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Short-term Effects of High CO_2 Concentrations on *Chlorella vulgaris* and *Scenedesmus regularis*

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ABSTRACT

High CO_2 concentration in the atmosphere can lead to climate change which can pose threats to ecosystems and living organisms. Use of microalgae as biomitigation tool for CO_2 sequestration has become an increasing interests. However, being photoautotrophs, too much of CO_2 can be detrimental to them as well. Thus, the aim of this study is to observe the responses of two microalgae, *Chlorella vulgaris* and *Scenedesmus regularis* after a short-term high CO_2 exposure (i.e. in 484 and 968 ppm CO_2). The algal maximal quantum yield (i.e. F_v/F_m) measured using a chlorophyll fluorometer; content of hydrogen peroxide (H_2O_2)and level of DNA damage using the Comet assay; were determined.In addition, the medium's pH value was also determined in order to observe the uptake of CO_2 by the algae. Recovery of the algae from the CO_2 stress was also observed. Results showed that supplementing the medium withadditional CO_2 induced an acidification reaching a pH between 5.4 and 6.1. After the recovery, the pH increased back into its original alkaline state. The F_v/F_m of *C. vulgaris* was reduced after treatments in both [CO_2] and these conditions did not mitigate after the recovery.Constrastingly, F_v/F_m of *S. regularis* was not affected in 484 ppm but showed an increase in 968 ppm CO_2.Further increase was observed after recovery. The content of H_2O_2 increased after treatments in *C. vulgaris* but was decreased in *S. regularis* for both [CO_2]. After recovery showed that the effect was lessen and reduced back to its original content in *C. vulgaris* but started to increase in *S. regularis*. Comet assay analysis showed that most of*C. vulgaris* cells had their DNA damage lies within the highest level of 4 (i.e. highly damaged) after the treatment. In contrast, *S. regularis*cells had their DNA damage at the highest level of 2 (i.e. moderately damaged). Repair activity of the DNA was observed in *C. vulgaris* and *S. regularis* cells treated with 484 ppm but not in 968 ppm CO_2.*C. vulgaris* was susceptible towards the high CO_2 while *S. regularis* was more tolerant. *S. regularis* has the potential to be effective as biomitigation tools of elevated CO_2.

KEYWORDS: Microalgae, Carbon Dioxide, F_v/F_m, H_2O_2, DNA Damage

INTRODUCTION

The problem of climate change arising mainly from carbon dioxide (CO_2) emission is currently a critical environmental issue. According to that stated in [1], atmospheric CO_2 concentrations have risen from 295 parts per million (ppm) to 380 ppm over the last 100 years. This is mainly contributed by combustion of fossil fuels and from reduced carbon uptake due to global deforestation and loss of arable land.Effects of climate change on the ecosystem are diverse and can be deleterious to living organisms [2, 3]. For example, the emission of high concentration of CO_2 into the atmosphere can cause the shift in carbonate chemistry of seawater or ocean acidification. Ocean acidification affects, among others,the feeding rates in a scleractinian coral [4], reproduction of coral reef fishes [5] and immune responses of oysters [6].Photosynthetic organisms rely on CO_2 to grow and develop, thus, they are also deemed to be highly affected by the high concentrations ofatmospheric CO_2. Growth of some C_3 plants such as *Caragana microphylla*, a leguminous shrub and *Stipagrandis*, a grass

was stimulated by elevated CO_2 but the biomass of a C_4 grass, *Cleistogenes squarrosa* was not affected by this [7]. Nitrate assimilation was also observed to be slower in wheat grown under elevated CO_2 [8]. Furthermore, elevated CO_2 enhanced the production of chlorophylls, proteins, phenolic compounds as well as carotenoids and alkaloids [9].

There is a growing interest in using seaweeds and microalgae as a tool for green technology in mitigating the effect of elevated CO_2 concentrations. Being photoautotrophs, they utilize CO_2 for photosynthesis which can be useful in CO_2 sequestration [1,10]. In addition, their capacity in fixing CO_2 is much greater than C_4 plants [10]. CO_2 fixation by the algae can also be combined with production of biomass which can be burned for heat and electricity [11, 12]. They can be good bioindicators as well since they are sedentary, easy to identify, have rapid reproduction rates, very short life span and are widely distributed in the aquatic ecosystem [13]. Moreover, algae are known to be able to adapt to harsh environments by inducing protective mechanisms which can be beneficial as bioindicator parameters [14]. This paper describes the biochemical and physiological responses of two microalgae, *Chlorella vulgaris* and *Scenedesmus regularis* against the effect of elevated CO_2 concentration under the laboratory conditions. From the observations, potential candidate for efficient CO_2 mitigation among the two algae was also determined.

MATERIALS AND METHODS

Algal Materials and CO_2 Treatments:

The green microalgae, *Chlorella vulgaris* (UMT-M1) and *Scenedesmusregularis*(KS-MC1) were cultured in f/2 media [15] and cultivated for treatment at log phase of growth. The algae in filtered seawater were aerated with two different concentrations of CO_2 (i.e. 484 and 968 ppm) for 6h at 25±1°C under a white fluorescent light. After the treatment, the CO_2 aeration was stopped and the algae were aerated with normal air and left at room temperature for a 24h recovery period. Control was subjected to similar conditions as treatment but with no additional CO_2.

Parameters Analyzed:

Chlorophyll (chl) a fluorescence was measured in terms of the dark-adapted maximal quantum yield (i.e. F_v/F_m) with a handheld chlfluorometer, AquaPen-P AP-P 100 (Photon Systems Instruments, Czech Republic). At the start of the measurement, a short, red, actinic pulse (~3000 µmol m^{-2} s^{-1} at 655 nm) was prompted for 5 s to ensure a stabilized fluorescence emission during the following F_m measurement. Then F_o was measured with a pulsed, blue measuring light (~900 µmol m^{-2} s^{-1}, 455 nm), and F_m was determined with a saturating white light pulse (~3000 µmol m^{-2} s^{-1}). The maximal quantum yield, F_v/F_m was calculated as $(F_m-F_o)/F_m$.

The H_2O_2 content in the algae was determined according to [16]. The treated cells were first harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The cells were washed once with distilled water and recentrifuged. The cell pellet was subjected to wet weight estimation and then dried in the oven for 2 h at 80°C. Algal material was homogenized with 10% (w/v) trichloroacetic acid in an ice bath. Then, the homogenate was centrifuged at 7,000 g for 10 min, and the supernatant was added with 50 mmol/L potassium phosphate buffer (pH 7.0) and 1mol/L potassium iodide. The absorbance of the mixture was measured at 390 nm. A series of 30% H_2O_2concentrations was used to construct a standard curve and the algal H_2O_2 content was determined from this.

Alkaline comet assay was performed according to [17]. After centrifugation at 2000 rpm for 5 min, cell pellets were embedded in a layer of 0.5% low-melting agarose on a fully frosted microscopic slide coated with 0.5% normal-melting agarose (NMA). The slide was placed in a lysis solution containing 300 mMNaOH, 30 mM Na_2EDTA and 0.01% SDS for 5 min and then in an electrophoresis buffer (300mM NaOHplus 1mM Na_2EDTA) for 20 min at 4°C to allow unwinding of DNA. After that, electrophoresis was conducted with the same buffer at 4°C for 20 min at 25V and 300 mA. The slides then were neutralized by immersing twice in a neutralizing buffer (400 mMTris pH 7.4) for 5 min. The DNA molecules then were stained with ethidium bromide for 5 mins and dipped in chilled distilled water to remove excess stain. The migrated DNA or comet images were observed under an epi-fluorescent microscope. Tail length and tail moment were determined using an image analysis software as an indication of DNA damage. Data was taken from 50 cells per sample. Scores of DNA damage were determined according to that categorized by [18]: score 0 (no damage, <1% DNA in tail), score 1 (low damage, 1-25% DNA in tail), score 2 (medium damage, >25-45% DNA in tail), score 3 (high damage, >45-70% DNA in tail) and score 4 (very high damage, >70% DNA in tail).

Statistical Analysis:

Values of all the parameters tested were related to 100% of controls for better comparison unless otherwise stated. Mean values and standard error were determined from three replicates of each treatment. The statistically significant differences between the CO_2 concentrations within similar algal species were analyzed using a Student's t-Test and one-way ANOVA followed by Tukey HSD *post-hoc* test at probability level of 0.05.

RESULTS AND DISCUSSION

CO_2 when diluted in aqueous solution forms carbonic acid (H_2CO_3). This H_2CO_3 dissociates in the water releasing hydrogen ions (H^+) and bicarbonate (HCO_3^-), thus, increasing acidity or a decrease in pH of the medium as shown by both algae after the 6h CO_2 treatment (Table 1). This condition creates acidification in the medium and can result in the decrease efficiency of bicarbonate uptake by the algae [19]. In response to low CO_2 in the cells, microalgaeactivate an active transport system known as the CO_2-concentrating mechanism (CCM) to enhance their photosynthetic performance in inadequate CO_2 supply [20]. However, it is expected that increases in dissolved CO_2 cause down-regulation of microalgal CCM capacity [21, 22]. Thus, this will generate stress in the cells. Being photoautotrophs, *C. vulgaris* and *S. regularis* use the CO_2 available in the medium for photosynthesis and photosynthesis performed in water will always cause an increase in pH since CO_2 in the water is withdrawn in the form of H^+ associated with HCO_3^-. Hence, an increase in pH was observed after the 24h recovery period (Table 1). This change in pH indicate that both algae were actively taking in the CO_2 in the medium during the recovery period.

Table 1: pH of medium after the 6h CO_2 treatment and after 24h recovery as compared with control. Data are mean±S.E. Different letters after value indicate statistically significant differences at p<0.05 (ANOVA, Tukey HSD *post-hoc* test, n=3).

Treatment Conditions	Algal Species and CO_2 Concentrations			
	C. vulgaris		*S. regularis*	
	484 ppm	968 ppm	484 ppm	968 ppm
Control	8.7 ± 0.04^{A}	8.5 ± 0.03^{b}	7.6 ± 0.03^{B}	7.7 ± 0.01^{b}
After 6h CO_2	6.0 ± 0.03^{B}	6.1 ± 0.02^{c}	5.6 ± 0.01^{C}	5.4 ± 0.01^{c}
After 24h recovery	8.8 ± 0.04^{A}	8.7 ± 0.04^{a}	7.8 ± 0.02^{A}	7.9 ± 0.05^{a}

F_v/F_m of *C. vulgaris* after the CO_2 treatment showed a significant reduction of about 27% and 17% from that of untreated algae in 484 (p=0.015) (Fig. 1a) and 968 (p=0.044) (Fig. 1c) ppm CO_2, respectively. Contrastingly, F_v/F_m of *S. regularis*increased significantly compared to untreated algae with 28% increment in 968 ppm (p=0.013) (Fig. 1c)but remained on par with that of untreated alga in 484 ppm CO_2(Fig. 1a). At the physiological level, the measurement of F_v/F_m is an effective parameter to monitor the efficiency of light utilization by the algae as well as to assess the efficiency and stability of the photosystem II (PSII), a major component of the photosynthetic machinery [23]. F_v/F_m values around 0.6–0.7 are expected for healthy microalgal cells, whereas lower values are observed if cells have been exposed to biotic or abiotic stress [24]. Therefore, it can be concluded that *C. vulgaris* cells were stressed under the high [CO_2] but not *S. regularis*and high [CO_2] can damaged the PSII of *C. vulgaris* resulting in inhibition of the transfer of electrons, leading to side effects such as oxidative stress [25]. In contrast, an increase in F_v/F_m as shown by *S. regularis*under both [CO_2] can indicate increase in the efficiency of PSII and also reduced risk of damage caused to PSII by oxidative stress. Similar increase in F_v/F_m was also reported by [26] with soybean under 1000 ppm CO_2, [27] with white birch under 700 ppm CO_2 and [9] with periwinkle under 560 ppm CO_2. The F_v/F_m did not increased back to normal after the 24h recovery period in *C. vulgaris* treated with both [CO_2] (Fig. 1a,c) indicating that the alga experienced chronic photoinhibition whereby the rate of PSII damage is faster than the rate of repairment[28]. In contrast, a further increment in F_v/F_m was observed significantly in *S. regularis* treated with both [CO_2] after the recovery period (Fig. 1a,c).

The content of H_2O_2 was observed to be significantly increased after the CO_2 treatment and decreased back to normal values after the 24h recovery treatment (Fig. 1b,d). Under stress conditions, the photosynthetic carbon fixation and electron transport rates are not of similar magnitude. This can create an electron excess that cannot be consumed, which will react with O_2, producing reactive oxygen species (ROS), including H_2O_2 [29]. Damaged PSII as indicated by lower F_v/F_m in *C. vulgaris* can also create ROS [25]. Thus, it is expected to observe an increase in H_2O_2 in this alga. It is reported that biological toxicity of H_2O_2 appears to be through oxidation of thiol (-SH) groups and can be enhanced [30]. Following oxidative conditions, algae,like many other living organisms respond by increasing antioxidant defences (e.g. [31, 32]. These antioxidants scavenge and neutralize the ROS lowering the effect of oxidative stress. Hence, the back to normal value observed in *C. vulgaris* after the recovery period (Fig. 1b,d). Contrastingly, a 17% and 24% reduction in H_2O_2 content was observed in *S. regularis* treated with 484 (Fig. 1b) and 968 (Fig. 1d) ppm CO_2, respectively.In addition to the results of F_v/F_m (Fig. 1a,c) and cell viability (data not shown), it can be assumed that the extra CO_2 was not a rate-limiting to the photosynthetic processes of *S. regularis* and the alga uses this CO_2 to increase its ability in maintaining life. Furthermore, at low concentration, H_2O_2 can act as a signal molecule involved in the regulation of specific biological processes and triggering tolerance against various environmental stresses [33].

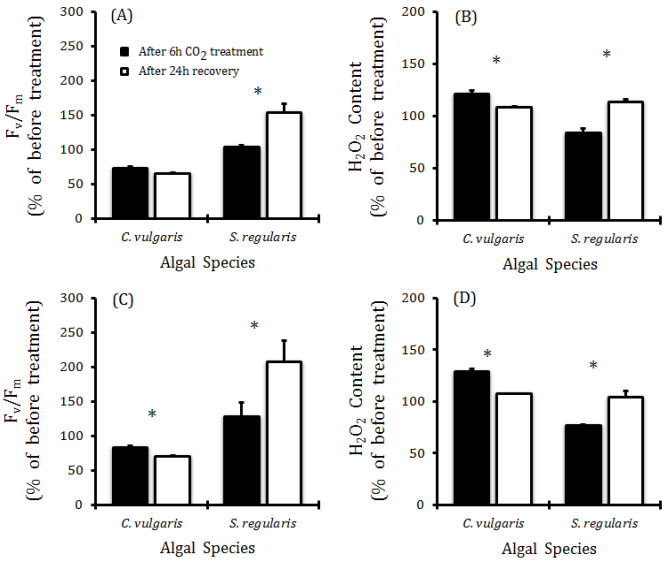

Fig. 1: Maximal quantum yield and H_2O_2 content of *C. vulgaris*and *S. regularis*after 6h treatment with 484 (*A-B*) and 968 (*C-D*) ppm CO_2 and after 24h recovery. Statistical analysis was done by comparing between treatment and recovery periods within similar species(Student's t-Test, n=3, $p<0.05$).

ROS are known to have damaging effects on many important biomolecules including DNA (as reviewed by [34]). In fact, H_2O_2, itself has been proven to induce DNA single and double-strand breaks in cells [35]. From the comet assay (Fig.2), it is evident that high [CO_2] induced DNA damage in both microalgae. Following exposure to high [CO_2], the number of comets belonging to scores of 3 and 4 increased significantly in *C. vulgaris* (Fig. 2a,b). It was observed that high [CO_2] induced the production of H_2O_2 in this algaas well (Fig. 1b,d). Thus, it can be said that the damage of DNA at scores 3 and 4 may be because of the H_2O_2 accumulated in the cells of *C. vulgaris*. After the recovery, the cells that belong to scores 3 and 4 decreased 8% in 484 ppm (Fig. 2a) compared to after the treatment. The reduction may indicate that the cells have repaired the H_2O_2-induced DNA single-strand breaks during the recovery or may also indicate that the highly damaged cells have gone into apoptosis and that the fragmented DNA has disappeared from the gel, leaving only relatively undamaged cells [36]. This condition, however, was not observed for cells treated with 968 ppm CO_2 since the % of cells belonging to scores 3 and 4 increased 2% after the recovery (Fig. 2b).In comparison, *S. regularis* treated with 484 (Fig. 2c) and 968 (Fig. 2d) ppm CO_2 showed significantly less DNA damage with the majority of the comets falling into scores 0, 1and 2.DNA repair activity was also observed during recovery for *S. regularis* cells treated with 484 ppm CO_2 whereby there was a 4% increase and a 6% decrease in cells belonging to score 0 and 2, respectively as compared to after treatment (Fig. 2c).

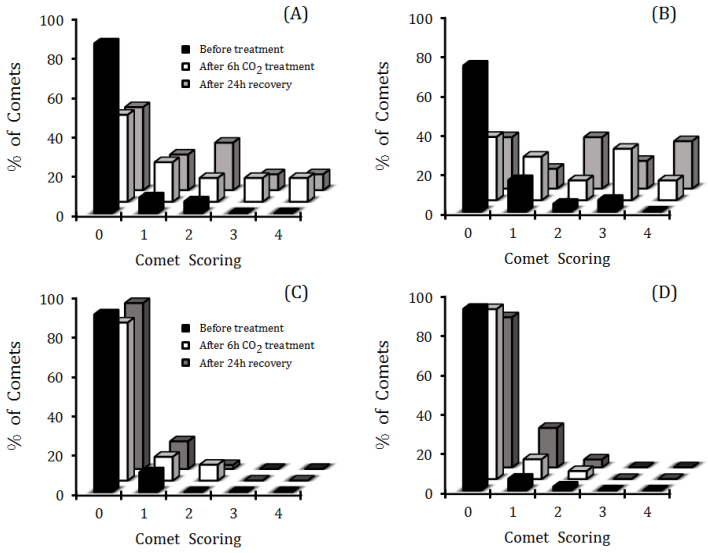

Fig. 2: Levels of DNA damaged in *C. vulgaris* cells after 6h treatment with 484 (*A*) and 968 (*B*) ppm CO_2 (*white bars*) and after 24h recovery (*grey bars*) compared to control (*black bars*) evaluated using Comet assay.

Conclusion:

In conclusion, *C. vulgaris* was stressed by the high [CO_2] as the CO_2 becomes toxic to the alga, inducing generation of H_2O_2 which in turn affecting the photosynthetic PSII apparatus and damaging the DNA. It also appeared that *C. vulgaris* was not able to fully recover even though the stress subsided. On the other hand, *S. regularis* responded diferently from *C. vulgaris*. *S. regularis* was not stressed by the presence of the 'extra' CO_2 and seemed to be growing healthily in these conditions. Therefore, it can also be concluded that while *S. regularis* is a high CO_2-tolerant species, *C. vulgaris* is more susceptible towards the CO_2. Additionally, *S. regularis* can be an efficient candidate as biomitigation tool of elevated CO_2.Furthermore, the findings from this study can also be used as bioindicators which can help environmentalists to keep track of the current increase in atmospheric CO_2.

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