Simultaneous photocalorimetric and oxygen polarographic measurements on *Dunaliella maritima* cells reveal a thermal discrepancy that could be due to nonphotochemical quenching

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Abstract

Photocalorimetry has rarely been employed to investigate the processes in photosynthesis and yet, especially when complemented by simultaneous oxygen polarographic measurements, it can probe the light-excited decay of chlorophyll molecules to the ground state by the photochemical process in photosynthesis, light emission as fluorescence and thermal dissipation. The possibility of detecting the latter two was examined by these methods. The halotolerant chlorophytic microalga, *Dunaliella maritima*, was grown routinely at 25 μmol photon m^{-2} s^{-1} incident light and in the experiments it was exposed to higher light intensity and salinity in order to observe the energetic consequences of stress in terms of the net heat and oxygen fluxes.

The results showed that at 50 and 90 μmol photon m^{-2} s^{-1} the net heat flow varied from rapidly negative (endothermic) to sharply positive (exothermic). This was different to the simultaneous data from the oxygen polarographic sensor that always showed oxygen evolution in the light. Energy balances to correct for the slight imbalance in the response to incident radiant light and to convert the oxygen evolution to energy values by the oxycaloric equivalent for glucose (–470 kJ mol^{-1} O_2) revealed an extra source (15.5 ± 3.3 (SE) and 9.4 ± 3.2 pW per cell for the control and treated cells, respectively, at 90 μmol photon m^{-2} s^{-1}) of heat which was thought to be due mainly to nonphotochemical quenching (NPQ) characteristically with a small contribution from chlorophyll fluorescence.

There was an insignificant (paired *t*-test: $p = 0.131$) difference in dark oxygen flux of the control (1% saline) and osmotically stressed (3%) cells, meaning that respiration could provide no more ATP to pump the greater quantity of Na^+ ions from the cell at higher salinity. Evidence is provided that cells in the higher salinity had a lower specific growth rate and this may have been due to so that ATP can be diverted from anabolism to the pump ATPases. The dark heat flux was significantly ($p = 0.036$) greater in the 3% treatment than in the 1% control whereas the dark oxygen flux was similar for both of them. This meant that the calorimetric/respirometric (CR) ratio in the control was more negative than the oxycaloric equivalent, and indicated the presence of an anaerobic pathway, presumably the glycolyic synthesis of glycerol that increased with higher salinity.

Keywords: Photocalorimetry, heat flux, oxygen flux, photosynthesis, non-photochemical quenching, glycerol synthesis

1. Introduction

The direct calorimetric measurement of energy flows as instantaneous heat (Q) flow ($\Phi = \mathrm{d}Q/\mathrm{d}t$) in plants and other photoautotrophs is rare [1-5] when compared with studies on microbes and animals [6]. The chemical reaction to form photosynthate and oxygen from carbon dioxide and water is equal and opposite to combustion, i.e. in terms of enthalpy change it is an endothermic process. Most of the calorimetric data seems to reflect this basic fact in that heat was absorbed from the heat sink [1-4] but the recent work by Janssen et al. [5] indicated that 1.5-L batch cultures of *Chlorella vulgaris* growing in the light in a Mettler-Toledo calorimeter produced heat, that is the overall process was exothermic in type.

As summarised by Govindjee [7], the photosynthetic process begins with the absorption of photons by macromolecules of the antenna complex causing the excitation of the chlorophyll molecules. These excited molecules decay to the ground state by (i) the transfer of the excitation energy to reaction centres, leading to the photochemical phase of photosynthesis; (ii) light emission as fluorescence; and (iii) thermal dissipation. The multitude of the antenna complexes functions not only in the above mentioned transfer of the excitation energy but also in the removal of excess absorbed light energy as heat in non-photochemical quenching (NPQ), commonly known as photoprotection [8] because it removes the excess energy which otherwise would damage the antennae, particularly the Photosystem 11 (PS11) D protein [9]. More recently, it has been realised that NPQ can be instituted at a relatively low photosynthetic photon flux density (PPFD) [10]. Lawson et al. [11] showed that NPQ was induced in Tradescantia leaves at PPFD less than 100 μmol m^{-2} s^{-1} compared to ~2000 μmol m^{-2} s^{-1} on the outer leaves of trees on a sunny day.

The photochemical events in photosynthesis take place in picoseconds [7]. For the charge separation in PS11, the positive charges oxidise water to molecular O_2 and the negative ones reduce plastoquinone. For PS1, electrons from the cytochrome b_6/f complex reduce the positive charges and the negative ones reduce $NADP^+$ to NADPH. The ATP synthase complex synthesises ATP using the energy stored in the proton gradient across the thylakoid membranes. The difficulty arises when photosynthesis is

saturated and there is excess excitation energy. Now NADPH and ATP are not synthesised sufficiently quickly to dissipate the proton gradient, causing a decrease in the pH of the thylakoid lumen. This acidification leads to the formation of singlet oxygen and peroxidation with the hydrogen peroxide causing the photodamage [12].

There are many mechanisms to remove electrons [10] which involve the consumption of oxygen. There is the high-risk strategy of PS1 forming active oxygen species which are then removed, via hydrogen peroxide, by ascorbate peroxidase [10]. Glutathione is another antioxidant in which the sulphydryl groups are oxidised by active oxygen radicals. Photo- and chlororespiration also remove electrons [13] but the capacity of the latter is small. However, the most significant photoprotective mechanism is NPQ and this does not involve oxygen consumption.

The majority of NPQ appears to occur in the PS11 antenna pigment bed [14] with at least most of it being due to the formation of zeaxanthin and antheraxanthin by the zanthophyll cycle [15,16], i.e. PS11 down regulation. It is currently a matter of dispute [9] whether the energy in singlet chlorophyll is transferred to and then dissipated thermally by zeaxanthin [17] or the latter promotes a conformational change in the Light Harvesting Complex 11b (LHC11) that favours quenching by altering the chlorophyll-chlorophyll interactions, an allosteric regulator [18]. For many years, the classical non-invasive way to probe photosynthesis has been chlorophyll fluorescence [7]. The extent of the NPQ is measured by the decrease in chlorophyll fluorescence [10] and it was from applying this technique that it was realised that NPQ can occur at relatively low PPFD [11].

The exothermic reaction of respiration takes place all the time, though it is a matter of dispute whether its rate is the same in the light as in the dark [19]. It should not be forgotten that there are three respiratory processes potentially in operation, classical mitorespiration, chlororespiration and photorespiration, with the first being the major [13]. It is possible to distinguish between them only by the use of inhibitors. Conventionally, the rate of respiration is measured by indirect calorimetry as oxygen uptake with a polarographic sensor (oxygraph) [20]. In the light, plants and other autophototrophs generally display net oxygen evolution because, in order for them to grow, there must be sufficient carbon compounds (photosynthate) for biomass production (p) as well as combustion (r), i.e. $p/r > 1$. Applying this logic to direct photocalorimetry means that photosynthetic material should absorb energy as heat to reflect the endothermic reaction (see [1-4]). However, the NPQ part of photoprotection

is accompanied by thermal dissipation which would manifest itself in the calorimeter as heat production. This would diminish or could even abolish the photosynthetic heat absorption to give net heat production. The contribution of photosynthesis to the net change in heat flow in the photocalorimeter can be calculated from the respirometric data in simultaneous experiments using the calculated oxycaloric equivalent which is –470 kJ mol^{-1} O_2 for the combustion of glucose and typical of all carbohydrates [21]. This was the approach employed for the current investigation. For the microalgal cultures, a photocalorimeter was used to measure the changes in heat flow, an oxygraph to measure the oxygen flow and the oxycaloric equivalent to calculate the possible heat flow from the thermal dissipation in NPQ photoprotection. In order to increase the probability of this phenomenon, the cells were subjected to incident light intensities greater than in the routine culture. Other environmental factors are said to influence the strength of the photoprotection [9,10,12]. The halophilic chlorophyte, *Dunaliella maritima*, was chosen for this investigation because the environment can easily be changed by varying the salinity as well as by raising the light intensity and observing the possible energetic consequences.

2. Experimental

2.1. Cell culture

The unicellular green chlorophyte alga, *Dunaliella maritima*, from the collection of the Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Moscow, Russia [22], was grown in T-75 vented flasks suspended in sterile Bold's Basal Medium (BM) in 1% and 3% saline buffered to pH 6.8 [23] in a constant temperature room at 25 °C. The only source of carbon was atmospheric CO_2.The illumination measured as PPFD with a Skye Quantum Sensor (Skye Instruments Ltd, Llandrindod Wells LD1 6DF, Wales, UK) for photosynthetically active radiation (PAR, 400-700 nm) was 25 μmol m^{-2} s^{-1} and the photoperiod was 12 h. Before each experiment, a sample of the algal culture was diluted 1:1 (v/v) with fresh medium of the same (control) or higher (treatment) NaCl concentration for calorimetric and polarographic measurements. Acute saline stress was achieved by increasing the saline concentration from 1% to 3%. As a comparison, cells were also cultured under chronic stress conditions at 3%. 0.5 cm^3 samples of the suspensions were taken at the start and finish of the experiments and the

cells fixed in 1 % (w/v) glutaraldehyde were counted using a modified Fuchs-Rosenthal haemocytometer on a Leitz Ortholux microscope equipped with a cross hatched graticule in the eyepiece. It was the small squares of the grid that aided the cell count rather than relatively large ones on the haemocytometer surface. The cell number concentration was calculated from the count with customised software and it varied in the experiments from 2.5 to 5.5 × 10^6 per cm^3. The specific growth rates of the cells in long term culture in 1% and 3% saline were determined by daily counts over 10 days from 2 × 10^6 per cm^3.

2.2. Analytical instrumentation

The photocalorimeter is a customised module of the commercial TAM heat conduction batch microcalorimeter (Thermometric AB, SE-17561 Järfälla, Sweden) [24]. It was modified from a design used for estimating the enthalpy changes of photochemical solutions [25] and the rate of photosynthesis in spinach leaves [4] but differs from the latter in several respects. Using the twin differential principle [26], 5 cm^3 of the cells suspended in Bold's BM containing 1% or 3% saline was placed in a 20-cm^3 stainless steel titration vessel (test) and stirred at 60 rev/min with a Thermometric KelF turbine [27] while the reference vessel contained Bold's BM in 1% saline. Measurements were undertaken at 25 °C and another cell count was taken at the end of the experiment to obtain the heat flux. The instrument was chemically calibrated by the heat flow of the slow hydrolysis of triacetin [28]. Light was introduced into each vessel through a 5-mm quartz rod (Suprasil 1, Hereaus Quartzglas, GmbH, D-63450 Hanau, Germany). As in earlier experiments [4], the optical part consisted of an Oriel 150 W Xe lamp in the housing (LOT-Oriel Ltd, Leatherhead, KT22 7BA UK). The light was focussed with a condenser and this also acted to attenuate the PPFD before it passed through a continuously cooled water filter and a glass filter to remove IR and UV radiation, respectively. Light was transferred from the housing exit slit through an Oriel bifurcated silica fibre bundle connected to the quartz rods which, unlike in the Johansson-Wadsö setup that employed two calorimeters [4], are in the test and reference vessels of one calorimeter.

It should be realised that even at the comparatively low PPDF radiance of 90 μmol m^{-2} s^{-1}, the energy potentially available for transformation to heat at the walls and in the liquid of the vessels is very high. By calculation from Planck's constant, the velocity of

light and the frequency, the above light energy is milliwatts per vessel, outside the range of a microwatt calorimeter [24]. Thus, even a minor imbalance in the radiance to the test and reference vessels produced a large shift from the zero baseline during the photocalorimetric measurements. It was caused by imperfect splitting of the light beam on its way from the source to the vessels. The imbalance was minimized by adjusting the position of the lamp in its housing but it was not possible to achieve the perfect balance. For the highest PPDF, 90 μmol photons m^{-2} s^{-1}, the effect was about 100 to 200 μW greater than the dark baseline, so the value was comparable with that produced by the culture. Before each set of experiments, therefore, a value for the radiance-induced heat flow was obtained from a calibration curve for each PPDF level and used as the anticipated radiance value for correcting the corresponding parts of the metabolic heat flow curves.

The rates of respirometric oxygen uptake in the dark and photosynthetic oxygen evolution in the light were measured in parallel to the heat flows using an Oroboros Oxygraph (Oroboros Instruments, A-6020 Innsbruck, Austria) two-chamber oxygen polarographic sensor [29,30] at 25 °C. A 2.3 cm^3 aliquot of the same cell suspension used in the calorimetry was added to the glass vessel of each chamber and magnetically stirred at 100 rev/min. The rate of oxygen flow was measured under the same light conditions as for calorimetery. It was corrected for the salinity of the medium and the cell count at the end of the experiment to express the results as the scalar flux.

From much of the available literature [1-4], it was anticipated that, for ideal conditions (balanced growth with unlimited nutrients and optimal light), the net heat flow would decrease after switching the light on with the quantity of it being the net value for the rates of endothermic photosynthesis and exothermic respiration. Oxygen flux data for dark and light conditions also provided the rate of photosynthesis and both the values obtained from photocalorimetric and respirometric measurements were compared after converting the oxygen flux to its energy equivalent using the calculated oxycaloric equivalent of –470 kJ mol^{-1} O_2 [21]. The calculations, abbreviations, symbols, quantities and units for the energy balances can be seen in Table 1. The actual value of the net heat flow in the light, termed the "observed response to light", corrected for any incident light-induced heat imbalance revealed by the prior calibration curve, was compared with the expected decrease in the heat flow due to photosynthesis calculated from the respirometric data. In the energy balance calculations, the rate of the heat flow produced by the algae in the dark (dark heat flux) was considered to be the

measure of the dark metabolic rate while the dark respiration rate (dark oxygen flux) was used only for calculating the CR ratio.

3. Results and Discussion

The heat and oxygen fluxes of *D. maritima* cells were measured at the same time after transferring them from the routine culture conditions of a 12-hour white light regime at 25 μmol photon m^{-2} s^{-1} to the dark and then to higher incident light intensity. It can be seen in the four experiments depicted in Fig. 1 that cells in both the1% control saline (curves a) and the 3% acute saline treatment (curves b) in the dark produced heat which, under aerobic conditions, is generally considered to be due mostly to respiration [6]. In contrast, the effect of radiant light energy at PPDF values of 50 and 90 μmol photon m^{-2} s^{-1}v was different depending on whether the cells were suspended in 1% or 3% saline (see Fig.1). In the experiments with the latter excepting that in Fig. 1D, light at 90 μmol photon m^{-2} s^{-1} caused net negative heat flux in which the endothermic heat flux due to photosynthesis was much greater than the exothermic heat flux due to respiration. Johansson and Wadsö [4] obtained similar result for discs of spinach leaf bathed in isotonic 0.9% saline [4]. They used a similar white light Xenon lamp to that used in the present experiments but, unlike these, the spinach plants in the greenhouse were maintained in a 16-hour photoperiod and a PPDF range of 120-180 μmol photon m^{-2} s^{-1}, while the experiments were conducted at 31-33 μmol photon m^{-2} s^{-1}. Thus the spinach chloroplasts were exposed to considerably less light than normal.

The situation on exposing the algal cells treated acutely with 3% saline to 50 μmol photon m^{-2} s^{-1} was more complex than at 90 μmol photon m^{-2} s^{-1}. The lower light intensity resulted in net heat flux values which were still negative (see Fig. 1C), positive but less than in the dark (Fig. 1B) or more than in the dark (Fig. 1A and D).This complicated pattern also held for the 1% controls. There was no case in which the result was a net negative heat flux, although on occasions 50 (see Fig. 1B) and 90 μmol photon m^{-2} s^{-1} ((Fig. 1A, C and D) caused a decrease in net heat production. Mostly, however, no change or even an *increase in the net heat flux* was observed in the light for the 1% controls. The meaning of all these findings firstly required analysis of the energy balances, bearing in mind that the light intensity in the culture room was lower than in the experiments.

Janssen et al. [5] had previously reported strong exothermic heat flux for cultures of *Chlorella vulgaris* growing for 80+ h in a 1.5 L bioreactor/calorimeter in which the PPDF for the continuous light at 660 nm averaged 145 μmol photon m^{-2} s^{-1} over the surface of the vessel The light conditions for the stock cultures are unknown. If the exothermic heat flux were due to mito-, chloro and/or photorespiration [13] in any experiments with photoautotrophs, then clearly there would be no cell growth, because all of the photosynthate would have been consumed in respiration and none in biosynthesis, an unsustainable condition. In fact, the simultaneous oxygraph data in the present experiments (Fig. 1) all showed that respiration was an untenable explanation for the cases of net heat production because, contrary to many of the above calorimetric results, aliquots of the same samples produced the expected "normal" findings: i.e. in the light, oxygen evolution (NOF > 0, see Table 1 for the balances) was at rates higher than dark respiration (NOF > DOF in terms of the absolute values), both for the control and under acute salt stress, meaning that sufficient photosynthate was synthesised to allow cell growth in terms of anabolic biomass as well as the catabolic processes to produce ATP as chemical energy.

Energy balances were constructed for the light conditions in order to try and assess the scale and possible origin of the discrepancy between the direct and indirect calorimetry. Figs. 2A and B represent the averaged data for light at 90 μmol photon m^{-2} s^{-1} and at 50 μmol photon m^{-2} s^{-1}, taken for the four sets of curves presented in Fig. 1. The balance was achieved as described in Table 1 by combining the photocalorimetric data, i.e. the dark heat flux as a measure of the integral metabolic rate and the change in the dark heat flux obtained after switching on the light (observed response to light), with the respirometric data, i.e. the photosynthesis rate converted into energy units using the oxycaloric equivalent [21]. As can be seen in Fig. 2, there was always a considerable imbalance between the expected net heat flux (DHF + PS) and the observed one (DHF + ORL), providing evidence of an extra source of heat which was called the "X-factor" . It was a surprise that in general the heat power quantities for this discrepancy on exposure to PPDF values of both 50 and 90 μmol photon m^{-2} s^{-1} were greater than the dark heat flux and comparable to photosynthesis. There are two biophysical sources for heat production in this system, chlorophyll fluorescence [7] and NPQ, as part of photoprotection [8], with the latter being in the majority and detected as a change in the quantity of the former [10,32]. It is possible that the net heat production of *C. vulgaris* cells growing in culture [5] was due to these phenomena. It is not known if stock

cultures were grown under the same light regime and thus adapted to it. As stated earlier, NPQ is essentially a mechanism to remove the excess electrons in the antennae, which otherwise would damage the photosynthetic apparatus, by thermal dissipation in the zanthophyll cycle [15-17]. Most recently, Pascal et al [33] has determined the atomic structure of the light-harvesting antenna protein, LHC11, by X-ray crystallography which switches its structure to a dissipative state when the excess of excited electrons lowers the pH of the thylakoid lumen. It appears that the cells had adapted to a certain photosynthetic rate at 25 μmol photon m^{-2} s^{-1} and at least in the short term cannot take full advantage of the increased supply of excited electrons to raise the photosynthetic rate sufficiently to avoid saturation at PPDF values of 50 and 90 μmol photon m^{-2} s^{-1} and so must dissipate the excess. This could imply that there are two populations in the cell suspensions, one which can respond to the increased photons by raising the photosynthetic rate and another which cannot and must thermally dissipate the excited electrons by NPQ. The cultures were not synchronised and so it is possible to envisage a sub-population of senescent cells and one of dividing cells, with the former unable to raise the rate of photosynthesis with increased incident light. This hypothesis is currently under investigation.

Some of the key factors in the energy balance calculations were plotted against each other as regression lines to show the range of them and to examine their relationships (see Fig. 3). For the dark heat flux, it is possible to envisage a strong direct relationship between it and the rate of photosynthesis as displayed by the control (see Fig. 3A) because of feedback control [34] exercised through the inorganic phosphate pool [35] but only in the event of demand in respiration controlling the rate of photosynthesis. Even so, it is not obvious why it should be so much more tenuous after the 3% treatment (Fig. 3B).

Regarding the regression line for the photosynthetic rate calculated from oxygen evolution against the X-factor (Fig. 3C), it is well known that the greater the light the more the fluorescence until the photochemistry is saturated [36] and therefore the greater the heat flow associated with that source as well as the greater the photosynthesis as measured by the oxygen evolution rate. Bearing in mind that the cell suspensions probably are not metabolically homogeneous, it is hypothesised that as the photosynthetic rate increased the X-factor also increased due to heat flow from the radiant fluorescent light but that, once the photochemical process was saturated, the increasing light energy was dissipated mostly as heat flow in NPQ, possibly with a

small continued contribution of 3-5% from the fluorescent light [37]. There is a strong correlation between photosynthesis and the X-factor for all but the cells treated with 3% saline in incident light at 50 μmol photon m^{-2} s^{-1}.

The effect of acute osmotic shock on cells grown in 1% saline was examined by exposing the cells to 3% saline and the probabilities for the paired *t*-tests on the results are given in Fig. 2. It is interesting that the dark heat flux which gives the integral rate of aerobic and anaerobic catabolic processes was significantly greater ($p < 0.05$) in the treated cells than in the controls whereas there was no significant difference ($p = 0.131$) in the dark oxygen flux which is a measure only of the respiratory rate. The average of the measurements for the rate of dark oxygen flux depicted in Fig. 1 for cells in the controls was $0.7 \pm 0.1 \times 10^{-17}$ mol O_2 s^{-1} per cell compared with $0.6 \pm 0.1 \times 10^{-17}$ mol O_2 s^{-1} per cell for the treated cells (Fig. 4). From these two sets of data, the experimental calorimetric-respirometric (CR) ratios, equivalent to the calculated oxycaloric equivalent, of the saline control were found to average -511 ± 82 kJ mol^{-1} O_2 but the acute exposure to increased saline (3% NaCl) caused an even more negative CR ratio of -838 ± 248 kJ mol^{-1} O_2 (Fig. 4). The paired *t*-test for the difference was not significant ($p = 0.072$) though there was a marked trend. Since the oxycaloric equivalent for the fully oxidative metabolism of glucose is –470 mol^{-1} O_2 [21] (see dashed line in Fig. 4), these results indicate that at least one anaerobic process occurred under the aerobic conditions of the cell culture.

Ben-Amotz and Avron [38] found that that the osmolyte present in halotolerant microalgae is glycerol and later discovered that the amount of it in the cytoplasm is a function of the salinity of the medium [39]. León and Galván [40] showed that it was synthesised from photosynthate in the glycolytic pathway, often at the expense of starch synthesis [41], when cells were exposed to hyperosmotic shock while Cronwright et al. [42] conducted a metabolic flux control analysis of its synthesis from dihydroxyacetone phosphate. In general, the glycolytic pathway contributes to the heat produced in the dark but not of course to the oxygen uptake rate and this would be the case for glycerol. As confirmed by the CR ratios, the requirement for it would be greater with increasing salinity. From Wilhout [43], the enthalpy change for the formation of glycerol was calculated to be –59 kJ mol^{-1}. This would contribute to the highly negative CR ratios obtained in this investigation and explain the more negative the CR ratio with greater salinity.

It has been shown that under fully aerobic conditions this rate is directly proportional to the turnover of ATP [44], the energy currency of the cell. Because of the thermodynamic Gibbs-Donnan effect, organisms must respond to hyperosmotic conditions by removing the greater concentration of Na^+ ions passively transported into the cells down the concentration gradient and by synthesising osmolytes to balance the external osmotic pressure. *D. maritima* is no exception to this rule in that it actively pumps out the Na^+ ions with both the large, multipass membrane Na^+,K^+-ATPases but also by the synthesis and operation of novel, relatively small Na^+-ATPases (100-140 kDa) induced by the presence of high salt [45]. The activity of these pumps and thus the ATP turnover obviously must be greater the higher the salinity of the culture medium and thus it would appear difficult to explain why there was so little change in the dark oxygen flux (see above).

A clue to the answer to this puzzle may lie in our unpublished data that cells adapted to culture in 3% saline had a slower specific growth rate ($\mu = 0.0087\ h^{-1}$), than those of the 1% control ($\mu = 0.0139\ h^{-1}$). By comparing the energy balances for cells in long term culture with 3% saline containing medium (Fig. 5) and those acutely exposed to 3% saline (Fig. 2, "treat" cells in A and B panels), it can be seen that photosynthesis and respiration were somewhat lower in the chronic condition. Nevertheless, the results suggest that the cells react to increased saline by reducing cell growth and thus the ATP demand inherent in it in order to devote more to the active pumping mechanisms.

Continuing to analyse the data in Fig. 2, photosynthesis measured by net oxygen evolution rate and converted to energy units, was slightly but not significantly higher with the increased salinity at both 50 (paired *t*-test, $p = 0.173$) and 90 μmol photon m^{-2} s^{-1} ($p = 0.139$). Given the known interactions between chloroplasts and mitochondria [13,19, 46], it is not surprising that the data for the rate of photosynthesis echoes that of oxygen flux in showing no response to increased salinity. However, as established earlier, it is reasonable that the photosynthetic rate would rise with increasing incident quantity of photons from 50 to 90 μmol photon m^{-2} s^{-1} (see Fig. 2). Statistical analysis of the data showed that there were significant rises in the rate of photosynthesis both in control (paired *t*-test, $p = 0.02$) and under saline stress ($p = 0.04$). The use of net oxygen evolution as a measure of the photosynthetic rate must be treated with caution because it relies on assuming that the mitorespiratory rate is the same in the light as in the dark and, although there is good evidence for this [47,48], it has still not been proved beyond

doubt [49]. It is also important that photorespiration [13,19] is absent or weak but this is not always the case – see for instance [49]. There is evidence, however, that microalgae act like C_4 plants in this respect by suppressing photorespiration, though by a different mechanism to C_4 plants that involves the intracellular accumulation of inorganic carbon by active uptake of HCO^{3-} [50] and CO_2 [51], the only sources of carbon in the Bold's medium used for the current investigation.

The standard error in the energy balance calculations was rather large. Although care was taken in the analysis, the light source gave potentially milliwatts heat, only a small percentage of which could produce errors [4,52]. Some of the variaton may be a reflection of the degree to which the cells have adapted to the medium and the light regime. For instance, it is known that the interception of incident light can be changed by reorientation and/or movement of chloroplasts within cells [53]. At the same time, there are molecular changes to the phosphorylation of proteins in the photosynthetic machinery, state transitions and adjustments to the photosystem stoichiometry on illumination [54] which can change with different physiological states. It would seem reasonable to hypothesise that adapted cells would not express NPQ and that cells in the senescent phase of growth also would give a different NPQ to those in exponential growth. In addition, long periods of time in culture without fresh sources of trace elements are known to affect the rate of photosynthesis [54]. So, the next stage of the study is to pay more attention to the physiological and environmental conditions for growing the cells and for the systematics of the growth curve per se. This is particularly important for dissecting chlorophyll fluorescence from NQP. Nevertheless, since the heat produced from the radiant energy of fluorescence is only 3-5% of NPQ, it is interesting to report that the NPQ component of photoprotection can be detected by direct calorimetry.

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Table 1
Components of the energy balance of *D. maritima*, their units, abbreviations and methods of determination.

Variable	Units	Abbreviation	Data source
PHOTOCALORIMETRY:			
Dark heat flux	$+10^{-12}$ W per cell	DHF	Measured
Net heat flux (in the light)	$+10^{-12}$ W per cell	$NHF_{50, 90}$[1]	Measured
Observed response to light	$\pm10^{-12}$ W per cell	$ORL_{50, 90}$	NHF−DHF
RESPIROMETRY			
Dark oxygen flux	-10^{-17} mol O_2 s^{-1} per cell	DOF,	Measured
Net oxygen flux (in the light)	$\pm10^{-17}$ mol O_2 s^{-1} per cell	$NOF_{50, 90}$	Measured
DOF/NOF energy equivalents	$\pm10^{-12}$ W per cell	DOF, $NOF_{50, 90}$	Calculated[2]
Photosynthesis	$+10^{-17}$ mol O_2 s^{-1} per cell	$PS_{50, 90}$	NOF−DOF
Photosynthesis energy equivalent	-10^{-12} W per cell	$PS_{50, 90}$	NOF−DOF
CR Ratio	-10^{3} J mol^{-1} O_2	CR	DHF/DOF
X-Factor	$\pm10^{-12}$ W per cell	$X_{50, 90}$	ORL−PS

[1]In incident light of either 50 or 90 μmol photon m^{-2} s^{-1}
[2]Calculated using the factor –470 kJ mol^{-1} O_2

Fig. 1. Results of four experiments in which dark/light heat and oxygen fluxes were measured at the same time for control (a) and treated (b) *D. maritima* in Bold's medium containing 1% and 3% NaCl, respectively. The oxygen flux measurements were made in two replicates. The arrows indicate the time points of switching on the light at 90 (1) and 50 (2) μmol photon m^{-2} s^{-1}, and switching off the light (3).

Fig. 2. Energy balances constructed from the data in Fig. 1 for control (chronic, 1% NaCl) and treated (acute, 3% NaCl) *D. maritima* in the light at 90 (A) and 50 (B) μmol photon m^{-2} s^{-1}. See the abbreviations and formulae in Table 1. The values are the mean ± SE (n=4) and the results of paired *t*-test (*p*). The discrepancy between the expected (DHF + PS) and measured (DHF + ORL) heat fluxes in the light is balanced with X-factor (X), an extra source of heat.

Fig. 3. Correlations with regression lines between the principal components of the energy balances shown in Fig. 2.

Fig. 4. Relationship between dark oxygen flux and dark heat flux in control (□) and treated (◆) *D. maritima* using the data from Fig. 1. The dashed line represents the oxycaloric equivalent of –470 kJ mol^{-1} O_2. The values are the mean ± SE (n=4) and the results of a paired *t*-test (*p*) comparing DOF and CR in the control and the treated cells.

Fig. 5. Energy balances constructed for *D. maritima* grown as a control in Bold's medium containing 3% NaCl, in the light at 90 (A) and 50 (B) μmol photon m^{-2} s^{-1}. See abbreviations and formulae in Table 1.

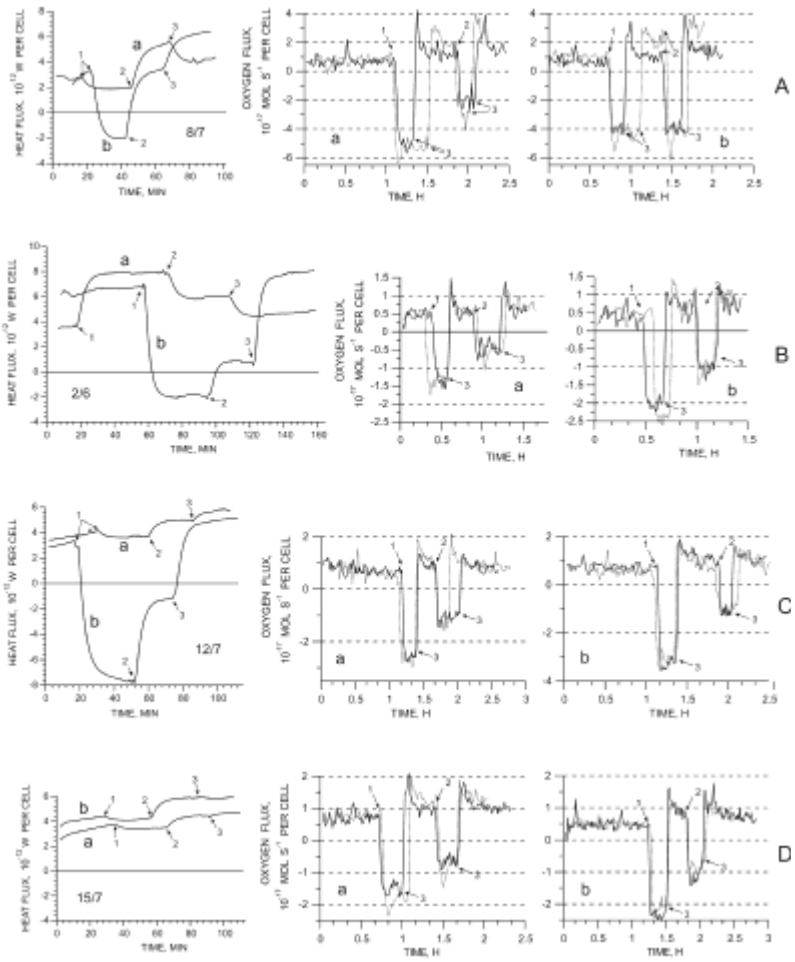

Fig. 1. Mukhanov & Kemp

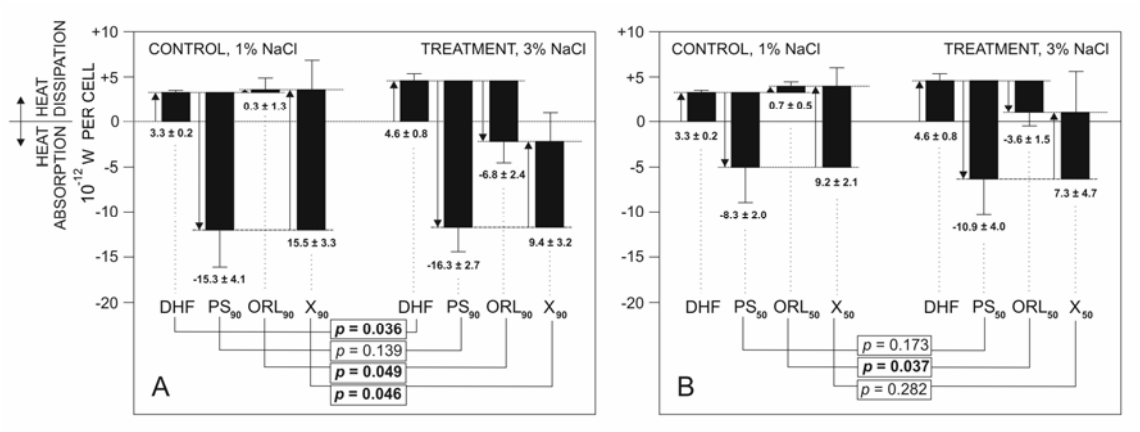

Fig. 2 Mukhanov & Kemp

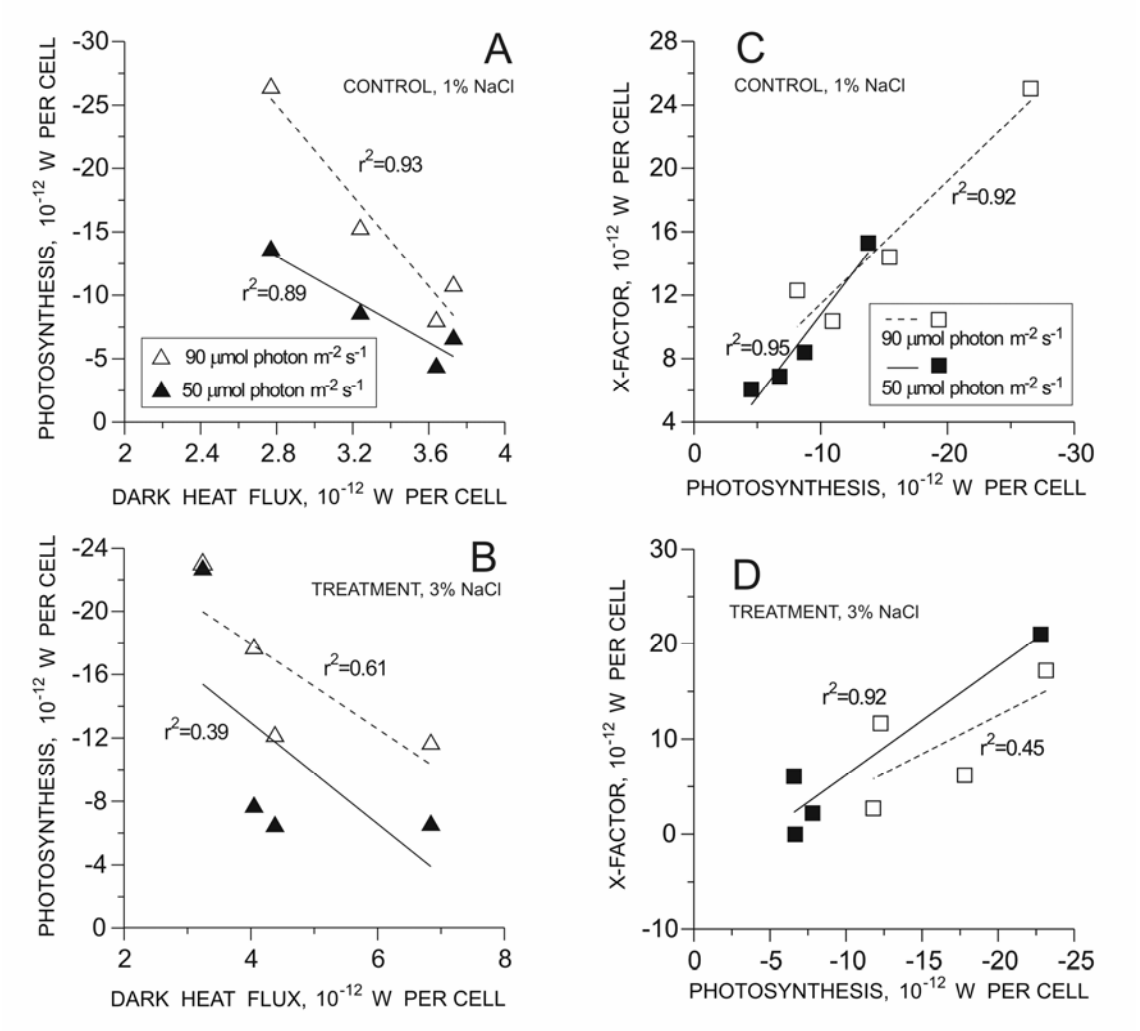

Fig. 3 Mukhanov & Kemp

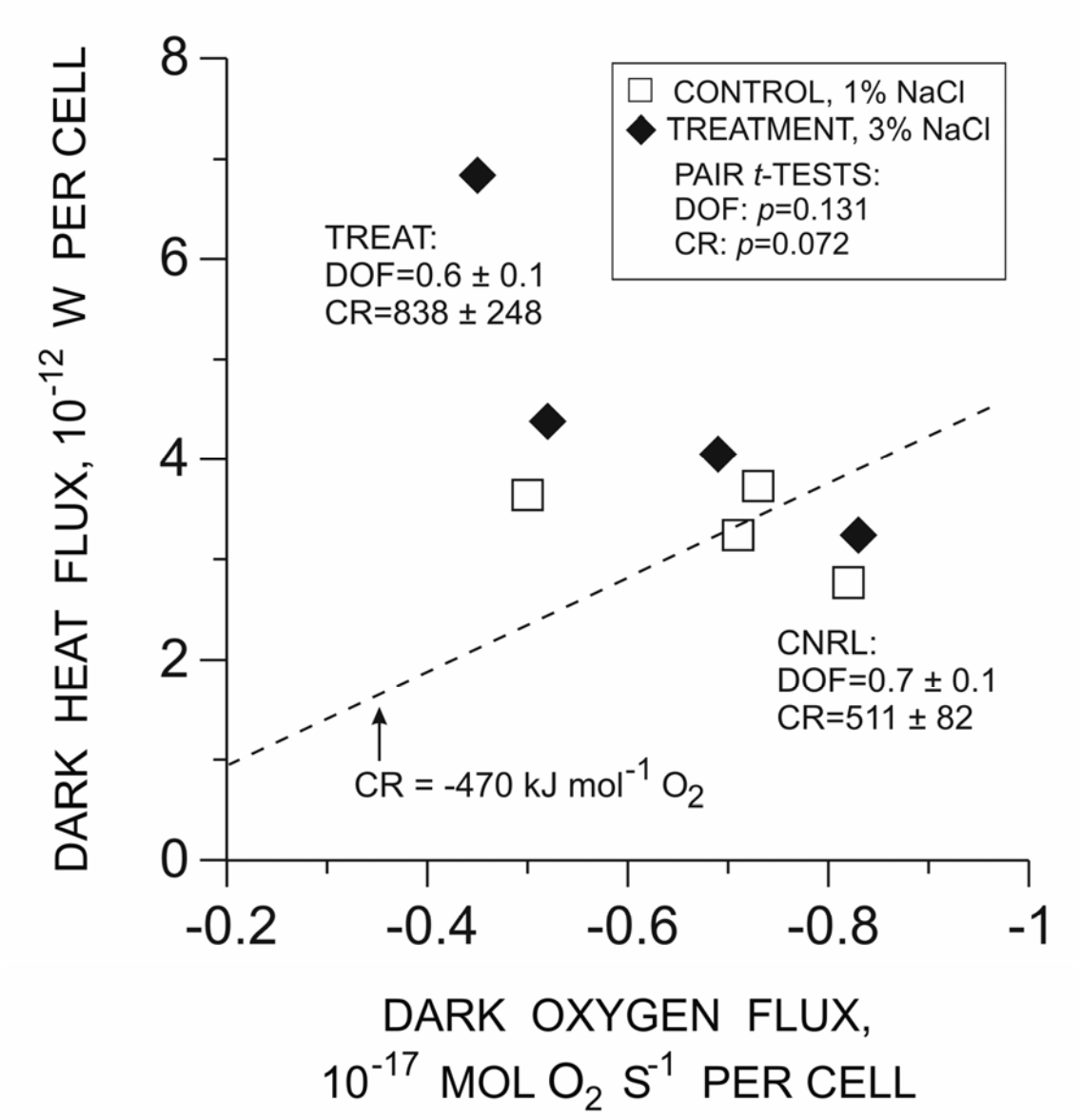

Fig. 4 Mukhanov & Kemp

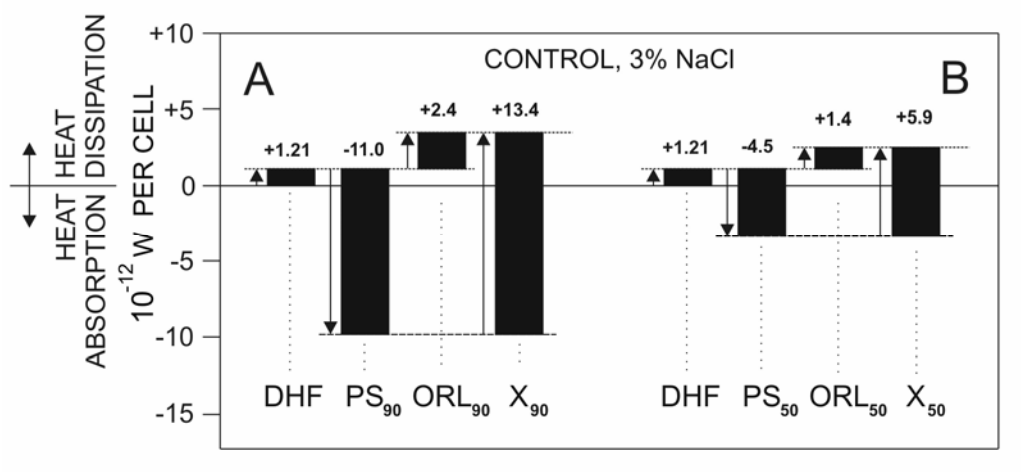

Fig. 5 Mukhanov & Kemp