# Inhibition of the water splitting system by sodium chloride stress in the green alga *Chlorella vulgaris*

**Mostafa M. EL-Sheekh**

*Botany Department, Faculty of Science, Tanta University, 31527 Tanta, Egypt. E-mail: msheekh@dec1.tanta.edu.eg*

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The effect of sodium chloride on the photosynthetic electron transport chain was investigated in the freshwater green alga *Chlorella vulgaris*. It was found that the rate of oxygen evolution under steady state and flash light conditions diminished by increasing salt concentrations. Salt treatment of cells also decreased the fluorescence and delayed luminescence yields. However, the fluorescence yield was almost completely restored by the addition of an artificial electron donor to the NaCl-treated cells. The oscillation pattern of the thermoluminescence B band as a function of flash number indicated that the $S_2 \rightarrow S_3$ transition of the water splitting system is inhibited by NaCl treatment.

**Key words:** *Chlorella*, electron transport, photosystem II, salinity.

**Inibição do sistema de fotooxidação da água por estresse com cloreto de sódio na alga verde *Chlorella vulgaris*:** O efeito do NaCl na cadeia transportadora de elétrons foi investigado na alga verde *Chlorella vulgaris*. Observou-se que a taxa de liberação de oxigênio sob condições de equilíbrio e de flash de luz diminuía à medida que se aumentava a concentração de sal. O tratamento com NaCl também diminuiu o rendimento da fluorescência e retardou o da luminescência. No entanto, o rendimento da fluorescência foi quase inteiramente restaurado pela adição de um doador artificial de elétrons. O padrão de oscilação da banda de termoluminescência B como função do números de flashes indicou que a transição $S_2 \rightarrow S_3$ do sistema de fotooxidação da água era inibida pelo tratamento com NaCl.

**Palavras-chave:** *Chlorella*, fotossistema II, salinidade, transporte de elétrons.

**Abbreviations:** Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCPIP, 2,6-dichlorophenolindophenol; Fm, F0, maximum and constant fluorescence; Fp, yield of fluorescence at transient Fp; Fv, variable chlorophyll fluorescence; MV, methyl viologen; QA, and QB, primary and secondary quinone acceptors of PSII; PS, photosystem

## INTRODUCTION

Salinity of soil and irrigation water is a serious problem in agriculture leading to severe crop losses. It is also considered an important ecological variable in the fresh water and marine environment. Salinity has been suggested as being a controlling factor for blooms of cyanobacteria in estuaries and is considered as one of the major constraints on species diversity and productivity of natural population of algae (Booth and Beardall, 1991; Chen and Plant, 1999). Particularly in estuarine water planktonic algae are often subjected to widely fluctuating salt concentrations (Guillard, 1962; Moisander et al. 2002). Such changes in the salinity of water often affect the growth, metabolism and photosynthesis of phytoplanktons (Moisander et al. 2002; Lartigue et al. 2003). Salt might have a direct effect upon processes involved in electron transport and/or photophosphorylation and result in a decrease in the quantum efficiency of photosynthesis (Seeman and Critchley, 1985). In this connection El-Sheekh and Omar (2002) indicated that ATP is severely affected by salt stress in *Chlorella vulgaris*, however NADPH was not affected. Other studies (Sharma and Hall, 1991) showed that the light saturating rate of $CO_2$ uptake and maximum quantum yield decreased with increasing salt concentrations in barley and sorghum seedling leaves. Shen and Katoh (1991) working

with chloroplasts from spinach localized the NaCl effect at photosystem II. However, there are several studies on the effect of salt stress on microorganisms, particularly in freshwater algae dealing with the inhibitory effect of NaCl on oxygen evolution, chlorophyll fluorescence, the photochemistry and function of photosystem II. (Joset et al. 1996; Murakami et al. 1997; Gonzales-Moreno et al. 1997; Lu and Vonshak; 1999, Lu and Zhang, 1999; Lu et al. 1999; Lu and Zhang, 2000; Lu and Vonshak, 2002). The work presented here deals with the effect of NaCl stress on photosynthesis of the freshwater green alga *Chlorella vulgaris*. Applying the techniques of fluorescence, delayed light emission and thermoluminescence measurements, we localized the inhibitory NaCl action at the $S_2 \rightarrow S_3$ transitions of the water splitting system in photosystem II.

## MATERIAL AND METHODS

*Organism and Growth Conditions*: *Chlorella vulgaris* was isolated from a water sample collected from the channels of Nile River in Egypt. Bacterial-free cultures were obtained by using the technique described by EL-Sheekh (1990). The medium used for cultivation was the freshwater algal medium recommended by Kuhl (1962). The cultures were continuously illuminated with fluorescent tubes, incubated at 27°C and aerated with a mixture of 95% air and 5% $CO_2$. The cells were incubated with different concentrations of NaCl for 10 min in the dark before measurements.

*Oxygen Evolution Measurements:* Oxygen evolution and /or consumption were measured with A Clark-type electrode. The actinic white light was obtained from a 150 W tungsten lamp. For oxygen evolution under flash light conditions, the algal cells were suspended in 50 mmol.$L^{-1}$ phosphate buffer pH 6.5 containing 50 mmol.$L^{-1}$ KCl. Flash-induced oxygen yield was measured with a Joliot-type electrode. 100 µl sample aliquots with a chlorophyll concentration of 70 µg chl.$ml^{-1}$ were illuminated with a sequence of short flashes after 5 min adaptation in the dark on the surface of the electrode. The flash frequency was 2 Hz.

*Fluorescence Induction Measurements:* Fluorescence induction transients of the intact algal cells (equivalent to 15 µg chl.$ml^{-1}$) were measured after excitation with white light (NARVA, TGL 10619, 10 W.$m^{-2}$). The emitted light was detected by an EMI 9558B photomultiplier perpendicular to the exciting light path. The fluorescence transients were recorded by a multichannel analyzer (ICA 70 KFKI).

*Delayed Luminescence Measurements:* For delayed luminescence measurements, algal cells were excited in a 1 cm cell as described by Hideg and Demeter (1985). The emitted light observed after opening of a Uniblitz shutter, was detected by an EMI 9558B photomultiplier. The signal was amplified and stored in the above mentioned multichannel analyzer connected to X-Y recorder.

*Thermoluminescence Measurements*: Thermoluminescence measurements were carried out as described by Demeter and Vass (1984). Samples containing 100 µg chl.$ml^{-1}$ were quickly cooled to -40°C. The samples were illuminated with white light (20 W.$m^{-2}$) for 30 sec before heating. Glow curves were then recorded between -40°C and 60°C at a heating rate of 20°C.$min^{-1}$.

## RESULTS

Figure 1 shows the effect of NaCl treatment on the oxygen evolution and on partial reactions of photosynthetic electron transport. A 90% inhibition of whole chain oxygen evolution was observed at 0.5 mol.$L^{-1}$ NaCl. The measurements of PSII-activated electron transport were carried out with DCQ as electron acceptor. It was strongly affected by increasing NaCl concentrations. At 0.5 mol.$L^{-1}$ NaCl the inhibition was about 70% of the control. PSI-mediated electron transport from $DCPIPH_2$ to methyl viologen was not affected by NaCl treatment in the applied concentration range. The flash light-induced oxygen production was drastically decreased with increasing NaCl concentrations but the pattern of oscillation did not change (figure 2). Similar to the results obtained during constant illumination, 0.5 mol.$L^{-1}$ NaCl inhibited the steady state oxygen evolution by about 70% (figure 2). The inhibitory effect of NaCl on PSII-mediated electron transfer was in addition investigated by thermoluminescence measurements. Figure 3A shows that the intensity of the B band is decreased with increasing NaCl concentrations and shifted by about 5°C from 25°C to 30°C. After addition of DCMU (figure 3B) the intensity decreasing effect of NaCl can also be seen with the Q band. These results provide evidence that the NaCl treatment decreases PSII activity. The inhibitory action of NaCl on the water-splitting system of photosynthesis was also seen with measurements of fluorescence induction of NaCl-treated cells. The $F_m$ level was markedly reduced (figure 4) but the $F_o$ level did not change, indicating a decrease of $F_v$ attributed to an inhibition of the electron flow at the water oxidizing side of PSII. Substitution of the inactivated water splitting system by the artificial electron donor $NH_2OH$ restored the fluorescence intensity to almost the control level (figure 5). This indicates that the action site of NaCl is before the $NH_2OH$ donation site and this is in agreement with our

results drawn from the thermoluminescence investigation. Measurements of the delayed light emission kinetics (figure 6), which also is a measure for PSII charge recombination, are in agreement with the above finding. Figure 7 shows the oscillation pattern of the thermoluminescence B band as a function of excitation flash number. In the NaCl-treated alga, the B band does not oscillate.

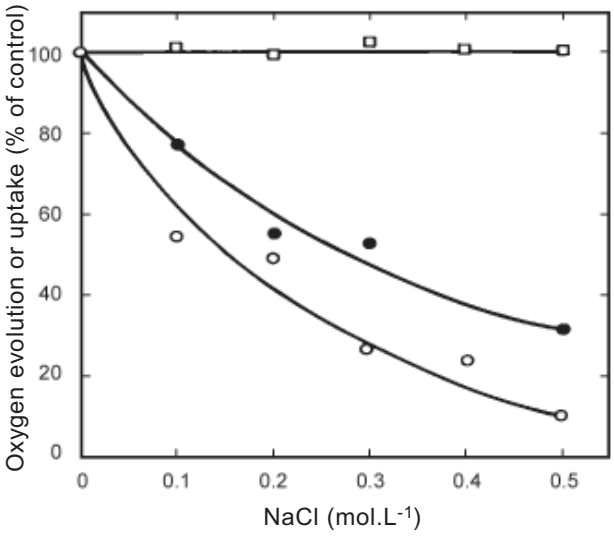


**Figure 1**. Effect of NaCl on various partial electron transport reactions of *Chlorella vulgaris*. Whole chain electron transport (○, open circle). PSI activity was measured from DCPIP (40 µmol.L$^{-1}$) to MV (2 mmol.L$^{-1}$) in the presence of 10 µmol.L$^{-1}$ DCMU and 2 mmol.L$^{-1}$ sodium ascorbate (□, open square). PSII activity was measured from $H_2O$ to 0.5 mmol.L$^{-1}$ DCQ (●, closed circle).

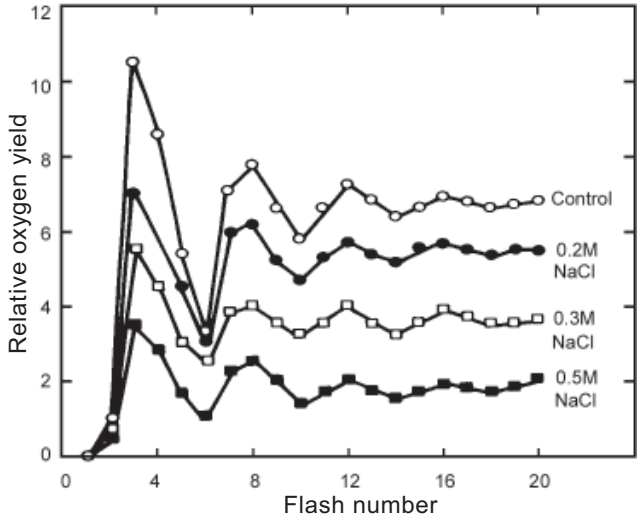


**Figure 2**. Effect of NaCl on the flash-induced oxygen yields of *Chlorella vulgaris* cells. After incubation with the salt, the cells were suspended in 50 mmol.L$^{-1}$ phosphate buffer, pH 6.5, containing 50 mmol.L$^{-1}$ KCl and incubated in the dark on the surface of the electrode for 5 min before flash excitation.

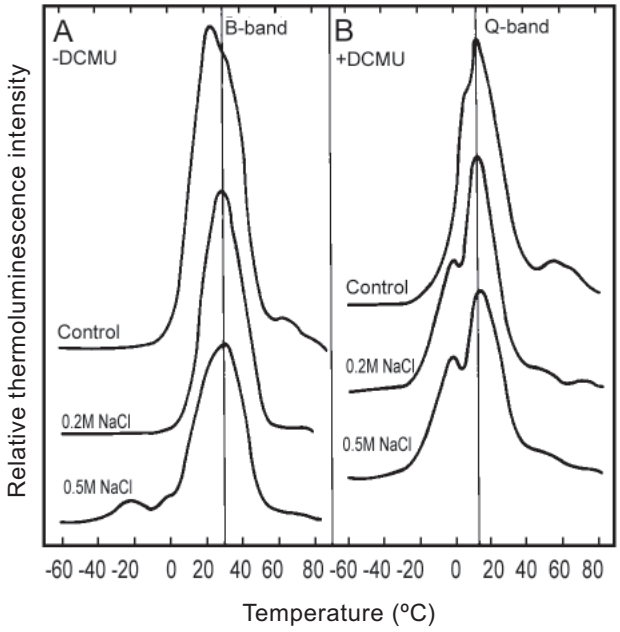


**Figure 3**. Effect of NaCl on the Q and B thermoluminescence bands of *Chlorella vulgaris* cells. Glow curves were obtained as described in Materials and Methods. The Q band was measured in the presence of 10 µmol.L$^{-1}$ DCMU. DCMU was added after incubation of the cells with the salt. The curves shown in the figures are representative of three independent measurements. (A) in the absence of DCMU, (B) in the presence of DCMU.

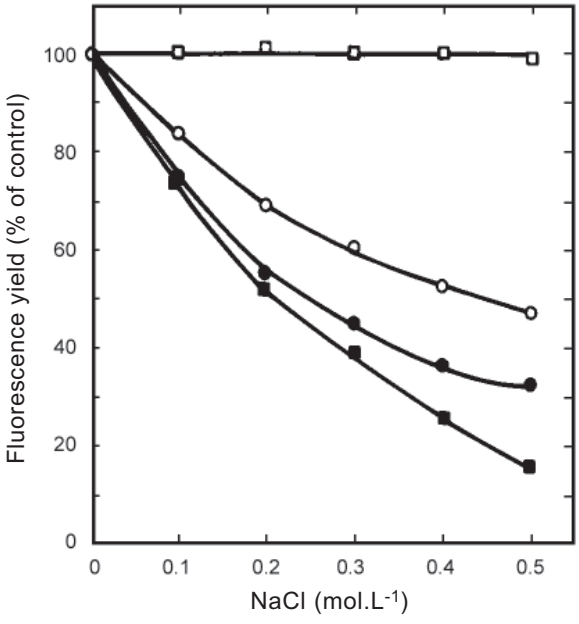


**Figure 4.** Effect of NaCl on the fluorescence induction transients of *Chlorella vulgaris* in the presence of DCMU, $F_m$ (○, open circle), $F_v$ (●, closed circle), $F_o$ (□, open square) and $F_p$ (□, closed square) in the absence of DCMU.

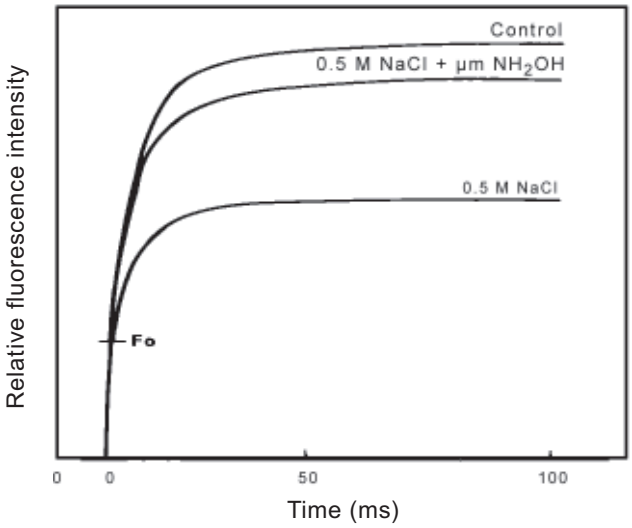


**Figure 5.** Fluorescence induction transient of untreated and NaCl-treated *Chlorella vulgaris* cells and its restoration by the artificial electron donor, 20 µmol.L$^{-1}$ hydroxylamine.

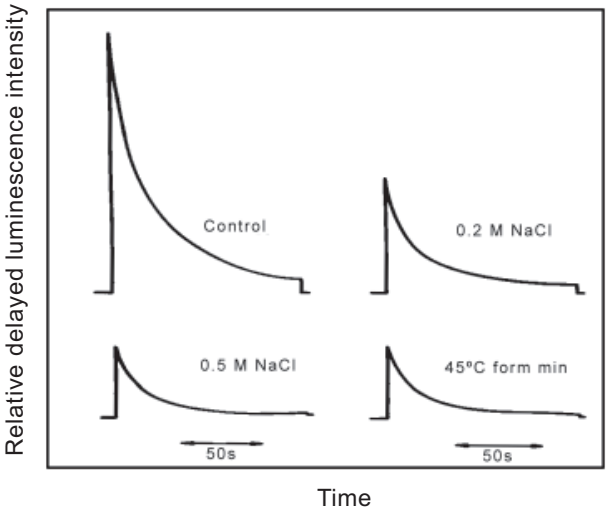


**Figure 6.** Influence of NaCl and heat treatment on delayed luminescence of *Chlorella vulgaris* cells. The curves shown in the figures are representative of four repetitions.

## DISCUSSION

The assay of the partial reaction of PSII using the artificial electron donor dichloro-p-benzoquinone indicated a rapid loss of oxygen evolution with increasing NaCl concentrations. However, the loss of PSI-mediated electron transport from DCPIPH$_2$ to methyl viologen was not affected by NaCl treatments. The inhibitory effect of NaCl on PSII-mediated electron transfer was also investigated by the thermoluminescence (TL) studies. The B band originates from $S_2Q_B^-$ and $S_3Q_B^-$ charge recombination and the Q band originates from $S_2Q_A^-$ charge recombination in the presence of DCMU (Demeter and Govindjee, 1989; Vass and Inoue,

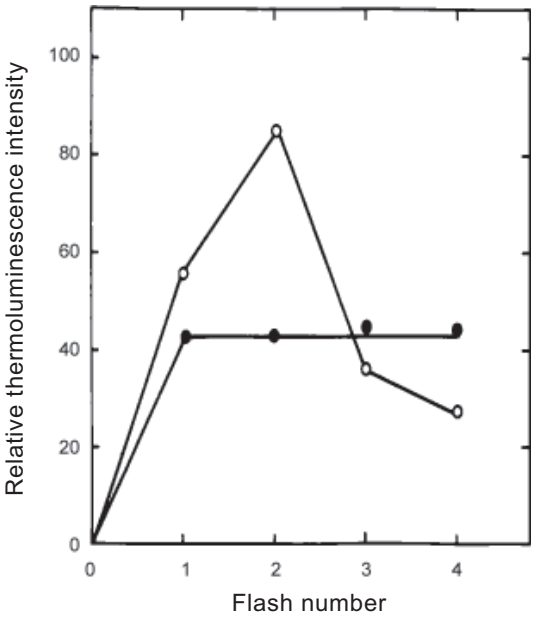


**Figure 7.** Oscillation of flash-induced thermoluminescence B band in control (○, open circle) and 0.5 mol.L$^{-1}$ NaCl-treated (●, closed circle) *Chlorella vulgaris*. Dark-adapted samples (5 min) were illuminated with a series of flashes at -5$^{o}$C and cooled quickly to -60$^{o}$C and the B band was recorded as described in the Materials and Methods.

1992). The magnitude of the two bands is diminished by NaCl treatments and this result supports the inhibitory effect of NaCl on PSII. Fluorescence induction was used to probe the pattern of inhibition (Murphy et al. 2003). In algal cells treated with different NaCl concentrations, both the maximum fluorescence ($F_m$) and the variable fluorescence ($F_v$) was markedly reduced. A decrease in fluorescence yield of the intact algae cells can be attributed to an inhibition of the electron flow at the oxidizing side of PSII (Lu and Vonshak, 2002). Substitution of the inactivated water splitting system by an artificial electron donor $NH_2OH$ (Canaani et al. 1986), restored the fluorescence intensity almost to the initial control. This result demonstrates that the action site of NaCl is before the $NH_2OH$ donation site. In this connection, Lu and Vonshak (2002) indicated that 0.8 mol.L$^{-1}$ NaCl decreased oxygen evolution which correlated with the decrease in the quantum yield of the PSII electron transport. They also suggested that salinity stress resulted in a decrease in the efficiency of electron transfer from $Q_A^-$ to $Q_B$. Delayed light emission is the result of charge reactions in PSII (Lavorel, 1975). The effect of heat, which is well known to inhibit the water splitting system, is comparable with the effect of NaCl and a similar decrease in the amplitude of delayed light emission was obtained. This result suggests that NaCl inhibits the electron

transport process at the water splitting system. Gonzales-Morono et al. (1997) also suggested that NaCl induced a drop in the $HCO_3^-$ - dependent water splitting activity of PSII of *Euglena gracilis*. The assignment of the B and Q thermoluminescence bands to the $S_2/S_3$ $Q_B^-$ and $S_2/Q_A^-$ recombination, respectively, opens up a new perspective in the study of both the water splitting system and the primary and secondary quinine acceptors. Since the S states participate in the generation of the B band, the oscillatory behavior of the B band provides an unique opportunity to investigate the transition of S states even if oxygen evolution is inhibited. In the salt-treated alga, the TL intensity could be changed by the first flash, but subsequent flashes did not change TL intensity. Since the water splitting system is in the $S_2$ state after the first flash, it can be concluded that NaCl inhibited the $S_2 \rightarrow S_3$ transition of the water splitting system. These results confirm the assumption that the mechanism of NaCl inhibition of PSII in the *in vivo* system at the low salt concentrations (0.1-0.5 mol.$L^{-1}$) used in this investigation is the same as has been observed by NaCl in *in vitro* experiments at high salt concentrations (1-2 mol.$L^{-1}$).

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