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IMPACT OF ENVIRONMENTAL CONDITIONS ON PHOTOSYNTHESIS, GROWTH AND CARBON ALLOCATION STRATEGIES OF HYPERSALINE SPECIES OF *DUNALIELLA*

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ABSTRACT
Hypersaline environments pose a number of ecological and metabolic challenges to the organisms that live in them. Primary producers, such as halotolerant species of the green microalgal genus *Dunaliella*, are no exception. In this paper we focus on the problems posed to the acquisition and metabolism of inorganic nutrients and on the consequences of exposure to high light and UV radiation. We show that not only does growth in high salinity environments have repercussions on the flow of carbon into osmolytes such as glycerol, it also affects speciation of inorganic carbon and the uptake of inorganic ions by the cells. The strategies that *Dunaliella* adopt to optimize resource utilization and the interactions among metabolic pathways are also discussed.

KEYWORDS: *Dunaliella*; photosynthesis; allocation strategies; inorganic carbon acquisition, nutrients, light, UVR.

1. INTRODUCTION

Chlorophyte algae of the genus *Dunaliella* are found in nature in many salt-water habitats. In most marine environments the genus never comprises a major component of algal populations. In hypersaline environments however, species such as *D. salina*, *D. parva* and *D. viridis* are found as the dominant microalgae in the water column. Examples of *Dunaliella* as the major microalga in natural populations are found from the Dead Sea, Israel (Nissenbaum, 1975), the Pink Lake in Australia (Borowitzka, 1981), and the Great Salt Lake in the USA (Post, 1981). *D. parva* and *D. salina* are the dominant spring phytoplankton species in the athalassic lake of Fuenta de Piedra in Spain (Jimenez *et al.,* 1990).

The taxonomy of *Dunaliella* species has, in the past, been disputed (Masyuk, 1973; Ginzburg, 1987; Preisig, 1992). Three major genera of *Dunaliella* capable of growth at high salinities are now generally accepted. *D. salina* appears red when cultured at high salinities, *D. parva* appears yellowish and has a lower carotenoid content, whereas *D. viridis* remains green even at elevated salinity. *D. tertiolecta* is mesohaline and stays green even at salinities above its optimum for growth (Borowitzka and Borowitzka, 1988). Because of their ability to grow and thrive at high salinities, *Dunaliella* species are the predominant algae in saltworks and naturally occurring hypersaline environments. *Dunaliella salina* for instance grows over a range of salinities from ~0.033 M to saturated salt solutions (5.8 M), with an optimum around 2 M NaCl (Ben Amotz and Avron, 1989). *D. viridis* can grow in up to 5 M NaCl with a broad optimum around 1.0 to 2 M NaCl (Borowitzka and Borowitzka, 1988; Jiménez and Niell, 1991), though the optimal salinity for *D. viridis* growth is dependent on temperature (Jiménez and Niell, 1991). In contrast, the mesohaline *D. tertiolecta* is only capable of growth up to 1.67 M. Isolates from hypersaline lakes in the Vestfold Hills, Australian Antarctic Territory showed

little effect of salinity on growth over the range 0.2 to 1.7 M (Xu, 1994). A more detailed description of the ecology of *Dunaliella* species is to be found in Borowitzka and Borowitzka (1988).

In addition to variations in salinity, *Dunalella* spp. in saltworks are exposed to high levels of light, as both photosynthetically active radiation (PAR 400-700nm) and UV radiation (280-320 and 320-400 nm, for the UVB and UVA ranges respectively) as well as variations in temperature and nutrients. The following paragraphs will deal with the effects of these environmental factors on the biology of *Dunaliella* spp.

2. LIGHT AND UV

Maximal growth rates of *D. euchlora* and *D. salina* have been reported as occurring at >180 µmol m^{-2} s^{-1} and 127 µmol m^{-2} s^{-1} respectively (Chang *et al.*, 1986), though these authors note a complex interaction between light, salinity and temperature in determining growth rates, with light being less important than the other two environmental parameters. In *D. tertiolecta*, growth saturates at ~150 µmol m^{-2} s^{-1}, and photoinhibition is not observed below 300 µmol m^{-2} s^{-1} (Quigg and Beardall, 2003).

Photosynthesis saturates at somewhat higher photon fluxes, with *D. parva* showing saturation at ~400-600 µmol m^{-2} s^{-1} depending on salinity (Jiménez *et al.*, 1990) and *D. tertiolecta* photosynthesis saturating at 100-200 µmol m^{-2} s^{-1}, depending on growth rate (e.g. Young and Beardall, 2005).

Although growth on high concentrations of NH_4^+ rather than NO_3^- as the sole N source leads to a stimulation of both maximal rates of photosynthesis (P_{max}) and apparent quantum yield, in *D. salina*, this has no obvious repercussions for the photon flux density required to saturate photosynthesis or on the light compensation point, nor does it impact on the susceptibility of cells to photoinhibition (Giordano and Bowes, 1997).

There is little information on the UV sensitivity of *D. salina* and other hypersaline tolerant *Dunaliella* species. Hermann *et al.* (1997) studied the effects of UV on *D. salina*, and reported that the inhibitory effects of the different wavelengths of solar radiation increased as UVB>UVA>PAR with the relative mean effectiveness of these classes being ~$2x10^{-4}$ (µmol m^{-2} s^{-1} $)^{-1}$ for UVB, 4 x 10^{-6} (µmol m^{-2} s^{-1} $)^{-1}$ for UVA and 2 x 10^{-7} (µmol m^{-2} s^{-1} $)^{-1}$ for PAR. Curiously, White and Jahnke (2002) report that whereas up to 24 h exposure to UVB had no effect on photosynthetic quantum yield of both *D. salina* and *D. bardawil*, UVA strongly decreased quantum efficiency, particularly in *D. bardawil* and in *D. salina* grown at low light. Beardall *et al.* (2002) have shown that quantum yield of photosynthesis in *D. tertiolecta* is inhibited by ~50 % by 60 min exposure to 2.8 W m^{-2} UVB. Inhibitory effects of UVB to photosynthesis of *D. tertiolecta* are enhanced by N-limitation (Shelly *et al.*, 2002) and P-starvation (Shelly *et al.*, 2005; Heraud *et al.*, 2005).

Light also plays a crucial role in one of the feature that makes *Dunaliella* a very important organism for biotechnology: carotenogenesis. The biosynthesis of β-carotene appears to be unrelated to light quality within the PAR region, but it is rather dependent on the overall amount of light received by the organism over one cell division (Ben-Amotz and Avron, 1989; Ben-Amotz and Shaish, 1992 and references therein). This may, to some extent, be related to the fact that, in carotenogenic *Dunaliella*, most β-carotene is located at the periphery of the chloroplasts and is not associated with the photosynthetic antenna (Borowitzka and Borowitzka, 1988). However, the isomeric composition of the β-carotene pool may not be totally unrelated to the wavelength composition of the incident radiation, since the *trans* to *cis* transition leads to a shift in the absorption maxima and the appearance of a peak in the UV region (Ben-Amotz and Shaish, 1992 and references therein).

3. NUTRIENTS

The range of environments that *Dunaliella salina* inhabits is characterized by large variations in nutrient availability. The impact of *D. salina* in saltworks, even in crystallizer basins, is highest when poor management of biological processes induces an increase of nutrients (Davis, 2000; Delapsakis *et al.,* 2005) and this can lead to substantial physiological changes

in *D. salina* cells (Giordano *et al.,* 1994; Giordano and Bowes, 1997). Nitrate concentration in the range 0.5 to 10 mM had little effect on growth rates of *D. viridis* (Jiménez and Niell, 1991). Although some authors reported that high concentrations of ammonium (>2.5 M) were lethal for *Dunaliella* species (Grant, 1968), *D. salina* (Giordano *et al.,* 1994) and *D. parva* (Giordano *et al.,* 2002) are capable of adaptation to growth on N concentrations up to 10 and 5 mM, respectively, regardless of whether the N source is NH_4^+ or NO_3^-. Cells acclimated to growth on high NH_4^+ concentrations usually showed similar division rates and larger cell size than their high [NO_3^-]-grown counterparts (Giordano and Bowes, 1997).

The storage capacity for NO_3^- seems to be relatively small (1 order of magnitude lower than in diatoms; Lomas and Gilbert, 2000). However, Del Rio *et al.* (1993) provided some evidence that nitrate reduction could be conducted even in the dark, thanks to the allegedly rather unusual (see Giordano *et al.*, 2005 and references therein) properties of *D. salina* nitrate reductase. The ability to take up and assimilate NO_3^- even at night could make a big difference in terms of the strategy for N utilization in this microalga; further studies are however needed to clarify this controversial aspect of NO_3^- reduction in *D. salina*. The ability to actively take up and store N as NH_4^+ is also still rather controversial (Pick *et al.,* 1991; Lomas and Gilbert, 2000, Giordano and Bowes, 1997; Giordano *et al.,* 2000). The presence of either form of inorganic N, especially at high concentrations, plays a major role in the photosynthetic performances of *D. salina* and on the pattern of resource allocation (see below) (Giordano *et al.,* 1994; 2002; Giordano and Bowes, 1997; Giordano, 2001; Norici *et al.,* 2002).

Of the other major nutrients, optimal concentrations of P for *D. salina* are 0.15 to 0.18 mM, with growth being inhibited above 36 mM (Borowitzka and Borowitzka, 1988), whereas sulfate saturates *D. salina* growth at around 0.30 mM (Giordano *et al.,* 2000).

To the best of our knowledge, no data are available on the kinetics of inorganic nitrogen uptake for hypersaline species. However, the ability of *Dunaliella* to take up nitrate, at least for the marine species *D. tertiolecta*, is characterized by a relatively low affinity for the substrate (Ks about 11 μM, ~5-30 times higher than that found for diatoms) and similar to that of some dinoflagellates (but ~0.5 times that in other dinoflagellate species), and a relatively small capacity (Lomas and Gilbert, 2000). Maximal uptake rates are reported as 18 fmol N h^{-1} $cell^{-1}$ (0.005 fmol N h^{-1} $fmol^{-1}$ C) (Lomas and Gilbert, 2000).

Half saturation for P uptake by *D. tertiolecta* is ~1 μM but maximal uptake rates vary from 14 to 26 ng P $(10^6 \text{ cells})^{-1}$ min^{-1} depending on the growth rate (Roberts *et al.*, 2008).

Limitation of growth by diminished supply of N, P, S and Fe all have effects on the ability of *Dunaliella* species to acquire carbon and allocation of C to cellular components. This is dealt with in more detail below.

Growth of *D. salina* on high concentrations of NH_4^+ leads to nearly a doubling of the chlorophyll per cell, when compared to growth on equal concentrations of NO_3^- (Giordano and Bowes, 1997). The effect of the N-source on cellular β-carotene content is much smaller, albeit noticeable (30-50% increase under NH_4^+), with a consequent increase in the chlorophyll to β-carotene ratio (Giordano and Bowes, 1997). The algae cultured on NH_4^+ rather than on NO_3^- increased not only the amount of chlorophyll per cell (~70%) but also the apparent chlorophyll concentration (~50%), despite the NH_4^+-related increase in cell size (~20%). The magnitude of the effect of the N-source on the cell content (~50%) and apparent concentration (~10%) of β-carotene was smaller, but this did not substantially affect the chlorophyll to β-carotene ratio (Giordano and Bowes, 1997). The amount of N available also plays an important role in the production of β-carotene. In combination with temperature, salinity and light, N-limitation is among the most potent inducers of carotenogenesis in the carotenogenic strains of *Dunaliella* (e.g. Ben-Amotz and Shaish, 1992). In general, factors causing a decline in the growth rate (primarily nutrient limitation) lead to the accumulation of β-carotene in *Dunaliella* cells (e.g. Ben-Amotz and Shaish, 1992).

The concentration of CO_2 for growth affects chlorophyll concentration in *D. salina* only indirectly, via a change in cell volume. When *D. salina* is cultured at ambient CO_2 or at 5% CO_2, no significant variation in the chlorophyll cell content can be observed, though the

increased volume of high-CO_2 grown cells dilutes the chlorophyll concentration by a factor of two (Giordano and Bowes, 1997). It should however be noted that it was not demonstrated that the change in whole cell volume corresponded to equal changes of chloroplast size.

4. INORGANIC CARBON ACQUISITION

Among the consequences of a high salinity environment are a decrease in CO_2 solubility and a shift in the equilibrium between CO_2 and HCO_3^-. *Dunaliella salina* responds to this decrease in CO_2 and increase in HCO_3^- at higher salinity by increasing the activity of a CO_2 concentrating mechanism (CCM) (Zenvirth and Kaplan, 1981; Booth and Beardall, 1991), though even at lower salinities, the CCM is as active as in the mesohaline *D. tertiolecta* (Zenvirth and Kaplan, 1981; Young *et al.,* 2001). Enhanced activity of the CCM of *D. salina* at high salinity is accompanied by elevated levels of an external carbonic anhydrase (CA_{ext}) (Booth and Beardall, 1991). *D. salina* adapted to high salinity shows induction of an unusual external α-type CA that retains its activity over the range of salinities from 0–4 M NaCl (Fisher *et al.,* 1996; Premkumar *et al.,* 2006). This CA_{ext} consists of two internally duplicated tandem repeated sequences fused into a single polypeptide of 60 kDa. This may be compared to the periplasmic CAs from the freshwater Chlorophyte, *Chlamydomonas,* that consist of 75 kDa heterotetramers of 2 large and 2 small subunits and which are 90% inhibited by 0.6 M NaCl (Premkumar *et al.,* 2006).

D. salina cells adapted for several generations to growth on high NH_4^+ concentrations (Giordano *et al.,* 1994) are more effective at utilizing light and CO_2, than those grown on NO_3^-, with NH_4^+ grown cells showing a halving of their photosynthetic $K_{1/2}(CO_2)$. This is indirect evidence of the fact that the chemical source of N modulates the effectiveness of *D. salina* CO_2 concentrating mechanisms. The high P_{max} and low $K_{1/2}(CO_2)$ of photosynthesis in NH_4^+ grown cells are associated with an increase of both Rubisco activity and Rubisco protein abundance and concentration. The increase of Rubisco protein under these growth conditions is not simply a result of a general increase of protein, since Rubisco abundance increases even relative to total protein (Giordano and Bowes, 1997). Periplasmic CA activity, which may also play an important role in facilitating CO_2 acquisition (e.g. Giordano *et al.*, 2005), also increases when N was available as NH_4^+ rather than as NO_3^-. This increase in activity is substantially larger than the cell volume increase under the same conditions; however, its relevance with respect to the photosynthetic response remains somewhat obscure, since the actual rates of CA-catalyzed dehydration of HCO_3^- to CO_2 are far above the photosynthetic requirements, even for NO_3^--grown cells (Giordano and Bowes, 1997).

In *D. salina*, the induction of a CCM subsequent to the transfer from 5% CO_2 to atmospheric CO_2 is associated with a decline of Rubisco abundance per cell by as much as 50%. However, low-CO_2-grown cells are much smaller (40 to 45%) than their high CO_2 counterparts; consequently, apparent Rubisco protein concentration is not compromised by the transfer to low CO_2. The concomitance between cell volume reduction and Rubisco down-regulation makes it difficult to understand whether the latter is part of the CCM induction process or is due to the change in cell size.

In contrast to the effects of N source on acquisition of inorganic carbon, in *D. tertioleta* at least, N-limitation leads to diminished Rubisco levels but enhanced CCM activity, leading to improved N-use efficiency of C assimilation under low N (Beardall *et al.*, 1991; Young and Beardall 2005). Although Fe-limitation of *D. tertiolecta* showed similar consequences to those of N-limitation (Young and Beardall, 2005), P-limitation of *D. tertiolecta* leads to a decreased CCM activity, presumably due to a lower availability of ATP to drive active transport processes (Beardall *et al.*, 2005).

5. THE FATE OF ASSIMILATED CARBON

It is interesting that *D. salina* is genotypically equipped to deal with high N concentration and is able not only to increase the activity of enzymes related to N-assimilation and C-skeleton construction, but also to induce specific isoforms of relevant enzymes when needed (Giordano *et al.,* 2002; Norici *et al.*, 2002). It is especially worthy of note, for instance, that a specific isoform of PEPc, different from those present at relatively low concentrations of both

NO_3^- and NH_4^+, is expressed when cells are exposed to high concentrations of NH_4^+ (Norici *et al.,* 2002). The importance of this anaplerotic enzyme (Norici and Giordano, 2002) in the detoxification of the cellular environment by excess NH_4^+ is consistent with the overall physiological patterns of high NH_4^+ grown cells (Giordano and Bowes, 1997). PEPC is not only up-regulated in cells acclimated to growth in NH_4^+, but it also appears pivotal in the short term (minutes to hours) and medium term (hours to few days; = from one to a few generations) responses to changes in N-source (Giordano *et al.*, 2007). This activity is in fact among the few physiological parameters that do not respond homeostatically (*sensu* Montechiaro *et al.,* 2006) to a transition from NO_3^- to NH_4^+, but is present at a higher level in the presence of incremental amounts of NH_4^+, compared to when NO_3^- is the sole N-source. Differences between cells acclimated/adapted to high NH_4^+ (Giordano and Bowes, 1997) and cells subjected to a gradual change of N-source from NO_3^- to NH_4^+ (Giordano *et al.,* 2006) are however obvious: while the carbon allocation in cells acclimated to high NH_4^+ undergoes substantial rearrangements with a large amount of C being invested in protein synthesis (Giordano and Bowes, 1997), the gradual transition causes only a transitory disturbance (within hours from change of N-source) in the ratio between protein and carbohydrates, which is brought back to the same relative proportions as had existed before the supply of NH_4^+. This occurs within about a day or two. This difference may have crucial repercussions for salt production in salt works. The quality of salt is negatively affected by the presence of organic matter in the crystallizer ponds. The amount of organic matter, possibly amino acids, released by *D. salina* can be substantial when cells are acclimated/adapted to high NH_4^+ concentrations (Giordano *et al.*, 1994); the maintenance of a balance between protein and carbohydrates during a medium term transition is instead suggestive of a rather moderate release of organic carbon, even if the peak in protein abundance that occurs soon after the change of N source could lead to a brief episode of organic release. This thus calls for careful management of the biological processes in saltworks to avoid excess production of NH_4^+, especially for a prolonged period of time.

In conditions of severe nutrient limitation, *D. salina* can undergo dramatic alterations to metabolism and composition to ensure maintenance of essential functions. In high salinity basins, glycerol production is paramount. It has been observed that a reduced supply of an essential nutrient like sulfur strongly affects the utilization of other nutrients, in particular C and N (Giordano *et al.,* 2000). The reduction of C assimilation causes a shortage of organic carbon in the cells. The inhibition of N assimilation on the other hand causes an imbalance in the C to N ratio. Under this circumstance, C is diverted from 4 and 5C compounds, possibly to ensure that sufficient C-skeletons are available for glycerol metabolism (Giordano *et al.,* 2000). Similar studies have not been conducted under limitation by other nutrients, but it can be expected that similar metabolic patterns are induced whenever, in *D. salina*, glycerol content per cell decreases when N is made available as NH_4^+ and when CO_2, concentration is high (5%) (Giordano and Bowes, 1997). Under these growth regimes, glycerol concentrations appear to be insufficient to provide the osmolarity needed to compensate for that in the external medium (Giordano and Bowes, 1997). An alternative osmolyte must therefore fill in for the shortage of glycerol.

6. CONCLUSIONS

In spite of the large amount of published information on the hyperhaline species of *Dunaliella* (e.g. Borowitzka and Borowitzka, 1988; Avron and Ben-Amotz, 1992), substantial gaps exist in the understanding of the biochemical and molecular mechanisms governing the ecophysiology of this organism. For instance, more studies are required before a satisfactory comprehension of the kinetics of nutrient uptake and of the strategies for the allocation of resources is attained. This information would help to decipher the ecological role of *Dunaliella* in hyperhaline ecosystems, especially in terms of its contribution to trophic chains and water quality. In particular, salt production in saltworks would greatly benefit from a more thorough understanding of the factors that control *Dunaliella* growth and modulate the release of organic matter from its cells. Due to the biotechnological relevance of this organism, it is possible that important information has not been made available to the scientific community for commercial reasons.

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