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MASTER´S THESIS

The enigma of nitrogen fixation in the non-heterocytous Cyanobacterium *Arthrospira fusiformis*

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1. Abstract

Nitrogen (N) is a key element for life on earth. Molecular N_2 contributes to 78% of the earth´s atmosphere, it however needs to be reduced before it can be further used for metabolism. One pathway is via biological N_2-fixation, which is performed by bacteria and archaea. Cyanobacteria are ubiquitous on our planet and play a major role for the fixation of N_2. N_2-fixing Cyanobacteria might be especially important in ecosystems with low N supply, such as the soda lakes of the East African Rift Valley. *Arthrospira fusiformis*, a non-heterocytous filamentous Cyanobacterium, forming almost unialgal blooms in East African soda lakes, is supposed to be a key driver in those ecosystems and is gaining increasing attention because of its nutritional value. In this study, we investigated the potential of *A. fusiformis* to fix molecular N_2, since a preliminary study showed some evidence. We carried out growth experiments under laboratory conditions with six *A. fusiformis* strains of different origin. Effects of N-limitation on *A. fusiformis* were consistent with already published effects of N-limitation on *Arthrospira* and other algae species; no evidence of N_2-fixation by *A. fusiformis* was found.

2. Introduction

First records of Cyanobacteria date back to more than 2.5 billion years (Bresinsky et al. 2008); they were the first group capable of pursuing oxygenic photosynthesis. Cyanobacteria, more than any other living organism, shaped the atmosphere of our planet by using water and solar radiation in photosynthesis to fix carbon dioxide and incidentally produce oxygen, which ultimately made the aerobic mode of heterotrophic metabolism possible (Fay 1992). Thus, essential for life on earth and even at the present, ubiquitous in all different ecosystems, ranging from barren deserts, deep oceans, hot springs and freezing glaciers, Cyanobacteria have long been of high interest for the scientific world (Kulasooriya 2016).

Cyanobacteria are prokaryotic organisms, representing a large taxonomic group within the domain of Eubacteria with around 2000 taxa currently valid (Fujisawa et al. 2010; Bresinsky et al. 2008). They can be morphologically divided into four different orders: Chroococcales (unicellular to colonies), Oscillatoriales (filamentous forms without heterocytes), Nostocales (filamentous forms with

heterocytes) and Stigonematales (multiserial filaments with true branching) (Bresinsky et al. 2008; Komárek 2013). Heterocytes, which occur in the orders Nostocales and Stigonematales, are cells specialized for the fixation of molecular N_2. The ability to fix atmospheric N_2 is limited to Prokaryotes (not encoded in any eukaryotic genome) and emerged during an early geological era, in which the reducing atmosphere provided perfect conditions for N_2-fixation (Berman-Frank et al. 2003; Kulasooriya 2016). The N_2-fixing enzyme system is highly conserved within the N_2-fixing organisms (diazotrophs) including Bacteria and Archaea and the ability is thought to have a common origin (Berman-Frank et al. 2003; Fay 1992). Since nitrogenase, the enzyme responsible for fixing molecular N_2, is irreversibly inactivated by oxygen, the genetic information for fixing N_2 was lost in many species during bacterial evolution as the atmosphere gradually became oxygenated (Fay 1992; Kulasooriya 2016; Raymond et al. 2004). Moreover, Cyanobacteria are photoautotrophs, carrying out oxygenic photosynthesis which poses a serious problem to the incompatible, because oxygen sensitive, process of N_2-fixation within a non-compartmented prokaryotic cell (Kulasooriya 2016). As a result, some Cyanobacteria species developed heterocytes, which are cells specialized for the functioning of nitrogenase (Ferimazova et al. 2013). Heterocytes show a considerably reduced content of photosynthetic pigments and are surrounded by a thick membrane, minimizing diffusion of oxygen into the cell and providing a perfect environment for N_2-fixation (Berman-Frank et al. 2003; Ferimazova et al. 2013). Spatial separation of the two processes (photosynthesis and N_2-fixation) by the formation of heterocytes was long thought to be the only strategy in autotrophic Cyanobacteria for fixing molecular N_2 and until 1960 it was believed, that only heterocytous Cyanobacteria are able to fix molecular N_2 (Bergman et al. 1997).

Today, we know that also temporal separation and some other physiological adaptations of non-heterocytous Cyanobacteria enable them to fix molecular N_2. Bergman et al. (1997) already reported a steadily growing list of 17 genera containing more than 70 strains of non-heterocytous Cyanobacteria that were known to be capable of N_2-fixation. Interestingly, there was not much research focused on potential N_2-fixation of non-heterocytous Cyanobacteria during the last 20 years and up to date information on this topic is relatively scarce.

Nitrogenase, the enzyme responsible for the fixation of atmospheric N_2 in all diazotrophs, consists of two protein components, dinitrogenase (MoFe-protein, heterotetramer of NifD and NifK proteins) and dinitrogenase reductase (Fe-protein, homodimer of NifH protein). Although, for some organisms, Mo-independent nitrogenases have been reported, no such reports exist for non-heterocytous Cyanobacteria (Bergman et al. 1997; Raymond et al. 2004). Nitrogenase is an ATP-hydrolyzing enzyme which catalyzes the reaction of the extremely unreactive gaseous N_2-molecule to the equivalent of ammonia (Berman-Frank et al. 2003). The reaction, given by Fay (1992) reads as follows: $N_2 + 6H^+ + 6e^- \rightarrow 2NH_3$.

The reaction is highly endergonic and 16 ATP are needed for each N_2-molecule that is fixed, making it one of the most metabolically expensive processes in biology (Bergman et al. 1997; Simpson and Burris 1984). Given its high cost, N_2-fixation is only carried out under N limiting conditions (Fay 1992; Vitousek et al. 2002). The presence of alternative sources of combined N (nitrate, ammonium, urea, etc.) not only inhibits nitrogenase activity, but also regulates nitrogenase synthesis to prevent wastage of energy and reductant (Dixon and Kahn 2004; Fay 1992). Moreover, in heterocyte forming Cyanobacteria like *Anabaena sp.*, the differentiation and development of heterocytes is controlled by the availability of combined nitrogen and heterocyte development is repressed in the presence of a rich source of combined nitrogen (Adams 2000; Herrero et al. 2016; Kumar et al. 2010; Zhang et al. 2006). The structural genes encoding for the nitrogenase subunits are organized within the *nif* operon and are called respectively *nif*H, *nif*D and *nif*K (Fay 1992; Raymond et al. 2004). Whole-genome shotgun sequencing of *A. platensis* revealed that the investigated strain carried the *nif* genes encoding for nitrogenase (Lefort et al. 2014). Also, genetic analyses of nine different strains of *A. fusiformis* from East African soda lakes showed that all of them carried the *nif*H gene and even in five of them, the *nif*H gene was expressed under N depleted conditions (Gschwandner, 2017).

A growing number of non-heterocytous Cyanobacteria which are capable of fixing molecular N_2, including *Trichodesmium*, *Cyanothece*, *Plectonema* and *Schizothrix,* indicate that also *Arthrospira* might be capable of N_2-fixation. Numerous studies have focused on the marine filamentous, non-heterocytous Cyanobacterium *Trichodesmium* which is able to fix molecular N_2 also during the photoperiod (Berman-Frank et al. 2001; Fredriksson and Bergman 1995; Levitan et al. 2007). *Trichodesmium* forms characteristic bundles of aggregated filaments in which 10 to 20% of the cells contain nitrogenase. In those cells N_2-fixation occurs also during the photoperiod, indicating a combined spatial and temporal separation of photosynthesis and N_2-fixation (Berman-Frank et al. 2003). *Plectonema boryanum* Gomont is another well-studied filamentous non-heterocytous Cyanobacterium that is able to fix molecular N_2 during the photoperiod under micro-oxic conditions (Misra and Tuli 2000; Weare and Benemann 1974). The temporal segregation of photosynthetic activity and N_2-fixation follows a circadian rhythm and is also reflected at transcriptional level, which was observed for the genus *Cyanothece* and also *Plectonema boryanum* (Berman-Frank et al. 2003; Misra and Tuli 2000). Very recently, Berrendero et al. (2016) reported N_2-fixation of *Schizothrix* mats (a non-heterocytous filamentous Cyanobacterium) from a mountain river in Spain similar to those of a heterocytous Cyanobacterium in the same river.

Besides temporal and spatial segregation, there are several other strategies to protect nitrogenase from oxygen which are especially important in non-heterocytous Cyanobacteria and Eubacteria. Those strategies include respiratory protection, conformational protection, hydrogenase activity and enzymes protecting against reactive oxygen species (Fay 1992). Respiratory protection is achieved by

enhanced rates of respiration to keep oxygen concentrations low, preventing nitrogenase from inactivation. For *Azotobacter* species, conformational protection of nitrogenase at high oxygen concentrations was reported, enabling the enzyme complex to fully recover when oxygen concentrations decrease again (Fay 1992). The enzyme unidirectional uptake hydrogenase catalyzes the Knallgas reaction (consuming H_2 and O_2) and can be considered as an oxygen-scavenging device which is present in all aerobic diazotrophs. Reactive oxygen species (ROS) are especially dangerous for all cells and various enzymes protecting against these reactive forms are known from non-heterocytous Cyanobacteria, complementing the other strategies for the protection of nitrogenase (Bergman et al. 1997; Fay 1992).

A. fusiformis (Voronichin) Komarek et Lund (1990) is a non-heterocytous, spirally coiled and filamentous Cyanobacterium which is also known under the commercial name *Spirulina* (*Arthrospira*) *platensis* (Sili et al. 2012). Since the first report of the genus *Arthrospira* in 1852, its classification has been extremely complicated and caused a lot of confusion in the scientific community. According to the modern taxonomy, *Arthrospira* and *Spirulina* are two distinct genera. Within the genus *Arthrospira*, species are classified into planktonic species of saline alkaline waterbodies with gas vacuoles that are forming blooms (*A. fusiformis*, *A. indica* and *A. maxima*) and benthic and periphytic species of freshwaters without gas vacuoles that are forming mats (*A. jenneri*, *A. platensis*) (Komarek and Lund 1990; Sili et al. 2012). *A. fusiformis* is found in Africa and Eurasia and is especially important in tropical soda lakes like those of the East African Rift Valley (Krienitz and Schagerl 2016; Schagerl et al. 2015). Soda lakes are extreme saline-alkaline environments with pH values up to 11, salinity up to 6 % and conductivity up to 70 mS cm^{-1} (Lanzén et al. 2013; Melack 1979; Oduor and Schagerl 2007b; Schagerl et al. 2015; Schagerl and Oduor 2008). They are usually shallow systems with high turbidity and limiting light availability for primary production. Nevertheless, those ecosystems belong to the world´s most productive water bodies (Oduor and Schagerl 2007a; Ogato et al. 2015). *A. fusiformis* can be considered as one of the major driving forces in those systems, forming almost unialgal blooms with extreme densities up to 5000 µg L^{-1} chlorophyll *a* (Kaggwa et al. 2013; Ogato et al. 2015). This high algal biomass of *A. fusiformis* serves as the main food source of the Lesser Flamingo and supports huge flocks of these birds, sometimes exceeding numbers of 2 Mio. birds for a single lake (Kaggwa et al. 2013; Krienitz et al. 2013; Vareschi 1978). Because of its high protein content and nutritional value, dried filaments of *Arthrospira* are also used as dietary supplements in human nutrition and recently even the European Space Agency has shown interest in *Arthrospira* as a food source for their astronauts (Janssen et al. 2010; Melack 1979; Ogato et al. 2014).

Interestingly, measurements of nutrient concentrations in soda lakes have repeatedly shown very low total nitrogen to total phosphorus (Ntot:Ptot) ratios with nitrate and ammonium levels often at the detection limit, indicating N-limitation of those systems (Oduor and Schagerl 2007b; Ogato et al. 2015).

Factors like internal cycling of nutrients can only to some extent describe the discrepancies between the high biomass and productivity and the low levels of inorganic N compounds observed for soda lakes (Ogato et al. 2015). Additionally, Sorokin et al. (2014) mentioned, that not much is known about the role of N_2-fixation at oxic conditions in soda lakes, which might influence the ecosystems N-cycle considerably. These circumstances and the growing number of non-heterocytous Cyanobacteria, which are now known to fix molecular N_2, as well as the outcomes of recent genetic analyses of *A. fusiformis* (Gschwandner, 2017), gave rise to the hypothesis, that *A. fusiformis* might be capable of molecular N_2-fixation.

To test this hypothesis, growth experiments under laboratory conditions with six different *A. fusiformis* strains of different origin were carried out. We tested the hypothesis that the ability of fixing molecular N_2 is reflected in continuing growth under nitrate-limiting conditions with an increase of total N in the medium.

3. Material and Methods

We tested six strains of *A. fusiformis* (provided by the ASW algae culture collection of the university of Vienna) originating from different soda lakes of the East African Rift Valley (**Figure 1**). The strains were labelled according to their origin (Error! Reference source not found.).

Growth experiments were carried out as batch cultures in a climate chamber at constant room temperature of 27°C. Cultures were grown in standard Zarrouk medium (Zarrouk 1966) as control group (**+N**) and under nitrate-limited conditions (**-N**) in Zarrouk medium without $NaNO_3$ addition (the equivalent amount of NaCl was added instead). 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. For experiments, cultures were prefiltered through a plankton net (mesh size 30µm), rinsed with new medium and resuspended.

5L glass reactors with 4 replicates for each treatment were used and illuminated via LED panels (~50µmol photons m^{-2} s^{-1}; light:dark cycle 12h:12h). The cultures were continuously bubbled with 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 d until final harvest.

Optical density (OD) of the cultures and dark yield was monitored daily by harvesting 25 ml of culture. 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: $\mu = (\ln N_t - \ln N_0)/t$ and $t_d = (\ln 2 - \ln 1)/\mu$ (N = OD surrogate parameter of biomass, t = time [d^{-1}]). Dark fluorescence yields (dark yields) of PSII (F_v/F_m)

were measured after 10 minutes of pre-darkening with a PAM-2500/US device (Walz company), given $F_v = F_m – F_0$ (F_0 = minimum fluorescence after dark adaption, F_m = maximum inducible fluorescence, F_v = variable fluorescence; Simis et al. 2012). F_v/F_m serves as a proxy of photosynthetic efficiency (Schuurmans et al. 2015).

Additionally to the monitoring variables mentioned above, a set of parameters including organic dry mass (DM), chlorophyll a (chl a) and total carotenoids, phycocyanin and phycoerythrin, nitrate-N and carbon/nitrogen (CN) contents were analyzed every 4th day during the experiment (day 1, 5, 9 and 13 (12)).

For pigment analyses (chl a, total carotenoid and phycobiline content), 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 carotenoid analyses) and PO_4-buffer (for phycocyanin and phycoerythrin content), homogenized by means of an ultrasonicator (Branson Sonifier 250) and extracted at 4°C for 12h (chl a and total carotenoids) and 24h (phycobilines), respectively. After extraction, the suspension was centrifuged and the supernatant measured spectrophotometrically at 750nm, 663nm, 520nm and 480nm (for chl a and total carotenoids) and 730nm, 618nm and 564nm (for phycobilines). Chl *a* content was calculated according to Lorenzen (1976), total carotenoids according to Strickland & Parsons (1972). Phycoerythrin and phycocyanin contents were estimated according to Sampath-Wiley & Neefus (2007).

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 24h 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 analyser Vario MICRO Cube.

Additionally, the cultures were microscopically checked for contamination every 4th day.

Statistical analyses were carried out in IBM´s SPSS Statistics 21 on a significance level of $p < 0,05$. A repeated measures ANOVA (rANOVA) was conducted to show significant differences for cellular pigment content over time and between treatments. Levene´s test was used as test for 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.

4. Results

Apparently, all cultures showed differences between the two treatments. Within a few days, +N treatments of all strains developed from pale green to very dense, dark-green cultures, whereas -N treatments turned from light green into yellow-green to almost colorless suspensions (**Figure 2**). Observations by light microscope confirmed this pattern, with 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 (**Figure 3**).

The linear regression model for the measured parameters of biomass (particulate C and DM) showed a significant and strong regression with an R^2 of 0,958 and no significant differences between treatments (**Figure 4**). The significant and strong linear regression between OD and DM within both treatments with an R^2 of 0,938 for +N treatments and an R^2 of 0,765 for -N treatments justifies using OD as a surrogate parameter of biomass. The test of homogeneity of regression slopes via an ANCOVA showed significant differences between the treatments ($p = 0,000$). Growth curves of the cultures (**Figure 5**) illustrate the continuous growth of +N treatments with increasing OD until the end of the experiment while -N treatments showed at maximum only slight growth throughout the experiments. t_d´s varied significantly between treatments of all strains (Mann Whitney U-test; $p < 0,05$; Abijata = 0,025; Arenguade = 0,000; Big Momella = 0,000; Nakuru = 0,000; Oloidien = 0,002; Simbi = 0,000) 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 (**Figure 6**).

F_v/F_m measurements (**Figure 7**) exhibit a similar pattern for all strains within the respective treatment. -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).

Regarding the phycobilin content, a significant and sharp decrease of phycocyanin from day 1 towards the end of the experiments was observed in all -N treatments (**Figure 8**). On the 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, 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), contents of -N cultures dropped to values below 3,1 mg g^{-1} DM. Results of the rANOVA 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 and treatment was also significant in most cases, except phycocyanin and phycoerythrin content of Big Momella and phycoerythrin content of Simbi, where

both time and treatment led to a decrease of pigment content. Phycoerythrin contents of algal biomass were rather low (compared to phycocyanin) with values of +N cultures ranging from 0,8 to 4,1 mg g^{-1} DM.

Similarly to phycobilins, a decrease of chl a and total carotenoids from the start to the end of the experiments was observed in all -N treatments (**Figure 9**). The decrease of cellular chl a and total carotenoids over time in -N treatments was significant, while the cellular chl a and total carotenoid content of the +N treatments significantly increased in most of the strains (except Abijata) (**Table 2**). An rANOVA 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 and treatment was significant for all cases. Maximum chl a content was recognized at the end of the experiment in +N treatments; which 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 for chl a content in -N treatments at the end of the experiments varied around 2 mg g^{-1} DM (1,3-3,2 mg g^{-1} DM) and total carotenoid content around 1 mg g^{-1} DM (0,6-1,2 mg g^{-1} DM). The linear regression between chl a and DM is shown in **Figure 4**. ANCOVA results revealed significant different regression slopes between the two treatments ($p = 0,000$) and underline the results mentioned above.

With exception of strain Simbi, total N content of -N treatments showed no significant increase from day 1 to the end of the experiment (Abijata: 4,8 to 4,8 mg L^{-1}; Arenguade: 4,0 to 4,1 mg L^{-1}; Big Momella: 2,5 to 2,4 mg L^{-1}; Nakuru: 3,5 to 3,8 mg L^{-1}; Oloidien: 4,7 to 4,3 mg L^{-1}; Simbi: 2,7 to 3,4 mg L^{-1}; **Table 3**). Also in +N treatments, no significant increase of total N content was detected. The development of N per unit DM throughout the experiments is given in **Figure 10**. 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. The significant decrease of N content of algal biomass in -N treatments from the start to the end of the experiments (**Table 3**) is also reflected in the increasing C:N ratios which are depicted in **Figure 11**. 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). A Mann Whitney U-test revealed significant differences of C:N ratios between treatments of all strains ($p < 0,05$; Abijata = 0,000; Arenguade = 0,000; Big Momella = 0,000; Nakuru = 0,000; Oloidien = 0,000; Simbi = 0,000). The significant different regression slopes of particulate N and particulate C within the treatments (tested via ANCOVA, $p = 0,000$) are shown in **Figure 4**.

5. Discussion

Macroscopic and microscopic observations, and continuous measurement of OD throughout the experiments yielded all the same, homogenous picture: +N treatments of all examined strains showed continuous growth until the end of the experiments, resulting in dense, dark-green to blue-green cultures with long, healthy green filaments, while -N treatments showed at maximum only slight growth during the first days of the experiments, resulting in stagnating cultures which turned from the initial typical green coloration to pale yellow-green, sometimes even almost colorless, decaying cultures with poor N-starving filaments being much shorter and yellowish brown in coloration. Opposing our hypothesis, none of the examined strains showed continuing growth under nitrate-depleted conditions, which stands also in contrast to the findings of Gschwandner (2017). The sustained growth of the -N treatments during the first days of the experiments can be explained by the maintenance of cell division and other vital functions through the usage of internal storage products.

Cyanophycin, a polypeptide consisting of two amino acids, aspartic acid and arginine (Simon 1971), is a well-known N-storage protein occurring in Cyanobacteria (Lawry and Simon 1982; Shively 1974). It was also observed in *A. fusiformis* by Van Eykelenburg (1980) but only at temperatures below 17°C and at times when cultures approached the stationary phase. Much more important at the growth conditions provided, phycocyanin acts as a N-storage protein in *A. fusiformis* (Boussiba and Richmond 1980). Phycocyanin, together with phycoerythrin, belongs to the phycobiliproteins, which are organized in phycobilisomes and are the major light harvesting complexes in Cyanobacteria, also accounting for their typical blue-green coloration (Fay 1992). Boussiba & Richmond (1980) and Gschwandner (2017) were already able to show, that N-limited cultures of *A. fusiformis* continued to grow unaffectedly for several days, using phycocyanin as an internal N-resource, which fits our results. Phycocyanin and phycoerythrin contents of -N treatments already reached minimum values on day five of the experiments, indicating severe N-starvation and almost complete consumption of the entire N-storage pool. Moreover, phycobilin content of -N treatments remained at minimum values until the end of the experiments and none of the strains were able to recover from N-starvation, which would have indicated the ability of N_2-fixation. The phycocyanin content of well supplied +N treatments varied highly between the different strains (7,5 – 83,7 mg g^{-1} DM) and lies at the lower end of the range of phycocyanin contents reported for *Arthrospira* between 5-18% DM (Sethu 1996; Xie et al. 2015). Given the importance of phycocyanin in various industries and it´s high market value (Sethu 1996) and the observed high variations of phycocyanin content even between closely related strains of *A. fusiformis* (Gschwandner 2017), a proper selection of adequate strains and certain culture conditions with special emphasis on irradiance supply are advised for biomass production.

The degradation of phycocyanin with advancing N-starvation in the -N treatments is also reflected by the results of pulse-amplitude modulated (PAM) fluorimetry measurements (**Figure 7**), which were conducted daily to monitor the overall photophysiological status of the cultures. In Cyanobacteria, phycobilins interfere with fluorescence yield measurements by increasing the background fluorescence (F_0) and leading to an underestimation of the quantum yield of PSII (Campbell et al. 1998). Reported dark yields for green algae are around 0,7 while those of Cyanobacteria are typically found at 0,4 (Schuurmans et al. 2015), which is consistent with our measured dark yields between 0,4 and 0,6 for well supplied +N treatments. For most of the cultures (especially Nakuru and Abijata), -N treatments showed an increase of dark yield during the first days of the experiments because of the degradation of phycocyanin as a consequence of N-starvation, resulting in lower background fluorescence and therefore higher dark yields. Around day 5 of the experiments, dark yields of the -N treatments however became greatly reduced. Considering other parameters such as chl a and microscopy, this strong decrease indicates severe N-starvation. According to Hockin et al. (2012), the down-regulation of photosynthesis is a universal response to N-starvation. Dark yields of +N treatments on the other hand showed only slight changes and are constant throughout the experiments, except for Big Momella, which showed an increase of dark yield from around 0,3 to 0,6. This increase can be explained by a decrease of phycobilin content from the start to the end of the experiment. The invariable dark yields of +N treatments around 0,5 indicate a good photophysiological status and acclimatization towards the optimal growth conditions. In conclusion, also PAM measurements revealed no hints of N_2-fixation by *A. fusiformis*.

Acclimatization of +N treatments towards the prevailing optimal growth conditions can also be observed by the even increasing chl a and total carotenoid content per unit DM throughout the experiments (**Figure 9**). Besides the light harvesting phycobilins in Cyanobacteria, chl a is the essential photosynthetic pigment and primary electron donor in all oxygenic photosynthetic organisms (McConnell 2002), containing four N atoms in the chlorine ring. Also carotenoids serve as light-harvesting pigments in Cyanobacteria but are only built up of C, hydrogen (H) and oxygen (O) and are responsible for various shades of yellow, orange and red (Hirschberg et al. 1994). While the increase of total carotenoids in +N treatments is only moderate, the more distinctive increase of chl a and phycocyanin per unit DM is a result of good growing conditions and N supply, which is also reflected in the strong, blue-green to dark-green coloration of the cultures. In -N treatments, the chl a content decreased significantly and continuously from the start towards the end of the experiments, but not as sharp and pronounced as observed for phycocyanin, with a sort of minimum threshold value of around 2 mg g^{-1} DM. This threshold can be explained by the importance of chl a as the primary electron donor in the photosynthetic electron transport chain. In -N treatments, with increasing N-starvation, carotenoids are probably taking over functions as accessory pigments since there is only a rather slight

decrease of total carotenoids from the start to the end of the experiments. This might be because carotenoids are only consisting of C, H and O (with carotenes being pure hydrocarbons) and therefore, the cyanobacterial cell should still be able to synthesize carotenoids under N-limited conditions. The stimulating effect of nitrogen deficiency to the synthesis of carbohydrate fractions and production of non-nitrogenous material was already described by Olguín et al. (2001) and Zhekisheva et al. (2002). 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 (1996). 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 (**Figure 2**). Richardson et al. (1969) and Kolber et al. (1988) already reported a decreasing chlorophyll content of different algae species which accompanied N-limitation. Also, the chl a content of around 10 mg g^{-1} DM of well supplied +N treatments is consistent with already reported chl a content of 1% DM (which equals 10 mg g^{-1} DM) for *Spirulina platensis* (Kebede 1997). The linear regression of chl a and DM for both treatments is shown in **Figure 4** and exhibits significant differences of the regression slopes between the two treatments, which is most likely an effect of N-limitation. Li et al. (2008) also hypothesized that the N rich compound chl a acts as intracellular N pool of algal cells and is utilized with increasing N starvation. Furthermore, since external N supply is limited, the internal concentration of N rich compounds like chl a is increasingly lower with each cell division. Summarizing the results from the pigment analyses, no evidence of N_2-fixation by *A. fusiformis* was found.

The strongest evidence for N_2-fixation would have been a significant increase of total N in the -N treatments from day 1 to 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. Besides N_2-fixation, an undetected contamination of the cultures with heterotrophic N_2-fixing bacteria or contamination by N_2-fixing *Synechococcus sp*. (which caused problems in the past in the ASW culture collection) could be a plausible explanation for this result. Considering all other measured parameters and the overall development of the culture, N_2-fixation by *A. fusiformis* is however unlikely.

A. fusiformis exhibits a quite high protein content of up to 70% of the biomass (Colla et al. 2007; Olguín et al. 2001; Sethu 1996) which explains its nutritional value. Lopez et al. (2010) reported a N content of around 12% of DM for *Arthrospira*, being the highest content of the five examined microalgae and Cyanobacteria in their study. 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 (= 6,5 to 8,9 % of DM) and are a little bit lower than the reported N content of *Arthrospira* by Lopez et al. (2010). As a result of excess N and good growing conditions, all +N treatments showed an increase of N per unit DM from the start to the end of the experiments (**Figure 10**). In contrast, N-limited -N treatments showed a significant decrease of

N per unit DM in the course of the experiments. Interestingly, the 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 *A. fusiformis* is comparably low (Markou et al. 2012). Redfield et al. (1963) described the classical Redfield ratio of C_{106}:N_{16}:P_1 (= $C_{6,6}$:N_1) which is a cornerstone of biogeochemistry (Sterner et al. 2008). The $C_{6,9}$:N_1 ratio (C_{69}:N_{10}:P_1) of the normal Zarrouk medium is quite close to the generally applicable Redfield ratio. Interestingly, particulate C:N ratios of +N treatments, grown in normal Zarrouk medium, deviate from the C:N ratio of the media (**Figure 11**). All +N treatments showed a rather constant C:N ratio of around 4,3:1 throughout the experiments. The comparably low C:N ratios of well supplied cultures reflect the high protein content of *A. fusiformis* and its ability to store large amounts of N in the form of phycocyanin when there is excess N in the surrounding media. In contrast, C:N ratios of all -N treatments significantly increased (**Table 3**) from around 4,3:1 at the start of the experiments to 8,3-17,3:1 at the end of the experiments. This increase of particulate C:N ratios is a clear result of N-limitation and integrates all other observations we made.

The different elemental composition of algal biomass in both treatments is also shown in the differing slopes of the regression lines of particulate C and N between the two treatments (**Figure 4**). The relation for both treatments is quite strong and significant and shows that with increasing C content, the increase of N is significantly higher in +N treatments. This clearly indicates that cells of -N treatments are facing N-deficiency as a result of N-limitation since N-uptake from the media is limited and N content is progressively decreasing with each cell division. The strong regression between particulate C and DM indicates a good application of both parameters as descriptors of biomass. Also OD, which we used as a surrogate parameter of biomass, correlates well with DM within the treatments, although ANCOVA results revealed significant differences between the regression slopes of the two treatments. OD, or also absorbance, is a measure influenced by cell size, shape and refractive index (Griffiths et al. 2011). Observations in the light microscope (**Figure 3**) showed that cells of -N treatments were increasingly shorter and less pigmented in the course of the experiment which leads to altered optical properties and is most probably reflected in the different regression slope of the -N treatments.

Summarizing up, no evidence of N_2-fixation by *A. fusiformis* was found and consequently, we have to reject our hypothesis. In contrast, Gschwandner (2017) assumed that *A. fusiformis* might be capable of N_2-fixation based on her results. Most probably, too much nitrate, which was introduced into the N-limited treatments of her experiment, led to a misinterpretation of the results. Besides that, she was able to show, that all the strains we were examining carried the *nif*H gene and that the strains

Arenguade, Nakuru, Big Momella and Abijata even expressed the *nif*H gene under N-limiting conditions. Since the *nif*H gene is only one of the structural genes within the *nif* operon encoding for nitrogenase (Fay 1992; Raymond et al. 2004), it is likely that the presence and expression of the *nif*H gene cannot be considered as an general indication of N_2-fixation. In contrast to the findings of Gschwandner (2017), genome sequencing of *A. platensis* by Cheevadhanarak et al. (2012) and Fujisawa et al. (2010) indicated that *A. platensis* is not able to fix N_2, because nitrogenase genes were lacking. Also genome data of *Arthrospira sp.* from Janssen et al. (2010) lacked essential *nif* genes. Therefore we presume that most probably only remnants of a fully functioning *nif* operon are conserved in *A. fusiformis*.

The effects of N-limitation on the cultures of *A. fusiformis* in our experiments are consistent with published effects of N-limitation on *Arthrospira* and other algae species, like the reduction of protein content (especially phycocyanin) and consequential reduction of N content, increase of carbohydrate content, down-regulation of photosynthesis and reduced synthesis of chl a (Boussiba and Richmond 1980; Hockin et al. 2012; Olguín et al. 2001; Sassano et al. 2010).

N_2-fixation of *A. fusiformis* could have explained some of the discrepancies between extremely high algal biomass and low levels of N sources in East African soda lakes. According to Sorokin et al. (2015), the highly productive, moderately saline soda lakes exhibit high microbial richness and activity with diverse haloalkaliphilic prokaryotes being responsible for the cycling of life-important elements, such as C, N and Sulfur, but a lot of questions regarding the biogeochemical cycles in soda lakes remain unanswered. Internal cycling of nutrients was found to have an overriding role in controlling the nutrients of lake Chitu (Ethiopia) and over 90% of the total N being in the algal biomass were reported for productive lakes like Chitu, which is regenerated during decomposition (Ogato et al. 2015). However, in hypersaline soda lakes, the N-cycle is partly inhibited by cessation of nitrification at high salt concentrations and those ecosystems (as well as moderately saline soda lakes) most probably rely on nitrate exports from surrounding soils or other externally supplied sources of N, like flamingo excreta or feather degradation (Ogato et al. 2015; Sorokin et al. 2014; Sorokin and Kuenen 2005). The overall high content of phycocyanin in *A. fusiformis*, which acts as an important N-storage product, could provide an explanation for its dominance in this N-limited ecosystems. Rhee & Gotham (1980) already reported that the storage pool size of Phosphorus (P) is much larger than that of N in planktonic algae. This fact and the potentially enhanced ability of *A. fusiformis* to store N, might give it an essential competitive advantage because it may react quickly to sudden inputs of N. Since *Arthrospira* preferentially utilizes NH_3 at high pH and is resistant to its toxicity, higher concentrations of NH_3 in the lower parts of the water column (as observed for lake Chitu) could serve as a N source for *Arthrospira* and represent an important link in the N-cycle of soda lakes (Boussiba 1989; Ogato et al. 2015).

Nevertheless, with our findings of *A. fusiformis* being not able to fix molecular N_2, the role of N_2-fixation at oxic conditions for the N-cycle of soda lakes remains unclear und still needs to be investigated.

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8. List of tables

Table 1: origin of examined strains

Lake	Country	Coordinates
Abijata	Ethiopia	7.617943, 38.599957
Arenguade	Ethiopia	8.695683, 38.976475
Big Momella	Tanzania	-3.223536, 36.909792
Nakuru	Kenya	-0.350799, 36.099956
Oloidien	Kenya	-0.813531, 36.277838
Simbi	Kenya	-0.367234, 34.628952

Table 2: results of repeated measures ANOVA for all analyzed pigments (per unit DM, significance level of $p < 0,05$)

Legend:

+... 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		total 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	-		-		-		-	

Table 3: summary of all results of Mann-Whitney-U-Test between start and end of experiments regarding the N content (significance level of $p < 0,05$)

Legend:

+... significant increase between start and end of experiment

-... significant decrease between start and end of experiment

=... no significant change between start and end of experiment

	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	+	=	+	-	+
Oloidien +N	+	-	=	+	=

9. List of figures

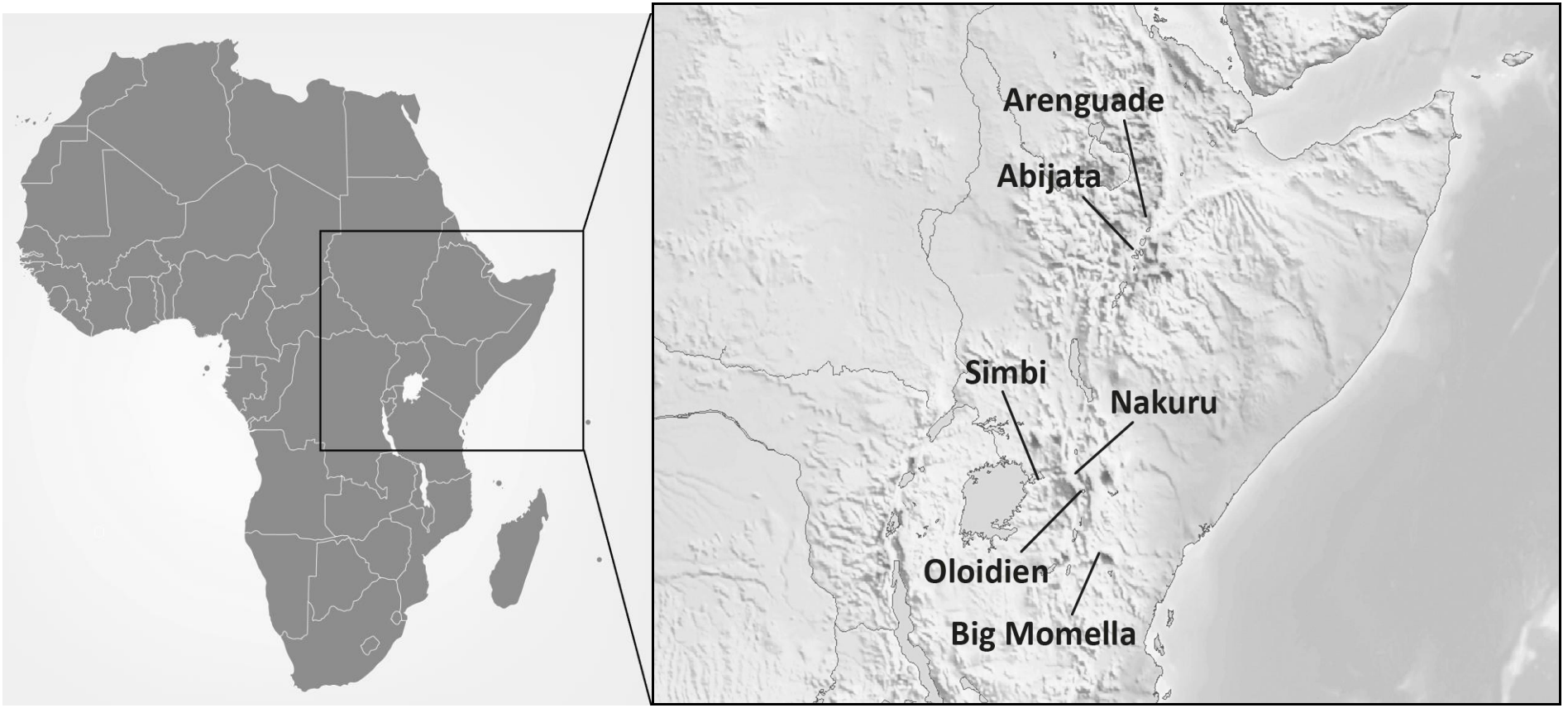


Figure 1: map of Africa (Created by Freepik) and location of lakes (edited, source: https://commons.wikimedia.org/wiki/File:Africa_topography_map.png)

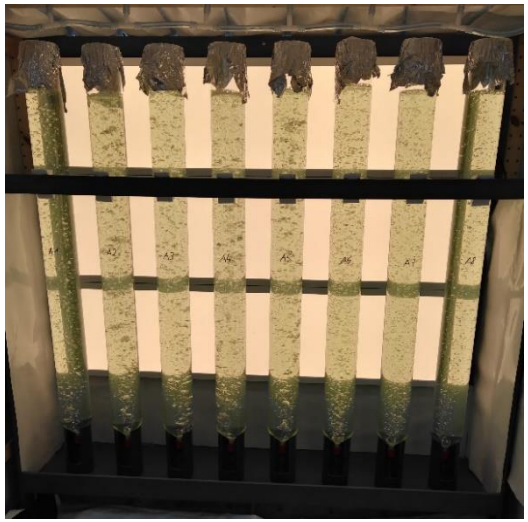
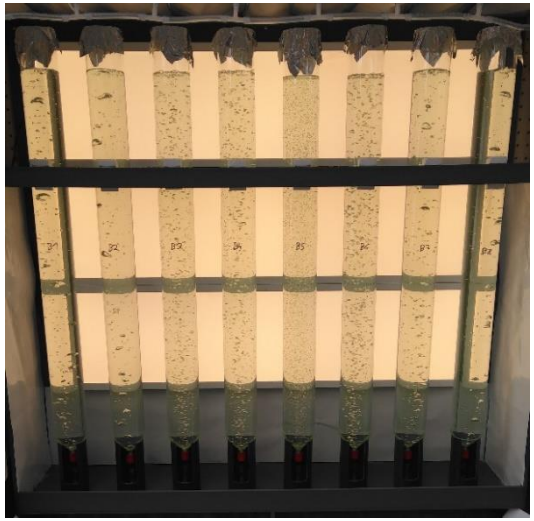
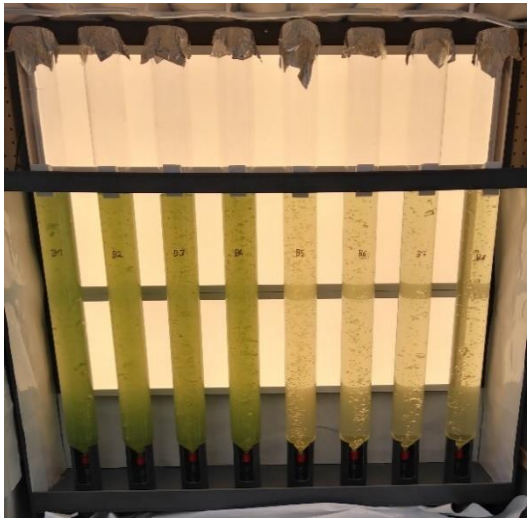
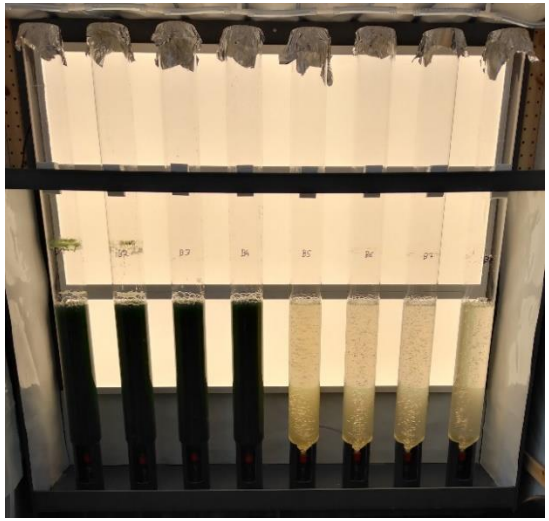
strain	start of experiment	mid of experiment	end of experiment
Abijata			
Nakuru			

Figure 2: macroscopic development of the cultures for two selected strains (Abijata and Nakuru)

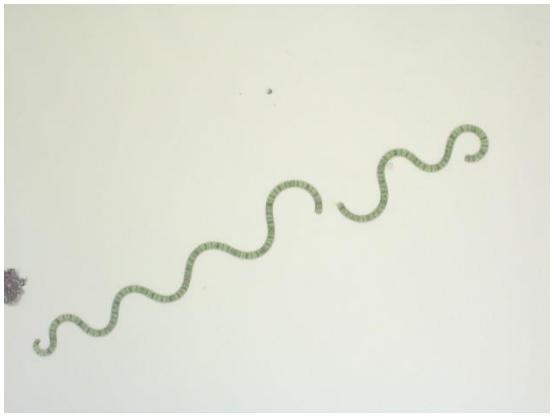
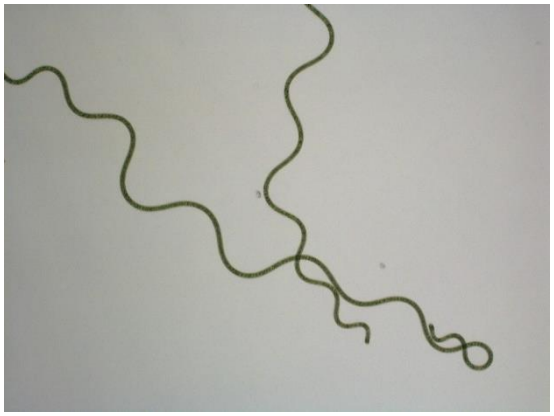
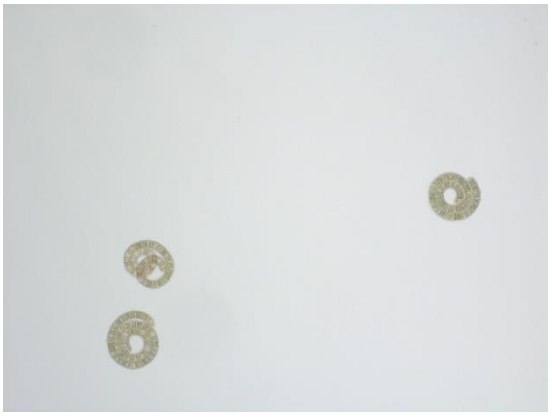
strain	start of experiment	end of experiment
Abijata		
Arenguade		
Nakuru		

Figure 3: microscopic development of -N treatments for three selected strains (Abijata, Arenguade and Nakuru)

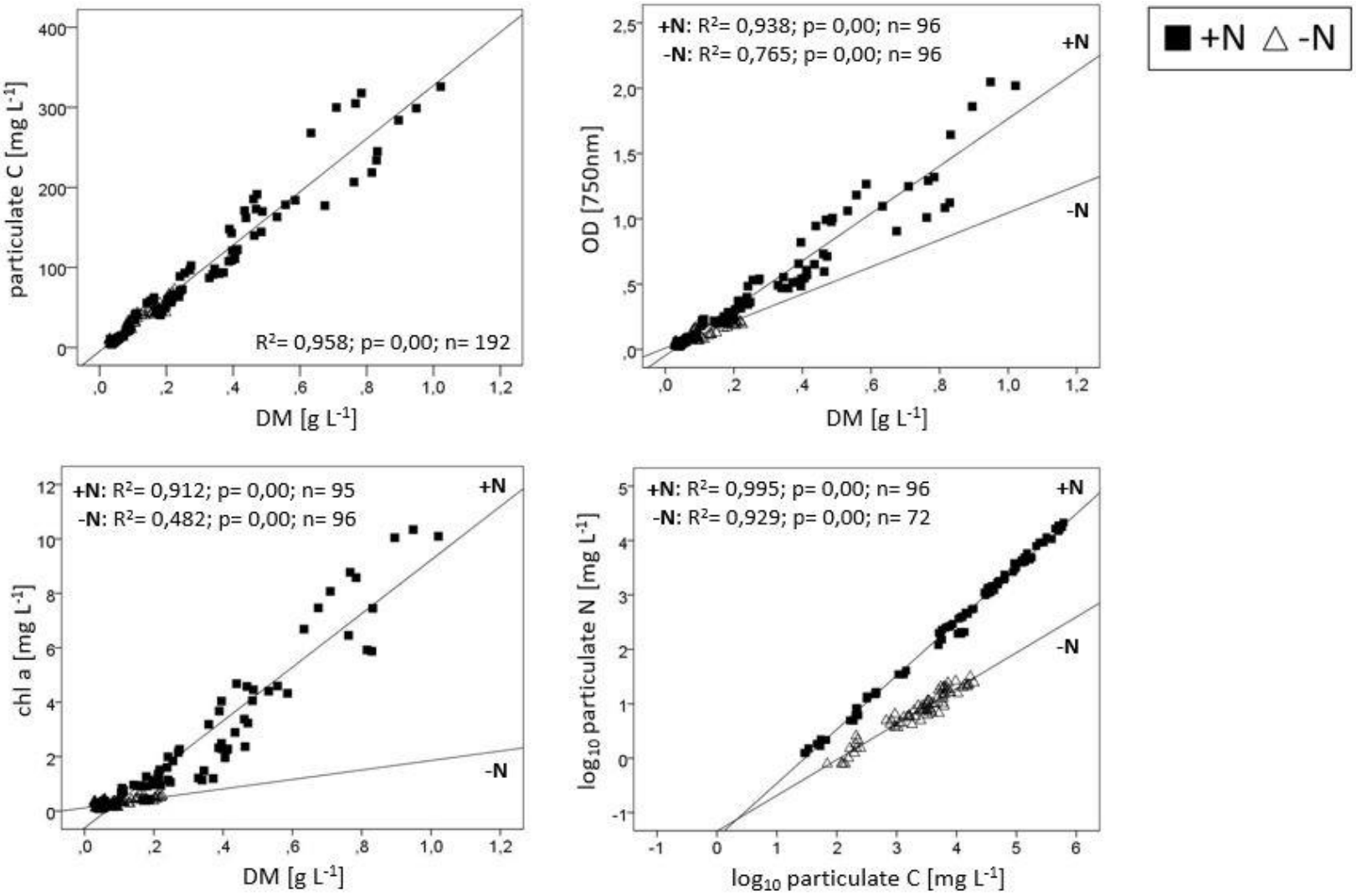


Figure 4: linear regression models between measured parameters of biomass (particulate C and DM) and measured indicators of biomass (OD and chl a) and log transformed linear regression between particulate C and particulate N. ANCOVA results showed significant ($p < 0,05$) differences between regression slopes of the two treatments for OD/DM, chl a/DM and particulate N/particulate C

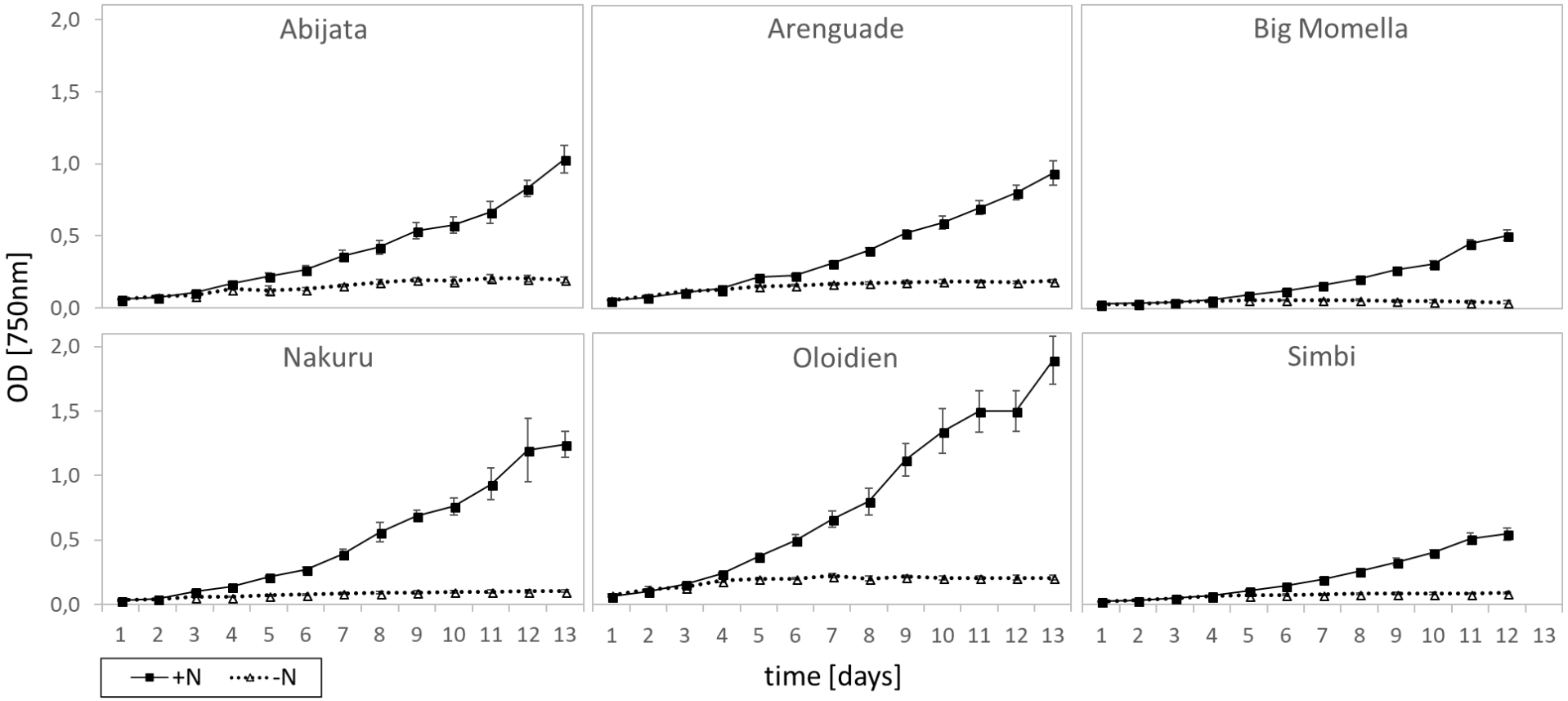


Figure 5: growth curves of examined strains based on OD measured every day during the experiments at 750nm, error bars = SD

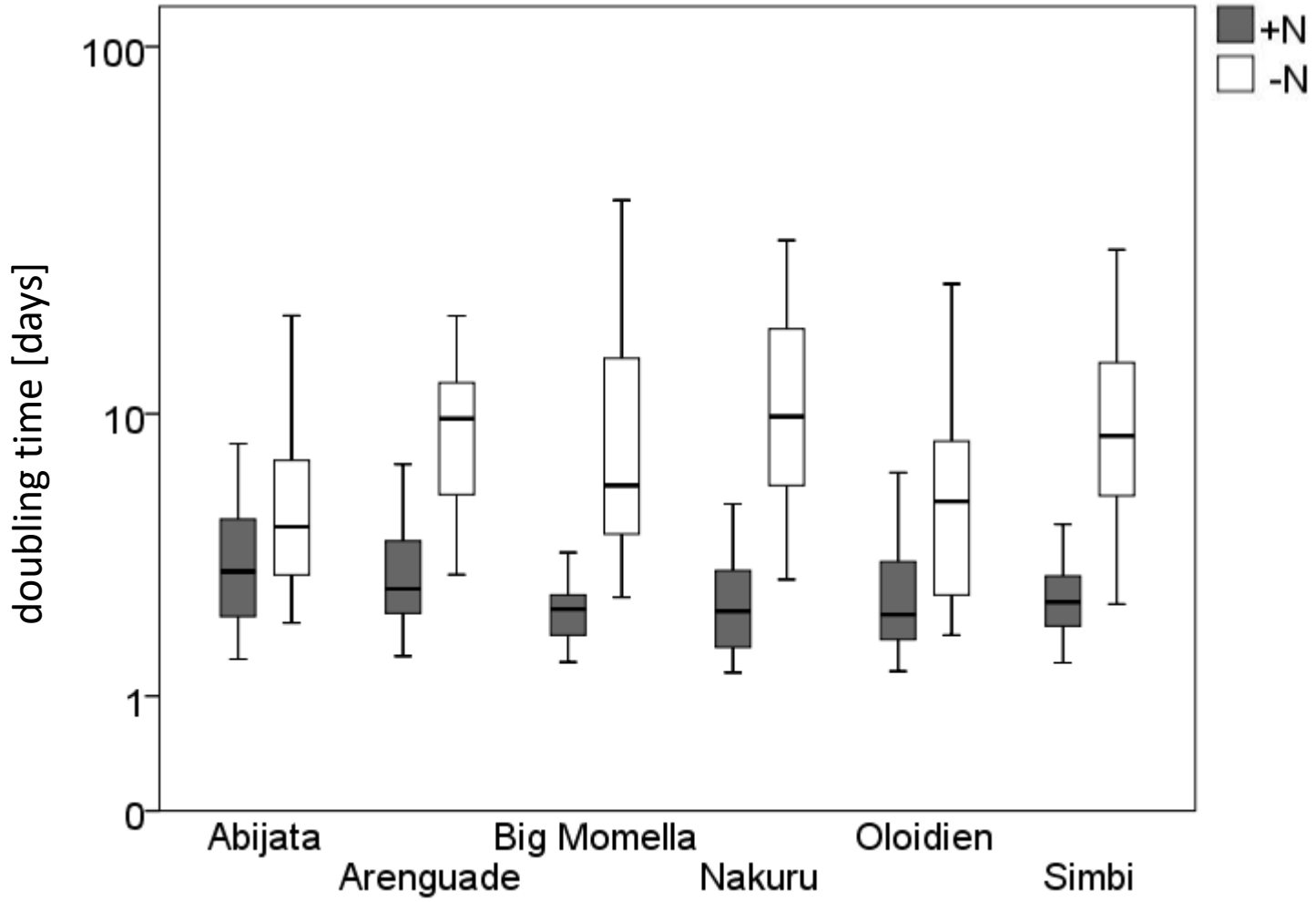


Figure 6: boxplot diagram of calculated doubling times (in days) for all examined strains

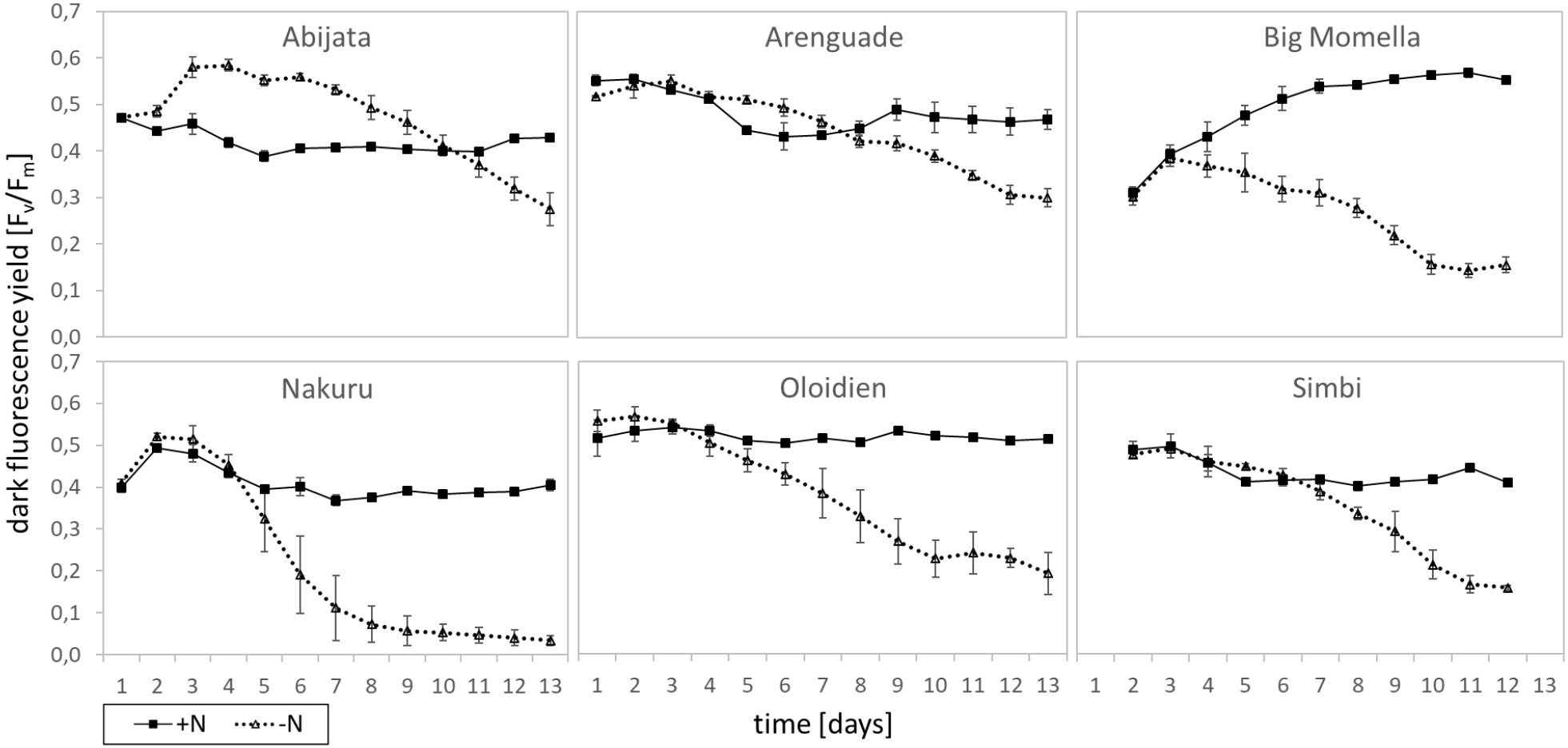


Figure 7: dark fluorescence yield of all examined strains measured every day during the experiments, error bars = SD

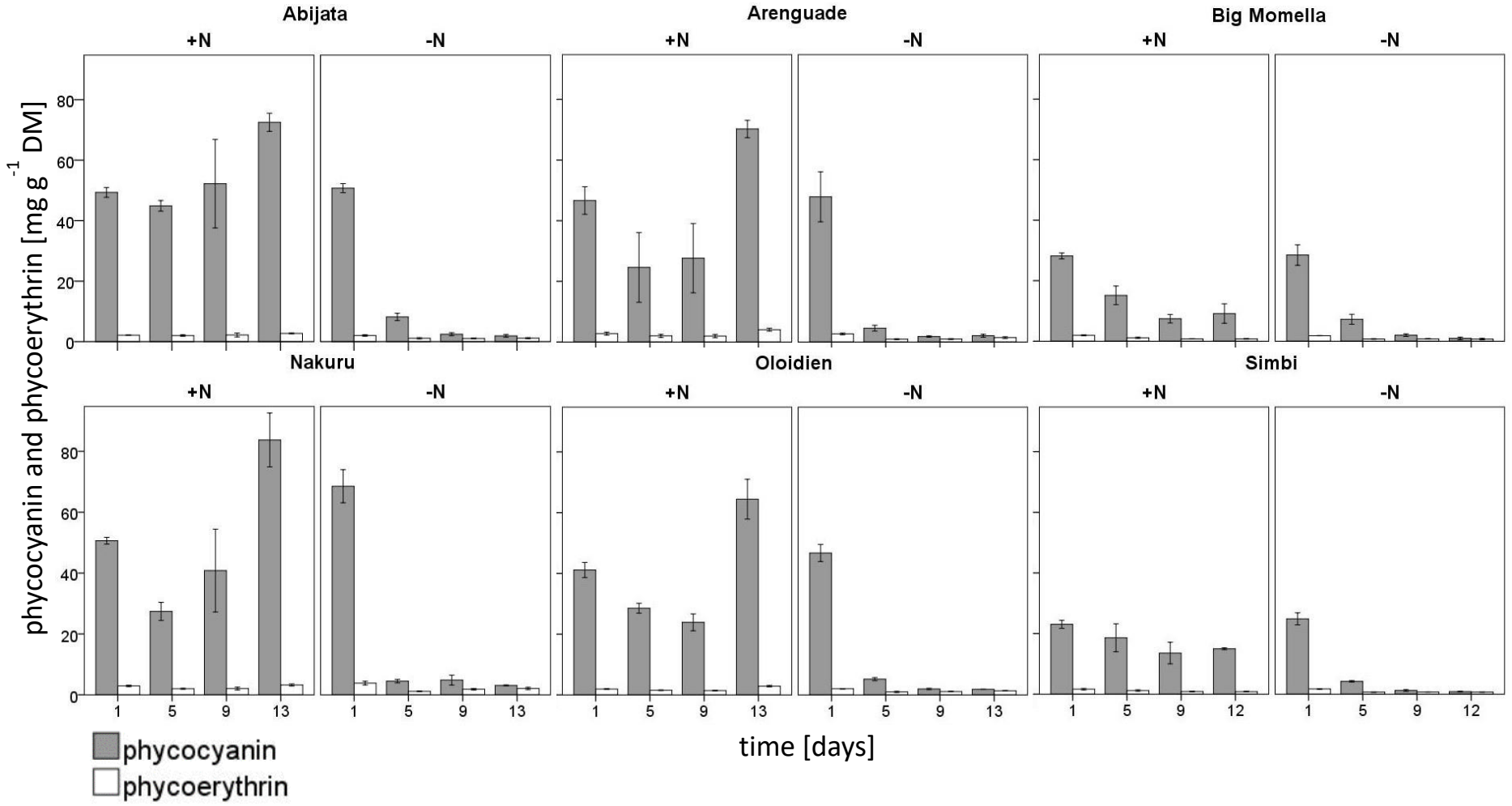


Figure 8: phycocyanin and phycoerythrin content of all examined strains measured 4 times during the experiment for +N treatments (left) and -N treatments (right), error bars = SD

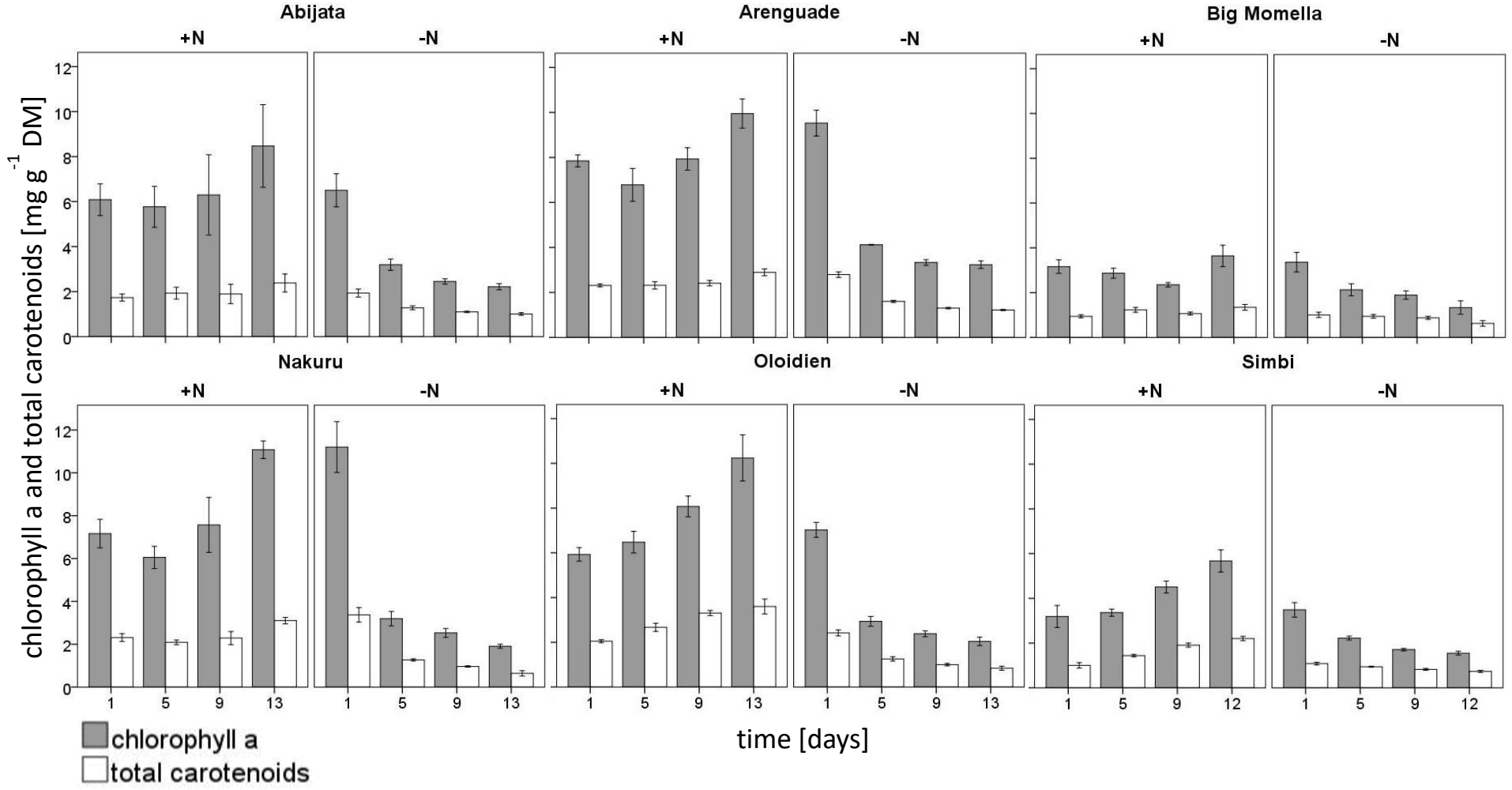


Figure 9: chlorophyll a and total carotenoid content per unit DM of all examined strains for +N treatments (left) and -N treatments (right) measured 4 times during the experiments (day 1, 5, 9, 13 (12)), error bars = SD

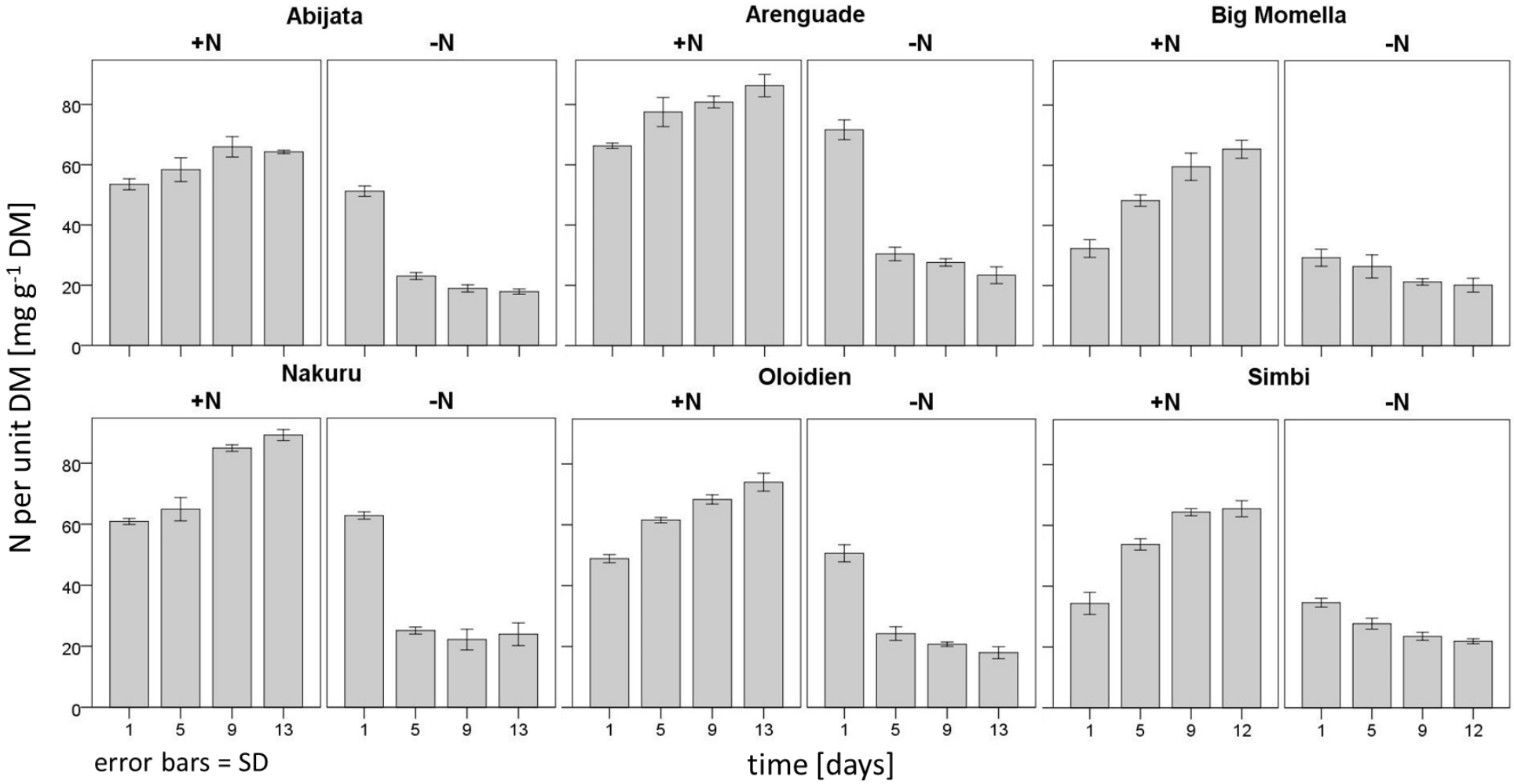


Figure 10: N per unit DM of all examined strains for +N and -N treatments measured 4 times during the experiment, error bars = SD

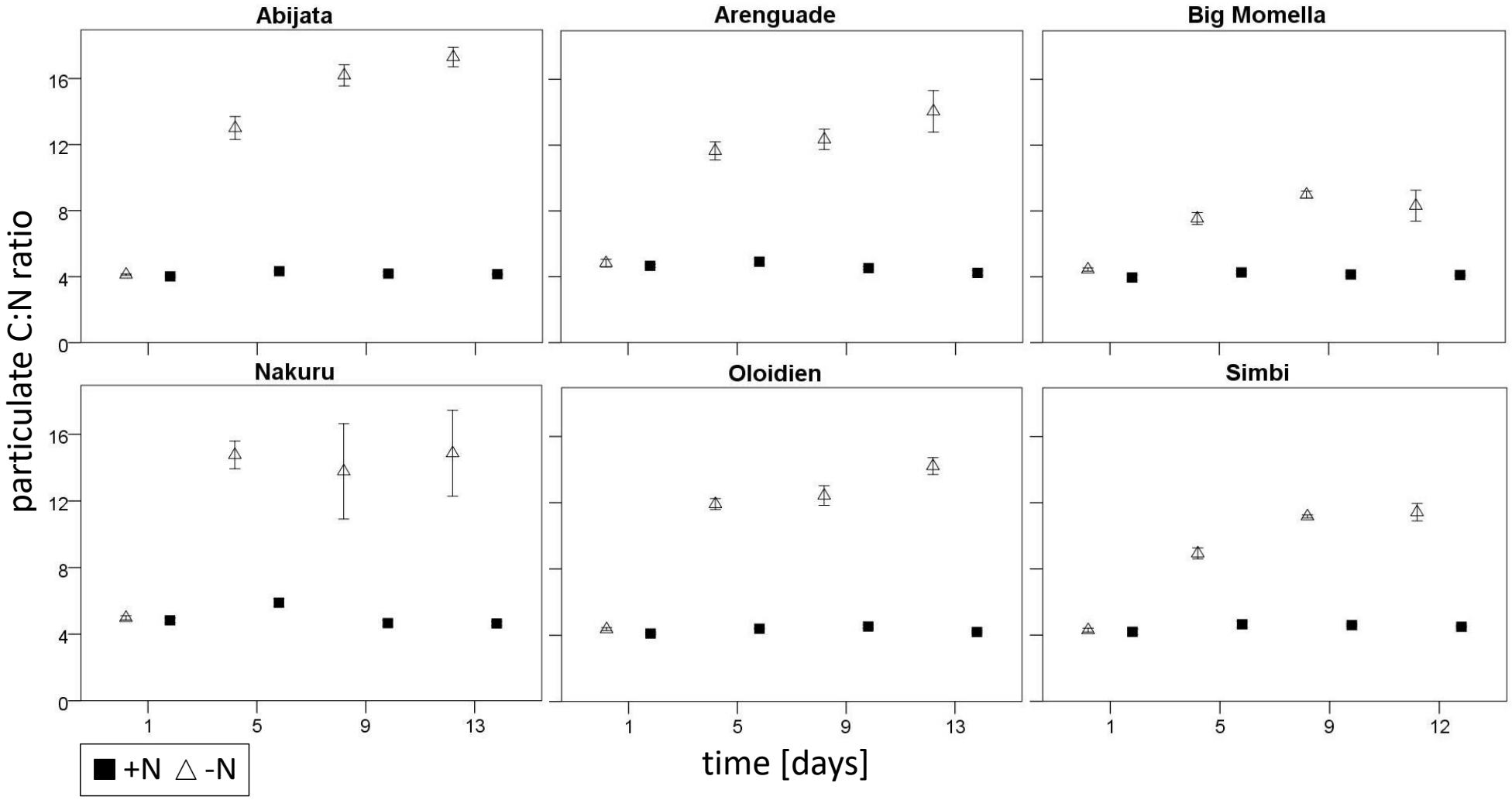


Figure 11: particulate C:N ratios of algal biomass of all examined strains for +N and -N treatments measured 4 times during the experiments (day 1, 5, 9, 13 (12)), error bars = SD