1 Algal glycerol accumulation and release as a sink for photosynthetic
2 electron transport

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ABSTRACT

Responses of freshwater and marine species of *Chlamydomonas* to salinity and light intensity were compared in the context of potential biomaterial and bioenergy production. While the freshwater species *C. reinhardtii* arrested growth at a salinity of 0.2 M NaCl, the marine species *C. euryale* showed vigorous growth and high levels of internal glycerol accumulation between 0.2 and 2 M NaCl. The freshwater species exhibited no downregulation of photosynthesis between 0 and 0.2 M NaCl; the marine species exhibited significant upregulation of photosynthesis between 0.2 and 2.0 M NaCl, indicating that internal glycerol, unlike sugars, does not induce photosynthetic downregulation. The freshwater species released considerably more glycerol than the marine species at the common salinity of 0.2 M NaCl, suggesting either possible limitations in the freshwater species to internal retention of glycerol as an osmoregulant, or formation and active release of high glycerol levels as a sink for photosynthetic electron transport when growth is not available as a sink. Continuous release of glycerol into the medium, or internal glycerol accumulation followed by periodic glycerol release via hypo-osmotic shock, are attractive potential options for continuous production of biomaterials and bioenergy from renewable sources in algae with high rates of solar energy conversion to photosynthetically produced energy carriers.

Keywords: *Chlamydomonas*, glycerol, osmoregulation, photosynthetic electron transport, salinity, source-sink regulation

Declaration of Author Contributions

BDA and WWA obtained funding for the work, conceived and designed the experiments, interpreted the results, and drafted the article with assistance from TAB. TAB carried out the experiments, collected and subjected the data to statistical analysis, and rendered the figures. JJS provided direction for, and participated in, the chlorophyll fluorescence measurements and provided comments on the manuscript. ELS assisted with some of the experiments.

1. Introduction

The overall efficiency of solar-energy conversion into energy-rich biomass by living organisms is relatively low[1,2], meaning that plant or algal biomass contains only a small fraction of the solar energy collected by the photosynthetic organism over its lifetime, because the vast majority of harvested energy is expended to fuel the myriad metabolic reactions the organism carries out during growth, cell division, and reproduction. In contrast, photosynthesizing organisms' efficiency of solar-energy conversion into the initial energy-rich chemical products of photosynthesis can be quite high[3-5]. Consequently, withdrawing energy carriers before the energy is utilized for the organisms' own growth and development is a promising approach for increasing overall efficiency and productivity of energy-carrier production by photosynthetic organisms.

Examples of such an approach can be found in nature in the symbioses between photosynthetic microbes and their non-photosynthetic host organisms. Such photosynthetic symbionts expend little energy for cell division and growth, instead releasing a large fraction of the energy-rich products of photosynthesis to the host in exchange for CO₂ and nutrients [6-11]. A

major energy carrier released by photosynthetic symbionts for utilization by their host is glycerol produced directly from photosynthesis; glycerol is, in turn, readily used as the main energy source by a multitude of non-photosynthetic organisms [12-14]. Attractive aspects of copying this process in a nature-inspired approach for producing biomaterials and bioenergy include (i) elimination of the need to harvest, extract, and regrow algae by virtue of simply harvesting energy carriers released by the algae to the surrounding medium and (ii) the prospect of driving up photosynthetic rates by circumventing feedback inhibition acting on photosynthesis.

A photosynthetic organism's maximal capacity for photosynthesis is controlled in a feedback loop by the organism's rate of utilization of energy carriers produced by photosynthesis [15,16]. When production exceeds utilization, resulting sugar accumulation in leaves or algae induces repression of multiple photosynthetic genes, which lowers (downregulates) photosynthetic capacity [17]. Conversely, efficient sugar removal from the site of photosynthesis leads to upregulation of photosynthetic genes and an increased maximal capacity for photosynthesis (for plants, see [15,16]; for algae, see [18]). This phenomenon is known as source-sink regulation, with photosynthesis representing the energy source and all processes that withdraw, store, and/or consume sugars produced in photosynthesis acting as sinks for photosynthate [19].

The present study focuses on green algae of the genus *Chlamydomonas* and their production of glycerol, which is strongly stimulated by salinity in the external medium [20-23]. Internal glycerol accumulation within the alga functions in osmotic adjustment, which lowers internal osmotic potential and thereby prevents excessive water loss from the alga to the saline external medium [24]. We test the hypothesis that formation and/or accumulation of glycerol, unlike that of sugars, is not associated with photosynthetic repression and may instead

act as a major sink for electrons from photosynthetic electron transport. This postulate is based on the notion that conversion of photosynthetically produced triose dihydroxyacetone phosphate to glycerol (via glycerol-phosphate dehydrogenase) produces an osmoregulant and energy carrier (glycerol) that, unlike glucose, is presumably not perceived as part of the pool of photosynthetic products (sugars) by the signaling network that exerts feedback on photosynthesis[25]. We predict that maximal capacity of photosynthetic electron transport can thus be positively correlated with internal glycerol accumulation and/or release from the algae into the external medium.

In addition, we compare and contrast the responses of a freshwater versus a marine species and show that the proportion of internally accumulated glycerol to released glycerol is higher in the marine compared to the freshwater species, while the freshwater species loses more glycerol to the external medium than the marine species at a common salinity. We place these results into the context of approaches to continuously siphon off released glycerol and of non-destructive removal of internally accumulated glycerol.

2. Materials and methods

2.1. Species

The freshwater species *Chlamydomonas reinhardtii* (UTEX #2244) and the marine species *Chlamydomonas euryale* (UTEX #2274) were obtained from the Culture Collection of Algae at the University of Texas, Austin (<https://utex.org>). Culture maintenance and pre-culturing in Roux bottles was performed as described in [23], except that the marine species was maintained in flasks and pre-cultured in Roux bottles at 0.5 M NaCl.

2.2. Experimental conditions and sampling protocol

After pre-culturing in Roux bottles, 30-mL culture volumes were standardized to an Optical Density at 750 nm (OD_{750}) of 2.25 and transferred to 50-mL test tubes. Test tubes and growing conditions were as described in [23], with a continuous stream of 5% CO_2 (in ambient air) delivered to cultures and an incident light intensity at the surface of the test tubes of either 200 or 500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Experimental media for *C. reinhardtii* contained 0.0, 0.1, or 0.2 M NaCl, while media for *C. euryale* contained 0.2, 0.5, or 2.0 M NaCl. Cultures were grown in test tubes for approximately 1.5 days for acclimation to the experimental conditions, and subsequently diluted (by visual assessment) to a similar greenness as initial cultures prior to the 1.5 days of growth. Thirty mL of these diluted cultures were transferred to sterile 50-mL centrifuge bottles, gently centrifuged (2 mins, 706 g), and pellets resuspended in 30 mL of fresh medium and returned to test tubes and experimental conditions for the duration of the subsequent 24-h experiment. Samples for OD_{750} , cell counts, chlorophyll, and intracellular and released glycerol were collected directly before and 24 hours after experimental exposure of diluted cultures in test tubes. Samples for determination of rates of CO_2 - and light-saturated photosynthetic oxygen evolution and respiration were collected after 24 hours. Samples for the quantification of intracellular glycerol levels were prepared from algal pellets using centrifugation, rinsing, and boiling protocols described by [18], but with a lower centrifugation g-force (10 mins, 1476 g) to avoid rupturing the cells. Supernatants were frozen for subsequent analysis of glycerol released into the medium. Samples were processed and analyzed as described in [23] for OD_{750} , cell count, chlorophyll, and rates of photosynthetic oxygen evolution and respiratory oxygen uptake.

2.3. *In-situ chlorophyll fluorescence*

In-situ chlorophyll fluorescence measurements were obtained with a portable PAM-2000 fluorometer (Walz, Effeltrich, Germany) by appressing the end of the fiber optic cable against the test tubes perpendicular to the incident growth light, and fluorescence yields were recorded on a chart recorder (Kipp & Zonen B.V., Delft, The Netherlands). Effective energy conversion efficiency of photosystem II under the incident light intensity ($[F_m' - F]/F_m'$, where F_m' is the maximal fluorescence yield during illumination and F is the steady-state yield of fluorescence under the incident light) was calculated as described in [3]. Measurements were taken 2.5 hours (200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ only) and 24 hours (for both 200 and 500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) after the start of the 24-h experimental period with diluted cultures in fresh medium.

2.4. *Glycerol analysis*

Glycerol concentration was determined colorimetrically using an EnzyChrom Glycerol Assay Kit (BioAssay Systems, Hayward, CA, USA). For assessment of glycerol in the external medium, 100 μL of supernatant was diluted 1:10 with 900 μL of deionized water in 2 mL-microcentrifuge tubes, thoroughly mixed, and 4 μL of each sample were placed in wells on a flat-bottom 96-well plate, with colorimetric readings obtained using a Tecan Safire² plate reader (Männedorf, Switzerland). For the determination of intracellular glycerol levels, samples were diluted 1:20 to account for the higher glycerol concentrations.

2.5. *Glycerol addition to the culture medium*

Cultures with glycerol added to the experimental medium were prepared as above, but after the cultures were diluted and gently centrifuged, they were resuspended in fresh sterile

medium containing 25 mM glycerol. Twenty-five mM glycerol was chosen to provide slightly more glycerol in the surrounding medium than would be accumulated after several days of glycerol release by a dense culture (data not shown), but was also a low enough concentration as to not significantly change the osmotic potential of the culture medium compared to cultures without glycerol added.

2.6. Statistical analyses

Data were subjected to ANOVAs coupled with Tukey-Kramer tests or non-paired, two-tailed *t*-tests in order to determine differences among mean values using JMP software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Response of the freshwater species *Chlamydomonas reinhardtii* to external salinity

The freshwater species *C. reinhardtii* was grown over a range of external salinities, from optimal growth conditions in the absence of NaCl to near-complete inhibition of algal growth. Algal growth rate (rate of cell divisions per day) was strongly inhibited by 0.2 M NaCl, which corresponds to approximately 40% of seawater salinity (Fig. 1A). Chlorophyll content per cell increased strongly with increasing salinity (Fig. 1B), and the efficiency of the conversion of light absorbed in photosystem II into photochemistry (measured via chlorophyll fluorescence as $[F_m' - F]/F_m'$) decreased with increasing salinity (Fig. 1C).

Both the maximal capacity of photosynthesis (light- and CO₂-saturated rates of linear electron transport as net oxygen evolution) and cellular respiration rate decreased with increasing salinity when expressed relative to the (increasing) algal chlorophyll content (Fig. 2A,C), but

was not significantly different under the different external salinity concentrations when expressed per cell (Fig. 2B,D). Intracellular glycerol content and the level of glycerol released into the external medium (expressed relative to chlorophyll –comparison on a cell basis is shown in Section 3.3) were both negligible in the absence of NaCl and increased with increasing salinity (Fig. 3A,B).

3.2. Response of the marine species *Chlamydomonas euryaleto* external salinity

Growth over a wide range of external salinities, from 0.2 to 2 M NaCl, lowered algal growth rates, but not severely so. Mean growth rates were the same in algae grown in the presence of 0.2 and 0.5 M NaCl, and were about half that in algae grown in the presence of 2 M NaCl, which is about 4 times the salinity of seawater (Fig. 4A). Chlorophyll content per cell was similar in algae grown in the presence of 0.2 and 0.5 M NaCl, and slightly greater in algae grown in the presence of 2 M NaCl (Fig. 4B). PSII energy conversion efficiency, $(F_m' - F)/F_m'$, was unaffected by salinity over the range from 0.2 to 2.0 M NaCl (Fig. 4C). Maximal photosynthetic capacity, as light- and CO₂-saturated rate of linear electron transport from net oxygen evolution, and, to a lesser extent, rates of cellular respiration both increased in the marine species with increasing salinity (Fig. 5). This increase was somewhat more pronounced when rates were expressed relative to algal cell number (Fig. 5B) than when expressed on a chlorophyll basis (Fig. 5A) for photosynthesis rate. For cellular respiration rate, only expression on a per cell basis resulted in a significantly greater rate in algae grown with 2.0 M versus 0.2 NaCl (Fig. 5C,D).

When expressed relative to chlorophyll, both intracellular glycerol accumulation (Fig. 6A) and glycerol release rates (Fig. 6B) increased with increasing salinity in the marine species (see the next section for expression on a cell basis), with very high levels of intracellular glycerol

in algae grown in the presence of 2 M NaCl, especially in comparison with either the marine (Fig. 6A) or the freshwater species (Fig. 3A) grown in the presence of 0.2 M NaCl.

3.3. Comparison of responses in the freshwater versus the marine species

While the freshwater species exhibited almost complete growth inhibition at an external salinity of 0.2 M NaCl, which corresponds to less than half of seawater salinity (Fig. 1A), the marine species maintained similar growth rates between 0.2 and 0.5 M NaCl and exhibited a growth-rate reduction of approximately 50% under four times the salinity of seawater (2.0 M NaCl; Fig. 4A). While the freshwater species responded to increased external salinity with strong increases in chlorophyll content (Fig. 1B) and strong decreases in PSII photochemical efficiency (Fig. 1C), the marine species exhibited only minor increases in chlorophyll content and no decreases in PSII efficiency with increasing salinity (Fig. 4B,C). Figure 7 shows that the freshwater and the marine species internally accumulated similar levels of glycerol when grown in the presence of a common external salinity of 0.2 M NaCl, and that the marine species accumulated, by comparison, very high levels of internal glycerol under 2.0 M NaCl (Fig. 7A). Conversely, the freshwater species released considerably more glycerol into the external medium than the marine species when grown in the presence of a common salinity level of 0.2 M NaCl (Fig. 7B). The ratio of internally accumulated glycerol to glycerol released into the external medium was consequently dramatically greater in the marine versus the freshwater species under all salinities tested after algal growth under the respective conditions for 24 hours (Fig. 7C).

Compared to the light intensity of $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ used for the growth experiments described in Figures 1-7, increasing light intensity to $500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ had little effect on algal growth rate (cell division rate) in either species (Fig. 8A). The higher light

intensity did, however, result in significantly greater intracellular glycerol accumulation in the freshwater species, but had no significant effect on the marine species under the common salinity of 0.2 M NaCl (Fig. 8B). The higher versus the lower light intensity, furthermore, resulted in greater glycerol release from the freshwater species grown with 0.2 M NaCl, as well as from the marine species grown with 0.5 M and 2.0 M NaCl (Fig. 8C). Lastly, the higher light intensity led to a further lowering of PSII energy conversion efficiency, $(F_m' - F)/F_m'$, in the freshwater species grown either without NaCl or with 0.1 M NaCl (where glycerol release rates were negligible or relatively low, respectively) but not in the presence of 0.2 M NaCl, where glycerol release rates were high (Fig. 8C,D). In the marine species, the higher light intensity led to a significant lowering of PSII energy conversion efficiency under all salinities tested (Fig. 8D).

3.3.4. Effect of experimental glycerol addition to the external medium

Addition of glycerol to the external medium did not affect chlorophyll content (Fig. 9A,B) or growth rate (Fig. 9C,D) in *C. reinhardtii* grown either without (Fig. 9A,C) or with 0.2 M NaCl (Fig. 9B,D) in the external medium.

4. Discussion

4.1. Differential proportions of glycerol accumulation versus glycerol leakage/release from cells in a freshwater versus a marine *Chlamydomonas* species

The freshwater *Chlamydomonas* species, *C. reinhardtii*, released twice as much glycerol to the external environment compared to the marine species, *C. euryale*, when both were grown under a common salinity of 0.2 M NaCl. The freshwater species is thus clearly not using all of the glycerol produced as an internal osmolyte; this species discontinued growth at 0.2 M NaCl,

248 which is less than half of seawater salinity. Similar conclusions were drawn by León and Gálvan
249 [20] and Husic and Tolbert [27]. In contrast, the marine species accumulated high internal levels
250 of glycerol, released comparatively low amounts of glycerol, and maintained comparatively high
251 growth rates even at the high salinity of 2 M NaCl. The effect of growth light intensity provides
252 further insight into these differences in the response of the freshwater and marine species to
253 salinity. The finding that glycerol release and internal glycerol accumulation in 0.2 M NaCl were
254 increased by growth at a higher versus lower light intensity in the freshwater species can be
255 viewed in two ways. On one hand, these findings could suggest that the freshwater species has a
256 limited ability to retain glycerol and thus (i) diverts energy from growth to continued glycerol
257 synthesis in the presence of 0.2 M NaCl and (ii) is unable to accumulate sufficient glycerol as an
258 osmoregulant to allow acclimation to salinities above 0.2 M NaCl. Such a limitation in internal
259 glycerol accumulation could be exacerbated by low rates of electron transport and glycerol
260 synthesis under the lower light intensity. Alternatively, growth impairment in the freshwater
261 species may be due primarily to other salinity-related limitations, and glycerol production and
262 release may have a significantly greater role in providing an actively regulated, photoprotective
263 sink for excitation energy and electron transport as an alternative to sinks provided by growth
264 processes. This scenario offers an explanation for the observations that (i) PSII efficiency is
265 further decreased under 500 versus 200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ only in the absence of NaCl (no
266 glycerol produced as a sink) but not in the presence of 0.2 M NaCl (ample glycerol produced as a
267 sink) in the freshwater species, and that (ii) the freshwater species actually produced, and
268 released, greater levels of glycerol than the marine species under the common salinity of 0.2 M
269 NaCl. At 0.2 M NaCl, these severely growth-impaired freshwater species would presumably require
270 higher glycerol production and release rates as a sink compared to the marine species with its

unaffected growth rate. In the latter scenario, higher versus lower light levels represent greater levels of excitation energy, which is consumed by glycerol production and release as a sink.

Other species of marine green algae exhibited similar responses as *C. euryale* to high levels of external salinity. For example, when grown in the presence of 2 M NaCl, the marine species *Dunaliella tertiolecta* [18] exhibited similar levels of both internal glycerol accumulation and glycerol release into the external medium as *C. euryale* grown with 2 M NaCl. Recalculation of Chow et al.'s results (Figures 1, 5, and 6 at 20 days) to express data on the same reference bases as used in the present paper yielded very similar levels of internal glycerol accumulation of an estimated 283 nmol glycerol per million cells for *D. tertiolecta* compared to 300 nmol glycerol per million cells for *C. euryale*, and comparable rates of glycerol release into the external medium of an estimated 54 nmol glycerol per million cells per day for *D. tertiolecta* versus 150 nmol glycerol per million cells per day for *C. euryale*.

It is currently unknown what mechanisms may regulate internal glycerol accumulation versus glycerol release into the external medium in green algae. However, a rather complete understanding is available for glycerol production as well as the regulation of internal glycerol concentration and glycerol release in salt-tolerant yeast that also accumulate glycerol as an internal osmoregulant in response to saline conditions [26]. In fact, glycerol pores (members of the trans-membrane aquaglyceroporin family) serve to drain excess glycerol to the external medium in yeast to adjust internal glycerol concentration as required to provide adequate osmoregulation, especially during transfer (hypo-osmotic shock) from high to low salinity in the external medium [26]. Members of the aquaporin/aquaglyceroporin family are also present in algae [see, e.g., 28,29], including *C. reinhardtii* [30]. Future research should address the possibility that such pores, serving as overflow valves, may function in photosynthetic algae not

only to facilitate glycerol release under hypo-osmotic shock but also to prevent a rise of internal glycerol concentrations beyond the appropriate level for osmoregulation at a given salinity under continuous light absorption (Fig. 10).

We reported previously that increasing salinity and growth light intensity were both positively correlated with glycerol release in both *C. reinhardtii* and *C. euryale*[23]. Further statistical analysis (two-way ANOVA; $p < 0.05$) of these previously published data reveals that there is also a significant synergistic interaction of salinity and light intensity on the levels of glycerol release in both species. The levels of glycerol release seen in either species may thus aid in the maintenance of appropriate internal glycerol pools and the release of glycerol produced above that level as a photosynthetic sink. As stated above, the high levels of glycerol release from the freshwater species seen even under modest external salinities may thus indicate a regulated release of glycerol formed as a major alternative sink for electrons in the freshwater algae with their severely curtailed growth rates. However, at this time, we cannot exclude the possibility of interspecies differences in the control of the opening/closing state of glycerol pores.

4.2. Differential response of photosynthetic electron transport to salinity in a freshwater versus a marine species

The higher light- and CO_2 -saturated photosynthesis rate (as rate of linear electron transport), and associated greater internal glycerol accumulation, in the marine species under higher salinity indicates that glycerol accumulation is not causing photosynthetic repression – unlike what would be expected from sugar accumulation (Fig. 11). Increasing glycerol synthesis over a wide range of increasing salinities in the marine species could represent a mechanism to facilitate the continued utilization of absorbed excitation energy, thereby preventing a backup of

electrons in the photosynthetic electron transport chain even when algal growth decreases or ceases. Absence of feedback inhibition of photosynthesis by glycerol accumulation would, in fact, make the putative regulated glycerol release pores a particularly attractive feature for prevention of excessively high internal glycerol concentrations under conditions of high salinity combined with high growth light intensities.

It is curious that the freshwater species lowers its photosystemII photochemical efficiency (the efficiency with which absorbed light is converted to photochemistry) in response to increasing salinity while at the same time increasing overall chlorophyll content and thus increasing apparent overall light absorption. In contrast to the situation in the marine species, the maximal rate of linear electron transport in the freshwater species did not increase with salinity, which makes it attractive to speculate that the combination of increased chlorophyll content with decreased photosystemII energy conversion efficiency may be due to an increase in cyclic electron flow around photosystem I in the freshwater species, with the increased chlorophyll level delivering excitation energy primarily to photosystemI. Unlike linear electron flow, cyclic flow is not detectable via the measurement of photosynthetic oxygen evolution presented here (and would also not be detectable as CO₂ uptake), but does increase the ratio of ATP to NADPH formed [31]. Increased cyclic electron flow around photosystemI in response to sudden salt stress was demonstrated in cyanobacteria, algae, and plants [31; see also 32,33]. Increased levels of cyclic electron flow would be a way to produce ATP under conditions where linear electron transport may be limited by electron acceptor availability in the freshwater species. Erickson et al. [34] reviewed recent progress in the study of cyclic electron flow in *C.reinhardtii* and pointed out that increased cyclic electron flow also increases the rate of photoprotective thermal energy dissipation typically assessed as non-photochemical quenching of chlorophyll fluorescence,

which, since increased energy dissipation lowers PSII efficiency [4], is consistent with our observation of lowered PSII photochemical efficiency in *C. reinhardtii* under elevated salinity.

Both the higher photosynthesis rate and greater internal glycerol accumulation in the marine species and the response of photosynthesis in the freshwater species under elevated salinity indicate that glycerol is not causing photosynthetic repression. The response of photosynthetic capacity in the freshwater species needs to be evaluated in the context of reference basis. Lower rates of photosynthetic capacity relative to the increased levels of chlorophyll, seen in the context of unchanged rates of photosynthetic capacity per cell, argue against any photosynthetic downregulation in response to elevated salinity despite the dramatic decline in algal growth rates, and are consistent with glycerol production acting as an alternative sink for electrons. These conclusions are consistent with the results of Chow et al. [18] for *D. tertiolecta* and are also consistent with the absence of an effect of experimental glycerol addition to the external medium on algal growth rate or chlorophyll content; either glycerol is not taken up from the medium into the algal cell, or if it is taken up, it exhibits no discernible internal effect. These findings give additional meaning to the term compatible solute – as a solute that does not interact with intracellular signaling networks modulating photosynthesis rate in response to the demand for photosynthate, in contrast to the downregulation of photosynthesis in response to sugar accumulation (Fig. 11). In addition to avoiding photosynthetic downregulation by build-up of reduced-carbon products of photosynthesis, utilization of electrons from photosynthetic electron transport for glycerol production presumably also prevents a build-up of excess energy in the light-harvesting antennae and the electron transport chain. This would allow glycerol production to act as an overflow valve for excitation energy and to increase algal tolerance of high light intensities.

363
364 4.3. Implications of the differential responses between a freshwater and a marine species in the
365 contexts of ecology and industrial application

366 The findings presented here support the view that osmoregulation via internal glycerol
367 accumulation under high salinities is achieved more readily by marine compared to freshwater
368 species of *Chlamydomonas*. Concerning glycerol release, exchange of energy carriers and
369 nutrients between photosynthetic and non-photosynthetic organisms is common in close
370 symbioses found in marine environments[6-11]. However, the greater glycerol release rates of
371 the freshwater species in response to minor increases in salinity level reported here suggest that
372 freshwater species could also be adept at exchanging energy carriers and nutrients with other
373 members of microbial communities.

374 Both internal accumulation and release of glycerol into the external medium offer
375 avenues for the use of algae for production of energy carriers or materials in an industrial
376 context. Unlike starch or lipid accumulation, internal accumulation of glycerol apparently avoids
377 photosynthetic downregulation in glycerol-producing *Chlamydomonas* (Fig. 11). Glycerol could
378 be harvested either via continuous release (the prime candidate of choice for this approach would
379 be the freshwater *Chlamydomonas* species, due to its high glycerol release rates) and, in addition,
380 via periodic draining of glycerol from cells (candidates for this additional approach would be the
381 marine *Chlamydomonas* species or algae from the genus *Dunaliella*). It has been demonstrated
382 that hypo-osmotic shock treatment can quickly and non-destructively drain large amounts of
383 glycerol from microbial cells [35,36].

384 Being able to harvest energy carriers released from the algae circumvents energy- and
385 resource-intensive harvesting, digestion, and regrowth of algae necessary in many other

approaches to the generation of feedstocks for biofuels and biomaterials. Glycerol can serve as the feedstock for a host of microbes that synthesize multiple products in industrial applications [37-43]. Future research is needed into the sustainability of glycerol production and release by *Chlamydomonas* species over time and its commercial prospects.

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Figure legends

Fig. 1. Effect of external salinity under the growth light intensity of $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ on (A) algal growth rate (cell division rate), (B) chlorophyll content per cell, and (C) the actual efficiency of the conversion of light absorbed in photosystem II into photochemistry, measured as $(F_m' - F)/F_m'$, in the freshwater species *C. reinhardtii*. Mean values $\pm$ standard deviation ($n = 3$) shown, with different lower-case letters indicating significant differences ($p < 0.05$).

Fig. 2. Effect of external salinity under the growth light intensity of $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ on (A,B) light- and CO_2 -saturated rate of linear electron transport (photosynthetic oxygen evolution) and (C,D) cellular respiration (oxygen uptake) in the freshwater species *C. reinhardtii*. Rates of photosynthesis and respiration are expressed on both a chlorophyll basis (A,C) and per cell

(B,D). Mean values $\pm$ standard deviation ($n = 3$) shown, with different lower-case letters indicating significant differences ($p < 0.05$).

Fig. 3. Effect of external salinity under the growth light intensity of $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ on (A) intracellular glycerol accumulation and (B) the rate of glycerol release into the external medium in the freshwater species *C. reinhardtii*. Both parameters are expressed on a chlorophyll basis here; for the same parameters expressed per cell, see Figure 7. Mean values $\pm$ standard deviation ($n = 3$) shown, with different lower-case letters indicating significant differences ($p < 0.05$).

Fig. 4. Effect of external salinity under the growth light intensity of $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ on (A) algal growth rate (cell division rate), (B) chlorophyll content per cell, and (C) the actual efficiency of the conversion of light absorbed in photosystem II into photochemistry, measured as $(F_m' - F)/F_m'$, in the marine species *C. euryale*. Mean values $\pm$ standard deviation ($n = 3$) shown, with different lower-case letters indicating significant differences ($p < 0.05$).

Fig. 5. Effect of external salinity under the growth light intensity of $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ on (A,B) light- and CO_2 -saturated rate of linear electron transport (photosynthetic oxygen evolution) and (C,D) cellular respiration (oxygen uptake) in the marine species *C. euryale*. Rates of photosynthesis and respiration are expressed on both a chlorophyll basis (A,C) and per cell (B,D). Mean values $\pm$ standard deviation ($n = 3$) shown, with different lower-case letters indicating significant differences ($p < 0.05$).

Fig. 6. Effect of external salinity under the growth light intensity of $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ on (A) intracellular glycerol accumulation and (B) the rate of glycerol release into the external medium in the marine species *C. euryale*. Both parameters are expressed on a chlorophyll basis here; for the same parameters expressed per cell, see Figure 7. Mean values $\pm$ standard deviation ($n = 3$) shown, with different lower-case letters indicating significant differences ($p < 0.05$).

Fig. 7. Comparison of the effect of external salinity under the growth light intensity of $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ on (A) intracellular glycerol accumulation per cell, (B) the rate of glycerol release into the external medium per cell, and (C) the ratio of intracellular to released glycerol for the freshwater species *C. reinhardtii* (left) and the marine species *C. euryale* (right). Mean values $\pm$ standard deviation ($n = 3$) shown, with different lower-case letters indicating significant differences ($p < 0.05$).

Fig. 8. Effect of growth light intensity (metrics determined for algae grown under $500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and expressed as a percentage of the metrics determined for algae grown under $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) on (A) algal growth rate (cell division rate), (B) intracellular glycerol accumulation, (C) glycerol release rate, and (D) the efficiency of the conversion of light absorbed in photosystem II into photochemistry, measured as $(F_m' - F)/F_m'$, at various external salinity levels in the freshwater species *C. reinhardtii* (left) and the marine species *C. euryale* (right). * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ from non-paired, two-tailed *t*-tests.

Fig. 9. Effect of addition of 25 mM glycerol to the external medium (+) under the growth light intensity of $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ on algal (A,B) chlorophyll content and (C,D) growth rate in

the absence (No salt = 0.0 M NaCl) or presence (Salt = 0.2 M NaCl) of external salinity in *C. reinhardtii*. n.s. = not significantly different.

Fig. 10. Schematic depiction of internal glycerol accumulation in a photosynthetic alga, as triggered by external salinity, fueled by photosynthesis, and regulated by pores (membrane transporters) through which glycerol can be released into the external medium. Based on concepts described in Hohmann [26] for osmoregulation in yeast.

Fig. 11. Schematic depiction of the effect of glycerol accumulation and/or release on photosynthesis compared to the effect of internal sugar accumulation. (A) Algal growth consumes the products of photosynthesis; (B) glycerol accumulation and release either does not affect linear electron transport rate on a per-cell basis (freshwater species) or results in elevated rates of photosynthesis (marine species); (C) sugar accumulation leads to feedback inhibition of photosynthesis by repression of genes serving in light processing and CO₂ fixation. GAP (glyceraldehyde phosphate) and DHAP (dihydroxyacetone phosphate), the triose-phosphate products of photosynthetic carbon fixation, are the immediate precursors of either sugars (GAP and DHAP) or glycerol (only DHAP).

Figure 1

Figure 2

Figure 3

C. reinhardtii

Intracellular glycerol,
 $\text{mol mol}^{-1} \text{ Chl}$

12

A

8

4

0

c

b

a

Released glycerol,
 $\text{mol mol}^{-1} \text{ Chl day}^{-1}$

60

B

40

20

0

c

b

a

0.0

0.1

0.2

NaCl, mol L^{-1}

Figure 4

Figure 5

Figure 6

C. euryale

Figure 7

Figure 8

Values at 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$,
 % of control values at 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$

Figure 9

Figure 10

Figure 11