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The effect of changes in salinity on the energy yielding processes of *Chlorella vulgaris* and *Dunaliella maritima* cells

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Abstract

The unicellular green halotolerant microalga *Dunaliella maritima* grown in medium containing 500 mM NaCl and the freshwater microalga *Chlorella vulgaris* were used as model systems to study adaptation of energy yielding mechanisms of cells in culture to changes in salinity.

A microcalorimetric method was used to study the alteration of the heat production rate of microalgae depending on the salt content in the culture medium. It was shown that the heat production of *Chlorella* at low salt concentration (50 mM) increased and that it decreased at high concentration (500 mM). *Chlorella* was unable to adapt to concentrations of 1 M NaCl and above—in this sense, this degree of salinity was a critical value for the adaptive processes of this microalga. On the other hand, the halotolerant *Dunaliella* readily adapts to very high salinity. At 1.5 M salt concentration there was a considerable increase in heat production rate up to 200% compared with the 500 mM control. The light reactions of the microalgae in salinity were measured by a photo-microcalorimetric method. *Dunaliella* cells absorbed heat to a greater extent than *Chlorella vulgaris* cells which can be interpreted as more stored light energy in photosynthate.

A polarographic method with a Clark-type electrode was employed in the dark to determine the respiration rate from the oxygen uptake rate; and photosynthesis was determined in the light from the oxygen evolution rate. At a salt concentration of 50 mM, there was some increase of the oxygen uptake rate by *Chlorella* cells and at high concentrations of salt in the medium from 500 mM to 1 M there was a sharp inhibition of this process. *Dunaliella* cells gave a different response to increasing salt concentrations in terms of the oxygen uptake rate. It considerably increased by 50–60% compared when the salt concentration in the medium was increased to 1.5 M. It is interesting to see that the rates of energy yielding processes in the *Dunaliella* control cells were significantly higher than those of *Chlorella* cells, presumably because of the increased rate of pumping out Na^+ ions.

The data support the hypothesis that an important feature of salt sensitive and salt tolerant microalgae is the increased energetic rate which ensures a quick and effective adaptation of them to stress.

Keywords: *Chlorella vulgaris*; *Dunaliella maritima*; Heat flow rate; Oxygen uptake rate; Oxygen evolution rate; Salinity changes

1. Introduction

Salt stress causes a multitude of bioenergetic and biochemical changes in photosynthetic organisms. Among the important non-specific changes of microalgal cells to salt stress are: (i) increased rates of biopolymers and lipid catabolism; (ii) changes in the rates of energy yielding processes; (iii) change of membrane permeability with interruption of ion homeostasis [1–4]. All of them are connected with bioenergetic aspects and are essential for understanding the adaptive mechanisms of microalgal cells to salinity. It is known that the energy demand of them cells increases under adverse conditions [1–5]. According to [5,6], the additional expenditure of metabolic energy under stress conditions is required for maintaining ion homeostasis and electrochemical gradients, for the biosynthesis of organic compounds which play an important role in protection and osmoregulation, and for supporting the maintenance of cellular structure. In this context, the increased rate of energy release can ensure the quick and effective microalgal adaptation to stress.

The unicellular green halotolerant microalga *Dunaliella maritima* and the freshwater microalga *Chlorella vulgaris* were used as suitable model systems in studies of the adaptation of energy mechanisms to changes in salinity. The unicellular microalga

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has been extensively used for biochemical and physiological work in many laboratories [7–11]. The authors consider that photosynthesis and respiration of these microalgae have a close resemblance with those in plants and thus they are valid models for them. It is supposed that an important feature of plants with respect to salt sensitivity and resistance is their alteration of energy yielding processes depending on the salt concentration. Direct microcalorimetry was used to study changes in the heat production rate of microalgae depending on the salt concentration in the culture medium. Its power as universal, integral, non-destructive and highly sensitive tool for many environmental problems has been widely acknowledged as well as its value in providing thermodynamic information [12–16].

The study of the alteration of energy yielding processes of the cells with different sensitivity to salt stress may be a key to the understanding of the complex mechanisms that play an important role in adaptation of algae to unfavorable conditions. Thus, the aim of this research is to study the key energetic processes of the model systems of *Chlorella* and *Dunaliella* cells by measuring the oxygen uptake and evolution rates using polarography [17] and the rate of heat absorption and thus the light-dependent rate of energetic processes employing photo-microcalorimetry [18–21]. In this way we estimated the energy costs of adaptation to salinity by these organisms.

2. Experimental

2.1. Biological materials

The unicellular green algae, *C. vulgaris* Beij. (from the collection of the Botany Institute, Saint Petersburg, Russia) and *D. maritima* (from the collection of the Timiryazev Institute of Plant Physiology, Russian Academy of Sciences) were the objects of the investigation.

Cells of both species were grown in Tamiya medium [22], pH 6.8–7.2, at 30 °C during the light period and at 22–24 °C in the night. The only differences between the two cultures was that 500 mM NaCl was added to the control *Dunaliella* cells and the *Chlorella* cell suspensions were bubbled with 0.3% CO_2 in air. Both cultures were illuminated at 155 μmol photon m^{-2} s^{-1} with a light/dark photoperiod of 12/12. All the experiments described below were carried out at 30 °C.. The cell concentration of *Chlorella* was maintained at 1–1.5 × 10^8 cells/ml and that of *Dunaliella* (1–2) × 10^7 cells/ml using an optical density method (photocolorimeter KFK-2MP; Zagorsk, Russia). The growth of *Chlorella* and *Dunaliella* cells was controlled by optical density measurements at A 670 nm (pathlength 1 mm). These results were recalculated by use of calibration curves or table data to obtain values in terms of dry weight or number of cells.

Appropriate amounts of NaCl were added to the *Chlorella* suspension for final NaCl concentrations of 50, 200, 500 mM and 1 M and of 700 mM, 1.5 M and 2 M for the *Dunaliella* suspension. The experimental samples were divided into aliquots in order to measure: (i) the rate of heat production; (ii) the rate of oxygen uptake/evolution; (iii) the rate of heat absorption. The measurements of growth were conducted for 24, 48 and 96 h. Calorimetric measurements were run over periods of 30 or 60 min.

2.2. Analytical methods

The rate of heat production was monitored by a heat conduction microcalorimeter (LKB Bio Activity Monitor BAM), the direct calorimetric predecessor of the thermal activity monitor (TAM) manufactured by Thermometric AB, Järfalla, Sweden) [23]. Suspensions of 1.5 ml were placed in unstirred 3 ml glass vessels that were hermetically sealed before thermal equilibration for 30 min. This means that the experimental zero time corresponded to 30 min after adding NaCl to the cultures.

Oxygen uptake and evolution rates were measured by polarography using a Clark-type electrode. Each 3.2 ml sample was placed in a measuring vessel located in a water bath and equilibrated for 5 min to 30 °C in the dark. A black box was fitted over the bath to allow measurements of the oxygen uptake rate in the dark for 5 min. Then the cells were illuminated to record the rate of oxygen evolution for a further 3–5 min period [17].

The rate of heat absorption was measured by photo-microcalorimetry. The necessary adaptations to a Tian-Calvet type microcalorimeter DAK-1-1A manufactured in Chernogolovka (Russia) were constructed in Kazan by Petrov [18,19]. The scheme for the experiments is depicted in Fig. 1 and intensively discussed in [19]. The baseline established in the dark as seen in Fig. 1(a) is the instrumental zero for the control, the dark zero D_0, when a thermal balance exists between the test vessel with medium and the reference vessel with a low-density oil inert to visible light. Then light (+*h*() is introduced to the test vessel; its radiance produces a heat flow, A, and a new level L_0 (see Fig. 1(a)). This flow is compensated by Joule heating of the oil in the reference vessel which reduces the heat flow to the so-called light zero, L_{0com}. Fig. 1(b) gives the idealized scheme for the changes of dark and light zero in the case when photosynthesizing microalgae are placed in the test vessel. In the dark, the change in heat flow from D_0 to D_{0res}, termed Δ_1, is

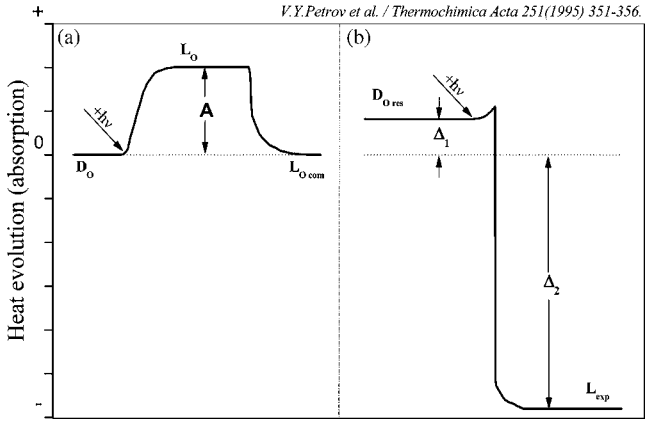

Fig. 1. Schematic presentation of the calorimetric response during illumination. (a) Effect A is compensated by setting the signal from level L_0 to level L_{0com}. (b) Calorimetric response of a *Chlorella* suspension in the dark (Δ_1) and during illumination (Δ_2). The light was switched on at the time marked by the arrow (+*h*().

considered to be due to dark respiration and any other exothermic metabolic processes. The test vessel is then illuminated and the incident light compensated by Joule heating in the reference vessel. The light-driven endothermic reaction of photosynthesis is opposite to the exothermic dark reaction. Heat is absorbed by the cells, Δ_2 to establish a new steady state, L_{exp}. Petrov [18,19] ascribed this change to the storage of energy in photosynthate macromolecules formed during photosynthesis. Since the heat capacity of the reference vessel is known, it is possible to calculate the quantity of stored energy.

As sources of illumination filament direct-current lamps were used: (a) in the photocalorimeter a lamp KGM 12-100; this is a quartz envelope halogen minilamp, supplied by 12 V at a power of 100 V A; (b) in the polarographic chamber a halogen lamp K 30–400 with a power of 400 V A at 30 V. The intensity and spectrum of light radiation were the same in the polarograph and the photo-microcalorimeter. Infrared and ultraviolet parts of the spectrum were cut off by optical filters (SZS-25 and JS-12), so that light was in a range of 380–660 nm in both vessels. The light intensity in both devices could be adjusted with the help of changing current and voltage; diaphragms and neutral optical glass filters (NS type) were used to choose the same light exposure as in the cultivator that means 155 μmol photon m^{-2} s^{-1}. It was possible to adjust the levels of light exposure in the calorimetric and polarographic vessels. This level was determined with the help of a photo-diode calibrated against a standard light meter.

All experiments were repeated five times. The standard error (S.E.) and *t*-test (at significance level $P = 0.05$) were calculated using the program Microcal Origin^{TM} V. 5.0.

3. Results

In the first place, it was necessary to determine the change of heat production of halophilic and non-halophilic algae in the dark depending on the salt content of the culture medium because the instantaneous heat flow rate is directly proportional to the metabolic rate of cells. It can be seen in Fig. 2 that the rate of heat production of *Chlorella* cells in 50 mM NaCl was up to 20% higher than in the control whereas the rate dropped considerably when cells were suspended in 500 mM NaCl; nevertheless, the cells were still alive.

In contrast to *Chlorella,* the halotolerant *Dunaliella* readily adapted to very high salinity (Fig. 3). At 700 mM salt concentration, there was a significant increase in the heat production rate of up to 40%, and at 1.5 M NaCl concentration the rate of this process was 100% higher than in the 500 mM control. As can be seen in Figs. 2 and 3, the heat production rate in the *Dunaliella* control was significantly higher than that of *Chlorella* (1.46 ± 0.16 against 0.197 ± 0.006 μW/10^6 cells, $t = 7.71$).

The effect of exposure to changed salinities on the rate of oxygen uptake and evolution of *Chlorella* cultures is presented in Fig. 4. At 50 mM NaCl as well as at 200 mM some increase of O_2 uptake was observed; a strong inhibition was found at the highest concentration (500 mM). In terms of photosynthesis measured by polarography, the data in Fig. 4 show that the rate of oxygen evolution of *Chlorella* cells decreased even at low

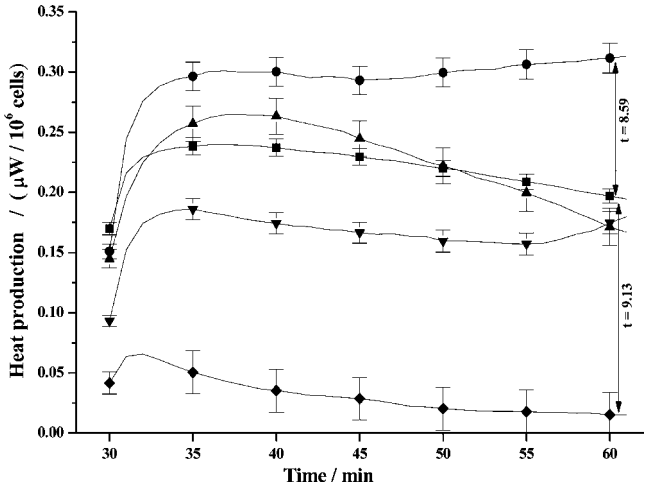

Fig. 2. Effect of NaCl in different concentrations on the rate of heat production of *Chlorella* cells. (■) Control (artificial freshwater); (●) 50 mM NaCl; (▲) 200 mM NaCl; (▼) 500 mM NaCl; (♦) 1 M NaCl (*t* is *t*-test value, calculated at $P = 0.05$ and $n = 5$; table score = 2.8).

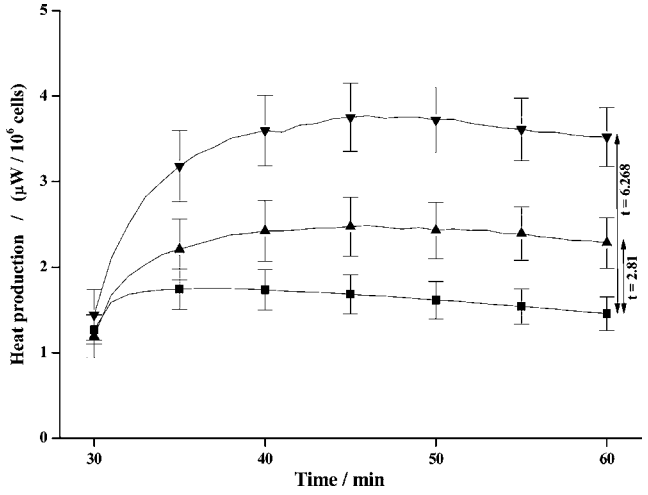

Fig. 3. Effect of NaCl in different concentrations on the rate of heat production of *Dunaliella* cells. (■) Control (500 mM); (▲) 700 mM; (▼) 1.5 M (*t* is *t*-test value, calculated at $P = 0.05$ and $n = 5$; table score = 2.8).

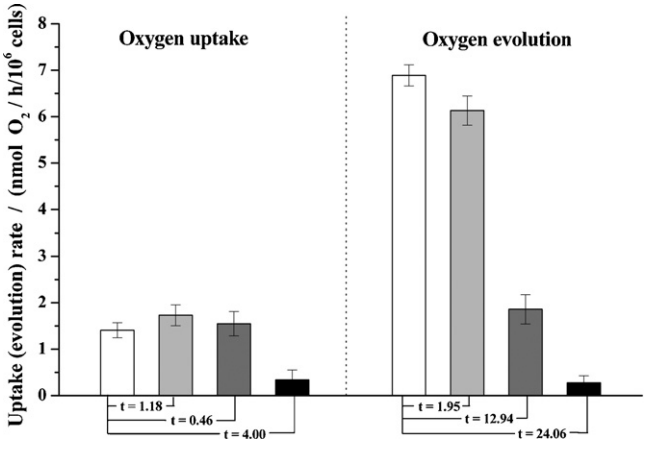

Fig. 4. Rates of oxygen uptake and evolution of *Chlorella* cells under the influence of different NaCl concentrations. (□) Control; (▨) 50 mM NaCl; (▩) 200 mM NaCl; (■) 500 mM NaCl (*t* is *t*-test value, calculated at $P = 0.05$ and $n = 5$; table score = 2.8).

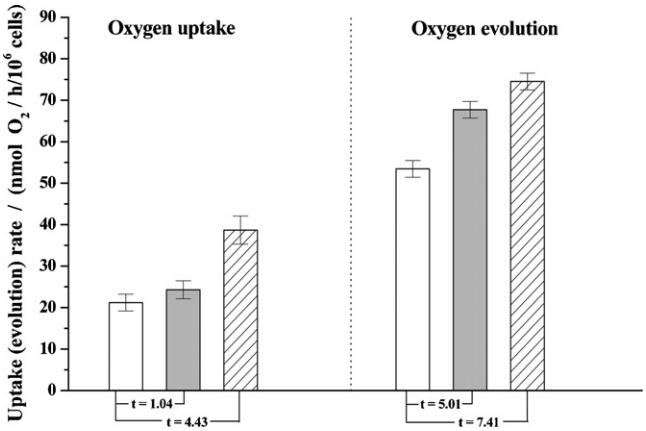

Fig. 5. Rates of oxygen uptake and evolution of *Dunaliella* cells under the influence of different NaCl concentrations. (□) Control; (■) 700 mM NaCl; (▨) 1.5 M NaCl (*t* is *t*-test value, calculated at $P = 0.05$ and $n = 5$; table score = 2.8).

NaCl concentrations (50 mM) and decreased to about 4% of the value for the control sample in 500 mM NaCl.

Dunaliella cells showed a different response to increasing salt concentrations in terms of the oxygen uptake rate (Fig. 5). It considerably increased by 60–80% in1.5 M NaCl. In general, it can be said that the microalgal heat production was in parallel with the oxygen uptake (Figs. 2 and 4; Figs. 3 and 5). After a slight stimulation with 50 mM NaCl, the heat production and oxygen uptake rates of *Chlorella* decreased at 500 mM (Fig. 4) and even more so at 1 M (Fig. 2), while *Dunaliella* showed an opposite behavior (Figs. 3 and 5). Correspondingly, the rate of O_2 evolution decreased for *Chlorella* and increased for *Dunaliella* cells by 30–40% in 1.5 M NaCl (Fig. 5).

Fig. 6 shows photo-microcalorimetric results of the effect of incident light on the energetic changes of *Chlorella* cells in terms of the heat absorption rate. It is important to note that at 500 mM NaCl this rate decreased when cells were exposed to light, only giving almost 50% of the control. To examine a possible influence of Na^+ ions on the electron-transport

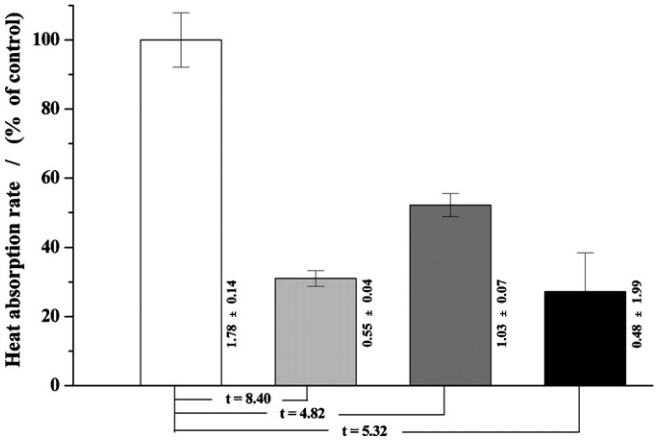

Fig. 6. Percentage change of the rate of heat absorption of *Chlorella* cells measured by photocalorimetry (after 1 h action of NaCl and DCMU). (□) Control; (▨) DCMU 1×10^{-7} M; (▩) NaCl 500 mM; (■) DCMU + 500 mM NaCl (*t* is *t*-test value, calculated at $P = 0.05$ and $n = 5$; table score = 2.8). The absolute values of the heat absorption rate ± S.E. (in μW/mg dry weight) are indicated at the columns.

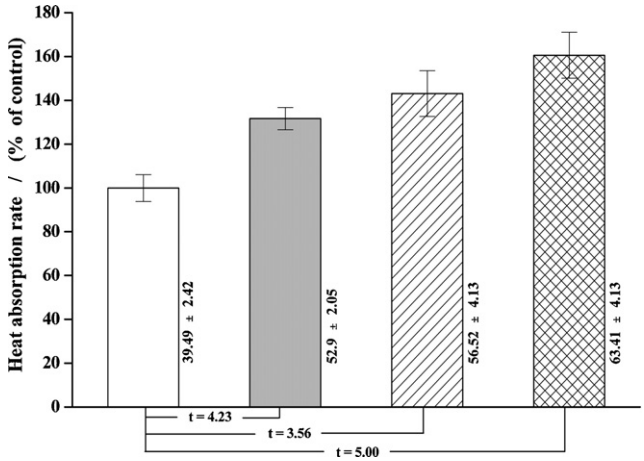

Fig. 7. Percentage change of the rate of heat absorption of *Dunaliella* cells measured by photocalorimetry (after 1 h action of NaCl). (□) Control; (▩) 1 M NaCl; (▨) 1.5 M NaCl; (▩) 2 M NaCl (*t* is *t*-test value, calculated at $P = 0.05$ and $n = 5$; table score = 2.8). The absolute values of the heat absorption rate ± S.E. (in μW/mg dry weight) are indicated at the columns.

chain of photosynthesis, we measured the action of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on this process. It was added to the cell suspension before the latter was placed in the calorimetric vessel. It can be seen from the data presented in Fig. 6 that the inhibition reduced the light energy uptake to about one third of the control. The additive action of NaCl (500 mM) and DCMU decreased the rate of heat absorption by cells by 27%.

Fig. 7 shows that the heat absorption rate of *Dunaliella* increased with the salt concentration. The apparent photosynthesis rate estimated from the rate of oxygen evolution showed a similar behavior (Fig. 5) as the heat absorption rate, measured by the direct method (Fig. 7).

Optical density measurements were performed as function of time to determine the effect of different salinities on the growth of both algae. It was shown (Fig. 8) that the amount

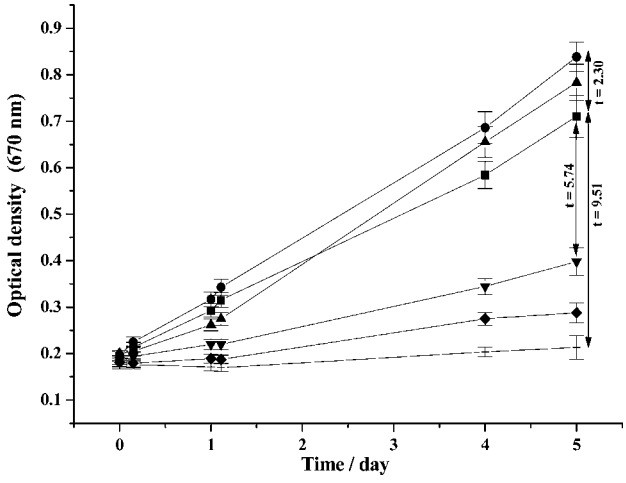

Fig. 8. Influence of increasing concentrations of NaCl on the growth of *Chlorella* cells. (■) Control; (●) 50 mM NaCl; (▲) 200 mM NaCl; (▼) 500 mM NaCl; (♦) 550 mM NaCl; (+) 600 mM NaCl (*t* is *t*-test value, calculated at $P = 0.05$ and n = 5; table value = 2.8).

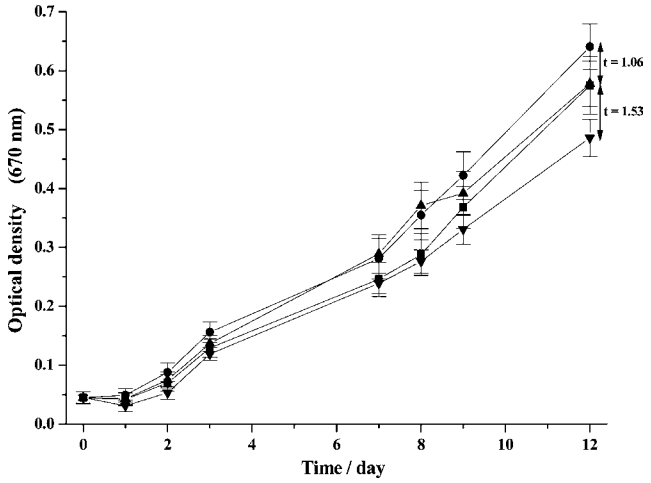

Fig. 9. Influence of increasing concentrations of NaCl on the growth of *Dunaliella* cells. (■) Control (0.5 M NaCl); (●) 1 M NaCl; (▲) 1.5 M NaCl; (▼) 2 M NaCl (t is t-test value, calculated at $P = 0.05$ and $n = 5$; table score = 2.8).

of *Chlorella* cells after 5 days was somewhat higher in the low salt concentration (50 mM) ($t = 2.30$) compared to the control in freshwater. In 200 mM NaCl, the growth rate was close to that of the control ($t = 1.21$) and at higher concentrations it decreased to levels below that of the control ($t = 5.74$; 8.40; 9.51) (Fig. 8). In contrast, growth of the halotolerant *Dunaliella* cells remained practically the same as the control ($t = 1.06$; 1.05; 1.53) over a wide range of NaCl concentrations (Fig. 9).

4. Discussion

Photosynthesizing organisms react to different extreme factors with changes in their bioenergetic processes. The determination of heat production rates of salt sensitive (*Chlorella*) and salt tolerant (*Dunaliella*) algae at different NaCl concentrations showed that the rate of heat production of *Dunaliella* cells increased up to 1.5 M NaCl in the growth medium, but that this rate decreased to 30% of the control in 0.5 M medium for *Chlorella* cells. Thus, the energy level in the latter cells was lower than in the control, possibly owing to an inhibition in metabolic reactions which is supposed to be helpful for organisms to survive for a relatively long time [24–25]). *Chlorella* was unable to adapt to concentrations of 1 M NaCl and above (Fig. 2)—a degree of salinity that indicates a critical concentration for the adaptive processes of this cell ($t = 9.13$).

The heat production rate, determined by direct calorimetry, is equal to the product of reaction rates and enthalpy changes and represents all ongoing processes in the organism. Therefore, the decrease in the oxygen uptake and evolution rates in *Chlorella* cells at salt stress could be observed in two ways (Figs. 2 and 4). This decrease of bioenergetic processes under high NaCl concentrations is connected with an inhibition of metabolic rates because Na^+ ions are toxic for plant cells. Proteins and nucleic acids can be targets of this ion [24]. Cells are able to decrease the salt toxicity by pumping Na^+ ions into the medium by means of the proton pump. Another picture was observed in *Dunaliella* cells under increasing salt concentrations. Oxygen uptake and evolution rates of *Dunaliella* increased compared with the control at high salt conditions in the medium. In this way, the rates of energy yielding processes ensure a fast adaptation of *Dunaliella* to salt stress.

Interesting data of the changes of the photosynthetic activities of *Chlorella* and *Dunaliella* cells were obtained at salt stress. The rate of photosynthesis was determined by two methods—polarography in terms of oxygen evolution and direct photocalorimetry as the effect of incident light on energetic changes. The apparent rate of photosynthesis in *Chlorella* measured under light as rate of oxygen evolution was depressed by 40% when 500 mM NaCl was present in the culture medium (Fig. 4). The heat absorption rate determined by photo-microcalorimetry decreased by 20–30% under the same conditions (Fig. 6). The difference between the obtained results may offer the possibility of using these methods further to investigate the influence of light on the cells.

It can be supposed that Na^+ ions decrease the effectivity of the electron transport chain and induce a toxic action on the cells. The classic inhibitor of photosystem PS11, DCMU, was used for confirmation of these assumptions. It reduced the rate of photosynthesis in *Chlorella* cells as indicated by the lower heat absorption rate for cells in freshwater as well as in medium with 500 mM NaCl (Fig. 6). The site of DCMU action is known to be the non-cyclic electron transport between the first acceptor of PSII and the incorporation into the chain of plastoquinone [26]. Probably, rather high rates of heat absorption in saline may be connected with the activation of the cyclic photophosphorylation which is not directly associated with oxygen evolution. It could be argued that cyclic photo-phosphorylation might also play a role in the generation of extra ATP for the survival of the organism under salt stress [27–31], as remarked by Chen and Arnon for stress conditions in general [26]. The cyclic electron transport *via* ferredoxine around the plastoquinon pool, the Cyt b_6–f complex and PSI are the driving forces for the generation of extra ATP needed for chloroplasts [26].

It is known that cyclic photo-phosphorylation is more resistant to unfavorable conditions than the non-cyclic photophosphorylation [18,27,28]. The experimental data allow us to suppose that the energetic status of *Chlorella* cells in saline conditions is due to the predominance of cyclic phosphorylation over the non-cyclic variant.

The rates of photosynthesis of *Dunaliella* cells, determined by polarographic and photo-microcalorimetric methods, were increased in the higher salinity conditions compared with the control. This is because *Dunaliella* has to adapt in nature as well as in these experiments to a broad range of NaCl concentrations. Therefore, it is reasonable to speculate on the mechanisms used by the cells to adapt to salt stress. Our data suggest that the function of organelles involved in bioenergetic processes in *D. maritima*, i.e. the chloroplasts and mitochondria, vary in their response to the level of salinity. It was estimated that *Dunaliella* cells had a photosynthesis to respiration activity ratio (P/R) of about 4:1 at 0.5 M NaCl; and a value of 22:1 at 1.5 M NaCl has been reported previously [7]. There are data where authors discuss the possibility of activation of carbonic anhydrase in CO_2 assimilation which may be an important protective mecha-

nism [32,33]. Pick and colleagues [32,33] identified two major plasma membrane proteins which were induced by high salt concentrations in *Dunaliella*. These proteins are transported to the chloroplasts. One of the proteins is homologue to the eukaryotic carbonic anhydrase which is involved in CO_2 acquisition. It is known that the activity of the carbonic anhydrase is more tolerant to higher concentrations of NaCl (and other stress conditions) than the carboxylase activity [32,34]. There is also a discussion in the literature on the mechanisms of the ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) redistribution between the pyrenoid and the stroma in *Dunaliella* cells in response to unfavorable conditions. It is supposed, that the modulation of Rubisco distribution between the pyrenoid and the stroma under changing regimes may be energetically more favorable than the degradation and synthesis of this enzyme [35]. The analysis of literature data about the defense reactions of *Dunaliella* to salinity helps to understand the adaptive mechanisms of the salt tolerance of bioenergetic processes of these algae.

5. Conclusion

Summarizing the data, it can be concluded that the most important feature of salt sensitive and salt tolerant organisms is how they can adapt to stress situations without using all their energy capabilities to maintain the structural–functional integrity of the organism. *Chlorella* was unable to adapt to concentrations of 500 mM NaCl and higher. This microalga has a metabolic rate which is significantly lower than that of *Dunaliella*. This halotolerant and cell wall-less microalga adapts to very high salinities. The tolerance is connected with the ability to maintain high rates of energy yielding processes. In the present study we showed that the heat production, O_2 uptake, and O_2 evolution rates increased in *Dunaliella* cells in conditions up to 2 M salt in the medium. The data support the opinion that an important feature of salt sensitive and salt tolerant algae is the increased rate of energy dissipation which ensures the quick and effective microalgal adaptation.

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