorophyll a and total carotenoid content per unit DM of all examined strains for +N treatments (left) and —N treatments (right) measured 4 times during the experiments (day 1, 5, 9, 13 (12)), error bars = SD.

**Table 3**
Summary of *nifH*-PCRs- that a PCR-product was not observed.

| | particulate N | dissolved N | total N | N per unit DM | C:N ratio |
|---|---|---|---|---|---|
| Abijata -N | + | − | = | − | + |
| Arenguade -N | + | = | = | − | + |
| Big Momella -N | = | = | = | − | + |
| Nakuru -N | + | = | = | − | + |
| Oloidien -N | + | − | − | − | + |
| Simbi -N | + | = | + | − | + |
| Abijata + N | + | − | = | + | = |
| Arenguade + N | + | − | = | + | = |
| Big Momella + N | + | − | = | + | = |
| Nakuru + N | + | − | = | + | = |
| Oloidien + N | + | − | = | + | = |
| Simbi + N | + | − | = | + | = |

phycobilin contents per unit DM, although they belong to the same species and were exposed to identical growth conditions. Given the importance of phycocyanin for commercial exploitation and its high market value [81], a proper selection of adequate strains and certain culture conditions with special emphasis on irradiance supply are highly advised.

Degradation of phycocyanin was also reflected by Fv/Fm (Fig. 3). Phycobilins interfere with fluorescence yield measurements by increasing the minimum fluorescence ($F_0$) thus leading to an underestimation of $F_v/F_m$ [83]. Reported dark yields for healthy, unstressed green algae are around 0.7, while those of cyanobacteria are typically around 0.4 [50]. These values are consistent with our measurements for +N treatments, and indicate a healthy photophysiological status. —N treatments showed first an increase of $F_v/F_m$ caused by degradation i.e. less interference of phycocyanin. With time, $F_v/F_m$ of —N treatments however dropped considerably below values of +N cultures due to N depletion and finally decay. Considering other parameters such as chl-a and microscopy, this strong decrease indicates severe N-starvation.

Acclimatization of +N treatments towards prevailing growth conditions was observed by increasing concentrations of pigments (Fig. 6). The substantial increase of chl-a and phycocyanin per unit DM is a result of suitable growing conditions and N supply, which is also reflected in the strong, blue-green to dark-green coloration of the cultures. Chl-a of around 10 mg $g^{-1}$ DM of well-supplied +N treatments is consistent with reported values [4]. In —N treatments, the chl-a content decreased significantly and continuously from the start towards the end of the experiments with a minimum threshold of around 2 mg $g^{-1}$ DM, but not as sharp and pronounced compared to phycocyanin. This threshold can be explained by the importance of chl-a as the primary pigment for photosynthesis. Observed values of total carotenoid content for +N treatments from 1.3 to 3.6 mg $g^{-1}$ DM are corresponding with the findings of Sethu [81]. The rather strong decrease of phycocyanin and chl-a in comparison to the total carotenoid content of the —N treatments is also expressed in the increasingly yellow coloration of the cultures (Fig. 1). Already Richardson et al. [84] and Kolber et al. [85] reported a strong decrease of chl in different algae groups caused by N-limitation. The linear regression of chl-a and DM for both treatments is shown in Fig. 4 and exhibits significant differences of the regression slopes between the two treatments due to of N-limitation. Since external N supply is limited, the internal concentration of N-rich compounds like chl-a is increasingly lowered with each cell division. Summarizing the results from the pigment analysis, no support for $N_2$ fixation by *L. fusiformis* was found.

Strong evidence for $N_2$ fixation would have been a significant increase of total N in the —N treatments from day 1 towards the end of the experiment. A Mann-Whitney-*U* test between the start and end of the experiments (Table 3) yielded no significant increase of total N for —N treatments, except for the strain Simbi, which showed only a slight but significant increase from 2.7 to 3.4 mg $L^{-1}$ N, probably caused by inaccuracy of particulate N analysis. Moreover, in $^{15}N_2$-incubation assays, no $^{15}N_2$ fixation activity was detected in any of the *L. fusiformis* strains (Fig. 8).

*L. fusiformis* exhibits a relatively high protein content of up to 70 % of the biomass [81,86,87], which explains its nutritional value. Lopez et al.

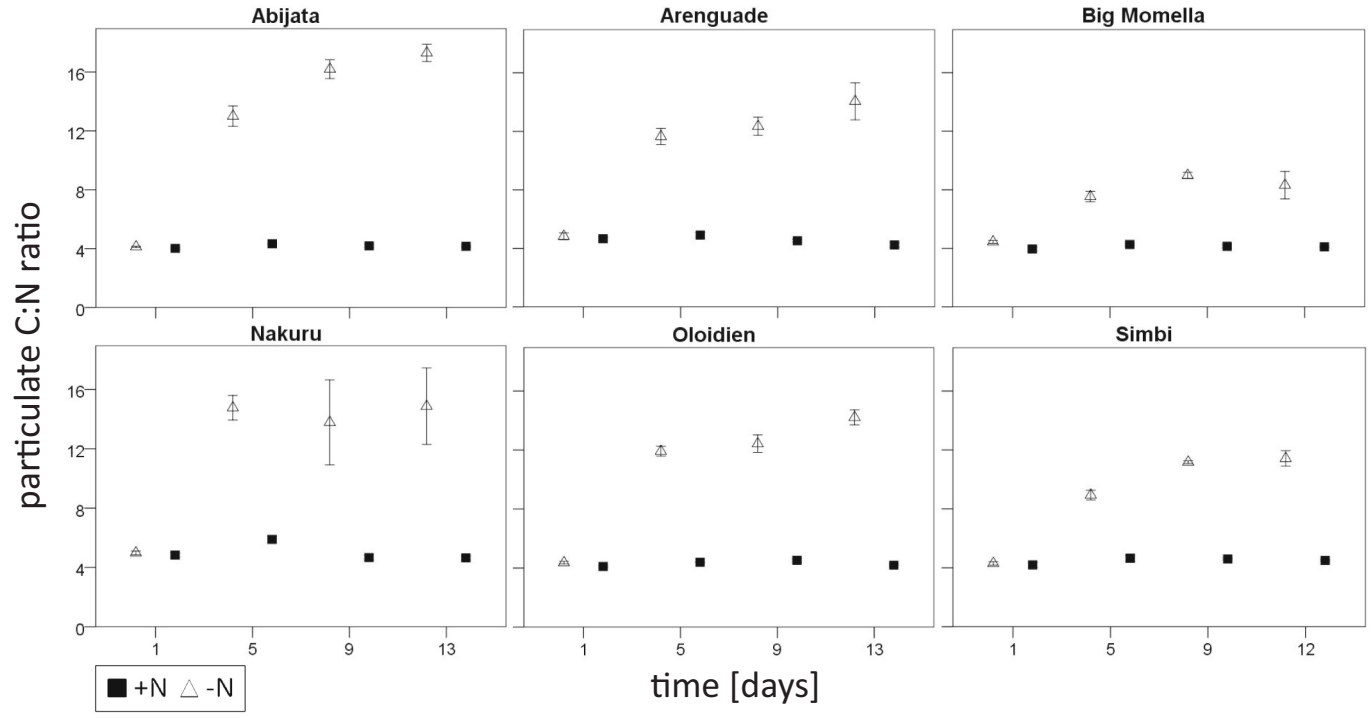


**Fig. 7.** Particulate C:N ratios of algal biomass of all examined strains for +N and —N treatments measured 4 times during the experiments (day 1, 5, 9, 13 (12)), error bars = SD.

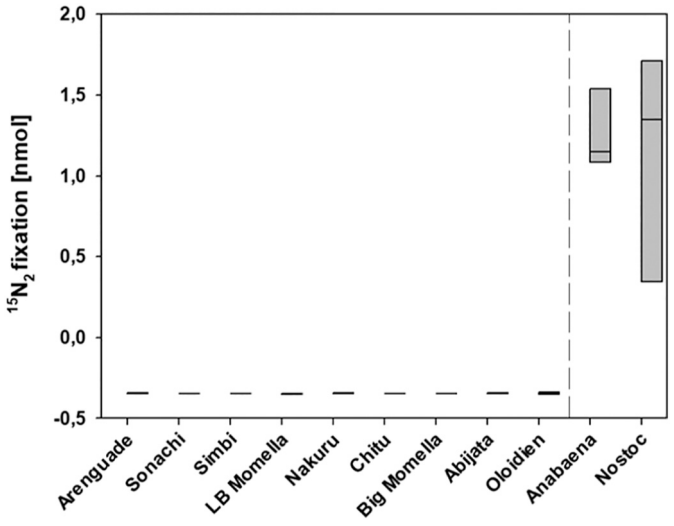


**Fig. 8.** Summary of $^{15}N_2$ incubation experiments (depicted as $^{15}N_2$ fixation in nmol) of all examined *L. fusiformis* strains in comparison to known diazotrophs (*Nostoc* sp., strain ASW 01020 and *Anabaena torulosa* ASW 01028). In none of the *L. fusiformis* strains was $^{15}N_2$ fixation activity detected.

[88] reported a N content of around 12 % of DM for '*Arthrospira*', being the highest content of five microalgae taxa examined. Maximum N content of well supplied +N treatments at the end of the experiments were in the range of 65 to 89 mg $g^{-1}$ DM, which is lower compared to Lopez et al. [88]. As a result of excess N and good growing conditions, all +N treatments significantly increased N contents per unit C compared to —N cultures (Fig. 4). N content of all —N treatments dropped to minimum values of around 20 mg $g^{-1}$ DM, which we propose as minimum threshold values of N that is necessary to maintain the vital functions of the cell. Given its high protein content and an accompanying low carbohydrate content of 12–20 %, the C:N ratio of *L. fusiformis* is comparably low [89]. Particulate C:N ratios of +N treatments showed a rather constant C:N ratio of around 4.3:1 throughout the experiments (Fig. 4), which reflects the high protein content and its ability to store large amounts of N. In contrast, C:N ratios of all —N treatments were significantly higher (C:*N* = 8 to −17:1). This increase is obviously the result of N-limitation and integrates all other observations.

In summary, our data indicate that the investigated *L. fusiformis* strains are not capable of $N_2$ fixation. This conclusion is supported by the fact that the identified *nifH* genes belong to cluster IV and that incubations with $^{15}N_2$ did not result in detectable $^{15}N_2$ fixation activity. The effects of N-limitation on our cultures are consistent with published effects of N-limitation on algae and cyanobacteria, like reduced protein content (especially phycocyanin) and reduction of cellular N, increase of carbohydrate content, down-regulation of photosynthesis and reduced synthesis of chl-a [77,87,90,91].

$N_2$ fixation of *L. fusiformis* would have explained some discrepancies between blooms of this taxon, low N:P ratios [92] and sometimes low nitrogen levels in ASL [13]. Until the 1980s, nutrients were analyzed only occasionally in ASL. Vareschi [93] considered P, silicate and inorganic carbon, while, at the same time did not analyze nitrogen compounds in his pioneering study on the limnology and ecology of Lake Nakuru. Later on, Vareschi and Jacobs [94] assumed that nitrogen limitation might cause a transition from *Limnospira* blooms towards other diazotrophic cyanobacteria. This was however not confirmed in a laborious study with weekly sampling over 18 months [6]. Mainly irradiance supply, grazing pressure and probably cyanophage infections structure the phytoplankton community in these turbid, hypertrophic systems [6,95].

Internal cycling of nutrients was found to have an overriding role in controlling nutrient availability of lake Chitu (Ethiopia), where >90 % of the total N are found in photoautotrophs [11]. However, the N-cycle might partly be inhibited in ASL due to reduced nitrification at high salt concentrations [96,97]. ASL most probably rely on nitrate imports from surrounding soils or other externally supplied sources of N, like flamingo excreta or feather degradation [11,18]. Additionally, other photoautotrophic taxa might play a pivotal role in $N_2$ fixation. According to

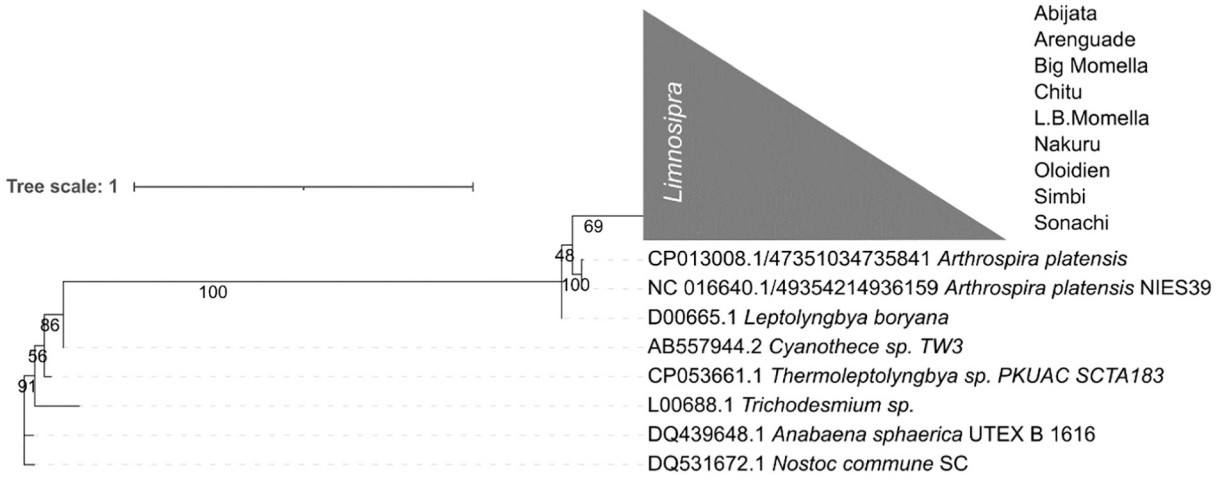


**Fig. 9.** Maximum likelihood phylogenetic tree based on translated amino-acid sequences of the amplified part of the *nifH* gene of the studied strains. Reference sequences were obtained from GenBank. Numbers at branches indicate the bootstrap support.

**Table 4**
Summary of *nifH*-PCR results, indicating whether a PCR-product was detected. +… gene present/expressed. – … gene absent/not expressed.

| | DNA (N-) | cDNA (N-) | cDNA (N+) |
|---|---|---|---|
| Arenguade | + | + | + |
| Sonachi | + | + | – |
| Simbi | + | + | + |
| Big Momella | + | + | + |
| Abijata | + | + | + |
| Nakuru | + | + | – |
| L.B. Momella | + | – | – |
| Chitu | + | – | – |
| L. Oloidien | + | – | – |

Sorokin et al. [98], the highly productive, moderately ASL exhibit high microbial richness and activity with diverse haloalkaliphilic prokaryotes being responsible for the cycling of elements such as, N, carbon and sulfur. For ASL in the Kulunda steppe (Russia) characterized by continental climate, non-heterocytous cyanobacteria located in biofilms might play an important role in light-dependent $N_2$ fixation specifically between a salinity of 60 and 100 [14,99]. If this holds also true for the highly turbid tropical ASL with attenuation coefficients usually beyond 10 [10], remains to be studied.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Roey Angel reports was provided by Austrian Science Fund.

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*CRediT authorship contribution statement*

MS designed experiments, modified the original manuscript and supported the culture experiments. RA supported the molecular work and calculated the phylogenetic trees. UD performed detailed culture experiments and evaluated data. AMG did molecular work, cultivated *Limnospira* and performed the $^{15}N_2$-incubations. DW supported $^{15}N_2$-incubations, assessed the results and worked on the final version of the manuscript. All authors report no commercial or proprietary interest in any product or concept discussed in this article and they declare no conflict of interest. No conflicts, informed consent, or human or animal rights are applicable to this study.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2022.102771.

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