

UREA FROM WASTE WATERS – PERSPECTIVE NITROGEN AND CARBON SOURCE FOR GREEN ALGAE *SCENEDESMUS* SP. CULTIVATION

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Abstract

The cultivation of Scenedesmus sp., isolated from a natural pond using urea as a nitrogen source was investigated. The results showed that the maximum cell density (about 3.10^7 cells/ml) in the medium with urea is at the 72nd hour of cultivation. Increasing the number of algal cells were 1.6 fold (2% CO₂) and 1.4 fold (0.04% CO₂) respectively, than those cultured in the control medium. Quantity of chlorophyll “a” was the highest in the cultures on the medium urea +0.04% CO₂ - about 5% of the dry weight. The best indication of the membrane stability and actual quantum yield (ϕ PSII) was registered on the medium with urea supplied with 0.04% CO₂ again. The repeated usage of urea as a carbon and a nitrogen source in the microalgal production can contribute not only to the reduction of environmental pollution, but also to considerably decrease the cost of cultivation.

Key words: *scenedesmus sp., urea, pigments, photosynthesis, ecology*

1. INTRODUCTION

The microalgae have received a renewed interest due to the wide field of their application. Several species of microalgae have been shown to produce high-value products such as proteins (Brown et al. 1997), polyunsaturated fatty acids (Zittelli et al 1994), antioxidants (Salguero, et al 2003), vitamins (Takeyama 1997) and other bioactive compounds (Pulz and Gross, 2004; Apt et al. 1999; Kabaivanova et al 2016). The advantages of algae are rapid growth rate and productivity as microalgae can produce 50 times more biomass compared to higher plants (Li et al. 2008). The accumulation of algal biomass varies depending on a number of environmental factors including temperature, nitrogen concentration, light intensity, CO₂, etc. (Jimenez 1991). The elements of culture medium are important to develop balanced media for optimal microalgal cultivation. Nitrogen is an important limiting nutrient in the marine environment (Edwards et al. 2006). Nitrogen can be found in the form of nitrate, ammonia and urea, which are the most common nitrogen sources (Becker 1994; Salguero 2003). It constitutes about 7 to 10% of microalgal cell dry weight (Richmond 2004). Urea as a source of nitrogen is cheaper than nitrate and has no effect on chlorophyll content of the cultures (Danesi et al. 2002). Previous experience using urea for microalgal growth showed good results (Choochote 2008). Moreover, among the organic nitrogen sources, urea is the best nitrogen source for cultivating *Chlorella sp.* (Becker, 1994). Another advantage associated with the usage of urea is that it can be obtained from sources of waste water while purifying them. Municipal wastewaters typically have organic ammonia nitrogen concentrations ranging from 25 to 45 mg/l (Tchobanoglous et al. 2003). Kim et al. (1998) reported 95.3% removal of nitrogen by *Chlorella vulgaris* in 25% secondarily treated swine wastewater after four days of incubation. The green algae of genus *Scenedesmus* are commonly found in fresh waters (Borowitzka & Borowitzka 1988). They are very suitable for both laboratory studies and mass intensive cultivation, since they have a high tolerance towards variation of the most important environmental factors (Ren et al. 2013). Research of new algae strains, especially Bulgarian, are important in order to find a local, competitive, fast growing and highly productive strain. Photosynthesis, oxygen respiration and chlorophyll fluorescence provide information on the degree of vitality of algae and plants (Lambers and Ribas-Carbo 2005). The most important results revealing the nature of the photosynthesis are obtained during the investigation of the photosynthetic oxygen production. Alterations in the shape of oxygen induction curve and pattern of oxygen flash

sequences are indicative for membrane stabilization (Zeinalov 2002). The highly sensitive to environmental changes method of prompt chlorophyll “a” fluorescence was applied to evaluate the state of the photosynthetic light phase directly and the dark phase indirectly (Strasser et al. 2004; Stirbet & Govindjee 2011) allowing estimation of the overall physiological state of microalgae cultured on different concentrations of CO₂ and cultivation media. The aims of this study were to assess the effects of urea on the cell density, pigments and photosynthetic parameters of *Scenedesmus* sp, grown under two different concentrations of CO₂. This will display actual physiological state of the algal cells.

2. MATERIALS AND METHODS

2.1. Algal material and cultivation conditions

Scenedesmus sp. strain BGP used in this study was isolated from a rainwater puddle (Sofia, Bulgaria). Monoalgal, non-axenic cultures of *Scenedesmus* sp. BGP are grown autotrophically on two different variations of the nutrient medium of Setlik (1967), modified by Georgiev et al. (1978) (standard recipe) and two carbon dioxide concentrations (2 and 0.04%).

2.2. Nutrient medium

1. control medium - 1/4 from standard recipe: NH₄NO₃ (0.4 g.L⁻¹), CO(NH₂)₂ (0.3 g.L⁻¹), MgSO₄.7H₂O (0.494 g.L⁻¹), KH₂PO₄ (0.17 g.L⁻¹), NaHCO₃ (2.0 g.L⁻¹), trace elements
2. medium with urea: NH₄NO₃ (0 g.L⁻¹), CO(NH₂)₂ (0.6 g.L⁻¹), MgSO₄.7H₂O (0.494 g.L⁻¹), KH₂PO₄ (0.17 g.L⁻¹), NaHCO₃ (1 g.L⁻¹), trace elements

2.2.1. Variants

- I. medium 2 (with urea) supplied with 2 % carbon dioxide
- II. medium 1(control) supplied with 2% CO₂ (control)
- III. medium 2 supplied with 0.04% CO₂
- IV. medium 1 supplied with 0.04% CO₂

The quantity of the nitrogen was kept the same as in the 1/4x standard recipe. The experiments were carried out on a specially equipped block. The cultures, in 200 ml flasks, were continuously supplied with carbon dioxide (2%) or (0.04 % CO₂) at constant temperature (about 24°C) and illumination (360 μmol m⁻² s⁻¹ photon flux density). pH was corrected every day with 0.1M (HCL). An initial culture cell density of ~ 0.8 g.L⁻¹ was used for all treatments. To analyze the pigment composition and photosynthetic activity experimental cultures were harvested at 72 hour of cultivation (middle exponential phase of growth). Cells were collected by centrifugation (5000×g, 10 min), rinsed three times with distilled water, frozen, and stored at -20°C.

2.3. Measurements and analyses

2.3.1. Algal growth The growth of *Scenedesmus* was evaluated by increase in biomass dry weight (DW). For this purpose, 10 ml of the algal suspension were centrifuged at 5000 g, 10 min (centrifuge rototfix 32 A). The cells were dried at 105°C for 16 h. The dry biomass concentration (g.L⁻¹) was calculated according to Makarevičienė et al. (2012).

2.3.2. Cell density Cell density was determined using Burger chamber and 40 x magnification power of the light microscope.

2.3.3. Pigment content Pigment content of algal strains was estimated spectrophotometrically (665 nm for chlorophyll “a”, 460 nm for carotenoids, 565 nm for phycoerythrin and 620-650 nm for phycocyanin). The pigments were measured spectrophotometrically after an extraction with boiling methanol and the quantity was calculated using the Mackiney coefficients (Mackiney 1941).

2.3.4. *Oxygen induction curves and flash yields oscillations of *Scenedesmus* sp. grown under different conditions* (variant from I to IV). The experiments were performed by a polarographic oxygen rate electrode (Zeinalov 2002) with short (10 μ s) saturating (4J) flash sequences and continuous white light (135 W.m⁻²). Dark adapted algae were equalized on the optical density BASED at a wavelength of 760nm.

2.3.5. *Fluorescence* The physiological state of four *Scenedesmus* sp. cultures was monitored by prompt chlorophyll “a” fluorescence (ChlF) induction transients recorded by fluorimeter M-PEA (Multi-Function Plant Efficiency Analyzer) Hansatech Instruments Ltd., King’s Lynn, UK. Before ChlF measurements the chlorophyll concentration of the algal suspension was determined spectroscopically and the suspensions were diluted to adjust the Chl concentration to 1 mg.ml⁻¹ in each sample. The protocol used was 0.3 s ChlF recording immediately followed by 0.03 s recording, which included dark intervals, both at 4000 μ mol.m⁻².s⁻¹ PPFD. The two successive recordings were repeated for 10 min every 30s during which period a PPFD of 100 μ mol.m⁻².s⁻¹ was applied. The data from the protocol were used in calculation of actual quantum yield of PSII (ϕ_{PSII}) by fluorescent quenching analysis according to Maxwell and Johnson (2000). To complete quenching analysis, a novel approach was applied for recording drops of ChlF after 1 ms dark intervals during the induction period, i.e. dark drops (DD) were applied (Goltsev 2013 ;Dimitrova 2015). The samples were dark-adapted for at least 1 hour before measurement. The measurements were repeated 4 times. The primary data processing was done by M-PEA Data Analyzer, program developed by Petko Chernev at the Department of Biophysics and Radiobiology, Faculty of Biology, Sofia University. The secondary processing, including calculation of quenching parameters, was performed on Microsoft Excel. The plots were done in Sigma Plot.

2.4. Statistical analysis

All data are presented as mean $\pm$ standard deviation. The significance of differences between the treatments was evaluated by one-way analysis of variance (ANOVA). Values of P\0.05 were considered significant.

3. RESULTS AND DISCUSSION

According to the literature data, urea has a wider availability and lower cost per nitrogen unit when compared to ammonium nitrate (Kallenbach et al. 2005). Our results show a better growth of microalgae when they are cultivated on medium containing urea, supplied with carbon dioxide 2% or 0.04%. (Photo 1, Figure 1). The intensity of the green color is an indicator of cell growth of the algal culture (Photo 1). The number of cells of *Scenedesmus* sp. cultivated on medium with urea and supplied with 2% carbon dioxide is about 30 million, or about ¼ more compared with those cultured under control conditions. Algae that are cultivated with the urea medium and blown with atmospheric air (0.04% CO₂) also showed very good growth comparable to that in the control medium.

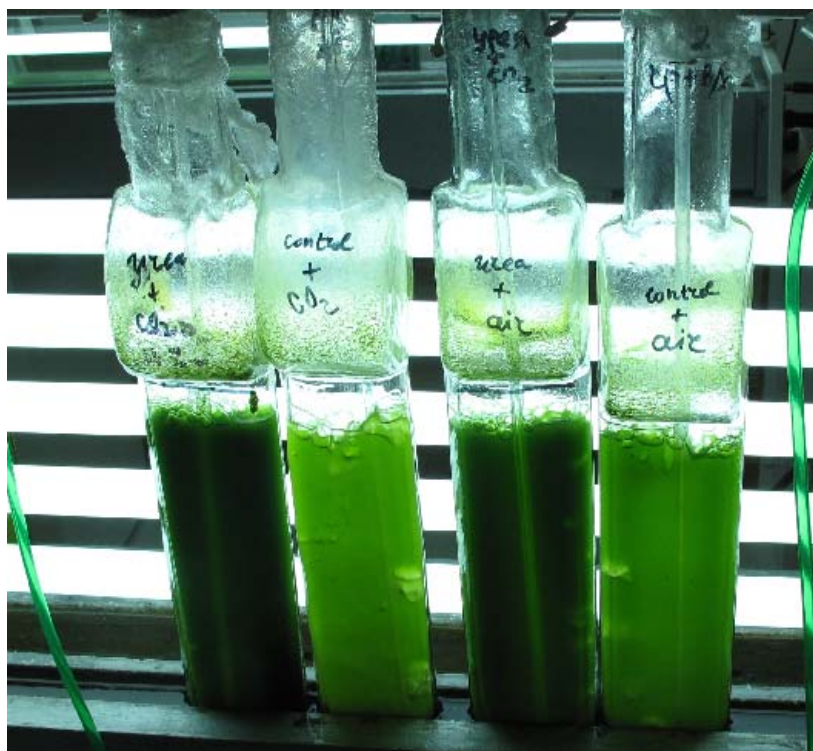


Photo 1. *Scenedesmus* sp. BGP cultures grown on different nutrient media and CO₂ concentrations – from left to the right: urea + 2% CO₂, control + 2% CO₂, urea + 0.04% CO₂, control + 0.04% CO₂

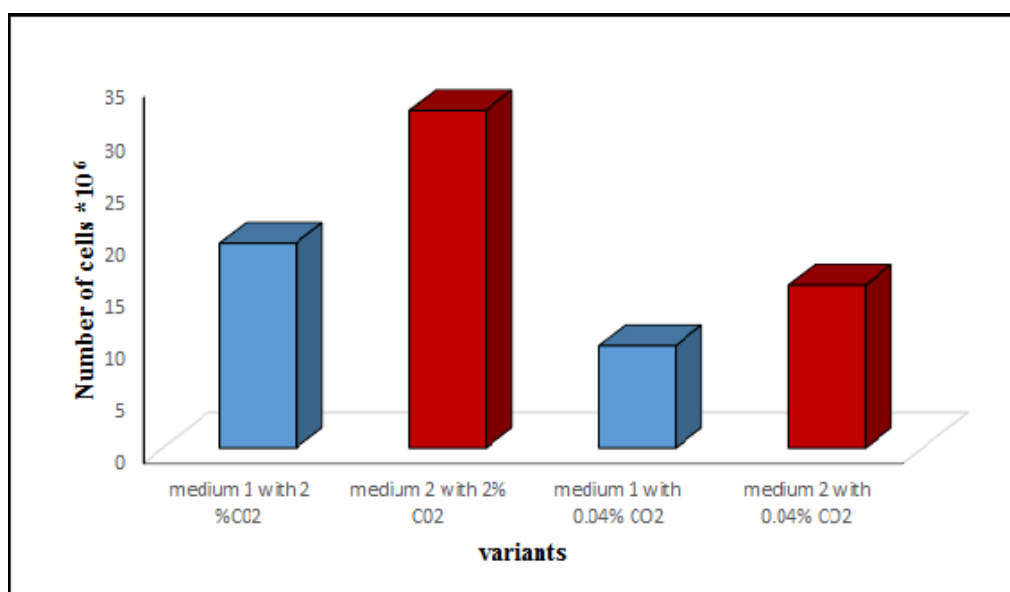


Figure 1. Number of algal cells during the exponential growth phase (at the 72nd hour).

Pigment content of algal cells is another indicator of the level of photosynthesis and cell vitality because chlorophyll “a” is the most readily and unambiguously measured indicator of microalgal abundance (Cullen 1982). It quantity of pigments in various test variants confirms that the algal culture develops better in medium with urea. The results once again show the highest content of chl “a” in samples growing on medium with urea (Fig.2). Best results here are shown when the alga is cultured on medium with urea and 0.04%CO₂. The amount of chlorophyll “a” in this sample is about 5% of algal DW or 2 times above this of the control medium with air. The interesting fact is that the

increased concentration (2%) of carbon dioxide ensures the best growth, but has a negative effect on the amount of chlorophyll "a". Similar results were obtained by Prabakaran & Ravindran (2012) when comparing the growth and pigment content of *Scenedesmus sp.* growing on medium with urea and ammonium nitrate.

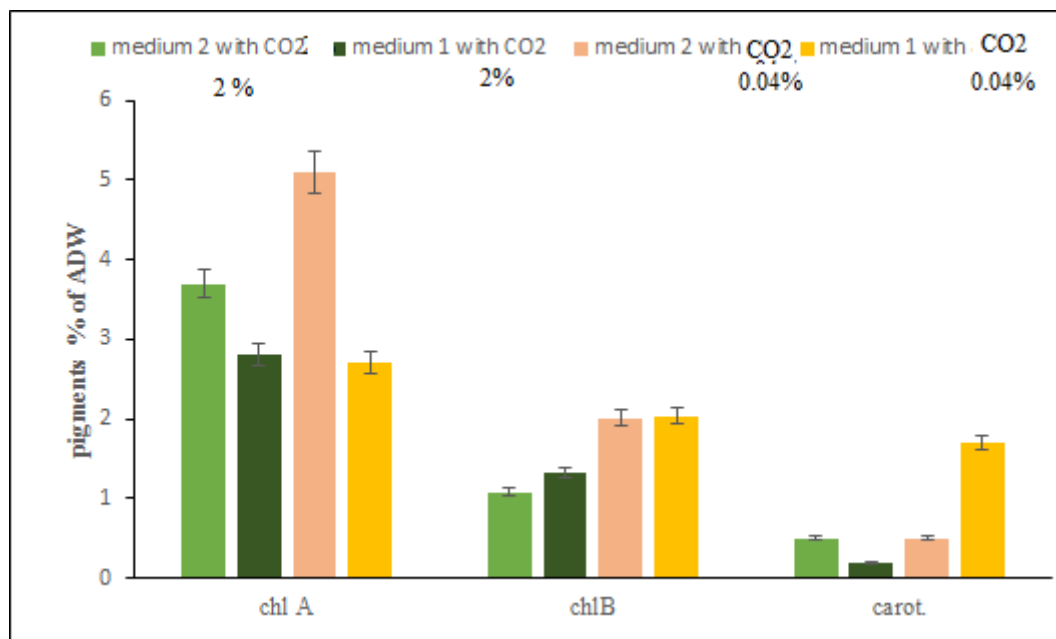


Figure 2. Pigments (chlorophyll "a", chlorophyll "b" and carotenoids) of *Scenedesmus sp.* cultivated on two different medium supplying with 2% CO₂ or 0.04%CO₂

The increased quantity of carotenoids is generally associated with overcoming the adverse conditions and/or stressors (Hu 2004). In our case, the amount of carotenoids in the sample cultured on control medium flushed with 0.04% CO₂, where the growth is the weakest (Figure 1). Probably the accumulation of carotenoids is a protective response of *Scenedesmus sp.* of these unfavorable growth conditions. Various environmental factors could achieve specific biotechnological goals. This fact can be used for the directed biosynthesis of cell metabolites. Alterations in the shape of oxygen induction curve and the pattern of oxygen flash sequences in different variants are indicative for membrane stabilization (Fig 3 and 4). The results show some differences in the various test samples. The best indication of membrane stability is registered in the sample with urea supplied with 0.04% CO₂. Lower membrane stability is registered when the algae are grown on a nutrient medium with urea flushed with carbon dioxide -2%. Apparently, the increased concentration of carbon dioxide is a stress factor for algal cell membrane.

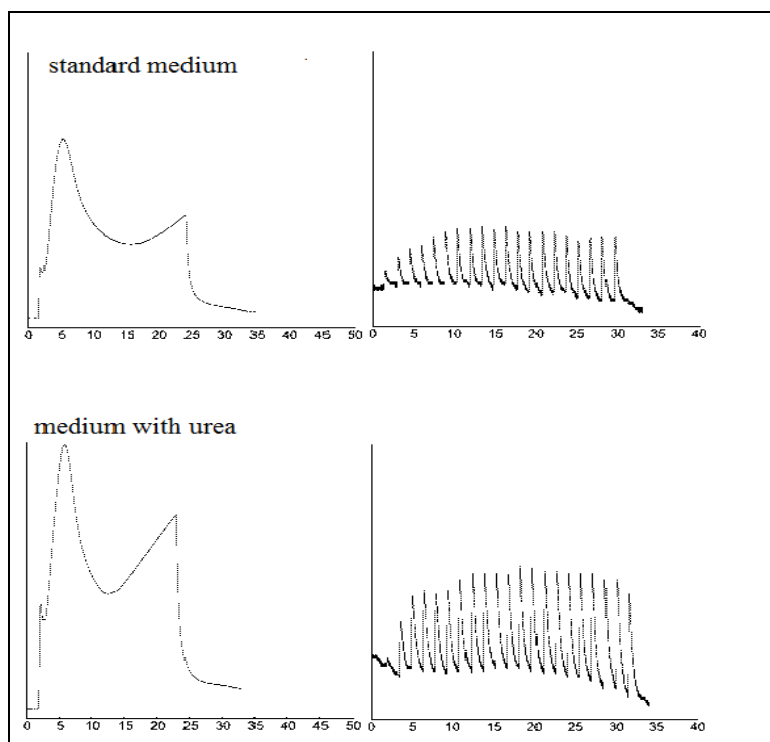


Figure 3. Oxygen induction curves and flash yields oscillations of *Scenedesmus sp.* grown under different media and 0.04% CO₂ (Variants 4 and 3).

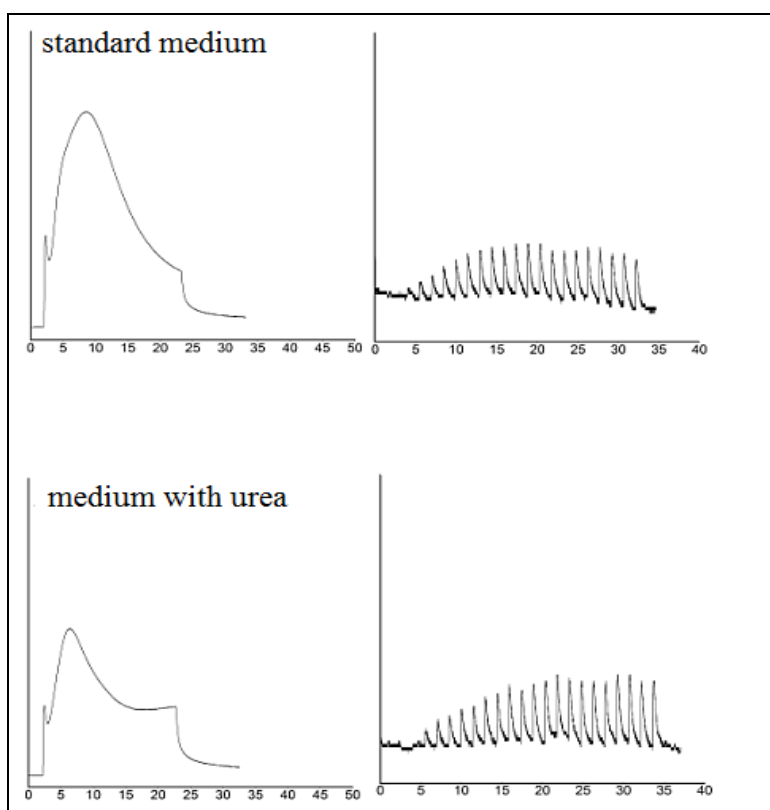


Figure 4. Oxygen induction curves and flash yields oscillations of *Scenedesmus sp.* grown under different media and 2% CO₂ (Variants 2 and 1)

In the last 20 years, chlorophyll “a” fluorescence measurement has become widespread approach to monitor photosynthetic performance of microalgal mass cultures (Malapascua et al. 2014).

The actual quantum yield (ϕ_{PSII}) gives information about the photochemical capacity of PSII in light-adapted state (Fig. 5, A). It is given by the formula: $\phi_{PSII} = \phi_{P_0} \cdot qP$. Urea, 0.04% CO₂ culture turned out to perform the best one as it sustained a steady high ϕ_{PSII} value in light. This fact is in concord with the high PI_{ABS} parameter and qP transient. NH₄NO₃, 0.04% CO₂ culture was inhibited in dark but positive trend was observed during light adaptation. In contrast, the 2% CO₂ cultures shows negative ϕ_{PSII} trend resembling their qP change, while the maximum quantum yield (ϕ_{P_0} – initial ϕ_{PSII} value indeed) is not changed. Dark drops (Fig. 5, B) showed fast and slow phase. The fast phase is related to the utilization of NADPH in the dark reactions after activation of FNR and Rubisco and the slow – to state transition. The difference is in the slow phase and once again the determining factor is CO₂. When its concentration is high a state 2-1 transition is observed, when low – state 1-2.

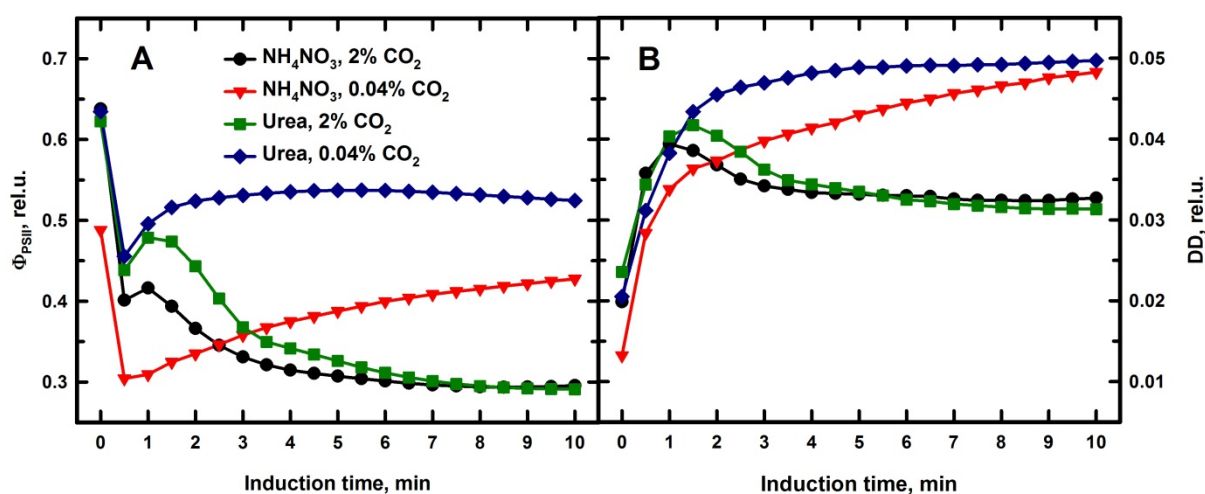


Figure 5. Effect of the different algal cell growth conditions on the change of actual quantum yield of PSII (ϕ_{PSII}) (A) and drops of the fluorescence after 1 ms dark periods, i.e. dark drops (DD), (B) during 10 min period of dark to light adaptation. The ϕ_{PSII} parameter are derived from 300 ms ChlF induction transients at 4000 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ PPFD repeating at 30 s intervals of 100 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ PPFD. DD are derived from 30 ms ChlF acquisitions at 4000 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, including 1 ms dark periods, recorded right after the 300 ms ChlF induction transients at 4000 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ PPFD repeating at 30 s intervals of 100 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ PPFD.

4. CONCLUSIONS

The usage of urea as a nitrogen source showed very good results concerning the vitality and photosynthetic parameters of cells of the newly isolated strain *Scenedesmus sp.* BGP. The combination of the new medium and reduced supply of CO₂ (only 0.04%) was of a particular interest. Implementation of these parameters in practice will reduce the cost of cultivation on one hand and contribute to environmental protection on the other.

ACKNOWLEDGEMENT

The authors are grateful for the financial support from the Bulgarian Academy of Sciences (BAS) by the Young Scientists Support Program (2016). We are also grateful to Dr. G. Marinova for the purification of *Scenedesmus sp.* as a monospecific, non-axenic culture. The help of Prof. Dr. L. Gigova with the editing of the article is gratefully acknowledged.

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