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Hydrogen production and metabolic response of *Spirulina platensis* , *Calothrix parteniana* and *Oscillatoria* sp to sulphur and /or nitrogen deficiency

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ABSTRACT

Spirulina platensis SAG obtained from (Sammlung von Algen culturing Gottingen), *Calothrix parteniana* and *Oscillatoria* sp. (isolated from Egyptian soil sample) were cultivated in BG11 medium either in the presence of nitrogen and sulphur (control culture condition) or without addition of nitrogen (N_0) or sulphur (S_0) and combined nitrogen and sulphur (N_0S_0) source to detect the effect of these element limitation on growth and hydrogen production of the tested algal isolates . The growth expressed as dry weight and chlorophyll content was generally inhibited in treated culture sample (N_0 and /or S_0) of all sp under study. Photosynthetic O_2 evolution expressed as μ mol O2↑/mg Chl.a /h. of *Spirulina sp and Calothrix parteniana*,*Oscillatoria sp* were severely dropped under nitrogen deficiency compared with control and sulphur limited growth condition. Respiratory oxygen uptake was however, increased with the above deficiency effect in all investigated spp. Assay of Hydrogen production using hydrogen electrode (expressed as nmol H_2↑/mg.chla/h) *revealed* that nitrogen or sulphur deficiency growth condition stimulated hydrogen production in all isolate under testing. Combination of both element deficiencies has an inhibition effect on hydrogen yield in *Calothrix parteniana* and *Oscillatoria sp.* Amazingly our results indicate that *Spirulina sp was the only culture able to produce hydrogen in combined nitrogen and sulphur deficiency growth condition,* Spirulina produce hydrogen in dark condition only, while the presence of light was very important for hydrogen production in other investigated cyanobacterial cultures. The above effect of nitrogen and sulphur deficiency demonstrate that both elements play a crucial role in growth and metabolism especially hydrogen production.

Key word : hydrogen production , cyanobacterial culture ,nitrogen and /or sulphur deficiency

1. INTRODUCTION

The worldwide energy need has been increasing exponentially, the reserves of fossil fuels have been decreasing, and the combustion of fossil fuels has serious negative effects on environment because of CO_2 emission. For these reasons, many researchers have been working on the exploration of new sustainable energy sources that could substitute fossil fuels. Hydrogen is considered as a viable alternative fuel and “energy carrier” of future. Hydrogen gas is clean fuel with no CO_2 emissions and can easily be used in fuel cells for generation of electricity. Besides, hydrogen has a high energy yield of122 kJ/g, which is 2.75 times greater than hydrocarbon fuels. The major problem in utilization of hydrogen gas as a fuel is its inavailability in nature and the need for inexpensive production methods, [1] Demand on hydrogen is not limited to utilization as a source of energy. Hydrogen gas is a widely used feedstock for the production of chemicals, hydrogenation of fats and oils in food industry, production of electronic devices, processing steel and also for desulfurization and re-formulation of gasoline in refineries.

Cyanobacteria, form a large and diverse group of oxygenic photoautotrophic prokaryotes, survive in a wide variety of environmental extremes ranging from ultra-oligotrophic oceans to nutrient-enriched estuarine, aphotic and anoxic waters, geothermal and sulfide-rich conditions for details See reviews [2,3].Many of Cyanobacteria have the ability to produce hydrogen ,Hydrogen production has been studied in a very wide variety of cyanobacterial species and strains for details see review [4].Hydrogen production occurs within at least 14 Cyanobacteria genera, under a vast range of culture conditions [5]. Since Cyanobacteria are of evolutionary interest, as they are highly adaptable to various environmental extremes recently, researchers were analyzing different cyanobacterial strains to find out a suitable strain for cyanobacterial biohydrogen production .Several studies, directly comparing the hydrogen evolution by different cyanobacterial strains have shown to be extremely useful. Our investigation aims to investigate and compare production of hydrogen gas from three blue green algae *Calothrix parteniana , Oscillatoria* sp isolated from Egyptian soil sample and *Spirulina platensis (SAG*) under nitrogen and /or sulphur deficiency condition trying to get and optimize the best condition for high hydrogen yield and spot on the effect of these elementary limitation on growth and some metabolic activity of these chosen algae .

2. MATERIAL AND METHODS

Cultivation of *Spirulina* sp. was done as described in [6] in 500 ml Erlenmeyer flask and the cultures were gassed with sterile air provided by a small air pump operating at a rate of 0.046 vvm (volumetric flow rate of air per volume of liquid per minute), at 30°C. The pH of the medium was adjusted to pH 8.3 prior to autoclaving .The cultivated flasks continuosly illuminated with continuous cool white fluorescent lamp at 48.4 mole photon.m-2.s-*Calothrix parteniana* and *Oscillatoria sp*. *were grown also* in BG11 modified medium [7] . The all three culture sample were grown either in the presence of nitrogen and sulphur (control culture condition), without addition of Nitrogen (N_0) or sulphur (S_0) and combined nitrogen and sulphur (N_0S_0). Growth has been assessed by measurement of the optical density, (absorbance of cyanobacterial cells was monitored throughout the growth period at 750 nm or by determination of dry mass (in case of Spirulina), and Chlorophyll content (for all investigated culture).Chlorophyll content was determined according to the method described by [8, 9] calculated and expressed as µg / ml algal suspensions. Photosynthetic activity and respiration of the control and treated sample was recorded using Clark type electrode computerized to an oxygraph (Hansatech,Inc., donation from the Alexander von Humboldt foundation to R. Abdel Basset). 2ml of culture samples were used and photosynthesis was recorded at light and then switches the light off for determination of Respiration. Photosynthesis is expressed as (µ mol $O_2\uparrow$/mg chlorophyll/h) and respiration expressed as (µ mol $O_2\downarrow$/mg chlorophyll/h). Hydrogen evolution was monitored using a Clark type oxygen electrode after being plated as recommended by Hansatech Inc.; 2ml of culture with known chlorophyll a content was added to the electrode chamber and hydrogen evolution was registered in the dark and then in the light.

3. RESULTS AND DISCUSSION

Nitrogen limitation reduced the growth of all cultures under investigation (absorbance of Calothrix and Oscilltoria) or dry weight of *Spirulina platensis* as shown in fig. 1(a,b,c) compared to growth in nitrogen-supplemented medium , In N-deficiency conditions, chlorophyll content were also reduced compared to regular growth condition as in Fig. 2 (a,b,c)**.**Nitrogen is an essential major element required for the synthesis of primary and secondary amino acids, proteins, nucleic acids, coenzymes, chlorophyll and other accessory photosynthetic pigments (phycobilins in cyanobacteria) [10]. Nitrogen comprises about 10% of cell dry weight in cyanobacteria. Kumer-Saha et al., [1] found that exclusion of combined nitrogen ($NaNO_3$) from the growth medium caused certain changes in metabolic processes leading to cessation in growth of the non-heterocystous, non nitrogen-fixing marine cyanobacterium Oscillatoria willei BDU 130511. Nitrogen limitation in O. willei BDU 130511 resulted in the reduction of photosynthetic pigments such as chlorophyll a, carotenoids and phycocyanin resulting in chlorosis [1,6] found that The growth of *Spirulina* sp. expressed as daily change in chl. a content were generally inhibited by nitrogen or sulphur deficiency growth condition

Photosynthetic oxygen evolution generally decreased in nitrogen deficiency sample compared to control culture (Fig.2). It is known that in photosynthetic organisms N limitation triggers ordered degradation of phycobilisomes, ribosomes and thylakoid membranes [11].N deficiency affects, to various extents, primary photosynthesis, sugar metabolism [12-14] The prominent effects observed during nitrogen starvation/limitation were reduction of major and accessory photosynthetic pigments, impairment of photosynthesis due to loss of one major Rubisco isoenzyme [1].

In contrast to reuction of photosynthesis, higher respiration rates were recorded in nitrogen omitted culture (Fig.3).This results were accordance with [15].

In N-starved cultures of Spirulina platensis, the rate of respiration was reported to have increased [16]. A high rate of respiration might be one of the reasons for induction of oxidative stress through the generation of active oxygen species [17]. Nitrogen limitation induced hydrogen production of the all culture under testing, Amazingly light is necessary for hydrogen production in case of Calothrix and Oscillatoria , Spirulina produce hydrogen only in dark condition Fig (4,5,6). Hifney and Abdel-Basset 2014 [15] ,examined the filamentous cyanobacterium *Nostoc* sp PCC 7120 (wild type), its hydrogen uptake deficient mutant Hup- 7120 and also *Nostoc* sp strain ATCC 29133 ,for their H2 production capacities under nitrogen and/or sulphur deficiency growth condition compared with control culture condition and found that hydrogen production of all tested Nostoc Spp with nitrogen and/or sulphur deficiency dependence on culture age and presence or absence of light.The highest amount of hydrogen produced under nitrogen deficiency was recorded in case of *Spirulina platensis* (136.1 nmol H_2↑/mgchla/h after 82.6min in dark condition) However,Calothrix and Oscillatoria produced only 6.2 and 45.5 nmol H_2↑/mgchla/h respectively as shown in fig 4 (a,b,c) Limited concentrations of macronutrients (nitrogen or sulphur) induce a set of general responses in cyanobacteria that could lead to elevated H_2 evolution. Nitrogen deprivation triggers cells to generate electron sink providing excess electron for hydrogenase [18].Our results agreements with [19] who found that high rates of hydrogen production were obtained in *Arthrospira* sp. PCC 8005 cells adapted in nitrogen-deprived medium with neutral and alkaline external pH. Antal and Lindblad [20] found that nitrogen-starved cells of *Anabaena cylindrica* produces highest amount of hydrogen (30 ml of H2/lit culture/hour)**.**

Data in Fig (1c, 2c) showed that The growth of *Spirulina sp*. expressed as dry weight, change in chl. a content were generally decreased when culture were grown without supplementation of sulphur or combined nitrogen and sulphur deficiency growth condition.The same trend was recorded in case of *Calothrix sp* and *Oscillatoria* sp. (growth also detected by measuring of absorbance and chlorophyll a content) (Fig.1a, b & 2a, b).

Our results are in agreements with those of [21], Sulfur starvation causes rapid chlorosis in *Synechococcus sp*.

All treated culture had photosynthetiac activity much less than the control culture otherwise showing increase of respiratory oxygen uptake (Fig. 3, 4). When cyanobacteria are maintained under conditions of starvation for an essential nutrient, they turn yellow .This process, termed chlorosis or bleaching, was described long ago [22] and has attracted manifold research activities. The common scheme of chlorosis is the degradation of photosynthetic pigments, in particular, the phycobiliprotiens, which constitute the major light-harvesting antenna in cyanobacteria, as well as chlorophyll a, the pigment in the reaction centers and core antenna of photosystem PSI and PSII. Zhang et al., 2002 [23] found that profile analysis of selected photosynthetic proteins showed a precipitous decline in the amount of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) as a function of time in S deprivation, a more gradual decline in the level of photosystem PS II and PSI proteins, and a change in the composition of the PSII light-harvesting complex (LHC-II). In the absence of S, rates of photosynthetic O_2 evolution drop below those of O2 consumption by respiration [23] .During growth under nutrient-sufficient conditions, the reducing potential produced by the photosynthetic electron transport chain is used for anabolic reactions. Nutrient limitation slows down the reoxidation of the final electron acceptors, and therefore electron transfer activity must be down-regulated [24, 25]. The adjustment of the photosynthetic apparatus to nutrient-limiting conditions is a process that causes apparent changes. The present study showed that hydrogen production was stimulated under sulphur deficiency condition in presence of light in case of *Calothrix* and *Oscillatoria* while *Spirulina* produce hydrogen only in dark condition in absence of sulphur (Fig 5).The highest amount of hydrogen produced was 103.4 nmol H_2↑/min at 76.3 min in dark condition in case of *Spirulina* while *Calothrix* and *Oscillatoria* produce (58.5 nmol H_2↑/mgchla/h and 11.3 nmol H_2↑/mgchla/h at 76.3 min and 130 min respectively when the light was turned on) . Unicellular non-diazotrophic Cyanobacteria *Gloeocapsa alpicola* under sulphur starvation shows increased hydrogen production [20]

Sulfur deprivation exerts distinct effects on hydrogen production. Sulfur deprivation stops the synthesis of methionine, and cysteine. As a result, oxygen evolution is restrained and respiration overrides the photosynthesis to produce hydrogen [26] Song et al.,[27] used S-deprived cells for hydrogen production. The duration of hydrogen production also prolongs in S-deprived condition of *Chlamydomones reunhardhii* without decreasing its rate [28]

Our results revealed that Spirulina produce hydrogen in dark condition only, while the presence of light was very important for hydrogen production in other investigated cyanobacterial cultures (Fig 5, 6, 7) .Sulfur deprivation under dark has been reported to be more efficient by some studies [29]. In dark condition, photosynthesis process stops and hydrognease activity starts due to the depletion of oxygen present in micro algae medium. During respiration, stored organic compounds generate electrons. The protons (Hþ) act as electron acceptor to produce hydrogen via ferredoxin [30]. In dark condition, hydrogen is produced by fermentation. Low oxygen production in dark doesnot affect hydrogeanse activity greatly, and thus, high hydrogen yield is obtained.Likewise,it is predictable that even under partial light condition; the hydrogen yield would be lesser than in dark condition.Growth and chlorophyll of the chosen cyanobacterial culture were severely dropped when grown under combined nitrogen and sulphur deficiency growth condition as shown in Fig. (1, 2).Generally photosynthetic oxygen evolution was decreased in contrast to increased respiration of culture grown without addition of both nitrogen and sulphur source (NoS_0) as figured in Fig. (3, 4)

Fig (6) showed that both of *Calothrix parteniana* and *Oscillatoria* sp failed to produce hydrogen when grown in BG11 media with combined nitrogen and sulphur (N_0S_0) deficiency growth condition either in presence or absence of light .The only culture was able to produce hydrogen in the above condition (N_0S_0) were *Spirulina platensis* .the highest amount of hydrogen production was 92.8 nmol $H_2\uparrow$/mgchla/h at 85.9 min

In conclusion our results revealed that sulphur or nitrogen deficiency enhanced hydrogen production in the studied sp *(Spirulina* sp, *Calothrix parteniana and Oscillatoria sp)* while combined nitrogen and sulphur limitation is only suitable condition for hydrogen production of Spirulina *platensis* especially at dark condition

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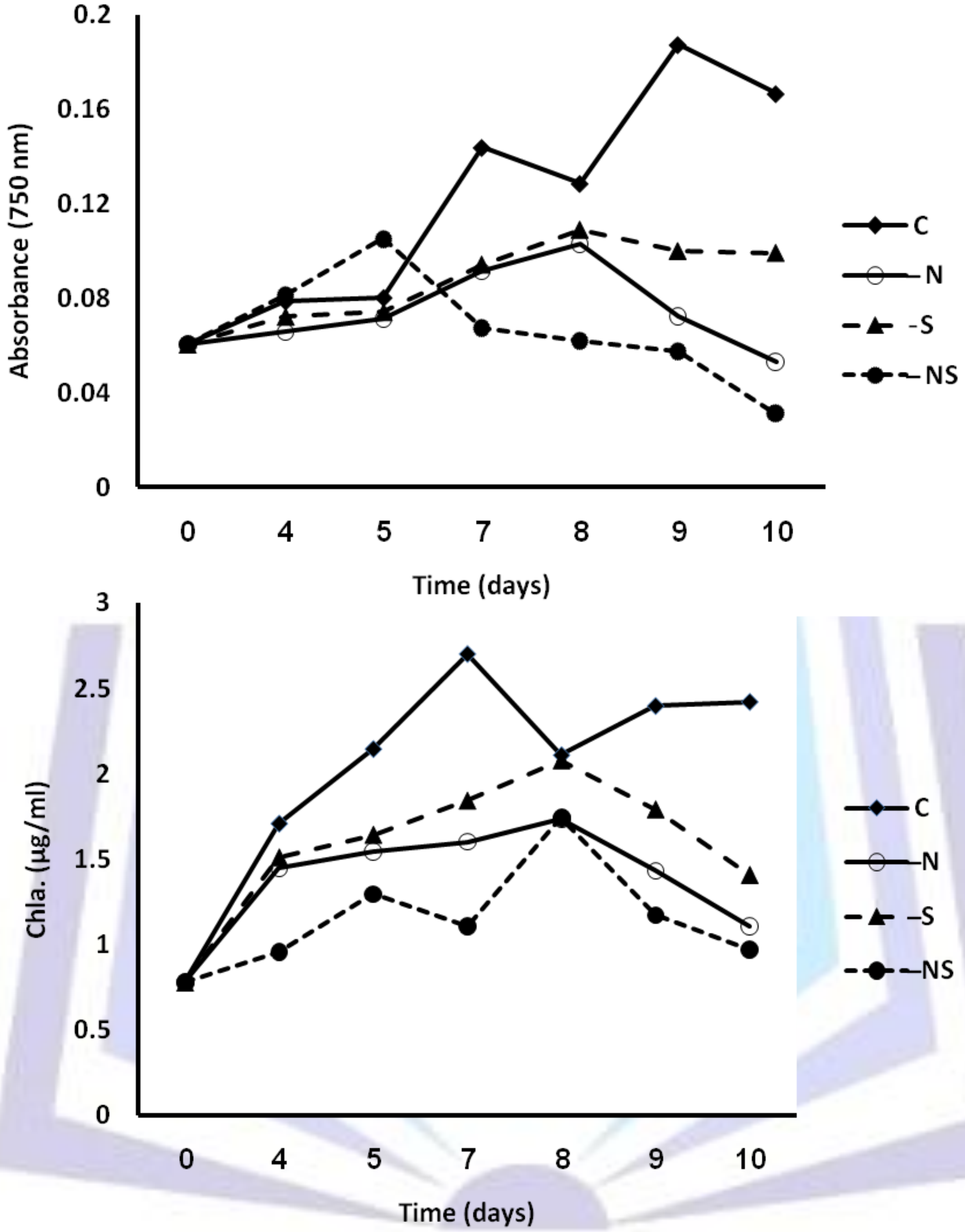

Figure 1a: Growth response (absorbance at 750 nm, Chlorophyl a Content) of *Calothrix* sp. To nitrogen (-N) or sulphur (-S) or combined nitrogen and sulphur (-NS) deficiency growth conditions, compared to regular growth conditions (control)

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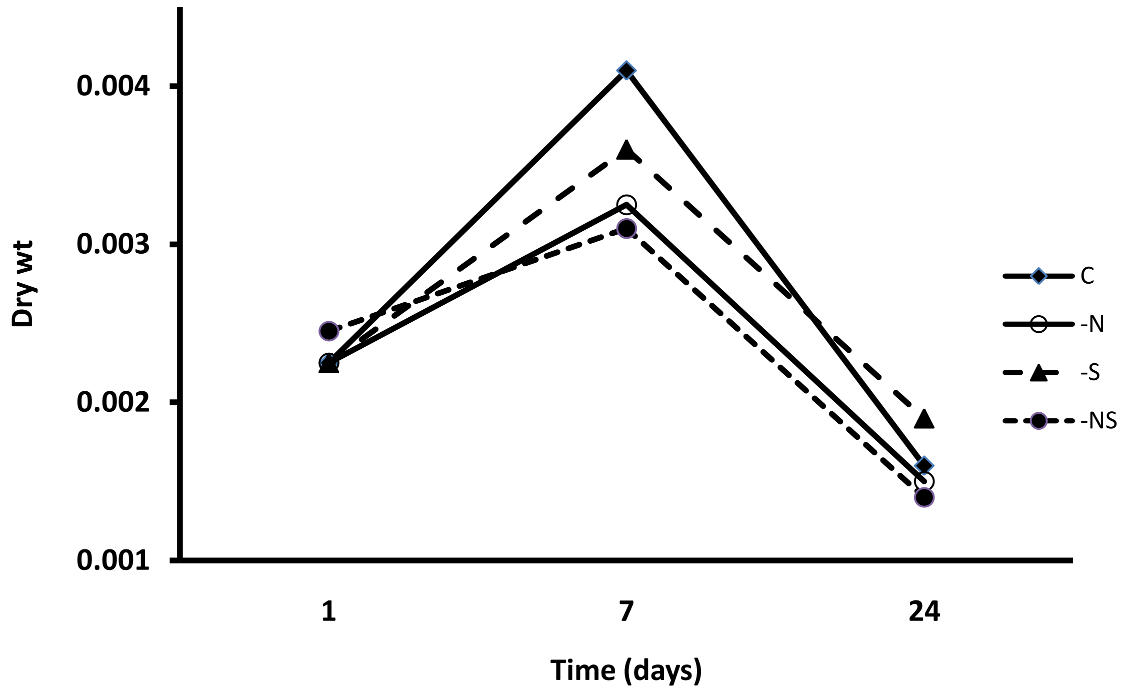

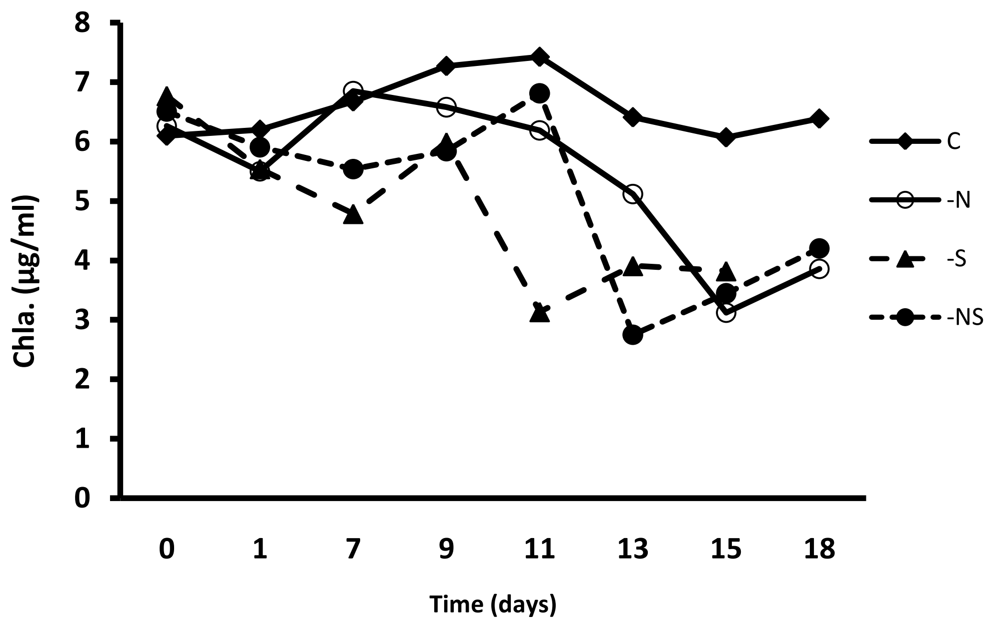

Figure 1b: Growth response (Dry weight, Chlorophyl a Content) of *Spirulina* sp. To nitrogen (-N) or sulphur (-S) and combined nitrogen and sulphur (-NS) deficiency growth conditions, compared to regular growth conditions (control)

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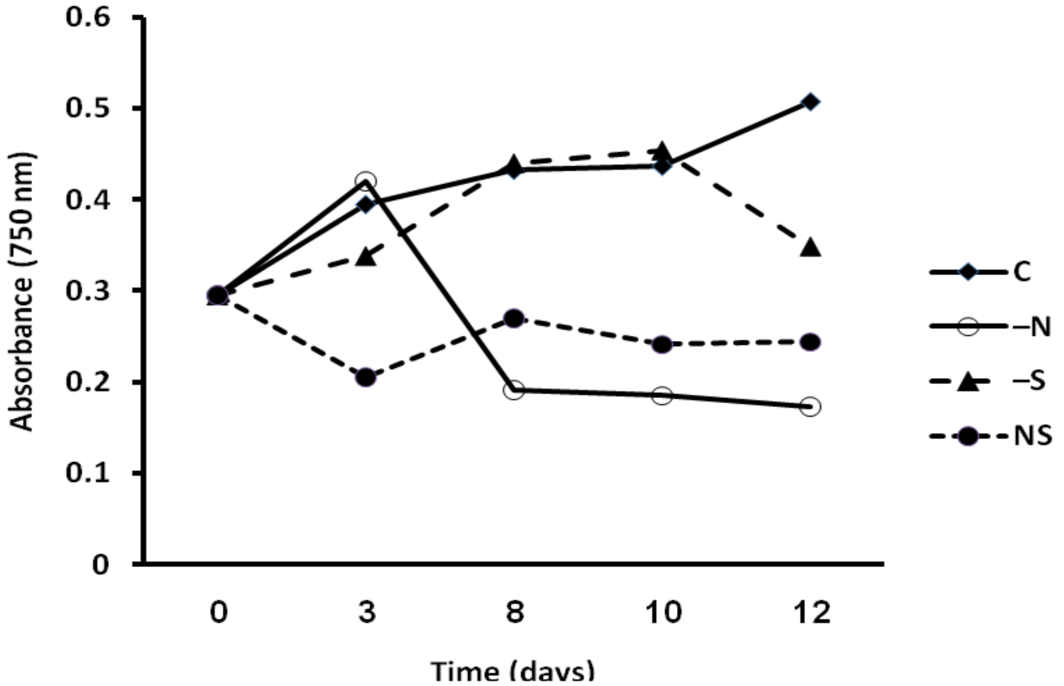

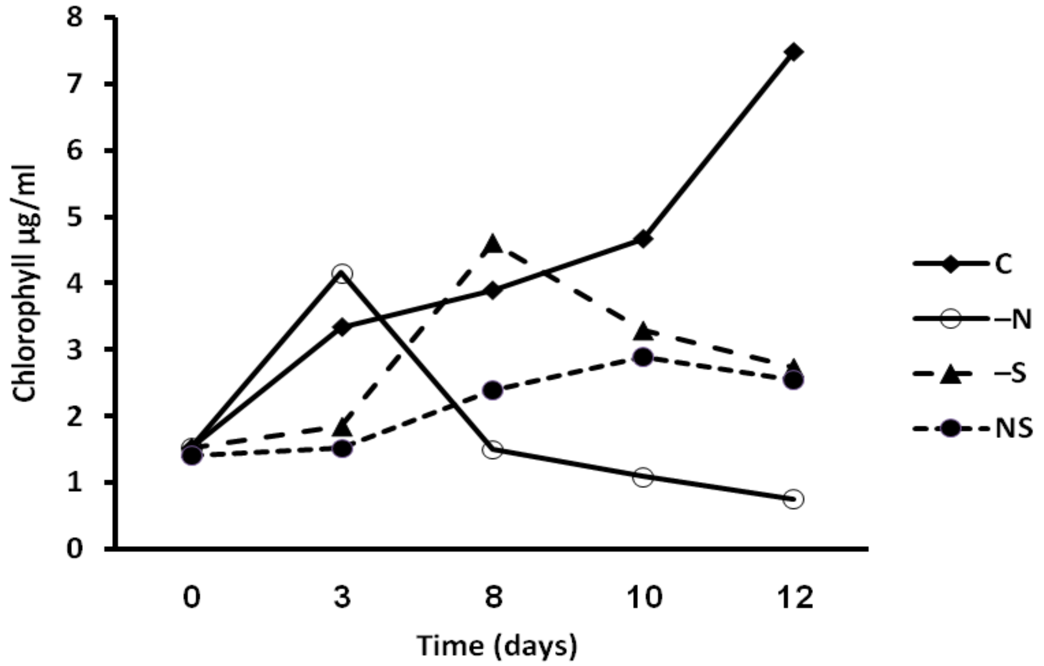

Figure 1C: Growth response (Absorbance (750nm), Chlorophyll a Content of *Oscillatoria* sp. To nitrogen (-N) or sulphur (-S) and combined nitrogen and sulphur (-NS) deficiency growth conditions, compared to regular growth conditions (control)

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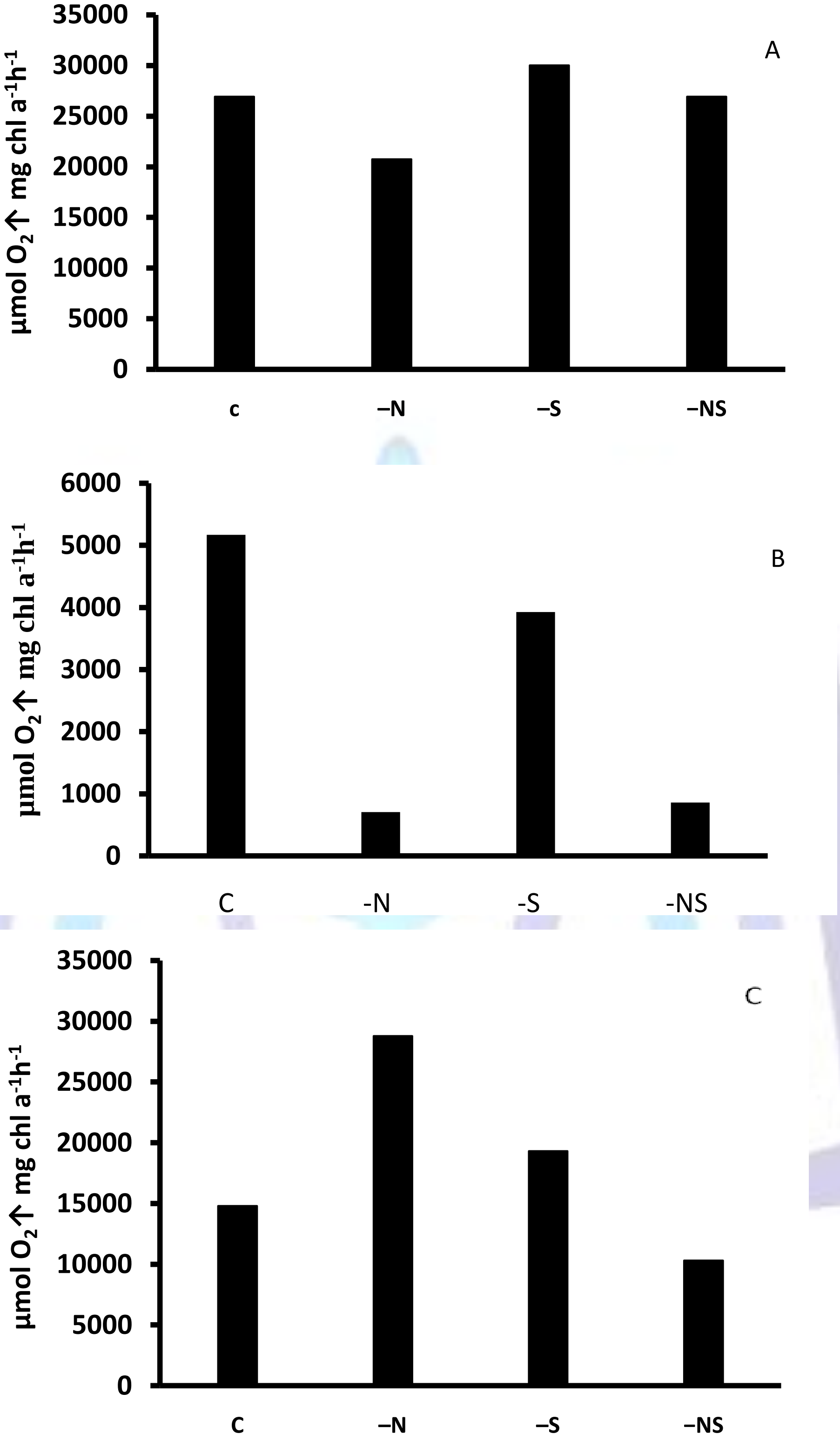

Figure 2: Effect of nitrogen and/or sulphur deficiency growth conditions on photosynthetic oxygen evolution of *Calothrix* sp (A) or *Spirulina* sp (B) and *Oscillatoria* sp (C) under testing as discuss in material and methods

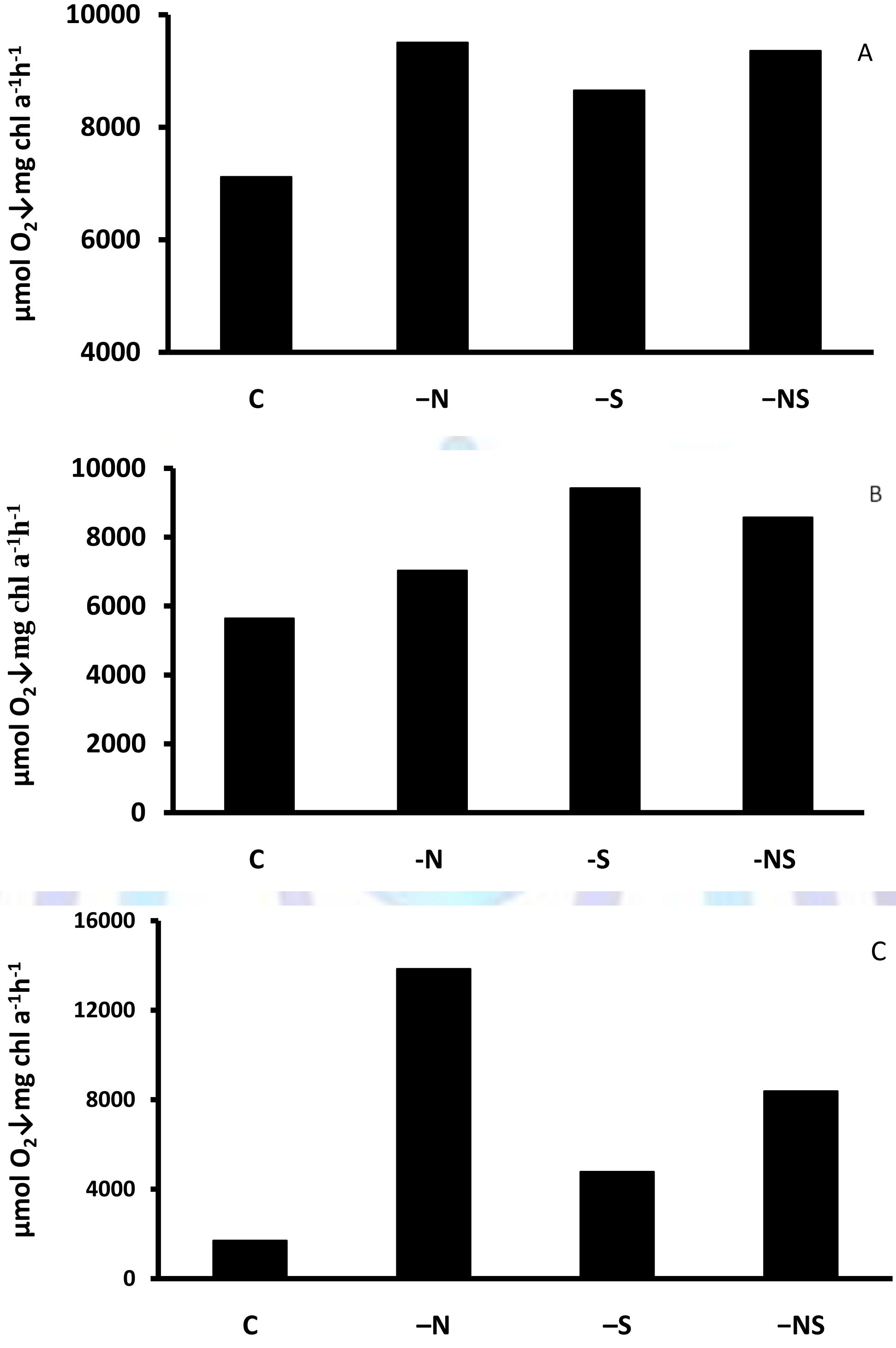

Figure 3: Effect of nitrogen and/or sulphur deficiency growth conditions on respiratory oxygen uptake of *Calothrix* sp (A) or *Spirulina* sp (B) and *Oscillatoria* sp (C) under testing as discuss in material and methods

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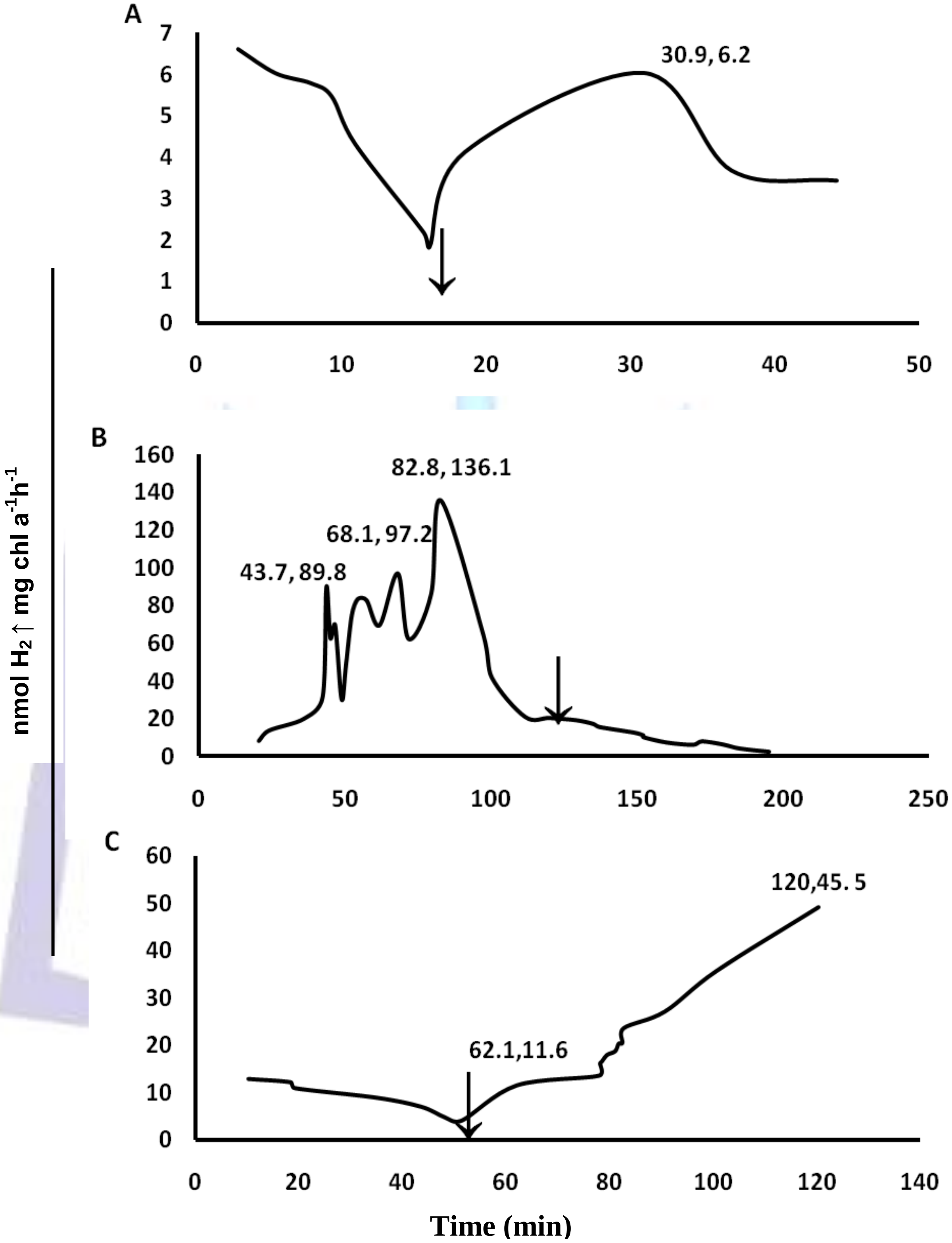

Figure 4: Hydrogen production (nmol H_2↑/mg chl $a^{-1}h^{-1}$) of *Calothrix* sp (A) or *Spirulina* sp (B) and *Oscillatoria* sp (C) grown under nitrogen deficiency growth conditions. Arrows indicate when the light was turned on.

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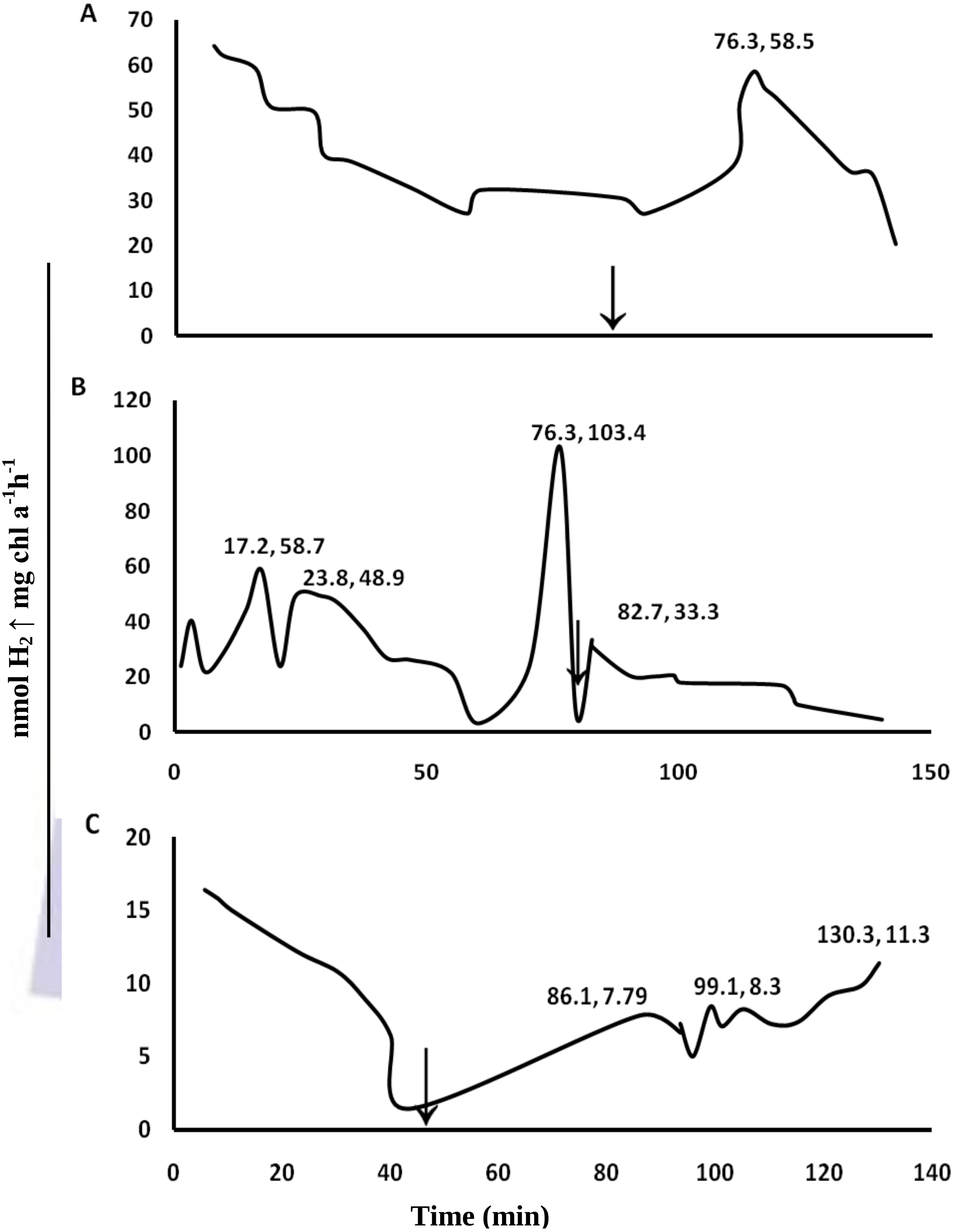

Figure 5: Hydrogen production (nmol H_2 ↑ mg chl $a^{-1}h^{-1}$) *of Calothrix* sp. (A) or *Spirulina* sp. (B) and *Oscillatoria* sp. (C) grown under sulphur deficiency growth conditions. Arrows indicate when the light was turned on.

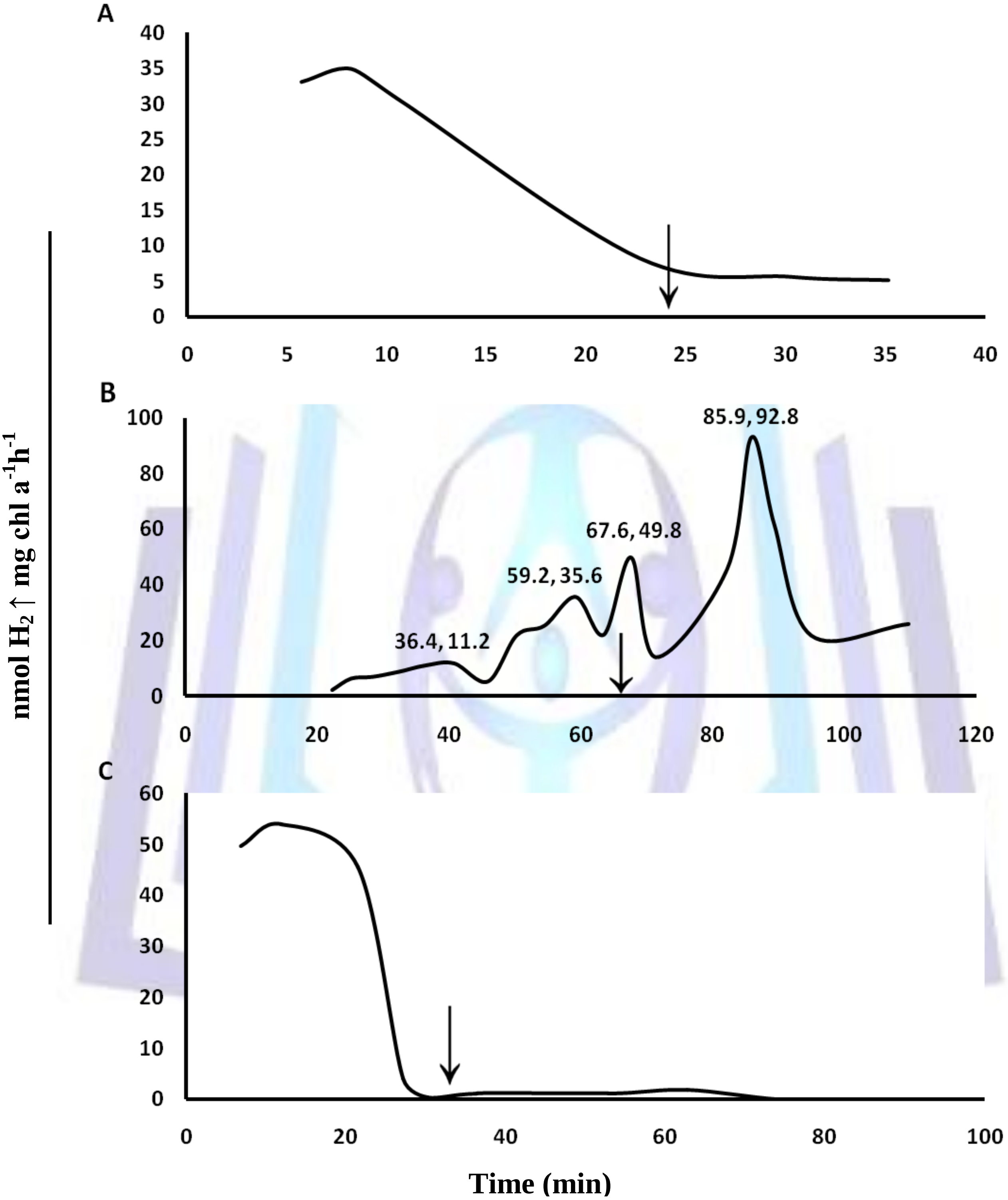

Figure 6: Hydrogen production (nmol H_2 ↑ mg chl $a^{-1}h^{-1}$) of *Calothrix* sp. (A) or *Spirulina* sp. (B) and *Oscillatoria* sp. (C) grown under nitrogen and sulphur deficiency growth conditions. Arrows indicate when the light was turned on.