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Effect of ammonia on the photosynthetic activity of *Arthrospira* and *Chlorella*: A study on chlorophyll fluorescence and electron transport

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ABSTRACT

Although ammoniacal nitrogen is the preferred nitrogen source for microalgae/cyanobacteria, at elevated concentrations and high pH values it may negatively impact photosynthesis and growth. Chlorophyll (Chl) fluorescence analysis is a useful tool to monitor the influence of various stress conditions on the photosynthetic activity of plants or microalgae/cyanobacteria. In this study, we investigated the effect of ammoniacal nitrogen on Chl fluorescence in microalgae/cyanobacteria. Chl fluorescence analysis revealed that the parameters related to flux ratios and specific energy fluxes of photochemistry were gradually inhibited as the free ammonia (FA) concentration increased. Photosynthetic electron transport activity was measured using artificial electron acceptors, donors or inhibitors. These analyses suggest that ammonia has multiple impacts on the photosynthetic apparatus; photosystems I (PSI) and II (PSII), the electron transport chain, the oxygen-evolution complex (OEC) as well the dark respiration were gradually inhibited by increasing FA concentration. At high FA concentration, the PSI/PSII activity increased, suggesting that PSI was more tolerant to FA than PSII. Non-photochemical quenching (NPQ) decreased to zero at elevated FA concentrations. The Chl fluorescence data obtained in the presence of DCMU (diuron) suggest that the decrease of NPQ under ammonia inhibition/toxicity is due to the increase of PSI/PSII activity. The rapid response of Chl fluorescence transients to increases in FA may allow one to use pulse amplitude modulation (PAM) fluorescence as a tool to monitor ammonia inhibition/toxicity in cultures of microalgae/cyanobacteria.

1. Introduction

Microalgae and cyanobacteria have recently attracted the interest because of their potential to produce various products ranging from biofuels (biodiesel, biogas, bioethanol etc.) to high value compounds (pigments, fatty acids, antioxidants, etc.) [1]. Furthermore, microalgae and cyanobacteria show great potential for wastewater treatment because they can remove nutrients (N, P, K, etc.) from wastewater down to very low concentrations [2].

All phototrophs, such as plants, algae and cyanobacteria require substantial amounts of nitrogen for the synthesis of proteins and other essential biomolecules (Chl, DNA, etc.). Nitrogen can be taken up by cells either in the form of ammonium/ammonia or of nitrogen oxides like nitrite and nitrate. Some cyanobacteria are capable of fixing N_2 nitrogen. However, ammoniacal nitrogen is the most preferred nitrogen source because it is already in reduced form and can be utilized directly for the synthesis of proteins. In contrast, nitrate (or nitrite) first has to be reduced intracellularly to ammonia before they can be used for protein synthesis [3]. Despite the fact that ammoniacal nitrogen is the preferred nitrogen source, it is toxic to cells at elevated concentrations and causes growth inhibition or even cell death [4,5]. The use of ammoniacal nitrogen as a nitrogen source for production of microalgae is of particular interest because synthetic ammonium-based fertilizers are cheaper than nitrate-based ones [6]. Moreover, ammoniacal nitrogen is the dominant nitrogen form in many types of wastewater, particularly those from the agro-industrial sector, and these could be utilized as a low-cost nutrient source for the cultivation of microalgae/cyanobacteria [7].

Ammonia toxicity is a well-known phenomenon. Most of the studies, however, have investigated ammonia toxicity in higher plants, fish and other animals [8], and less in bacteria, phytoplankton and yeasts [9,10]. In higher plants some of the ammonia toxicity mechanisms that have been suggested are cation depression (Ca, K and Mg) and changes in the ionic balance of tissues, intracellular pH disturbance, changes in the hormonal balance or saturation of membrane lipids resulting in an increase in membrane permeability (plasmolysis and necrosis) [4,11].

In aqueous solutions, ammonia is present in two types: the protonated cation NH_4^+ (ammonium), and the gaseous form NH_3 (free ammonia or FA). The equilibrium between these two forms is dictated by the pH of the solution. As the pK_a of the ion equilibrium of NH_4^+/NH_3 is 9.25, ammonium dominates at pH < 9.25 and FA above this pH. Ammonium

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does not diffuse through the cell membrane and its uptake rate can be regulated by the cells. In contrast, FA diffuses passively the cell membrane and its uptake rate cannot be regulated, resulting in high ammonium/ammonia intracellular concentrations affecting cell growth and viability [12]. In microalgal or cyanobacterial cultures without pH regulation, the pH of the medium changes relative fast, and can increase up to values above 9. In such environments, ammonium is converted to the FA form that may have a great toxic effect on the cells. The ammoniacal nitrogen concentration in wastewater may vary significantly over time or between the batches [13] and these fluctuations in ammoniacal nitrogen may render the microalgal/cyanobacterial culture susceptible to ammonia toxicity. Although the occurrence of ammonia toxicity is frequently considered an important issue in microalgae cultivation [7,14,15], information on the cause and effects of ammonia on microalgal or cyanobacterial cells is fragmentary. It is known that ammonia influences photosynthesis by uncoupling the ΔpH across the photosynthetic membrane [16,17] or damages the Mn cluster of OEC of PSII [4,18–23]. Also, it has been demonstrated that ammonia toxicity increases the sensitivity of PSII to photodamage [18], but without affecting the PSII repair rate [24]. FA was also shown to promote oxidative stress in the aquatic macrophyte *Myriophyllum mattogrossense* [25].

Chl fluorescence is a widely used technique to monitor photosynthetic performance (mainly in PSII) because it is easy, fast, non-invasive and provides plenty of information about the fundamental performance of the PSII of higher plants, eukaryotic microalgae and cyanobacteria under various environmental conditions [26–30]. In mass culture of microalgae, it has been proposed as a tool to estimate biomass productivity [31]. The stress factors that have been studied most intensively using fluorescence analysis in microalgae and cyanobacteria are temperature [32], salinity [33], light intensity [34,35] or heavy metal toxicity [36,37] while studies on ammonia toxicity are limited. To the best knowledge of the authors, there are no available data about the fluorescence transients of two economically important species, namely of *Arthrospira platensis* and *Chlorella vulgaris* under elevated FA concentration.

The aim of this study was to study the effect on the photosynthetic performance of the cyanobacterium *A. platensis* and the Chlorophyte *C. vulgaris* using Chl fluorescence studies, and to examine whether Chl fluorescence analysis could be used as a tool to monitor ammonia inhibition/toxicity. In addition, photosynthetic electron transport activity was estimated by using specific electron donors/acceptors and inhibitors that interact with specific sites of the photosynthetic apparatus and the photosynthetic electron transport chain [38–40].

2. Materials and methods

2.1. Microorganisms and cultivation conditions

The cyanobacterium *A. platensis* SAG 21.99 and the green microalga *C. vulgaris* SAG 211-11b were used to study the effect of ammonia concentration on the photosynthetic apparatus. These species were selected as model organisms for this study because they are the two species that are cultivated on the largest scale worldwide. *A. platensis* was cultured in a modified Zarrouk medium with the following composition: 16.8 g/L $NaHCO_3$, 2.5 g/L $NaNO_3$, 0.5 g/L KH_2PO_4, 1.0 g/L K_2SO_4, 1.0 g/L NaCl, 40 mg/L $CaCl_2$, 80 mg/L Na_2EDTA, 200 mg/L $MgSO_4 \cdot 7H_2O$, 10 mg/L $FeSO_4 \cdot 7H_2O$ and 1.0 mL of trace elements stock solutions: 2.86 g/L H_3BO_3, 20 mg/L $(NH_4)6Mo_7O_{24}$, 1.8 g/L $MnCl_2 \cdot 4H_2O$, 80 mg/L $CuSO_4$ and 220 mg/L $ZnSO_4 \cdot 7H_2O$. The green alga *C. vulgaris* was cultured in Wright's Cryptophyte (WC) medium with the following composition: 85.1 mg/L $NaNO_3$, 36.76 mg/L $CaCl_2$ $2H_2O$, 36.97 mg/L $MgSO_4 \cdot 7H_2O$, 12.6 mg/L $NaHCO_3$, 28.42 mg/L $Na_2SiO_3 \cdot 9H_2O$, 8.71 mg/L K_2HPO_4, 24 mg/L H_3BO_3 and 1 mL/L of trace elements stock solutions: 4.36 g/L $Na_2EDTA \cdot 2H_2O$, 3.15 g/L $FeCl_3 \cdot 6H_2O$, 2.5 mg/L $CuSO_4 \cdot 5H_2O$, 22 mg/L $ZnSO_4 \cdot 7H_2O$, 10 mg/L $CoCl_2 \cdot 6H_2O$, 180 mg/L $MnCl_2 \cdot 4H_2O$, 6.3 mg/L $Na_2MoO_4 \cdot 2H_2O$, and 18 mg/L Na_3VO_4. Both species were cultured in 2 L glass bottles illuminated with 75 μmol photons/m^2/s and agitated through aeration.

Subsamples from the cultures were exposed to ammonia treatments in 20 mL transparent disposable plastic beakers for fluorescence analysis or 100 mL beakers for electron transport activity analyses. The beakers were illuminated using 30 W cool fluorescent tubes providing a light intensity of 150 μmol photons/m^2/s. The experiments were conducted in an air-conditioned room with constant temperature of 22 °C. For the fluorescence experiments, the biomass was harvested by filtration (20 μm pore size filter) for *A. platensis* and by centrifugation (3000 rpm for 5 min) for *C. vulgaris*. The cells were re-suspended at a constant optical density ($OD_{660\ nm} = 0.1 \pm 0.05$) in fresh cultivation media supplemented with the various ammonia concentrations. Ammoniacal nitrogen was added as NH_4Cl in concentrations ranging from 12.5 to 200 mg-N/L. The pH was adjusted using NaOH to 9.25 in all treatments (including the controls). At this pH value, 50% of the total ammoniacal concentration is in the form of FA (NH_4^+/NH_3 equilibrium has an acid dissociation constant (pK_a) of 9.25). This resulted in FA concentration ranging from 6.25 to 100 mg-N/L. The pH of 9.25 was chosen in order to achieve increased FA concentrations while keeping total ammoniacal concentrations relative low. Nevertheless, the intention of the present study was not to simulate real or optimum cultivation conditions and therefore the optimum pH or temperature for each microorganism was not taken into consideration. The pH of the stock cultures ranged from 9.5 to 10 for *C. vulgaris* and 10 to 10.5 for *A. platensis*. We assumed that the adjustment of the pH to 9.25 would not have a significant impact on the cells. All experiments were performed in triplicate.

2.2. Analytical methods

2.2.1. Fluorescence transient (OJIP test)

The polyphasic fluorescence transients were measured using the portable PAM fluorometer AquaPen-C AP-C 100 (PSI, Czech). In this test, the increase in fluorescence in response to a saturating light intensity is recorded. The general behavior of the oxygenic phototrophs is that the fluorescence transient starts at an initial fluorescence F_0 (phase O), passes through two intermediate steps F_J and F_I (phases J and I, respectively) before it reaches a maximum F_M (phase P). The fluorescence transient reflects the kinetics and heterogeneity involved in the reduction of the plastoquinone (PQ) [27]. Briefly, as it is illustrated in Fig. 1, photons are absorbed by the antennae pigments (ABS, absorption flux) resulting in their excitation. A part of the excitation energy is transferred to the reaction center (RC) called trapping flux (TR), while the other part is dissipated either as fluorescence or as heat. The trapped flux (TR) is converted in the RC to redox energy by reducing the electron acceptors Q_A to Q_A^-, which is then re-oxidized to Q_A leading to an

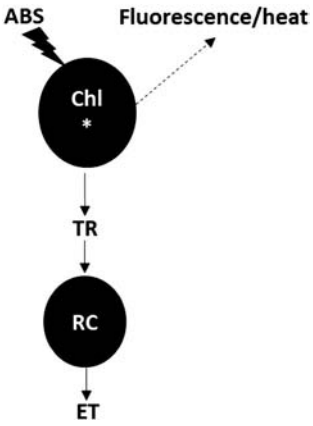

Fig. 1. Schematic representation of the energy-flux model of PSII based on Strasser et al. [41]. ABS: light absorption flux; TR: energy flux trapped by PSII; RC: reaction centers; ET: electron transport flux.