



Osmo-induced proline accumulation in *Chlorella vulgaris* SAG 211-11p

Simone Barera^{1,2} · Giuseppe Forlani¹

Received: 26 May 2025 / Revised: 10 July 2025 / Accepted: 11 July 2025 / Published online: 20 August 2025
© The Author(s) 2025

Abstract

Increasing evidence shows that proline accumulation may contribute to acclimation of microalgae to environmental stress such as excess salt or heavy metals. However, significant variability does exist among algal strains concerning the conditions that promote proline synthesis, as well as the intracellular levels of the amino acid that are reached and maintained under stress. Here we report on osmo-induced proline accumulation in the high biomass-yielding strain SAG 211-11p of *Chlorella vulgaris*. Algal cultures were grown mixotrophically and subjected to osmotic stress by the addition to the culture medium of increasing concentrations of either salts or non-ionic, non-permeant osmolytes. The intracellular concentration of proline was then measured in cells grown under conditions under which the growth rate had been found steadily reduced. A remarkable tolerance toward molar concentrations of NaCl was found, which was accompanied by an up to 24-fold increase of proline over basal levels. Results suggest the possibility of using marine or partially desalinated water sources for the cultivation of this strain.

Keywords Microalgae · Osmotic stress · Proline accumulation · Salt stress · *Chlorella vulgaris*

Introduction

Eukaryotic microalgae, a diverse group of photosynthetic microorganisms, have been proposed as a source for renewable fuels, valuable bio-products and nutraceuticals (Borowitzka 1995, 2013; Saide et al. 2021; Dutta et al. 2025). They possess the potential to address various global demands, particularly in the context of sustainable development and environmental conservation (Brennan and Owende 2010; Mata et al. 2010). Despite their inherent adaptability to environmental conditions, the cultivation of microalgae in outdoor photo-bioreactors is limited to a few robust species (Muller-Feuga 2003). Most microalgal strains are indeed highly sensitive to environmental fluctuations, such as changes in solar radiation, temperature, osmolarity, and nutrient availability. These fluctuations can significantly affect biomass yield (Beardall and

Raven 2013). Moreover, in recent years climate change and human activities are leading to a global salinization of freshwater resources. This has prompted the search for halotolerant microalgal strains, given the substantial freshwater demands of large-scale microalgal cultivation. Freshwater microalgal strains are particularly vulnerable to high salinity, which perturbs ionic homeostasis and induces the generation of reactive oxygen species (ROS) disrupting photosynthesis (Fal et al. 2022). To survive hyperosmotic stress microalgae employ various physiological, metabolic and molecular responses, including the accumulation of carbohydrates and lipids as storage molecules (Hagemann 2016; Wang et al. 2018; Anand et al. 2019). Some microalgae, like brown algae, utilize mannitol and sorbitol for osmotic acclimation (Wegmann 1986). Halotolerant microalgal species, such as *Chlamydomonas* spp., *Scenedesmus* spp. and *Chlorella* spp., have been isolated that are capable of thriving in saline environments. In most cases, proline accumulation seems to play a crucial role in their response to excess salt, acting as a compatible osmolyte to recover positive cell turgor and potentially participating in ROS scavenging (da Silva et al. 2009; Mastrobuoni et al. 2012; Singh et al. 2019). In desiccation-prone environments, microalgae like *Pseudostichococcus* and *Deuterostichococcus* exhibit resilience, possibly due to a higher proline

✉ Giuseppe Forlani
flg@unife.it

¹ Department of Life Science and Biotechnology, University of Ferrara, Via L. Borsari 46, 44121 Ferrara, Italy

² Present Address: Department of Biology and Biotechnology “Lazzaro Spallanzani”, University of Pavia, Via A. Ferrata 9, 27100 Pavia, Italy

content, suggesting a role for proline also in desiccation resistance (Van and Glaser 2022).

Accumulation of various compatible osmolytes and protectant molecules has been widely reported in *Chlorella* spp. grown under salt stress (Wang et al. 2018), as well as the production of ROS-detoxifying enzymes such as superoxide dismutase, ascorbate peroxidase, catalase, glutathione reductase and peroxidase (Ismail et al. 2018). An increase in the intracellular level of free proline represents one of the earlier responses to a wide range of abiotic stressors in both algae (Krell et al. 2007; Barera and Forlani 2023) and higher plants (Trovato et al. 2019). Its possible roles in stress tolerance include stabilization of proteins and membranes, protection of cellular structures, ROS scavenging, and balancing of cellular redox status (Li et al. 2018; Forlani et al. 2019). Consistently, increased intracellular levels of proline have been reported in most studies in which *Chlorella* spp. have been grown under hyperosmotic stress. However, marked variability is evident in the scientific literature regarding the threshold over which proline accumulation takes place, as well as the intracellular concentration of the amino acid that is reached and maintained under a given condition. *Chlorella vulgaris* was reported to accumulate free proline under salinity stress up to 1.6-fold the basal level, whereas no changes were found in *Chlorella salina* (Farghl et al. 2015). *Chlorella autotrophica* accumulated proline in both nitrate- and ammonia-supplemented media, with 8- and 10.5-fold increase over basal level, respectively (Ahmad and Hellebust 1988). Another study comparing *C. vulgaris* and *Chlorococcum humicola* showed in the former a proline level that was proportional to the concentration of salt, peaking at 500 mM NaCl with a twofold increase over controls (Singh et al. 2018). When *C. vulgaris* was treated with NaCl for 30 days, an up to fivefold increase in proline content was found, with an adaptive response involving multiple solutes (Hiremath and Mathad 2010). Under excess NaCl (0–400 mM), *C. vulgaris* exhibited a decrease in growth, protein, chlorophyll, carbohydrate, and total organic carbon, whereas proline content increased up to 1.6-fold (Yadav et al. 2022). Under similar conditions (0–600 mM NaCl), *C. vulgaris* strain YH703 exhibited a 5.5-fold increase in proline compared to untreated cells (Yun et al. 2019). The treatment of *C. vulgaris* CCAP 211/11B with 150 and 300 mM NaCl did not have significant effects on algal growth rate, while resulting in a slight (< twofold) increase of proline content (Pinto et al. 2025). *Chlorella* spp. also exhibited a rapid increase in free proline when exposed to heavy metals. The strain 2350 accumulated proline up to sixfold the basal level in the presence of excess Cu^{2+} (Wu et al. 1998). Furthermore, *C. vulgaris* exposed to UV-B light showed proline

concentrations up to fourfold higher than untreated controls (Singh et al. 2019).

To address these issues and obtain a more comprehensive picture of osmo-induced proline accumulation in *C. vulgaris*, we at first determined the effect of increasing concentrations of either ionic or non-ionic osmolytes on the growth of the *C. vulgaris* SAG 211-11p strain. Proline content was then measured in algal cultures at increasing time after the exposure to hyperosmotic stress conditions causing progressive inhibition of cell growth.

Materials and methods

Strain and culture conditions

Chlorella vulgaris wild-type strain 211-11p was obtained from the SAG Culture Collection of Algae (Göttingen University, Germany). The strain was routinely maintained on TAP-agar plates (Kropat et al. 2011) and grown at 25 °C under a photoperiod of 16/8 h light/dark at 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PAR using warm-white fluorescent lamps. Growth in liquid TAP medium was performed under the same conditions in Erlenmeyer flasks of various volumes, maintaining a fixed ratio (1:4) between the volume of the medium and that of the flask, on a rotary shaker at 120 rpm, with illumination from the top.

Growth rate measurement

Cells in the late exponential phase of growth were used to inoculate 96-well plates (0.2 mL per well) to an initial density of approximately 2×10^6 cells mL^{-1} . Osmotic or ionic stress conditions were imposed by supplementing TAP medium with increasing concentrations of either salts (NaCl, KCl, Na_2SO_4 or K_2SO_4), or non-permeant non-ionic osmolytes (mannitol, sorbitol or polyethylene glycol (PEG) 6000). Every treatment was performed in quadruplicate. Cell growth in each well was monitored over a week by daily absorbance measurements using a Ledetect 96 plate reader (Labexim, Austria) equipped with a LED plug-in at 750 nm (Griffiths et al. 2011). After logarithmic data transformation, growth constants were calculated and expressed as percentage of the mean value for untreated controls. The concentrations causing 25, 50 and 75% inhibition (IC_{25} , IC_{50} , IC_{75}) and their confidence intervals were computed by non-linear regression using Prism 6 for Windows, version 6.03 (GraphPad Software, USA). For PEG 6000, osmotic potentials were calculated according to Money (1989). To evaluate the effect of amiloride on the growth of control or salt-stressed cells, the medium was added with a proper dilution of a 100 mM

solution in DMSO. Controls received the same amount of the solvent.

Total free amino acids and proline measurement

Cells in the late exponential phase of growth ($\sim 1 \cdot 10^8$ cells mL^{-1}) were pelleted by centrifugation 10 min at $3000 \times g$ and resuspended in the same volume of TAP medium supplemented or not with a given osmolyte concentration. At increasing time after the resuspension and up to 48 h, 30-mL aliquots (corresponding to ~ 0.25 g FW) were harvested from each flask and used for amino acid extractions according to Laliberté and Hellebust (1989), with minor modifications. Cells were pelleted as above and extracted with 300 μL of methanol-chloroform-water (12:5:3, v/v) for 20 min. Following centrifugation, the supernatant was transferred into a clean Eppendorf tube, and the pellet was further extracted with 300 μL of methanol-chloroform-water. The two supernatants were combined, added with 150 μL of chloroform and 150 μL of water and carefully mixed by vortexing. After centrifugation for 5 min at $12,000 \times g$, the chloroform bottom layer was discarded and the methanol in the top layer was evaporated under air flow until a residual volume of 250 μL was obtained. The resulting samples were analysed for proline and total amino acid content by the acid ninhydrin method as described (Forlani and Funck 2020). All treatments were carried out in triplication, and mean values \pm SE were reported.

Cell viability test

Cell viability was determined by the Evans Blue method, with minor modifications. Cell culture aliquots corresponding to 50 mg FW were harvested, centrifuged at $3000 \times g$ for 5 min, and washed twice with distilled water. Cells were then resuspended in 1 mL of 0.2 M K phosphate buffer, pH 6.5, and added with 20 μL of a 2.5% (w/v) aqueous solution of Evans Blue. After incubation for 15 min at room temperature with occasional stirring, cells were washed for 4–5 times with distilled water to remove unbound dye. The cells were then resuspended in 1.2 mL of decolorizing solution (50% [v/v] methanol and 1% [w/v] sodium dodecyl sulphate) for 15 min at 50°C to solubilize the bound dye. After centrifugation 3 min at $12,000 \times g$, the supernatant was diluted 1:5 with distilled water and read at 600 nm. Exponentially growing cells were used as negative controls (0% dead cells), while cultures treated for 8 min in a microwave oven at maximal power were used as positive controls (100% dead cells). Presented data are mean \pm SE over three replicates.

Results

The mixotrophic growth of *Chlorella vulgaris* SAG 211-11p is progressively affected by the presence of either ionic or non-ionic osmolytes in the medium at concentrations causing a Ψ_{medium} decrease of 1 MPa or higher, yet a remarkable tolerance to NaCl is evident

To elucidate the effect of osmotic and ionic stress conditions on the growth of *C. vulgaris*, an array of several permeant or not-permeant, ionic or non-ionic osmolytes at increasing concentrations was added to the culture medium and the resulting growth rates were measured and expressed as percent values of that for untreated controls. The treatment with Na^+ or K^+ cations, added either as chlorides or sulphates, had differential effects (Fig. 1). Even if considering that the molality of KCl solutions is slightly higher than that of equimolar solutions of NaCl, K^+ salts were significantly more inhibitory than the Na^+ counterparts. In the case of KCl, cell viability was rapidly lost over a threshold concentration of about 200 mM, while for NaCl at least twofold higher levels were required to induce similar effects. The difference was even more evident for sulphates (Fig. 1A). The corresponding concentrations causing 25, 50 or 75% reduction of the growth rate are reported in Table 1. When data were plotted as a function of cation concentration, the effect of K^+ salts was very similar, whereas those of Na^+ salt were not only lower, but different from each other, chloride being more inhibitory than sulphate (Fig. 1B). Remarkably, *C. vulgaris* cells retained a significant capability to grow in the presence of salt levels similar to those in seawater, with a growth rate of $43.5 \pm 1.3\%$ with respect to controls at a NaCl concentration of 0.625 M. If the osmotic component was considered and data expressed as a function of the resulting decrease of medium water potential (Ψ_{medium}), once again the effect of K^+ salts was significantly higher than that of Na^+ salts (Fig. 1C). Because data were suggestive of the ability of the algal strain to actively extrude Na^+ ions, the effect of amiloride, a potent inhibitor of *E. coli* Na^+/H^+ antiporter NhaB (Pinner et al. 1995), was considered. The addition of micromolar concentrations of amiloride to the medium was found to steadily inhibit algal growth, which was suppressed at 0.7 mM. The effect was completely reverted when amiloride was added to the medium containing NaCl concentrations able to inhibit growth by about 10 or 50% (250 or 500 mM, respectively; Fig. 1D), suggesting the salt-induced expression of an amiloride insensitive antiporter.

To discriminate osmotic from ion toxicity, parallel experiments were carried out using non-permeant

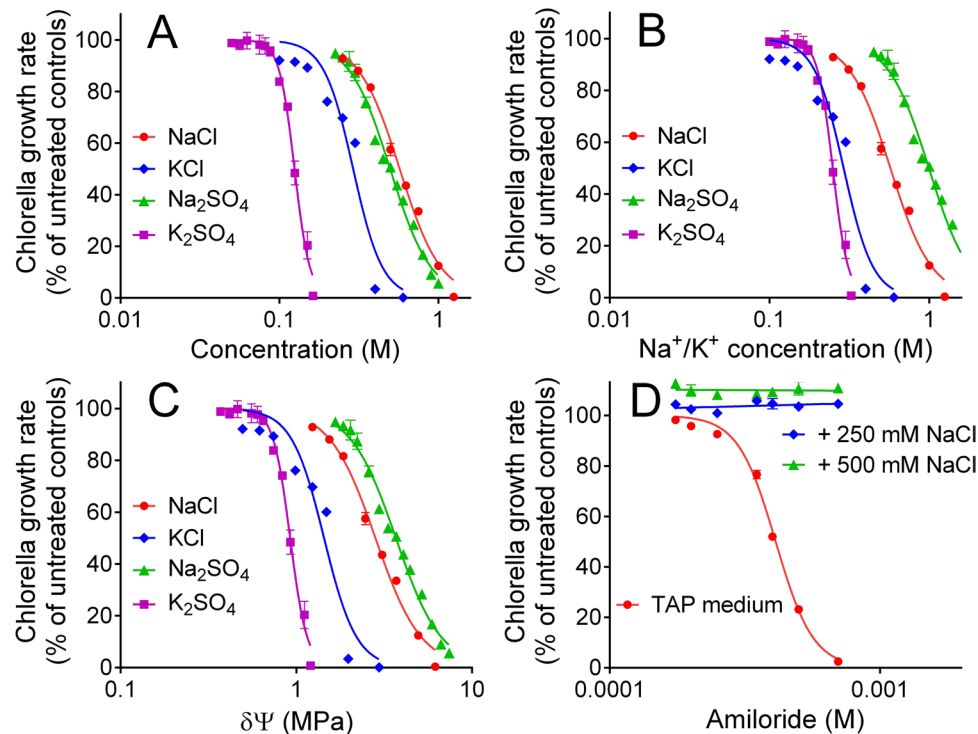


Fig. 1 Effect of salt stress conditions upon the growth of *Chlorella vulgaris* SAG 211-11p. TAP medium was supplemented with increasing concentrations of various salts, as indicated, and the resulting growth rate was calculated and expressed as percent of that in untreated controls. Data are means \pm SE over at least three replicates. To compare the effects, data were plotted on the basis of either salt molarity (A), cation concentration (B) or the consequent decrease of

water potential ($\delta\Psi$; C). When micromolar concentrations of amiloride, a potent inhibitor of *E. coli* Na^+/H^+ antiporter NhaB, were added to the medium, growth was strongly reduced. The effect was completely reverted in the presence of inhibitory levels of NaCl, suggesting salt stress-induced expression of an amiloride-resistant antiporter (D)

Table 1 Concentrations of ionic and non-ionic osmolytes able to inhibit by 25% (IC_{25}), 50% (IC_{50}) or 75% (IC_{75}) the growth of *Chlorella vulgaris* SAG 211-11p

Osmolyte	IC_{25}	IC_{50}	IC_{75}
NaCl	0.411 ± 0.016 M	0.572 ± 0.015 M	0.794 ± 0.031 M
KCl	0.231 ± 0.017 M	0.291 ± 0.016 M	0.368 ± 0.031 M
Na_2SO_4	0.360 ± 0.011 M	0.498 ± 0.010 M	0.689 ± 0.021 M
K_2SO_4	0.110 ± 0.003 M	0.125 ± 0.002 M	0.141 ± 0.004 M
mannitol	0.464 ± 0.026 M	0.785 ± 0.060 M	1.331 ± 0.193 M
sorbitol	0.494 ± 0.046 M	0.871 ± 0.061 M	1.535 ± 0.185 M
PEG 6000	$8.2 \pm 0.5\%$	$17.3 \pm 0.7\%$	$37.9 \pm 2.8\%$

osmolytes, namely mannitol, sorbitol and polyethylene glycol (PEG). With the former, results were almost overlapping, the growth rate of *C. vulgaris* being progressively reduced at concentrations exceeding 0.4 M (Fig. 2). When data were plotted as a function of the corresponding decrease of Ψ_{medium} (Fig. 2B), the two patterns were similar to those obtained for K^+ salts (Fig. 1C). Completely different results were obtained in the case of

PEG. When increasing concentrations of PEG were added to the medium, 50%-inhibition of growth was found at 17.3% (w/v) PEG 6000 (Table 1), corresponding to about 29 mM. Because osmotic pressure of aqueous PEG solutions does not increase linearly with the concentration, data were expressed as a function of the resulting decrease of Ψ_{medium} using an empirical formula based on experimental measurements (Money 1989). The resulting graph (Fig. 2B) showed that PEG causes 50%-inhibition of *C. vulgaris* growth rate at Ψ values at which sorbitol and mannitol are substantially ineffective. Furthermore, the slope of the function that interpolates the experimental data (Hill slope) was strikingly different (-2.083 , -1.937 and -0.6426 for mannitol, sorbitol and PEG 6000, respectively). To verify whether such difference may be due to toxic effect(s) of PEG that are unrelated to the resulting osmotic imbalance, the viability of *C. vulgaris* cells treated with 20% (w/v) PEG6000 was measured by means of the Evans Blue test. Results (Supplementary Fig. S1) showed that, at least up to 48 h from the treatment, the resulting loss of cell viability was marginal.

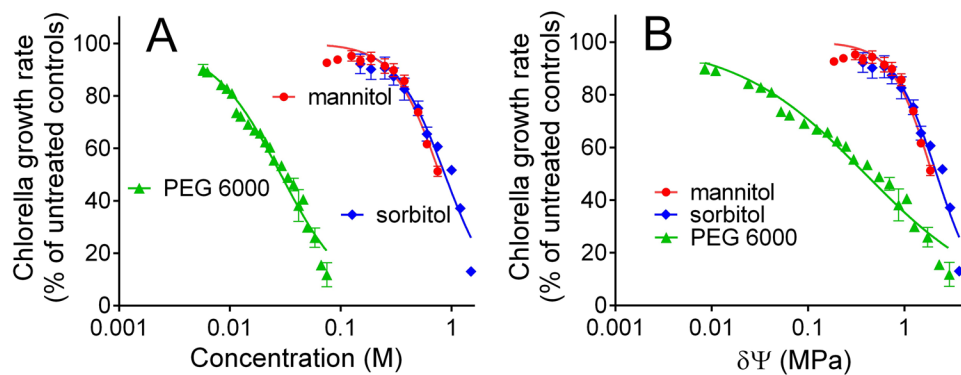


Fig. 2 Effect of osmotic stress conditions upon the growth of *Chlorella vulgaris* SAG 211-11p. TAP medium was supplemented with increasing concentrations of various non-ionic, non-permeant osmolytes, as indicated, and the resulting growth rate was calcu-

lated and expressed as percent of that in untreated controls. Data are means \pm SE over at least three replicates. To compare the effects, data were plotted on the basis of either osmolyte concentration (**A**), or the consequent decrease of water potential ($\delta\Psi$; **B**)

When exposed to decreased values of Ψ_{medium} exceeding -0.5 MPa, *Chlorella vulgaris* SAG 211-11p accumulates free proline levels that are proportional to the stress, while the overall concentration of other free amino acids shows only minor changes

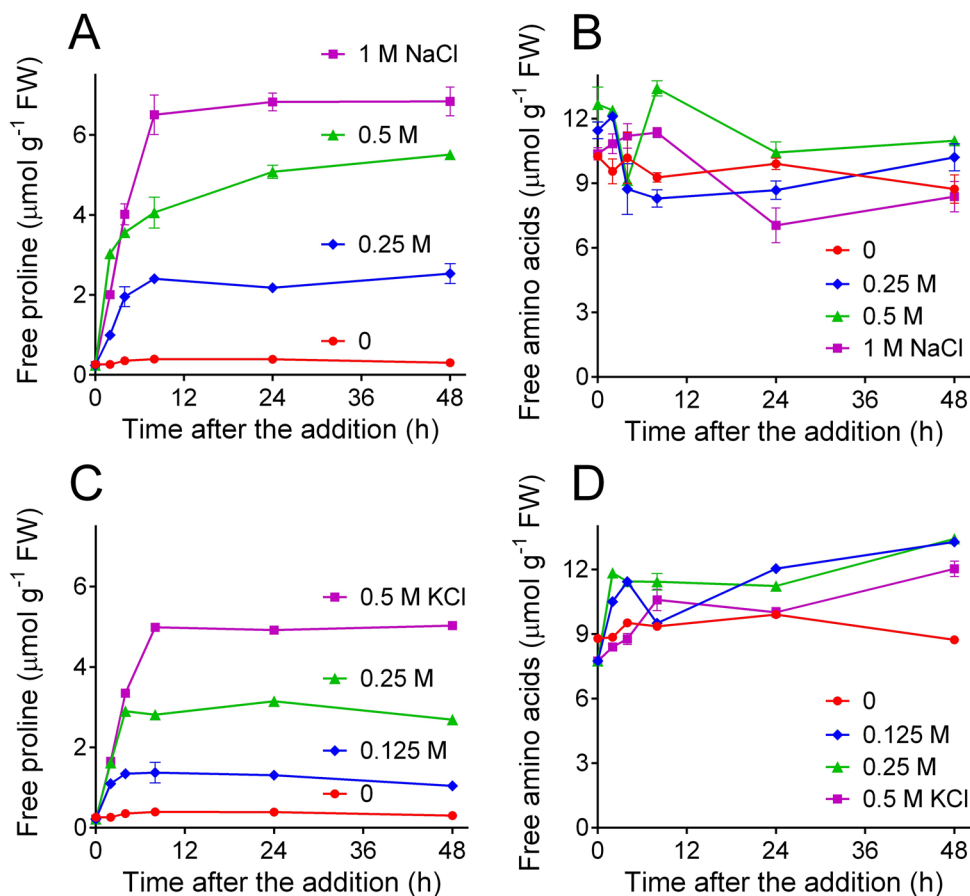
The occurrence of proline accumulation in osmotically stressed *C. vulgaris* cells was investigated by adding concentrations of either ionic or non-ionic osmolytes that had been previously found to slightly (10 to 20%), moderately (50%) or severely (80 to 90%) affect the growth rate to the culture medium. Under normo-osmotic conditions the intracellular content of free proline was found relatively stable, with levels ranging from 0.25 to 0.40 $\mu\text{mol g}^{-1}$ FW. Following salt treatments with either NaCl or KCl, a rapid increase was found that was proportional to the severity of the stress. Proline levels reached maximal values about 8 h after the treatment, and such levels remained relatively constant thereafter (Fig. 3A and C for NaCl and KCl, respectively). To rule out the possibility that such an increase might be at least in part due to cell dehydration, the overall levels of other free amino acids were also quantified in the same samples. Total free amino acid content (excluding proline) indeed showed a slight increase soon after the treatment, but the variations never exceeded 40% with respect to controls (Fig. 3B and D), and at least in the case of the most severe treatment (1 M NaCl) an opposite effect was evident. For proline, on the contrary, a consistent and striking increase was found in all cases, corresponding to 4 to 24-fold the control levels. As a consequence, the relative content of proline showed a marked increase, rising from 2–4% to almost 40% of total free amino acids.

Similar results were obtained when osmotic stress conditions were imposed by the addition of the non-permeant, non-ionic osmolyte mannitol (Fig. 4A and B).

In this case also, other free amino acid content showed only a moderate increase over control levels (up to 30%), and without a clear relationship with the severity of the stress (Fig. 4B), whereas free proline increased several fold and proportionally to the treatment intensity (Fig. 4A). From a quantitative point of view, the resulting proline levels were lower than those obtained with salt treatments. However, this was simply a consequence of milder treatments, since the concentration of mannitol theoretically causing 75% of growth inhibition (~ 1.3 M; Table 1) could not be obtained due to its limited solubility in water. Apparently inconsistent patterns were found when osmotic stress was alternatively induced with PEG 6000, added to final concentrations of 10%, 15% or 20% (w/v), which had previously found to cause about 33%, 45% and 51% inhibition of cell growth, respectively. The effect of higher doses could not be investigated because the resulting high viscosity of the medium hampered uniform cell resuspension and culture shaking. Under these conditions, a general slight decrease of free amino acid content was found early after the treatment, while thereafter the trend was inverted, resulting into a moderate increase 48 h after PEG addition (Fig. 4D). Concerning proline, the treatment did not cause its accumulation but, on the contrary, resulted in a marked decrease of its intracellular content (Fig. 4C).

To shed light on the overall relationship between the severity of the stress and the amount of stress-induced proline accumulation, the increase of its intracellular content, measured 48 h after the treatment, was plotted against the resulting lowering in the Ψ_{medium} independently of the osmolyte that had been used. The results, depicted in Fig. 5, showed a highly significant ($P < 0.001$) correlation. Free proline level started to raise at about 0.5 MPa $\delta\Psi$, and increased linearly with the extent of the osmotic imbalance at least up to 3 MPa $\delta\Psi$.

Fig. 3 Effect of salt stress conditions upon free amino acid content of *Chlorella vulgaris* SAG 211-11p. Cells in the late exponential phase of growth were pelleted and resuspended in the same volume of TAP medium supplemented or not with sublethal concentrations of either NaCl or KCl. At increasing time after the addition, cells were extracted and free proline levels were quantified by the acid ninhydrin method (A and C for NaCl and KCl, respectively). To rule out the possibility that variations may be –at least in part– due to cell dehydration, total free amino acid concentration was also measured (B and D). Data are means \pm SE over three replicates

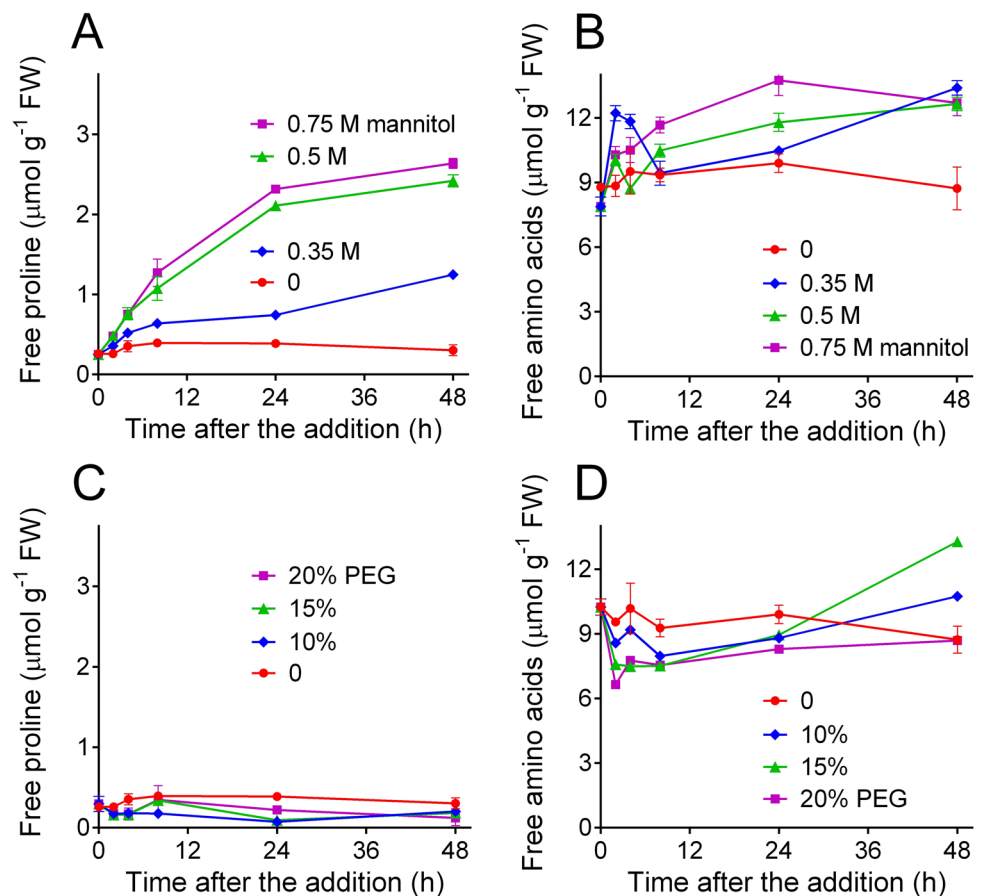


Discussion

The addition of increasing concentrations of non-permeant osmolytes causes a decrease of the medium water potential. In the absence of mechanisms for osmotic adjustment, when the Ψ_{medium} lowers below that inside the cell, water is withdrawn from the cell, resulting in growth inhibition. If salts are used instead, ionic toxicity is also exerted, because ion homeostasis between cell and the environment is altered. As a consequence, at the same $\delta\Psi$ value the inhibitory effect of salts is usually higher than that of non-ionic osmolytes, mainly in the case of Na^+ salts. The results obtained in this study seem therefore inconsistent, since in no case the effects of salts were higher than those of mannitol or sorbitol, which have been found unable to permeate the plasma membrane of *C. vulgaris* (Colman et al. 1986). In the case of K^+ salts, results were very similar to those obtained with mannitol or sorbitol (Fig. 1C and 2B). This implies that the inhibition brought about by K^+ salts depends almost exclusively on their osmotic effect. This is not unexpected, since K^+ ions are usually taken up or secreted by the cell according to the transmembrane electrochemical potential (Trombolla 1978), and do not exert toxic effects on cell structures or functions, being used for turgor provision and water homeostasis

(Maathuis 2009). On the contrary, high intracellular Na^+ concentrations usually exert negative effects, by disrupting macromolecule native conformation and interfering with several enzymatic activities (Kronzucker et al. 2013). This notwithstanding, Na^+ salts were found significantly less inhibitory than K^+ salts or non-ionic osmolytes (Fig. 1C and 2B). Such results strongly suggest the occurrence of efficient mechanism(s) for Na^+ extrusion. *C. vulgaris* has been reported to quickly take up external Na^+ ions (Barahoei et al. 2021). The initial osmotic imbalance leads to water loss and plasmolysis, providing the algal cell with sufficient time to osmoacclimate through efflux of excess Na^+ (Mountourakis et al. 2023). Being extrusion concomitant with a slight alkalisation of the medium, the involvement of Na^+/H^+ antiporters has been hypothesized (Mountourakis et al. 2023), as in plants (Bhatt et al. 2020) and in some halotolerant algal species (Katz et al. 1992; Phipps et al. 2021). To verify such possibility, the effect of amiloride, a potent inhibitor of *E. coli* (Pinner et al. 1995) and *Dunaliella salina* (Katz et al. 1994) Na^+/H^+ antiporters was investigated. Amiloride was found to strongly inhibit growth when added to *C. vulgaris* cultures in TAP medium (Fig. 1D), but the effect was completely reverted if mildly inhibitory concentrations of NaCl were added at the same time. A similar result had

Fig. 4 Effect of osmotic stress conditions upon free amino acid content of *Chlorella vulgaris* SAG 211-11p. Cells in the late exponential phase of growth were pelleted and resuspended in the same volume of TAP medium supplemented or not with sublethal concentrations of either mannitol or PEG 6000. At increasing time after the addition, cells were extracted, and free proline levels were quantified by the acid ninhydrin method (A and C for mannitol and PEG 6000, respectively). To rule out the possibility that variations may be—at least in part—due to cell dehydration, total free amino acid concentration was also measured (B and D). Data are means \pm SE over three replicates



been previously described for a psychrophilic strain of *Xanthonema* sp. (Tartari and Forlani 2008). Although further experimental evidence is required to draw a conclusion, data suggest that an amiloride-sensitive Na^+/H^+ antiporter is expressed in *C. vulgaris* under normo-osmotic conditions, whereas the exposure to salt stress induces the production of an amiloride-resistant Na^+/H^+ antiporter able to extrude excess Na^+ . Whatever the mechanism, *C. vulgaris* SAG 211-11p retained a growth rate of more than 40% of the value for untreated controls at NaCl concentrations near to those in seawater, suggesting the possibility of using marine or partially desalinated water sources for its cultivation.

An unexpected result was obtained when osmotic stress conditions were imposed by the addition of increasing levels of PEG 6000 to the growth medium. In this case growth inhibition was evident at concentrations corresponding to a much lower decrease of Ψ_{medium} . Moreover, the effect showed a different pattern, with the inhibition curve spanning over four orders of magnitude to achieve complete growth suppression (Fig. 2B). Various and somehow inconsistent results have been described concerning the effect of PEG on microalgal cells. At concentrations below 5% (w/v), PEG was found to stimulate growth and lipid accumulation in some halotolerant microalgal species (Lee and Yeh

2014). If added at higher concentrations, PEG was shown to inhibit gas exchange in the culture medium, thereby limiting *Chlorella* growth (Greenway et al. 1968). Our results seem consistent with the last finding. PEG did not compromise cell viability, since treated cells showed only a slight increase in Blue Evans binding (Supplementary Fig. S1), but mechanism(s) other than osmotic imbalance seem to affect *Chlorella* growth. Based on the reported limiting effect on gas diffusion, an inhibition of the respiratory chain and/or a stimulation of the photorespiratory rate could take place. In any case, data herein reported strongly suggest that PEG may be not suitable for osmotic studies, and that other non-permeant osmolytes should be preferred.

Among the mechanisms evolved for cell osmoacclimation, proline accumulation plays a main role and is widely distributed among plants and microorganisms (Forlani et al. 2019; Barera and Forlani 2023). In *C. vulgaris*, glycine betaine was also found to be accumulated under salt stress conditions, showing that acclimation can involve other compatible solutes. However, glycine betaine content increased only up to 0.3 M NaCl external concentration, a threshold over which the role of proline became predominant (Hiremath and Mathad 2010). When proline content was determined in *C. vulgaris* SAG 211-11p cells at increasing

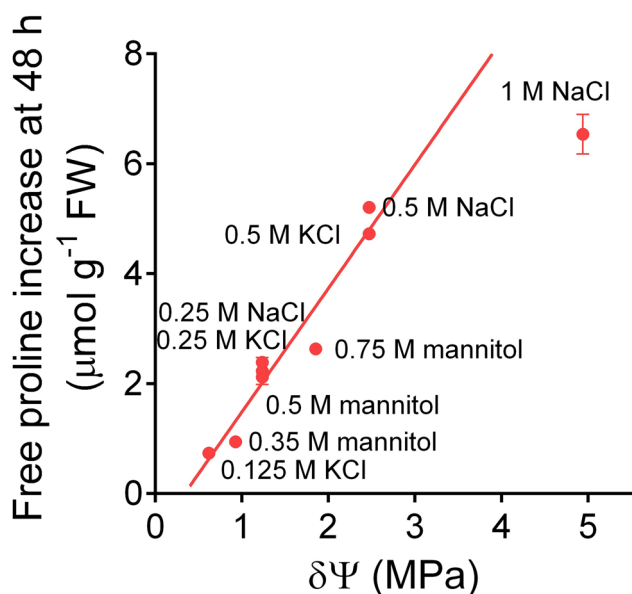


Fig. 5 Relationship between the severity of osmotic stress conditions and the intracellular accumulation of free proline. *Chlorella* cells in the late exponential phase of growth were pelleted and resuspended in the same volume of TAP medium supplemented or not with various ionic or non-ionic osmolytes, as indicated. Free proline levels were quantified in cell extracts 48 h after the exposure to stress, and expressed as the difference with the mean concentration in untreated controls

time after the exposure to osmotic stress, a rapid increase was found over basal rates, and a different homeostatic level was reached 6–8 h after the addition of salts to the medium (Fig. 3AC). When the stress was imposed by using the non-ionic osmolyte mannitol, the attainment of the new steady state level required more time, about 24–48 h. Such a difference might be due to the occurrence in salt-stressed cells of secondary toxic effects caused by the consequent ROS formation (Sharma et al. 2012; Yun et al. 2019). The beneficial effect of proline under stress has been hypothesized to depend also on its ability to scavenge some ROS species (Forlani et al. 2019). Therefore, a quicker synthesis of this amino acid may be functional to cope with ionic more than with osmotic stress conditions. A mild increase of proline content in osmotically stressed cells may simply depend upon their shrinkage due to water loss in the presence of low Ψ_{medium} values. This seems not to be the case for *Chlorella*. The homeostatic levels that were reached are remarkably high, up to 24-fold the basal concentration (Fig. 3AC, 4A). Moreover, when the content of all the other free amino acids was also quantified, results pointed out only minor variations (Fig. 3BD, 4B). A slight increase was in fact found soon after the exposure to stress, but such increase never exceeded 40%, and was partially reverted thereafter. Data thus confirm a specific role for proline synthesis and accumulation within the *C. vulgaris* defence system against hyperosmotic stress.

Interestingly, the intracellular concentrations of free proline that were attained in stressed cells resulted strictly proportional to the severity of the stress. When the new proline homeostatic levels were plotted as a function of the lowering of the Ψ_{medium} , a highly significant correlation was evident (Fig. 5). The equation of the interpolating straight line allowed us to calculate the lowest stress conditions at which proline accumulation seems induced, which is equivalent to about 0.5 MPa $\delta\Psi$. This also explains why in PEG-stressed cells proline content was found unaffected. Despite causing 51% inhibition of cell growth, the highest concentration used (20% PEG [w/v]) corresponds to a $\delta\Psi$ of about 0.546 MPa, a value not high enough to produce a significant effect on proline steady state level. On this basis, the use of PEG as the osmotic stressor seems unsuitable also for studies on stress-induced proline accumulation.

In summary, this study presents the results of a systematic analysis of the effects of hyperosmotic stress conditions on growth rate and proline accumulation in a high biomass-yielding strain of *C. vulgaris*. Such information could represent a useful basis for further studies aiming at the optimization of growth conditions for large-scale sustainable production of biomass for various industrial processes, and potential applications in biofuel production and wastewater treatment. Moreover, to the best of our knowledge this is the first study to investigate the quantitative response of proline accumulation as a function of the severity of the osmotic stress in *Chlorella* spp. The achieved identification of the threshold condition over which proline synthesis is activated may help planning future research on this topic.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10811-025-03607-9>.

Author's Contribution G.F. conceived the research, performed part of the experiments and analysed data. S.B. performed part of the experiments, acquired funding and drafted the paper. Both authors contributed to the final manuscript, and approved it for publication.

Funding Open access funding provided by Università degli Studi di Ferrara within the CRUI-CARE Agreement. Project funded by the University of Ferrara (FIRD 2023). S.B. also acknowledges support from the National Recovery and Resilience Plan (NRRP), Mission 04 Component 2 Investment 1.5 – NextGenerationEU, Call for tender n. 3277 dated 30/12/2021. Award Number: 0001052 dated 23/06/2022.

Data availability The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are

included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Ahmad I, Hellebust JA (1988) The relationship between inorganic nitrogen metabolism and proline accumulation in osmoregulatory responses of two euryhaline microalgae. *Plant Physiol* 88:348–354
- Anand V, Kashyap M, Samadhiya K, Ghosh A, Kiran B (2019) Salinity driven stress to enhance lipid production in *Scenedesmus vacuolatus*: a biodiesel trigger? *Biomass Bioenergy* 127:105252
- Barahoei M, Hatamipour MS, Afsharzadeh S (2021) Direct brackish water desalination using *Chlorella vulgaris* microalgae. *Process Safety Environ Protect* 148:237–248
- Barera S, Forlani G (2023) The role of proline in the adaptation of eukaryotic microalgae to environmental stress: an underestimated tool for the optimization of algal growth. *J Appl Phycol* 35:1635–1648
- Beardall J, Raven JA (2013) Limits to phototrophic growth in dense culture: CO₂ supply and light. In: Borowitzka MA, Moheimani NR (eds) *Algae for biofuels and energy*. Springer, Dordrecht, pp 91–97
- Bhatt T, Sharma A, Puri S, Minhas AP (2020) Salt tolerance mechanisms and approaches: future scope of halotolerant genes and rice landraces. *Rice Sci* 27:368–383
- Borowitzka MA (1995) Microalgae as sources of pharmaceuticals and other biologically active compounds. *J Appl Phycol* 7:3–15
- Borowitzka MA (2013) Energy from microalgae: a short history. In: Borowitzka MA, Moheimani NR (eds) *Algae for biofuels and energy*. Springer, Dordrecht, pp 1–15
- Brennan L, Owende P (2010) Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renew Sustain Energy Rev* 14:557–577
- Colman B, Brickell PC, Gehl KA (1986) The uptake of mannitol and sorbitol by a species of *Chlorella* (Chlorophyceae). *J Phycol* 22:436–440
- da Silva TL, Reis A, Medeiros R, Oliveira AC, Gouveia L (2009) Oil production towards biofuel from autotrophic microalgae semi-continuous cultivations monitored by flow cytometry. *Appl Biochem Biotechnol* 159:568–578
- Dutta S, Kataki S, Banerjee I, Pohrmen CB, Jaiswal KK, Jaiswal AK (2025) Microalgal biorefineries in sustainable biofuel production and other high-value products. *N Biotech* 87:39–59
- Fal S, Aasfar A, Rabie R, Smouni A, Arroussi HE (2022) Salt induced oxidative stress alters physiological, biochemical and metabolomic responses of green microalga *Chlamydomonas reinhardtii*. *Heliyon* 8:e08811
- Farghl AM, Shaddad MAK, Galal HR, Hassan EA (2015) Effect of salt stress on growth, antioxidant enzymes, lipid peroxidation and some metabolic activities in some fresh water and marine algae. *Egypt J Bot* 55:1–15
- Forlani G, Funck D (2020) A specific and sensitive enzymatic assay for the quantitation of L-proline. *Front Plant Sci* 11:582026
- Forlani G, Trovato M, Funck D, Signorelli S (2019) Regulation of proline accumulation and its molecular and physiological functions in stress defence. In: Hossain MA, Kumar V, Burritt DJ, Fujita M, Mäkelä PSA (eds) *Osmoprotectant-mediated abiotic stress tolerance in plants: recent advances and future perspectives*. Springer, Cham, pp 73–97
- Greenway H, Hiller RG, Flowers T (1968) Respiratory inhibition in *Chlorella* produced by “purified” polyethylene glycol 1540. *Science* 159:984–985
- Griffiths MJ, Garcin C, van Hille RP, Harrison ST (2011) Interference by pigment in the estimation of microalgal biomass concentration by optical density. *J Microbiol Meth* 85:119–123
- Hagemann M (2016) Coping with high and variable salinity: molecular aspects of compatible solute accumulation. In: Borowitzka MA, Beardall J, Raven JA (eds) *The physiology of microalgae*. Springer, Cham, pp 359–372
- Hiremath S, Mathad P (2010) Impact of salinity on the physiological and biochemical traits of *Chlorella vulgaris* Beijerinck. *J Algal Biomass Utln* 1:51–59
- Ismaiel MMS, Piercey-Normore MD, Rampitsch C (2018) Proteomic analyses of the cyanobacterium *Arthrospira (Spirulina) platensis* under iron and salinity stress. *Environ Exp Bot* 147:63–74
- Katz A, Kleyman TR, Pick U (1994) Utilization of amiloride analogs for characterization and labeling of the plasma membrane Na⁺/H⁺ antiporter from *Dunaliella salina*. *Biochemistry* 33:2389–2393
- Katz A, Pick U, Avron M (1992) Modulation of Na⁺/H⁺ antiporter activity by extreme pH and salt in the halotolerant alga *Dunaliella salina*. *Plant Physiol* 100:1224–1229
- Krell A, Funck D, Plettner I, John U, Dieckmann G (2007) Regulation of proline metabolism under salt stress in the psychrophilic diatom *Fragilariopsis cylindrus* (Bacillariophyceae). *J Phycol* 43:753–762
- Kronzucker HJ, Coskun D, Schulze LM, Wong JR, Britto DT (2013) Sodium as nutrient and toxicant. *Plant Soil* 369:1–23
- Kropat J, Hong-Hermesdorf A, Casero D, Ent P, Castruita M, Pellegrini M, Merchant SS, Malasarn D (2011) A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*. *Plant J* 66:770–780
- Laliberté G, Hellebust J (1989) Regulation of proline content of *Chlorella autotrophica* in response to changes in salinity. *Can J Bot* 67:1959–1965
- Lee YH, Yeh YL (2014) Using polyethylene glycol as nonionic osmoticum to promote growth and lipid production of marine microalgae *Nannochloropsis oculata*. *Bioproc Biosyst Eng* 37:1669–1677
- Li X, Yuan Y, Cheng D, Gao J, Kong L, Zhao Q, Wei W, Sun Y (2018) Exploring stress tolerance mechanism of evolved freshwater strain *Chlorella* sp. S30 under 30 g/L salt. *Bioresour Technol* 250:495–504
- Maathuis FJM (2009) Physiological functions of mineral macronutrients. *Curr Opin Plant Biol* 12:250–258
- Mastrobuoni G, Irgang S, Pietzke M, Assmus HE, Wenzel M, Schulze WX, Kempa S (2012) Proteome dynamics and early salt stress response of the photosynthetic organism *Chlamydomonas reinhardtii*. *BMC Genomics* 13:215
- Mata TM, Martins AA, Caetano NS (2010) Microalgae for biodiesel production and other applications: a review. *Renew Sustain Energy Rev* 14:217–232
- Money NP (1989) Osmotic pressure of aqueous polyethylene glycols: relationship between molecular weight and vapor pressure deficit. *Plant Physiol* 91:766–769
- Mountourakis F, Papazi A, Maragkoudakis A, Stamatis N, Kotzabasis K (2023) Evidence of physiological adaptation of *Chlorella vulgaris* under extreme salinity – new insights into a potential halotolerance strategy. *Environ Exp Bot* 216:105543
- Muller-Feuga A (2003) Microalgae for aquaculture: the current global situation future trends. In: Richmond A (ed) *Microalgal culture: biotechnology and applied phycology*. Blackwell Science, Oxford, pp 352–364

- Phipps S, Goodman CA, Delwiche CF, Bisson MA (2021) The role of ion-transporting proteins in the evolution of salt tolerance in charophyte algae. *J Phycol* 57:1014–1025
- Pinner E, Padan E, Schuldiner S (1995) Amiloride and harmaline are potent inhibitors of NhaB, a Na⁺/H⁺ antiporter from *Escherichia coli*. *FEBS Lett* 365:18–22
- Pinto AS, Maia C, Sousa SA, Tavares T, Pires JCM (2025) Amino acid and carotenoid profiles of *Chlorella vulgaris* during two-stage cultivation at different salinities. *Bioengineering* 12:284
- Saide A, Martínez KA, Ianora A, Lauritano C (2021) Unlocking the health potential of microalgae as sustainable sources of bioactive compounds. *Int J Mol Sci* 22:4383
- Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J Bot* 2012:217037
- Singh R, Upadhyay AK, Chandra P, Singh DP (2018) Sodium chloride incites reactive oxygen species in green algae *Chlorococcum humicola* and *Chlorella vulgaris*: implication on lipid synthesis, mineral nutrients and antioxidant system. *Bioresour Technol* 270:489–497
- Singh R, Upadhyay AK, Singh DV, Singh JS, Singh DP (2019) Photosynthetic performance, nutrient status and lipid yield of microalgae *Chlorella vulgaris* and *Chlorococcum humicola* under UV-B exposure. *Curr Res Biotechnol* 1:65–77
- Tartari A, Forlani G (2008) Osmotic adjustments in a psychrophilic alga, *Xanthonema* sp. (Xanthophyceae). *Env Exp Bot* 63:342–350
- Tromballa HW (1978) Influence of permeant acids and bases on net potassium uptake by *Chlorella*. *Planta* 138:243–248
- Trovato M, Forlani G, Signorelli S, Funck D (2019) Proline metabolism and its functions in development and stress tolerance. In: Hossain MA, Kumar V, Burritt DJ, Fujita M, Mäkelä PSA (eds) *Osmoprotectant-mediated abiotic stress tolerance in plants: recent advances and future perspectives*. Springer, Cham, pp 41–72
- Van AT, Glaser K (2022) *Pseudostichococcus* stands out from its siblings due to high salinity and desiccation tolerance. *Phycology* 2:108–119
- Wang N, Qian Z, Luo M, Fan S, Zhang X, Zhang L (2018) Identification of salt stress responding genes using transcriptome analysis in green alga *Chlamydomonas reinhardtii*. *Int J Mol Sci* 19:3359
- Wegmann K (1986) Osmoregulation in eukaryotic algae. *FEMS Microbiol Rev* 2:37–43
- Wu J-T, Hsieh M-T, Kow L-C (1998) Role of proline accumulation in response to toxic copper in *Chlorella* sp. (Chlorophyceae) cells. *J Phycol* 34:113–117
- Yadav N, Gupta N, Singh DP (2022) Ameliorating effect of bicarbonate on salinity induced changes in the growth, nutrient status, cell constituents and photosynthetic attributes of microalga *Chlorella vulgaris*. *Bull Environ Contam Toxicol* 108:491–499
- Yun C-J, Hwang K-O, Han S-S, Ri H-G (2019) The effect of salinity stress on the biofuel production potential of freshwater microalgae *Chlorella vulgaris* YH703. *Biomass Bioenergy* 127:105277

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.