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Negative effects of P-buffering and pH on photosynthetic activity of planktonic desmid species

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Abstract

The photosynthetic activities of three planktonic desmid species (*Staurastrum brachiatum*, *Staurodesmus cuspidatus* var. *curvatus*, and *Staurastrum chaetoceras*) were compared after adaptation to medium enriched with either a 20 mM Na^+-phosphate (P) or HEPES buffer. Incubations up to 2 d were carried out at pH 6 or 8 under normal air or air enriched with 5 % CO_2. Gross maximum photosynthetic rate (P_{max}) and growth rate were decreased in both *S. brachiatum* and *Std. cuspidatus* at higher pH when using the HEPES buffer and this effect was independent of CO_2 concentration, indicating that pH had an inhibitory effect on photosynthesis and growth in these species. The P-buffer at pH 8 caused a large decrease in P_{max} and quantum yield for charge separation in photosystem 2 (PS2), compared to HEPES-buffered algae. This effect was very large in both *S. brachiatum* and *Std. cuspidatus*, two species characteristic of soft water lakes, but also significant in *S. chaetoceras*, a species dominant in eutrophic, hard water lakes. The decreased P_{max} in P-buffer could not be related to a significant increase in cellular P content known to be responsible for inhibition in isolated chloroplasts. Experiments at pH 6 and 8 showed that two conditions, high pH and high Na^+ concentration, both contributed to the decreased P_{max} and quantum yield in the desmids. Effects of a P-buffer were less pronounced by using K^+-P buffer. The use of P-buffer at pH 8 possibly resulted in high irradiance stress in all species, indicated by damage in the PS2 core complex. In the soft water species pH 8 resulted in increased non-photochemical quenching together with a high de-epoxidation state of the xanthophyll cycle pigments.

Additional key words: *Desmidiaceae*; high irradiance stress; inorganic carbon limitation; Na^+; non-photochemical quenching; photosystem 2; pH-stress; *Staurastrum*; *Staurodesmus*.

Introduction

Most representatives of the algal family Desmidiaceae grow in slightly acidic, oligotrophic environments. A limited number of species are, however, more often found in eutrophic environments (Brook 1981), and several of these species can be characterized as 'eutrophic', only occurring in eutrophic lakes with high pH (Coesel 1994). During 10 d experiments, Coesel (1993) observed that the growth of planktonic desmid species was influenced by the buffer used. Therefore, in order to study the physiological mechanisms of inorganic carbon uptake in desmids (*Chlorophyceae*) as an important factor in their distribution pattern in the field, we searched for an appropriately buffered medium with a minimum effect on photosynthesis. A Tris/HCl buffer (in a strength of 4.0 mM) at pH 8.5 was lethal for *Closterium acutum* var. *variabilis* whereas 2.0 mM HEPES/NaOH or 4.5 mM (Na^+)bicarbonate at that same pH resulted in 50 and 70 % growth reduction, respectively (Coesel 1993). Moreover, photosynthetic rates in *Cl. acutum* were already slightly decreased immediately after incubation into pH 8.5 as compared to pH 7.0, being their pre-culture pH. Because photosynthetic activity is more or less coupled to CO_2 concentration (Miyairi 1998) and pH is very closely related to CO_2 concentration (Stumm and Morgan 1970), photosynthesis

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was determined in a well buffered medium at high and low CO_2 concentration at two different pH conditions. A HEPES and (Na^+)-phosphorus buffer were chosen for this purpose. Buffers were used in strength of 20 mM, being a minimum strength to counterbalance the acidifying effect of CO_2-gassing.

Although phosphate (P)-buffer inhibits photosynthesis in isolated chloroplasts of several green algal species (Klein *et al.* 1983, Goyal *et al.* 1988, Rotatore and Colman 1990, Moroney and Mason 1991), they are commonly used in physiological experiments with algae (Birmingham and Colman 1979, Rotatore and Colman 1991). Amoroso *et al.* (1998) observed that 20 mM of a P-buffer did not have a negative effect on whole cells of the green alga *Chlamydomonas reinhardtii*. Birmingham and Colman (1979) reported that for various green algal species a 50 mM P-buffer (and also HEPES buffer) had no negative effect on the maximal photosynthetic rate or carbon compensation concentration. As a result of these inorganic carbon related studies, P and HEPES were considered appropriate buffers for our future investigation.

We used three desmid species characteristic of either soft or alkaline lakes, namely *Staurastrum brachiatum* and *Staurodesmus cuspidatus* var. *curvatus* isolated from soft water bodies and *Staurastrum chaetoceras* from an alkaline lake. Photosynthesis was measured after culturing them at pH 6 and 8 in medium enriched with 20 mM P or HEPES for 1 or 2 d.

Materials and methods

Three planktonic desmid species were tested: *Staurastrum brachiatum* Ralfs (clone 232, isolated from Glennicmurrin, Ireland), *Staurodesmus cuspidatus* (Bréb.) Teil. var. *curvatus* (W. West) Teil. (clone 137, isolated from Lough Bofin, Connemara, Ireland), and *Staurastrum chaetoceras* (Schr.) Smith (clone 233, isolated from Lake IJmeer, The Netherlands). The first two species were isolated from soft water lakes and the latter from an alkaline lake. The isolates originated from the desmid culture collection of the Department of Evolutionary Botany, University of Amsterdam.

Continuous cultures (for design and conditions, see Coesel and Wardenaar 1994) growing at growth rates about half the species specific maximum growth rate, μ_{max} were maintained in continuous irradiation (50–100 μmol m^{-2} s^{-1}) and aerated with normal air at 20 °C. The culture medium consisted of Woods Hole medium (Nichols 1973), where TRIS-buffer was replaced by 2 mM HEPES-NaOH, pH 7.0. For the buffer experiments, cells were collected by centrifugation (1 500×*g*, 2 min) and re-suspended in Woods Hole medium enriched with a 20 mM HEPES-NaOH or Na_2HPO_4/NaH_2PO_4 (P) buffer at pH 6.0 and 8.0. Cultures were aerated with normal air. The buffer concentration used raised inorganic P (P_i) concentration 400 times and HEPES concentration 10 times compared to the concentration in the original culture medium. For CO_2-experiments, cells were re-suspended in medium enriched with 20 mM HEPES buffer at pH 6.0 and 8.0. Cultures were either aerated with normal air (low CO_2) or with air enriched with 5 % CO_2 (high CO_2). High CO_2 conditions did not change the pH of the culture at pH 6.0, but lowered the pH of the 8.0-culture to ~ 7.7. Both incubations were performed at 150 μmol m^{-2} s^{-1} incident irradiance (continuous). Although HEPES is not the optimal buffer at pH 6, its use was justified, since the pH remained stable during the pH 6 experiments using this buffer.

Cell concentrations were determined on a Coulter Counter multi-sizer. All incubations were started with a cell volume density of 250 cm^3 m^{-3}, using the volume estimation from the Coulter Counter. This is approximately $4x10^{10}$, $8x10^{10}$, and $5x10^{10}$ cells m^{-3} for *S. brachiatum*, *Std. cuspidatus*, and *S. chaetoceras*, respectively.

Pulsed Amplitude Modulator (*PAM*): Photosystem 2 (PS2) fluorescence emission was measured in a pulse amplitude modulated fluorimeter (*PAM-101*, *H. Walz*, Effeltrich, Germany) equipped with the *ED-101US* emitter-detector cuvette. F_0 and F'_0, the minimal fluorescence emission in darkness and with actinic irradiation, respectively, were recorded with the measuring beam at 1.6 kHz (450 nm LED emission). A strong saturating pulse (*Schott Kl-1500* lamp; 12 000 μmol m^{-2} s^{-1}) was applied to fully reduce Q_A and measure maximum fluorescence emission F_m (dark-acclimated samples) or F'_m (during actinic irradiation, being 150 μmol m^{-2} s^{-1}). The maximum quantum yield of charge separation in the dark ($\Phi_{II,d}$) was expressed as $(F_m - F_0)/F_m$, whereas the quantum yield in the light ($\Phi_{II,l}$) was calculated as $(F'_m - F'_0)/F'_m$. The reduction of F_m in light was taken as a measure of non-photochemical quenching (NPQ), using the Stern-Volmer equation: $F_m - F'_m/F'_m$.

77 K emission fluorescence: Dark-acclimated samples (min. 30 min) were taken by glass pipettes (2 mm internal diameter) and immediately frozen in liquid nitrogen. Low temperature fluorescence emission measurements were performed on an *Aminco-Bowman* series *2* luminescence spectrometer with a Dewar flask filled with liquid nitrogen. Fluorescence emission was recorded from 650 to 750 nm exciting chlorophyll at 440 nm with a band width of 4 nm. Photosystem 1 and 2 absorption maxima were obtained between 720–730 and 680–695 nm, respectively.

Photosynthesis *vs.* irradiance curves (*P*/*I*): Oxygen production was measured with a polarographic oxygen electrode at six varying photosynthetic photon flux densities (PPFD) from 0 to 1 500 μmol m^{-2} s^{-1} in a temperature controlled oxygen chamber (Dubinsky *et al.* 1987). In

buffer experiments, *P-I* curves were recorded after 1-d incubation because after prolonged incubation in pH 8 P-buffer only respiration could be determined. In CO_2 experiments, measurements were performed after 2 d. Oxygen production or consumption rates were recorded over a 3 min period for every PPFD. Results were fitted to an exponential model (Webb *et al.* 1974):

$$P = P_{max}\,(1 - e^{-\alpha I/Pm}) + R_D,$$

in which α is the slope of the initial part of the curve that is related to the maximum quantum yield for photosynthesis, P_{max} is maximum gross oxygen production rate, R_D is dark respiration rate, and I is irradiance. Data were fitted to the model using the non-linear module in *SPSS* software. *SPSS* software was also used to perform statistical tests. CA was purchased from *Sigma* [(6 240 W-A units per mg(protein); 83 % protein].

Chemical analyses: For cellular P determinations, cells were collected on acid-drained filters (cellulose acetate, *Sartorius*, 12 μm) and washed several times with demineralized water. Filters were disrupted in 0.15 M H_2SO_4 plus 56 mM $K_2S_2O_8$ at 100 °C for 1 h. Total cellular phosphate was then determined spectrophotometrically according to Murphy and Riley (1962). Pigments were analysed using high performance liquid chromatography (HPLC) following Kraay *et al*. (1992). Algal cells were filtered through a *Whatman GF/C* paper and frozen immediately in liquid nitrogen. Pigments were extracted in 90 % acetone using glass beads. Immediately before injection into the HPLC, samples were diluted with water to 70 % acetone. Pigment quantification was done by reverse phase C-18 high liquid chromatography, in an *Isco 77* system equipped with a 5 μM size particle *Hypersil ODS-2* column (250.0×3.2 mm). 90 % acetronitrile in water was used as solvent A and 100 % ethylacetate as solvent B. Pigment absorption was recorded at 436 nm in an *Isco* detector and at 452 and 485 nm in a Diode Array Detector (*Kontron Instruments, DAD 440*) connected in series with the *Isco* system. De-epoxidation state of xanthophyll cycle pigments (A = antheraxanthin, V = violaxanthin, Z = zeaxanthin) was calculated by (V + 0.5 A)/(V + A + Z).

Addition of test substances: Algal cells adapted to P-buffer at pH 8 for 5 or 24 h were incubated in the oxygen chamber (optical density of 0.1 at 750 nm) and adapted to 150 μmol m^{-2} s^{-1} irradiance for a few minutes. In some cases 3-phosphoglycerate (3-PGA) was added to a final concentration of 2 mM. The addition of 3-PGA compensates for inhibiting effects of increased P concentrations on photosynthesis in isolated chloroplasts (Goyal *et al.* 1988). Prior to the addition of 3-PGA, nystatin (0.5 mg cm^{-3} final concentration) was added, as 3-PGA cannot be taken up by whole cells (Moroney and Mason 1991). This concentration of nystatin was found to permeabilize the plasma membrane without any other visible damage to other membranes within a time span of 15 min (results not shown). O_2 production was followed throughout the experiment. Experiments did not exceed a total of 15 min, since during longer incubations chloroplast membranes were also permeabilized and O_2 production diminished. As another alternative, mastoparan (up to a concentration of 4 μM) was added to P-buffered algae, incubated in the oxygen chamber. Because mastoparan is a G-protein activator (Takahashi *et al.* 1998), triose-P concentrations in the cytoplasm likely increase (Legendre *et al.* 1993).

Likewise, experiments were performed with the addition of carbonic anhydrase (CA, 10 μg cm^{-1} final concentration). Oxygen evolution was recorded in the cell suspension already containing 150 mM HCO_3^- that will be quickly converted to CO_2 after addition of CA. CA was purchased from *Sigma* [6 240 W-A units mg^{-1}(protein), 83 % protein].

Results

Photosynthesis: P_{max} values were significantly decreased in cells of *S. chaetoceras*, *S. brachiatum,* and *Std. cuspidatus* after a 1 d period at pH 8 in a P-buffer compared to pH 6 in a HEPES buffer (Table 1, *t*-test, $p<0.01$). In addition, photosynthetic performance was higher in every pH 6-buffered culture when compared to pH 8, and in the HEPES-buffered as compared to the P-buffered cultures. The observed P_{max} values in all three species in the HEPES buffer at pH 6 were higher than those observed in cells taken from 0.5 μ_{max} growing continuous cultures (pH 7, HEPES buffer, Table 1). Aeration with CO_2 did not result in significant differences in P_{max} within a certain pH in all three desmid species, whereas in contrast, differences in P_{max} at pH 6 and 8 were pronounced in *S. brachiatum* and *Std. cuspidatus* (Table 1, *t*-test, $p<0.05$). In *S. chaetoceras* no significant difference in P_{max} could be detected between cultures grown at pH 6 and 8 (*t*-test, $p = 0.09$), although the values acquired at pH 8 were smaller than those at pH 6. Therefore, photosynthesis declined in all three desmid species as a result of the higher pH and the use of a P-buffer, without any influence of the CO_2 concentration in the culture when using a HEPES buffer. The major adaptations to buffer and pH occurred during the first 5 h of incubation as is shown in a decrease in P_{max} in the P-buffer at pH 8 in *Std. cuspidatus* (Fig. 1). After approximately 5 h, P_{max} only slowly decreased and remained about equal to measurements performed after 1 d. After adaptation to pH 8, the photosynthetic reaction to the addition of CA (10 mg m^{-3} final concentration) was tested in all three desmid species. In all cases (over 5 separate times for every species) no significant change in oxygen evolution was recorded

Table 1. Maximum gross photosynthetic rate, P_{max} [g(O_2) kg^{-1} (Chl *a*) s^{-1}] of three desmid species in continuous flow culture (pH 7), adapted to pH 6 or 8 and high or low CO_2 conditions for 2 d using a HEPES buffer and adapted to pH 6 or 8 in a HEPES or P-buffer for 1 d. Values gathered from at least 3 independent incubations±STD.

pH	*S. chaetoceras*	*S. brachiatum*	*Std. cuspidatus*
7	2.11±0.70	1.74±0.49	2.35±0.78
6, high CO_2	2.98±0.54	3.52±0.92	4.36±1.96
6, low CO_2	9.05±2.73	3.77±1.68	3.90±2.19
8, high CO_2	2.51±0.78	1.48±0.69	1.35±1.49
8, low CO_2	2,29±0.73	0.95±0.61	1.69±0.10
6, HEPES-buffer	3.08±0.60	3.52±0.98	2.88±1.10
6, P-buffer	1.19±0.37	2.04±0.22	1.41±0.29
8, HEPES-buffer	2.52±0.24	1.61±0.85	1.96±0.67
8, P-buffer	0.99±0.65	0.74±0.51	0.46±0.34

although 150 mM HCO_3^- was present in the medium (results not shown). These results complement the results from the high and low CO_2 adapted cultures showing that the CO_2 concentration did not play any role in the decreased P_{max} at higher pH.

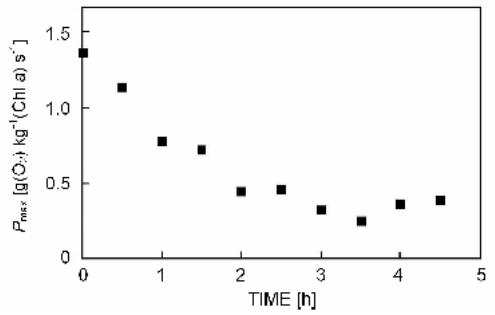

Fig. 1. Maximum O_2 production, P_{max} in cells of *Std. cuspidatus* var. *curvatus* incubated in a 20 mM phosphate buffered medium at pH 8 over a period of 5 h.

Growth rates: Hardly any growth was measured in the P-buffered cultures (Table 2), particularly at pH 8. *S. chaetoceras* grew substantially in the pH 8 HEPES-buffered culture, whereas *S. brachiatum* and *Std. cuspidatus* barely did. The growth rates were more or less comparable to the photosynthetic characteristics shown in Table 1, although some differences are obvious. For example, in *S. brachiatum* substantial O_2 production was measured in pH 6 P-buffered and pH 8 HEPES-buffered conditions, but hardly any growth was observed. As growth rates were calculated over one day of incubation, these values were greatly influenced by the lag phase in growth occurring after the incubation of cells in new culture conditions. This lag phase is very variable and is normally not included in growth rate calculations, but is included here as the experiments only lasted 1 d and cell counts were only executed before and after incubation. As a result of the high variability in the growth rates

Table 2. Growth rates, μ [per d] in three desmid species in continuous flow culture (pH 7), adapted to pH 6 or 8 and high or low CO_2 conditions over 2 d using a HEPES buffer and adapted to pH 6 or 8 in a HEPES or P-buffer over 1 d. Values gathered from at least 3 independent incubations ± STD. N.d. = not determined.

pH	*S. chaetoceras*	*S. brachiatum*	*Std. cuspidatus*
7	0.72	0.43	0.43
6, high CO_2	0.35±0.16	0.18±0.05	0.28±0.08
6, low CO_2	0.30±0.07	0.29±0.08	n.d.
8, high CO_2	0±0	0.07±0.13	0.06±0.10
8, low CO_2	0.15±0.05	0.12±0.19	0.02±0.02
6, HEPES-buffer	0.35±0.15	0.29±0.10	0.24±0.08
6, P-buffer	0.09±0.12	0.10±0.13	0.18±0.15
8, HEPES-buffer	0.34±0.19	0.06±0.05	0.10±0.07
8, P-buffer	0.07±0.07	0.08±0.05	0.04±0.06

obtained, the photosynthesis values are considered more reliable than growth rates in reflecting the effect of pH and buffer.

Cellular P contents: To see whether the negative effect on photosynthesis of the increased P content as applied by the P-buffer at pH 8 was caused by an increased internal P concentration, cellular P contents were determined after 1 d adaptation (Table 3). Cellular P contents were significantly increased in *S. chaetoceras* incubated in the P-buffer at pH 8 compared to cells in the HEPES buffer at pH 6 (ANOVA, $p<0.05$), but did not differ significantly in *S. brachiatum* and *Std. cuspidatus* due to high standard deviations (ANOVA, $p = 0.29$ and 0.11, respectively). Besides measuring cellular P-content, 3-PGA or mastoparan were added in an attempt to increase cytoplasmic 3-PGA contents. An increased cytoplasmic 3-PGA content can compensate for decreased photosynthesis caused by a high external P-content in isolated chloroplasts. Neither the addition of 3-PGA or mastoparan altered oxygen production (results not shown).

Fluorescence measurements: After 1 d of adaptation to the P-buffer and pH 8, fluorescence emission from PS2 had decreased compared to the other 3 treatments in both *S. brachiatum* and *Std. cuspidatus* (Kruskal-Wallis, $p<0.05$), but not in *S. chaetoceras*. This is shown in the fluorescence emission measured as PS1 : PS2 ratio (Fig. 2). When cells were transferred from a P-buffer to a HEPES buffer, recovery of PS2 fluorescence emission only started after about 4 h. This indicated that the observed decrease in PS2 fluorescence was the result of irreversible changes (results not shown). The transfer of pH 8 P-buffered cells of *Std. cuspidatus* to pH 6 HEPES buffer allowed partial recovery in the PS2 emission fluorescence after 1 d (Fig. 3).

Independent of the buffer used, a decrease in

Table 3. Cellular P content [pg per cell] of three desmid species after 1 d incubation in either 20 mM HEPES or P-buffer at pH 6 or 8. Average of 4 independent measurements ± STD.

pH	*S. chaetoceras*		*S. brachiatum*		*Std. cuspidatus*	
	HEPES	P-buffer	HEPES	P-buffer	HEPES	P-buffer
6	5.0±0.5	6.4±1.3	11.1±1.5	10.6±2.3	6.6±0.5	6.4±1.7
8	5.4±1.2	7.0±1.6	10.6±2.4	12.7±3.2	6.2±2.1	7.5±1.0

fluorescence emission around the peak at 689–690 nm was observed at pH 8 when compared with pH 6 in all three species. Only the results for *Std. cuspidatus* are shown (Fig. 3). The fluorescence in this part of the spectrum is believed to come from the PS2 core complex (Šiffel and Braunová 1999) suggesting that fluorescence from PS2, and not from the antenna complexes, was affected during adaptation to higher pH's.

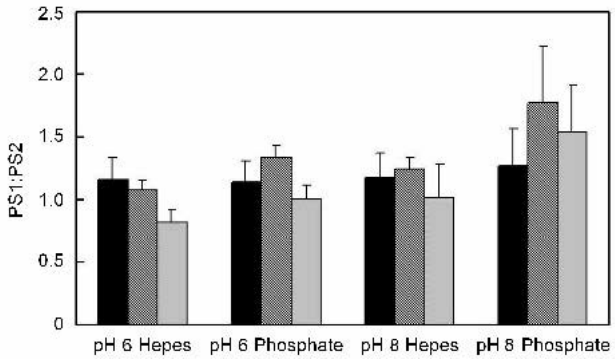

Fig. 2. PS1/PS2 fluorescence emission ratio measured at 77 K in dark adapted samples after 1 d incubation in either a 20 mM HEPES or P-buffer at pH 6 or 8 in *S. chaetoceras* (*black*), *S. brachiatum* (*gray*), and *Std. cuspidatus* (*light gray*). *Error bars* indicate STD.

Corresponding with maximum photosynthetic O_2 evolution data, quantum yield of charge separation in light acclimated cells ($\Phi_{II,l}$) in all three species was lowest at pH 8 P-buffered conditions (Table 4). This decrease was most pronounced in *S. brachiatum* and *Std. cuspidatus*. Furthermore, pH 8 HEPES and pH 6 P-buffered cultures had lower quantum yields than pH 6 HEPES incubations in all three species, and this decrease was significantly correlated with P_{max} (paired *t*-test, $p<0.001$). The quantum yield of charge separation in dark acclimated cells ($\Phi_{II,d}$) showed a similar trend and was significantly correlated with α (Fig. 4, paired *t*-test, $p<0.001$). Remarkably, a large decline of 50 % was observed in $\Phi_{II,l}$ as compared to $\Phi_{II,d}$ in pH 8 HEPES-adapted cells of *S. brachiatum*, suggesting that ribulose-1,5-bisphosphate carboxylaxe/oxygenase (RuBPCO) activity was seriously declined in this species under these conditions (Table 4). In *S. chaetoceras* and *Std. cuspidatus* this decrease was 15–20 %, comparable to decreases found in pH 6 HEPES buffer. Non-photochemical quenching in the light (NPQ) was highest in pH 8 HEPES-buffered cultures of *S. brachiatum* (Table 4). In pH 8 HEPES-buffered conditions *Std. cuspidatus* also had increased NPQ as compared to *S. chaetoceras*. As a result of the largely decreased F_m values in pH 8 P-buffered cultures, no significant NPQ could be detected in these cultures.

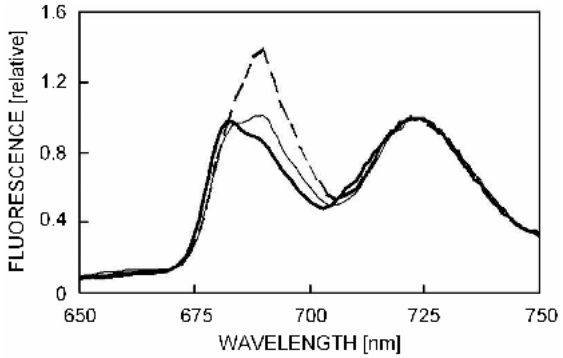

Fig. 3. Recovery in chlorophyll fluorescence shown as 77 K emission fluorescence spectra of dark adapted samples of *Std. cuspidatus*. Spectra after 1 d exposure to 20 mM HEPES buffer at pH 6 (- - -), P-buffer at pH 8 (——), or at pH 6 H-buffer 1 d after transfer from pH 8 P-buffer (——). Curves normalized to PS1 peak at 720 nm.

To check whether the experimental results described above could be the result of increased Na^+ concentrations in *Std. cuspidatus* and *S. chaetoceras*, experiments with a K^+-P-buffer and increased Na^+ concentrations in the

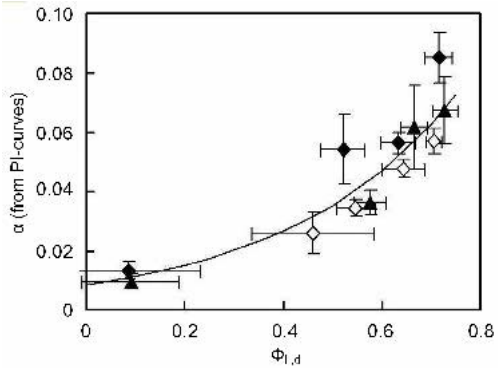

Fig. 4. Quantum yield of charge separation in the dark ($\Phi_{II,d}$) in relation to the slope of the initial part of the PI-curve (α) in *S. brachiatum* (♦), *S. chaetoceras* (◊), and *Std. cuspidatus* var. *curvatus* (▲) after 1 d incubation in a 20 mM HEPES or P-buffered medium at pH 6 or 8. Symbols are averages ± STD from at least 3 independent treatments.

Table 4. Quantum yield (Φ_{II}) in dark (d) and light (l) adapted cells and non-photochemical quenching (NPQ) in three desmid species after 1 d incubation in four combinations of pH and buffer. F_m and F_0 were determined after a minimum of 30 min dark adaptation and F'_m and F'_0 in light-adapted cells (150 µmol m^{-2} s^{-1}). Average ± STD of at least 3 independent measurements.

pH		*S. chaetoceras*		*S. brachiatum*		*Std. cuspidatus*	
		HEPES	P-buffer	HEPES	P-buffer	HEPES	P-buffer
6	$\Phi_{II,d}$	0.71±0.02	0.55±0.04	0.72±0.03	0.63±0.03	0.73±0.03	0.58±0.03
	$\Phi_{II,l}$	0.62±0.02	0.40±0.05	0.67±0.02	0.49±0.03	0.65±0.02	0.45±0.04
	NPQ	0.11±0.03	0.20±0.07	0.06±0.08	0.24±0.05	0.06±0.03	0.21±0.12
8	$\Phi_{II,d}$	0.64±0.04	0.46±0.12	0.52±0.04	0.09±0.14	0.67±0.03	0.09±0.10
	$\Phi_{II,l}$	0.56±0.04	0.34±0.12	0.25±0.03	0.04±0.08	0.56±0.06	0.03±0.05
	NPQ	0.09±0.02	0.16±0.09	0.46±0.08	0.06±0.09	0.21±0.07	0.08±0.04

HEPES buffer were carried out. Quantum yields ($\Phi_{II,d}$) decreased slightly from Na^+ addition to the HEPES buffer, at both pH 6 and pH 8. After adaptation of the cells to 20 mM K^+-P-buffer (instead of Na^+-P) $\Phi_{II,d}$ was hardly affected at pH 6, but increased markedly at pH 8 (Table 5). These results indicate that the inhibitory effect of the P-buffer is based on an additive effect of high concentrations of Na^+ and PO_4^{3-} at high pH.

Table 5. Relative change in quantum yield ($\Phi_{II,d}$) in dark-adapted cells of *S. chaetoceras* and *Std. cuspidatus* after 1 d incubation in a K^+-P-buffer or a HEPES buffer enriched with 20 mM Na^+ compared to the data given in Table 4. The value of the counterwise value within each species is set to 100 %.

	pH 6 Na^+-HEPES	pH 6 K^+-Phosphate	pH 8 Na^+-HEPES	pH 8 K^+-Phosphate
S. chaetoceras	92	113	75	160
Std. cuspidatus	84	102	87	186

Table 6. De-epoxidation state in *S. brachiatum*, *S. chaetoceras*, and *Std. cuspidatus* after 1 d incubation in either HEPES or P-buffer at pH 6 and 8 expressed as zeaxanthin + 0.5 antheraxanthin/(violaxanthin + antheraxanthin + zeaxanthin) and after 3 h dark incubation. Average values of 2 independent measurements (STD < 5 %).

		pH 6 HEPES	pH 6 P-buffer	pH 8 HEPES	pH 8 P-buffer
DPS (light)	*S. brachiatum*	0.94	0.96	0.90	0.91
	S. chaetoceras	0.73	0.72	0.54	0.73
	Std. cuspidatus	0.96	0.97	0.88	0.94
DPS (3 h dark)	*S. brachiatum*	0.40	0.46	0.53	0.89
	S. chaetoceras	0.35	0.34	0.26	0.40
	Std. cuspidatus	0.41	0.43	0.51	0.76

The de-epoxidation state in both *S. brachiatum* and *Std. cuspidatus* were very close to 1 after 1 d incubation in all of the different culture conditions tested, whereas it was remarkably lower in *S. chaetoceras* (Table 6). Unlike any other species known, hardly any V could be detected in both desmids characteristic of soft water lakes growing in an irradiance of only 150 µmol m^{-2} s^{-1}. Upon dark incubation, Z was epoxidated to A and further to V at a faster rate in all pH 6 cultures as compared to pH 8 cultures in all 3 desmid species. Rates were not different from zero in pH 8 P-buffer cultures in both *S. brachiatum* and *Std. cuspidatus* (rates not shown). Table 6 shows the results of this process being the DPS after 3 h of dark incubation. A change in content of total xanthophylls related to total chlorophyll content between pH 6 and 8 incubations or between cultures with HEPES or P-buffer could not be detected in any culture, average values being 0.07±0.01, 0.10±0.01, and 0.09±0.02 Σg(xanthophylls)/Σg(chlorophylls) for *S. chaetoceras*, *S. brachiatum*, and *Std. cuspidatus*, respectively.

Discussion

Our results show that *Std. cuspidatus*, *S. brachiatum*, and *S. chaetoceras* have increased P_{max} at pH 6 as compared to pH 8. This effect is most pronounced in *Std. cuspidatus* and *S. brachiatum* that also realise higher growth rates at pH 6. *S. chaetoceras*, on the contrary, has comparable growth rates and P_{max} at pH 6 and 8. The observed decrease in P_{max} as a result of high pH in all three species is clearly independent of CO_2 availability and must therefore be a result of pH *per se*. These results fit nicely to the species distribution pattern in the field: *S. chaetoceras* is a species common in alkaline, eutrophic lakes (often confronted with pH values up to 8.0–8.5), whereas *S. brachiatum* and *Std. cuspidatus* are both characteristic of soft water lakes (pH 5–6, Coesel 1994). In addition, an unexpected decrease in both P_{max} and $\Phi_{II,d}$ caused by high pH and the use of P-buffer is described. As far as we know, this has not been observed in green algae before, at least not in entire, P-replete cells. Since the decrease in these to photosynthesis related parameters occurred in all three species, it could be a characteristic specific of desmids. The decrease seems to be an additive effect of increased P_i and Na^+ concentrations which only occurs at a relatively high pH. The sensitivity to the P-buffer was largest in *S. brachiatum* and *Std. cuspidatus*, but also the effect in *S. chaetoceras* was pronounced.

The buffer capacity of the medium influences photosynthesis in marine macro-algae, red algae, and freshwater characeans (*e.g*. Choo *et al*. 2002). In certain freshwater red algae the uptake of CO_2 by algal cells in a high pH environment is stimulated by the HCO_3^- to CO_2 conversion in extra-cellular acid zones on the cell surface (Raven and Beardall 1981). Increased buffering capacity will prohibit the formation of these acid zones thereby decreasing carbon acquisition and consequently photosynthesis, as has been shown in some characeans (Price and Badger 1985). This strategy of carbon acquisition is only possible in algae that posses a large surface area, and is therefore only known in macro-algae. The P_{max} measured in the desmids under consideration were not significantly decreased by the increased buffer capacity when HEPES was used (compare data in Table 1, pH 7 with 2 mM HEPES buffer and all other HEPES treatments, 20 mM), and therefore a possible influence of buffering capacity on P_{max} is likely to be excluded.

P_{max} characterizes the maximum processing capacity of the cell, and is therefore likely to be determined by the content of enzymes of the Calvin cycle, especially the enzyme RuBPCO (Sukenik *et al.* 1987, Zonneveld 1997) and the availability of the substrate (CO_2) to RuBPCO. Whole-cell carbon-fixation rate and P_{max} are related to RuBPCO-content or -activity (Miyairi 1998, Bailey *et al.* 2001, Föstner *et al.* 2001). As P_{max} was decreased in pH 8 HEPES-buffer in all three desmid species as compared to rates measured at pH 6, one could argue that this is the result of decreased CO_2 availability to RuBPCO at pH 8 as compared to pH 6. However, given the fact that neither CO_2 aeration nor CA addition caused any significant increase in P_{max} in the desmids at pH 8 excludes this explanation. We therefore conclude that pH by itself inhibited photosynthesis and growth in the species characteristic of soft water lakes (*S. brachiatum* and *Std. cuspidatus*) whereas it did only to a lesser extend in the species characteristic of eutrophic lakes (*S. chaetoceras*). Data acquired *via* PAM-fluorescence indicate that a structural and functional damage of the PS2 antenna and core complex (shown by a decrease in $\Phi_{II,d}$ and $\Phi_{II,l}$, respectively) as effected by pH was only observed in *S. brachiatum*. Obviously, structural and functional changes caused by increased pH were largest in this species and might reflect a decreased carbon fixation rate at pH 8. Remarkably, electron transport rates and photosynthesis did not relate linearly: large differences in P_{max} resulted in a minor decrease in quantum yield. Decreases in electron transport rates as a result of changing pH were therefore not as large as changes in photosynthesis. De-epoxidation processes resumed in approximately the same rates in both pH 6 and 8 HEPES buffered cultures, and therefore the effect of pH on electron transport was likely based in quenching *via* ΔpH or state transitions (excluding the effect of the P-buffer).

From growth experiments lasting 10 d, a Tris/HCl buffer (in a strength of 4.0 mM) at pH 8.5 was lethal for *Closterium acutum* var. *variabilis* whereas 2.0 mM HEPES/NaOH or 4.5 mM (Na^+)bicarbonate at that same pH resulted in 50 and 70 % growth reduction for HEPES and bicarbonate, respectively (Coesel 1993). So, also in another species (*Cl. acutum* is a species often observed in alkaline habitats) a higher pH can significantly decrease growth. One possible explanation for the negative effect of high pH on growth and photosynthesis might be the inability of a species to generate its membrane potential when ΔpH over the plasma membrane changes and the external pH becomes higher than the internal one. The H^+-ATPases are among the first to be inhibited (Gimmler and Degenhardt 2001) and many nutrient uptake processes are hampered.

Under the conditions of increased P_i and/or Na^+ concentrations, the photosynthetic capacity is decreased in all three desmid species, the effect being more pronounced at pH 8 than at pH 6. Experiments with increased [Na^+] in the HEPES buffer and identical experiments with a K^+-P-buffer (instead of the Na^+-P-buffer), indicated that the combination of increased P_i and Na^+ concentrations in combination with a high pH in particular, decreased quantum yield ($\Phi_{II,e}$) of charge separation at PS2. As early as 1943, Pratt observed that the Na^+ salts of HCO_3^- and CO_3^{2-} inhibited growth of *Chlorella vulgaris* (Pratt 1943). This inhibition in *C. vulgaris* was later confirmed using 13 mM Na^+, and was in their case not related to CO_2 availability or pH (Goldman and Graham 1981).

Concentrations of 100 mM NaCl resulted in lower photosynthetic rates (75 %) and increased quenching in F'_m, in *Chlamydomonas reinhardtii*, although total recovery of F'_m could be obtained in 3 h (Endo *et al.* 1995). These studies do not provide a physiological explanation, but in many eukaryotes both H^+/Na^+ and H^+/K^+ exchangers are present in the plasma membrane, their activity being regulated by phosphorylation (H^+-ATPases; Bakker 1990, Kalampanayil and Wimmers 2001). Interestingly, the effect of the P-buffer on both P_{max} and $\Phi_{II,d}$ at pH 8 was very large in both soft water isolates *S. brachiatum* and *Std. cuspidatus*, whereas the effect in *S. cheatoceras* was less pronounced. At pH 8 the molarity of Na^+ in the P-buffer will have risen to 40 mM which is well above the K_m values reported for Na^+/H^+ exchangers on the cytoplasmic membrane involved in osmoregulation (Qiu *et al.* 2003). High P_i and Na^+ concentrations will likely enhance the activity of these exchangers and cause increased proton excretion (Kaplan *et al.* 1989). The most pronounced effect of increased pH and Na^+ concentrations will be a consequent impending of internal pH regulation.

The strong and fast decrease in P_{max} upon incubation in a P-buffer suggests that high P_i and Na^+ concentrations have a direct effect upon photosynthetic processes. Stressful conditions can overexcite PS2, causing photoinhibition under low irradiances and resulting in damage to the D1 protein (Osmond 1981, Färber *et al.* 1997, Kaplan and Reinhold 1999). Low temperature fluorescence emission data from Fig. 3 clearly support the occurrence of photoinhibition in pH 8 P-buffered desmid cells. Transferring cells incubated at pH 8 to 6 did not result in stimulation in P_{max} during the first hours, indicating that proteins were damaged by culturing the cells in pH 8 as confirmed by the fixation into the de-epoxidation state at pH 8 P-buffer as compared to pH 6 in the P-buffer or pH 8 HEPES-buffer.

Regarding the negative effect of the high P_i concentrations, an analogy to the effects of P_i on isolated chloroplasts was made. The inhibition of O_2 production caused by high P_i concentrations is a well-known phenomenon in isolated chloroplasts of green algae (Klein *et al.* 1983, Goyal *et al.* 1988, Flügge 1995, Amorosso *et al.* 1998). An increased P_i concentration surrounding the chloroplast causes an exchange of P into the chloroplast with triose-P *via* the triose-P translocator (Hall and Rao 1994). The observed decrease in photosynthetic O_2 evolution was a direct result of increased P_i content inside the isolated chloroplasts and the indirect result of the decreased pool of triose-P that is filled from 3-PGA (Goyal *et al.* 1988). Lower contents of 3-PGA and increased P_i contents in the stroma result in a lower rate of regeneration of RuBPCO (Flügge 1995, Gauthier and Turpin 1997). The latter effect is the cause of decreased photosynthetic rates after the addition of P_i to P_i-limited cells (Gauthier and Turpin 1997). In the desmid species under consideration, cellular P_i concentrations in pH 8 P-buffer were slightly increased, when compared with those in HEPES buffer. In contrast to effects known in isolated chloroplasts, no stimulation of O_2 production was found after supply of 3-PGA to nystatin-treated cells or after addition of mastoparan. Because it is not known if cytoplasmic 3-PGA contents were increased upon these treatments, the effect of high P_i contents on the photosynthesis of desmids can only be speculated. Unfortunately, we were not able to isolate chloroplasts to elucidate this problem. Addition of P_i in a well-buffered environment while measuring O_2 production revealed that whole cells of *S. chaetoceras* decreased their O_2 production above concentrations of 10 mM P_i, *Chlamydomonas noctigama* above 40 mM, and *Tetraedron minimum* only above 400 mM P_i (E. van Hunnik, personal communication). These results underline the susceptibility of desmid species to high P_i concentrations when compared with other green alga.

Overall, although P-buffers are widely used by plant physiologists, one has to be prepared for detrimental side-effects. The acknowledged effect of P-buffers on isolated chloroplasts or P-limited cells could not be related to the results described in this paper. From the presented data it is concluded that high Na^+ and P_i concentrations in combination with a high pH may cause photoinhibition and, as far as we know, this has not been described before. It is also shown that high pH decreases photosynthesis in two out of three species of desmids and that this is independent of CO_2 availability. The inhibition in photosynthesis caused by high Na^+ concentrations is one possible physiological explanation for the total absence of species of the Desmidiaceae in marine environments.

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