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Dunaliella salina (Chlorophyceae) affects the quality of NaCl crystals

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Abstract – The green alga *Dunaliella salina* is a common inhabitant of crystallizers in solar saltworks. Although its presence has been associated with low salt quality, little is known about the mechanism through which *Dunaliella* causes such effect. It has been suggested that the release of organic matter from healthy *D. salina* cells interferes with NaCl crystallization. With the intention to cast some light on this matter, we grew *D. salina* cells at different [NaCl] (0.3, 1.0, 2.0 and 3.0 M), in the presence of either NO_3^- or NH_4^+, in conditions that, although strongly hyperaline, were mild enough to minimize cell lysis and substantial spill over of cytosolic sap. The two N treatments were done because the N-source was shown to influence organic release from *D. salina*. We then attempted to link this physiological information to the information to the quality of NaCl crystals. In order to reduce the impact of inter-crystal inclusion and focus on the structure of the crystalline reticulum, the volume of water used for the production of crystals was as small as possible. Photosynthesis (as chlorophyll variable fluorescence associated with photosystem II), cell organic (as determined by Fourier Transform Infrared spectroscopy, FTIR) and elemental (measured by total reflection X ray fluorescence spectrometry, TXRF) composition were analyzed at the beginning and in the middle of the exponential growth phase, and in the stationary phase. The quality of the crystals obtained from sterile media and from *D. salina* growth media was studied by optical microscopy (to determine the shape of the crystals) and by X-ray diffractometry.
Our results demonstrate that the presence of *D. salina* cells is directly connected to alterations of NaCl crystalline structure (specifically of crystallite size) and with the quantity and quality of contaminants in the salt.

Cell composition / *Dunaliella* / elemental stoichiometry / FTIR / NaCl / photosynthesis / salt / saltworks / TXRF / X-ray crystallography

INTRODUCTION

Dunaliella salina is a member of the Chlorophyta able to live and grow over a wide range of salinities, up to the saturation limit for NaCl solutions (Ben-Amotz & Avron, 1989). The ability of this alga to cope with high and changing salinities is largely due to a peculiar glycerol metabolism that allows a fine control of the cell osmotic equilibrium with the external medium (Avron, 1992).

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Dunaliella salina is a very important component of the planktonic community of the ponds of solar saltworks where NaCl precipitation occurs (crystallizers; Davis, 2000); substantial amounts of *D. salina* cells can be also found on the floor of these ponds (Davis, 2009). The presence of *D. salina* in the crystallizers has been associated with a low quality of NaCl crystals and overall low productivity of solar saltworks (Davis & Giordano, 1995; Malik *et al.*, 2011). It has been suggested that the impact of *D. salina* on NaCl crystal quality depends on the interference of the organic matter released by the alga on the crystallization process (Davis & Giordano, 1995). Even healthy cells of *D. salina* have been shown to release organic molecules, and to do so to different extents depending on the growth conditions and specifically as a function of the N-source (Giordano *et al.*, 1994; Giordano & Beardall, 2009). Unfortunately, no data exist on the mode of interaction of this organic matter with the crystals. Also the influence of *D. salina* on the inorganic contaminants of NaCl crystals has not been studied; yet, it is reasonable to expect that the uptake of nutrients by *D. salina* may impoverish the brine of certain elements, whereas the release of ions, as a consequence of extrusion of metallo-enzymes such as Zn-containing carbonic anhydrases (Giordano & Bowes, 1997), may enrich the crystallizer of such components.

In this study, we intend to make a first step towards the comprehension of the mechanistic link between the presence of *D. salina* and the quality of NaCl crystals.

MATERIALS AND METHODS

Cultures

Batch cultures of *Dunaliella salina* CCAP 19/25 were maintained in 250 mL Erlenmeyer flasks containing 200 mL of Artificial Multipurpose Complement for the Nutrition of Algae (AMCONA) medium (Fanesi *et al.*, 2014). Depending on the treatment, the AMCONA composition was modified by the addition of NaCl to final concentrations of 0.3, 1.0, 2.0 and 3.0 mol L^{-1}, and by the substitution of NO_3^- with an equimolar concentration of NH_4^+ (final concentration 550 μM). The medium was buffered with 10 mM Tris-HCl and the pH was kept constant at 8.4 for all treatments. The growth temperature was 20°C, and light was supplied at a continuous photon flux density (PFD) of 100 μmol photons m^{-2} s^{-1} (PAR).

Cell number and volume

The number of *Dunaliella salina* cells and their volume were determined using a CASY TT cell counter (Innovatis AG, Reutlingen, Germany) according to Palmucci *et al.* (2011). Counts were effected daily.

Experimental design

Dunaliella salina was grown in batch cultures, in the presence of either NO_3^- or NH_4^+, at all the above mentioned salinities. The cultures were allowed to acclimate to each condition for at least four generations. Cells from the early exponential phase of these "acclimation" cultures were re-inoculated in fresh media equal to those to which they had acclimated. The inoculum was such to give an approximate initial cell density of $2{\cdot}10^5$ cells mL^{-1}. Samples were then collected

immediately after inoculation (T_0), in the middle of the exponential growth phase (T_e), and in the stationary phase (T_s). The cells were separated from the medium by centrifugation at 2,000·g for 10 min. The medium was used to generate NaCl crystals. Fresh medium, treated exactly as the cultures, was used as a control for crystal analysis.

Chlorophyll determination

Chlorophylls *a* and *b* were determined spectrophotometrically according to Norici *et al.* (2011), using the equation by Jeffrey and Humphrey (1975).

Chlorophyll variable fluorescence

Samples containing approximately $4{\cdot}10^7$ cells were collected and centrifuged (2,000·g, 10 min). The pellet was resuspended in 1.5 mL of fresh medium and then dark-adapted for 15 minutes, at the same temperature used for growth. Samples were then transferred into a quartz cuvette and the variable fluorescence was determined with a Dual-PAM fluorometer (Walz GmbH, Effeltrich, Germany), under continuous stirring, as described in Cosgrove & Borowitzka (2006). The pulse modulated measuring light had a photon flux density PFD of 12 µmol photons m^{-2} s^{-1} and did not induce electron transport. The minimal fluorescence yield of dark-adapted cells (F_0) was determined. Subsequently, the sample was subjected to a saturation pulse (10,000 µmol photons·m^{-2}·s^{-1}) and the maximum fluorescence yield of dark-adapted cells (Fm) was measured. After the first pulse, actinic light (i.e. light capable of driving electron transport) was provided at a PFD approximately equal to that used for growth. From this time on, saturating pulses were supplied with a 20 seconds frequency and the maximal (F_m') and minimal (F_0') fluorescence yields of non dark-adapted cells were determined. Indirect parameters, such as the maximum PSII quantum yield (Fv/Fm), the coefficients of non photochemical quenching (NPQ and qN) and photochemical quenching (qP and qL) were automatically calculated by the Dual PAM software (Walz GmbH, Effeltrich, Germany). A precise description of the various parameters can be found in Maxwell and Johnson (2000).

Organic composition

Organic composition of the cells and organic contaminants in the NaCl crystals were evaluated according to Palmucci *et al.* (2011), using a Tensor 27 FTIR Spectrometer (Bruker Optik GmbH, Ettlingen, Germany). Sample preparation, spectra acquisition and spectra normalization were carried out according to Domenighini & Giordano (2009). The determination of proteins was conducted according to Peterson (1977); the semi-quantification of the lipid and carbohydrate pool was done according to Palmucci *et al.* (2011). For the analysis of salt crystals, 30 µL aliquots of growth or fresh (control) medium were put on a silicon slide (Crystran Ltd, Poole, UK) and allowed to dry at room temperature. The FTIR spectra of cells and crystals were analyzed using the OPUS 6.5 software (Bruker Optik GmbH, Ettlingen, Germany). A ''rubberband'' baseline correction was applied, using 200 baseline points and excluding the CO_2 bands.

Elemental composition

Cells were collected by centrifugation (2,000·g, 10 min). The pellet was washed once in 10 mL and three times in 1.5 mL of an isosmotic ammonium formate solution. It was then resuspended in 1 mL of milliQ water, with an approximate cell concentration of $2{\cdot}10^6$ cells mL^{-1}.

For crystal analysis, samples of the medium separated from cells by centrifugation (2,000·g, 10 min) were mixed with a solution of polyvinyl alcohol (0.3 g L^{-1}) in a 9:1 volumetric ratio. This was done to obtain a more homogeneous layer of salt crystals. In all cases, a solution of Ga in 5% HNO_3 was added as internal standard to a final concentration of 5 mg L^{-1}. Aliquots of 10 (cell suspension) or 5 (medium) µL were deposited on a quartz sample holder (Bruker AXS Microanalysis GmbH, Berlin, Germany) and dried on a hot plate under a chemical hood for 10 minutes. The X-ray fluorescence of elements with an atomic number higher than 10 was measured for 1000 seconds with a S2 Picofox total reflection X-ray fluorescence (TXRF) spectrometer (Bruker AXS Microanalysis GmbH, Berlin, Germany; Bruker 2008). The spectra were deconvoluted and analyzed using the software SPECTRA 6.1 (Bruker AXS Microanalysis GmbH, Berlin, Germany).

Microscope analysis of salt crystals

The optical analysis of salt crystals was carried out on NaCl crystals obtained from the dehydration of *Dunaliella salina* growth medium, after separation of the cells by centrifugation (2,000·g, 10 min). Fresh medium was used as a control. Ten microliters of medium were deposited on a microscope slide; the water was allowed to evaporate at a temperature of 30°C and a relative humidity of approximately 75%. The crystal number and surface area were measured under a stereoscopic microscope, using a micrometric scale. Crystals were generated from the smallest possible volume of water in order to minimize the formation of large inter-crystal inclusion and facilitate the interpretation of the FTIR and TXRF data with respect to the hypothesized alteration of the fine crystalline structure associated with *Dunaliella* presence.

X-ray crystallography

The structure of the crystalline reticulum was studied by X-ray diffractometry. The spent medium and the fresh medium (control) were dried and NaCl crystals were collected. A 1.6 kW X-ray source (Philips PW1830, Philips, Almelo, The Netherlands) equipped with a Germanium monochromator and image plate was used. The observed peaks depend on the crystalline structure of the NaCl crystal cell and follows Bragg's law:

$$n \cdot \lambda = 2d \cdot \sin\theta$$

where n is a natural positive number, λ is the wavelength of the incident radiation (1.54 Å in this case), d is the distance from two different crystallographic planes, θ is the angle between the incident radiation and the plane (Bragg *et al.*, 1913).

The diffraction image was processed with the softwares Image J and Igor Pro 6.1.2.1 to evaluate the θ value from the diffraction pattern and subsequently calculate d. A tricosane standard was used as reference.

The mean size of the ordered crystalline domains (crystallites) was defined using Debye-Scherrer's law:

$$\tau = K \cdot \lambda \cdot \beta \cdot \cos\theta$$

where τ is the mean size of the ordered crystalline domains, K is the shape factor (a typical value of 0.9 was used here) and β is the width of the peaks in the diffractogram at half maximum intensity.

A cubic crystalline structure contains a large number of planes, and thus generates a large number of diffraction peaks. In this work, we confined our

analysis to the two main peaks present in the diffraction profiles, corresponding to plane orders 200 and 220. Fitting analysis was conducted according to Franz *et al.* (1994).

Statistics

All results were expressed as the mean and standard deviations of measurements obtained from three biological replicates (i.e. three different cultures). In the case of comparison of two means, the significance of differences was determined with a 2-tailed t-test. When three means were compared, a one-way ANOVA was applied. The level of significance was always set at 95% (i.e. $p < 0.05$ for all differences mentioned in the result section). The software GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA) was used.

RESULTS

Growth rates and cell volume

Cells grown at 0.3 M NaCl showed a specific growth rate (μ) similar to that of cells grown in the presence of 1 M NaCl. A halving of μ was observed at 2 M NaCl. The growth rate of cells cultured at 3 M NaCl was similar to that of 2 M NaCl-grown cells (Fig. 1). The growth rates of cells cultured in the presence of different N-source were not significantly different ($p > 0.05$).

The volume of *Dunaliella salina* cells followed an opposite trend to that showed by the growth rate: the cells grown at 0.3 M and 1 M NaCl had similar volumes and were approximately half the size of the cells grown at 2 and 3 M NaCl (Fig. 2). No volume differences were observed in relation to the growth phase and the N source.

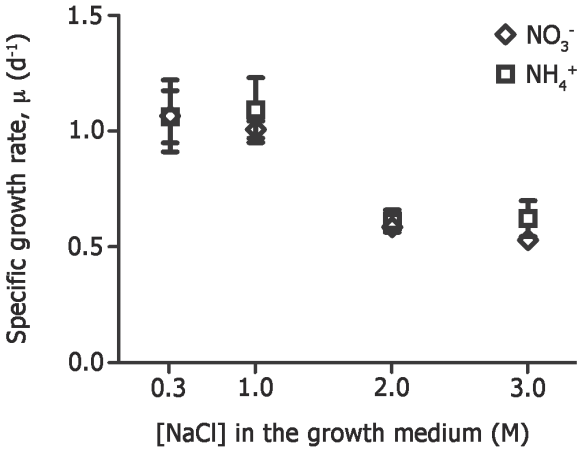

Fig. 1. Specific growth rate of *Dunaliella salina* acclimated to growth at different NaCl concentrations (0.3, 1.0, 2.0 and 3.0 M) and in the presence of 550 μM either NO_3^- or NH_4^+ as the N source. The error bars indicate the standard deviations (n = 3).

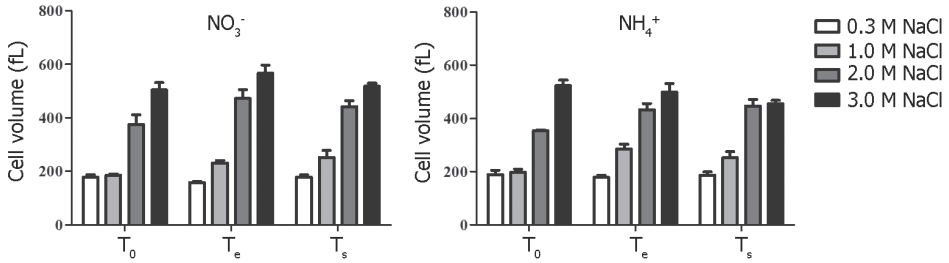

Fig. 2. Volume of *Dunaliella salina* cells grown in the presence of either NO_3^- or NH_4^+ as the sole N source and at different NaCl concentrations (0.3, 1.0, 2.0 and 3.0 M). Samples were collected in the early exponential growth phase (T_0), middle exponential growth phase (T_e) and stationary phase (T_s). The error bars represent the standard deviations (n = 3).

Photosynthetic performance

No differences were observed in the maximum quantum yield (Fv/Fm) as a function of the NaCl concentration, growth phase and N source ($p > 0.05$ Tab. 1). Cells cultured at 3 M NaCl showed higher values of NPQ and qP then cells grown at all other salinities, regardless of the growth phase and the N source (Tab. 1). At 3 M NaCl, NPQ was higher when NO_3^- was used as the N source than when N was supplied as NH_4^+ ($p < 0.05$, Tab. 1).

Chlorophyll cell content

The chlorophyll content increased in the exponential growth phase (Tab. 2). The only exception were the samples collected from 3 M NaCl cultures in NO_3^-; in this case the amount of chlorophyll was the same at the three different time points ($p > 0.05$). The N source did not affect the chlorophyll content ($p > 0.05$).

Organic cell composition

Proteins were less abundant when cells were cultured at 0.3 M NaCl in comparison to all other salinities ($p < 0.05$, Tab. 3). Carbohydrates and lipids were most abundant in cells grown at 3 M NaCl and in the presence of NH_4^+ ($p < 0.05$, Tab. 2).

Elemental composition

P, S, Fe and Zn cell quotas were higher in cells grown at 3 M NaCl than in cells acclimated to lower salinities ($p < 0.05$, Tab. 4). When NO_3^- was the N source, Cl, K and Ca were more abundant in 3 M NaCl-grown cells (Tab. 4). Other elements were either only occasionally detected or their cell quotas were not affected by the growth treatments (data not shown).

Organic contaminants of NaCl crystals

The FTIR spectra of salt revealed the presence of a water peak at 1632 cm^{-1}, whose shape suggests that it was due to bound water. The water peak was evaluated relatively to the SO_4^{2-} peak (1150 cm^{-1}), which was assumed to be constant. This assumption was based on the fact that, considering the SO_4^{2-} content of the medium, the S cell quotas, and the growth rates, it could be confidently concluded that S acquisition by cells had a negligible influence on the

Table 1. Photosynthetic parameters of *Dunaliella salina* cells grown in the presence of 550 µm either NO_3^- or NH_4^+ as the N source, at 0.3 M, 1.0 M, 2.0 M, or 3.0 M NaCl, in initial exponential phase (T_0), middle exponential phase (T_e), and stationary phase (T_s). Fv/Fm, maximum quantum yield of photosystem II in dark-adapted cells; NPQ, coefficient of non-photochemical quenching; qP, coefficient of photochemical quenching; qL, fraction of PSII centers that are "open"; qN, coefficient of non photochemical quenching. The standard deviations are shown in parentheses (n = 3)

Growth phase	*Growth [NaCl]*	*NO_3^--grown cells*					*NH_4^+-grown cells*				
		Fv/Fm	*NPQ*	*qP*	*qL*	*qN*	*Fv/Fm*	*NPQ*	*qP*	*qL*	*qN*
T0	0.3	0.58 (0.02)	0.12 (0.06)	0.92 (0.02)	0.85 (0.05)	0.15 (0.07)	0.56 (0.03)	0.07 (0.02)	0.92 (0.01)	0.84 (0.02)	0.09 (0.03)
	1.0	0.41 (0.09)	0.05 (0.06)	0.74 (0.01)	0.64 (0.04)	0.07 (0.01)	0.30 (0.13)	0.00 (0.00)	0.54 (0.41)	0.49 (0.37)	0.00 (0.00)
	2.0	0.54 (0.01)	0.12 (0.04)	0.91 (0.02)	0.84 (0.04)	0.16 (0.04)	0.53 (0.07)	0.05 (0.02)	0.92 (0.02)	0.85 (0.01)	0.07 (0.02)
	3.0	0.57 (0.08)	1.13 (0.30)	0.49 (0.02)	0.38 (0.03)	0.67 (0.07)	0.47 (0.07)	0.44 (0.17)	0.50 (0.09)	0.38 (0.07)	0.42 (0.10)
Te	0.3	0.65 (0.01)	0.11 (0.03)	0.92 (0.00)	0.86 (0.06)	0.14 (0.02)	0.61 (0.01)	0.09 (0.01)	0.93 (0.01)	0.84 (0.01)	0.11 (0.01)
	1.0	0.61 (0.08)	0.07 (0.03)	0.93 (0.02)	0.85 (0.03)	0.08 (0.03)	0.68 (0.02)	0.10 (0.02)	0.95 (0.00)	0.88 (0.01)	0.12 (0.02)
	2.0	0.61 (0.06)	0.09 (0.01)	0.89 (0.01)	0.79 (0.03)	0.11 (0.02)	0.61 (0.05)	0.08 (0.02)	0.95 (0.00)	0.88 (0.02)	0.10 (0.02)
	3.0	0.64 (0.04)	0.30 (0.09)	0.75 (0.03)	0.55 (0.02)	0.31 (0.06)	0.67 (0.03)	0.14 (0.03)	0.74 (0.02)	0.51 (0.02)	0.17 (0.03)
Ts	0.3	0.57 (0.02)	0.12 (0.02)	0.91 (0.01)	0.84 (0.02)	0.15 (0.02)	0.44 (0.05)	0.04 (0.01)	0.80 (0.06)	0.70 (0.06)	0.06 (0.01)
	1.0	0.35 (0.08)	0.03 (0.03)	0.79 (0.05)	0.72 (0.04)	0.04 (0.04)	0.33 (0.04)	0.01 (0.01)	0.77 (0.03)	0.70 (0.02)	0.02 (0.02)
	2.0	0.41 (0.09)	0.09 (0.10)	0.83 (0.06)	0.75 (0.07)	0.11 (0.13)	0.31 (0.02)	0.04 (0.02)	0.78 (0.02)	0.71 (0.02)	0.06 (0.04)
	3.0	0.41 (0.07)	0.56 (0.07)	0.42 (0.17)	0.35 (0.16)	0.50 (0.07)	0.29 (0.02)	0.25 (0.11)	0.38 (0.05)	0.32 (0.05)	0.31 (0.11)

Table 2. Chlorophyll *a* and *b* and total (pg cell^{-1}) of *Dunaliella salina* cells grown in the presence of 550 µm either NO_3^- or NH_4^+ as the N source, at 0.3 M, 1.0 M, 2.0 M, or 3.0 M NaCl, in initial exponential phase (T_0), middle exponential phase (T_e), and stationary phase (T_s). The standard deviations are shown in parentheses (n = 3)

Growth phase	*Growth [NaCl]*	*NO_3^--grown cells*			*NH_4^+-grown cells*		
		Chl a	*Chl* b	*Chl tot*	*Chl* a	*Chl* b	*Chl tot*
T_0	0.3	0.88 (0.05)	0.18 (0.02)	1.05 (0.06)	0.78 (0.03)	0.16 (0.01)	0.93 (0.04)
	1.0	0.89 (0.05)	0.15 (0.01)	1.03 (0.05)	0.68 (0.03)	0.11 (0.02)	0.79 (0.05)
	2.0	1.00 (0.02)	0.18 (0.01)	1.18 (0.03)	0.89 (0.03)	0.23 (0.12)	1.12 (0.15)
	3.0	1.01 (0.10)	0.22 (0.05)	1.23 (0.13)	1.13 (0.15)	0.27 (0.07)	1.40 (0.12)
T_e	0.3	0.88 (0.20)	0.19 (0.04)	1.06 (0.16)	0.99 (0.06)	0.20 (0.03)	1.19 (0.08)
	1.0	1.566 (0.24)	0.27 (0.04)	1.83 (0.27)	1.43 (0.12)	0.29 (0.26)	1.72 (0.27)
	2.0	1.81 (0.13)	0.37 (0.03)	2.17 (0.10)	1.57 (0.23)	0.32 (0.06)	1.90 (0.21)
	3.0	1.12 (0.18)	0.18 (0.02)	0.64 (0.04)	1.40 (0.08)	0.31 (0.12)	1.71 (0.19)
T_s	0.3	0.84 (0.10)	0.15 (0.02)	0.57 (0.02)	0.81 (0.13)	0.15 (0.03)	0.96 (0.15)
	1.0	1.18 (0.43)	0.25 (0.08)	0.35 (0.08)	1.00 (0.26)	0.21 (0.04)	1.17 (0.30)
	2.0	1.07 (0.05)	0.14 (0.02)	0.41 (0.09)	0.87 (0.02)	0.11 (0.02)	0.98 (0.01)
	3.0	1.05 (0.20)	0.29 (0.09)	0.41 (0.07)	1.07 (0.05)	0.45 (0.18)	1.52 (0.23)

SO_4^{2-} concentration of the medium (and thus in the salt crystals), over the duration of the experiments. Consequently, the ratio between the bound water peak and the sulfate peak provides a good indication of the relative water content of the crystals. The crystals obtained from the medium collected from algal cultures tended to have a higher value of this ratio, although with a high variance, than the crystals from the control medium (Fig. 3).

The amount of organic matter in the crystals was mostly below the limit of detection by FTIR spectroscopy; furthermore, the water and the sulfate peaks obscured some of the diagnostic regions of the spectra.

Trace elements contaminations of NaCl crystals. The crystals obtained from algal cultures were impoverished of Fe when compared to the controls ($p < 0.05$; Fig. 4). Zn, on the contrary, was detected only in the culture medium and not in the control medium, with rare exceptions (Fig. 4). The abundance of other elements in the salt crystals was either independent of the cell presence or did not show any clear trend (Tab. 4).

Table 3. Organic composition of *Dunaliella salina* cells grown with 550 µm either NO_3^- or NH_4^+ as the N source, at 0.3 M, 1.0 M, 2.0 M, or 3.0 M NaCl, in early exponential phase (T_0), middle exponential phase (T_e), and stationary phase (T_s). "Carb/Lip", ratio between the FTIR absorbance of carbohydrates and lipids; "Carb/Prot", ratio between the FTIR absorbance of carbohydrates and proteins; "Lip/Prot", ratio between the FTIR absorbance of lipids and proteins. The values in the column "Protein content" refer to the total absolute cell protein content and is expressed in pg cell^{-1}. "Carb content" and "Lipid content" are the sizes of the cell carbohydrate and lipid pools relative to those in the cells where the pool sizes were the smallest (set equal to 1). The standard deviations are shown in parentheses (n = 3)

Growth phase	*Growth [NaCl]*	*NO_3^--grown cells*						*NH_4^+-grown cells*					
		Carb/ Lip	*Carb/ Prot*	*Lip/ Prot*	*Proteinco ntent*	*Carb. content*	*Lipid content*	*Carb/ Lip*	*Carb/ Prot*	*Lip/ Prot*	*Prot content*	*Carb content*	*Lipid content*
T_0	0.3	86.6 (30.7)	10.1 (4.63)	0.13 (0.06)	9.60 (5.82)	1.25 (0.95)	1.00 (0.77)	54.8 (21.5)	11.1 (2.39)	0.25 (0.16)	11.9 (4.95)	2.45 (1.14)	2.53 (1.89)
	1.0	38.0 (4.55)	7.19 (0.93)	0.16 (0.02)	29.5 (0.95)	2.75 (0.37)	3.80 (0.43)	37.8 (4.11)	8.65 (2.66)	0.23 (0.07)	27.7 (2.57)	4.40 (1.42)	5.34 (1.61)
	2.0	39.1 (11.5)	5.66 (0.81)	0.15 (0.04)	28.5 (2.92)	2.09 (0.37)	3.56 (1.46)	62.8 (13.8)	7.75 (1.53)	0.13 (0.02)	27.6 (8.95)	3.94 (1.49)	2.92 (1.09)
	3.0	29.5 (22.3)	9.62 (4.68)	0.38 (0.18)	27.3 (0.43)	3.41 (1.66)	8.52 (4.11)	71.8 (22.8)	14.1 (5.00)	0.21 (0.09)	23.7 (2.00)	6.15 (2.25)	4.15 (1.87)
T_e	0.3	67.1 (47.1)	9.10 (4.92)	0.18 (0.11)	9.93 (2.36)	1.17 (0.69)	1.43 (0.92)	80.6 (47.4)	4.49 (3.60)	0.07 (0.05)	16.2 (4.22)	1.34 (1.13)	1.00 (0.76)
	1.0	34.0 (8.96)	2.40 (0.34)	0.07 (0.02)	38.9 (3.51)	1.21 (0.20)	2.35 (0.55)	46.0 (13.4)	1.49 (0.29)	0.04 (0.01)	36.3 (9.41)	1.00 (0.32)	1.08 (0.49)
	2.0	40.0 (18.8)	1.93 (0.69)	0.08 (0.07)	46.6 (5.39)	1.16 (0.44)	2.84 (2.75)	58.9 (9.46)	4.18 (0.71)	0.07 (0.02)	40.3 (5.60)	3.09 (0.68)	2.46 (0.71)
	3.0	153 (67.7)	9.80 (5.58)	0.08 (0.06)	32.14 (5.60)	4.08 (2.44)	2.07 (1.70)	67.9 (51.2)	19.0 (9.48)	0.44 (0.39)	34.7 (7.69)	12.2 (6.61)	12.8 (11.7)
T_s	0.3	45.0 (17.2)	5.92 (1.73)	0.17 (0.08)	13.0 (1.15)	1.00 (0.31)	1.81 (0.89)	44.3 (17.6)	7.62 (4.89)	0.18 (0.09)	9.74 (2.09)	1.37 (0.92)	1.50 (0.81)
	1.0	88.3 (19.4)	9.64 (2.72)	0.12 (0.04)	30.4 (8.65)	3.80 (1.53)	2.88 (1.33)	61.3 (10.2)	11.4 (1.51)	0.19 (0.03)	28.8 (7.44)	6.02 (1.75)	4.53 (1.32)
	2.0	38.1 (10.6)	6.71 (1.00)	0.19 (0.08)	35.3 (4.34)	3.07 (0.59)	5.61 (2.30)	52.2 (10.4)	9.01 (2.05)	0.18 (0.04)	38.4 (4.06)	6.37 (1.60)	5.64 (1.39)
	3.0	32.0 (23.5)	10.39 (3.04)	0.64 (0.54)	24.4 (5.77)	3.29 (1.24)	12.8 (11.3)	46.4 (29.1)	15.7 (7.79)	0.50 (0.40)	25.9 (7.60)	7.46 (4.31)	10.9 (9.16)

Table 4. Elemental composition (*pg cell*$^{-1}$) of *Dunaliella salina* cells grown with 550 µm either NO_3^- or NH_4^+ as the N source, at 0.3 M, 1.0 M, 2.0 M, or 3.0 M NaCl, in initial exponential phase (T_0), middle exponential phase (T_e), and stationary phase (T_s). The standard deviations are shown in parenthesis (n = 3)

Sampling time	*[NaCl]*	*NO_3^- grown cells*							*NH_4^+ grown cells*						
		P	*S*	*Cl*	*K*	*Ca*	*Fe*	*Zn*	*P*	*S*	*Cl*	*K*	*Ca*	*Fe*	*Zn*
T_0	0.3	0.32 (0.09)	0.73 (0.25)	0.18 (0.13)	0.06 (0.01)	0.08 (0.02)	0.14 (0.06)	0.23 (0.04)	0.45 (0.03)	0.85 (0.14)	0.19 (0.05)	0.12 (0.03)	0.12 (0.04)	9.83 (1.93)	0.65 (0.52)
	1.0	0.63 (0.23)	1.25 (0.50)	0.37 (0.21)	0.12 (0.05)	0.15 (0.06)	0.23 (0.13)	0.03 (0.02)	0.67 (0.35)	1.36 (0.67)	0.54 (0.71)	0.12 (0.11)	0.22 (0.26)	0.51 (0.34)	0.03 (0.01)
	2.0	1.18 (0.51)	2.52 (0.80)	0.72 (0.37)	0.18 (0.03)	0.10 (0.01)	0.41 (0.07)	0.05 (0.02)	0.61 (0.24)	1.39 (0.70)	0.194 (0.13)	0.08 (0.08)	0.07 (0.07)	0.87 (0.32)	0.05 (0.01)
	3.0	4.08 (1.17)	6.25 (1.15)	5.12 (3.35)	0.37 (0.02)	1.06 (0.52)	7.27 (2.87)	0.03 (0.01)	3.72 (0.74)	5.15 (1.28)	1.09 (0.67)	0.15 (0.08)	1.00 (0.70)	0.19 (0.09)	0.03 (0.01)
T_e	0.3	0.41 (0.13)	0.70 (0.31)	0.33 (0.20)	0.16 (0.08)	0.17 (0.07)	0.11 (0.05)	0.45 (0.05)	0.34 (0.05)	0.84 (0.16)	0.22 (0.03)	0.19 (0.09)	0.12 (0.02)	13.54 (1.37)	0.69 (0.47)
	1.0	1.61 (0.24)	2.03 (0.43)	0.32 (0.08)	0.23 (0.05)	0.31 (0.03)	0.90 (0.20)	0.03 (0.01)	1.47 (0.21)	2.30 (0.52)	0.16 (0.05)	0.32 (0.05)	0.25 (0.07)	0.97 (0.15)	0.05 (0.01)
	2.0	1.12 (0.56)	1.88 (1.29)	0.33 (0.13)	0.07 (0.05)	0.05 (0.02)	0.74 (0.25)	0.06 (0.01)	1.05 (0.11)	1.72 (0.25)	0.59 (0.05)	0.08 (0.03)	0.10 (0.01)	0.96 (0.28)	0.07 (0.01)
	3.0	8.63 (0.77)	5.95 (1.14)	2.09 (2.48)	0.45 (0.05)	0.68 (0.10)	18.3 (2.25)	0.03 (0.01)	9.46 (1.32)	5. 44 (0.57)	0.72 (0.44)	0.10 (0.01)	0.44 (0.03)	0.13 (0.05)	0.03 (0.01)
T_s	0.3	0.43 (0.07)	0.89 (0.16)	0.21 (0.07)	0.14 (0.04)	0.09 (0.03)	0.08 (0.02)	0.25 (0.11)	0.50 (0.09)	1.27 (0.48)	0.91 (1.26)	0.07 (0.05)	0.08 (0.05)	5.65 (1.99)	0.14 (0.09)
	1.0	0.93 (0.09)	2.40 (1.11)	0.90 (1.10)	0.20 (0.12)	0.14 (0.09)	0.52 (0.06)	0.04 (0.01)	1.24 (0.37)	1.98 (0.61)	0.25 (0.04)	0.09 (0.02)	0.12 (0.05)	0.77 (0.46)	0.034 (0.02)
	2.0	0.76 (0.27)	1.50 (0.58)	0.43 (0.13)	0.06 (0.03)	0.05 (0.04)	0.51 (0.14)	0.04 (0.01)	0.63 (0.38)	1.13 (0.72)	0.45 (0.29)	0.14 (0.10)	0.10 (0.05)	0.19 (0.06)	0.03 (0.02)
	3.0	4.57 (0.31)	4.64 (0.73)	2.63 (0.79)	0.22 (0.07)	1.26 (0.16)	7.86 (1.51)	0.02 (0.01)	6.00 (0.97)	4.08 (0.54)	1.06 (0.11)	0.13 (0.03)	0.91 (0.06)	0.13 (0.03)	0.02 (0.01)

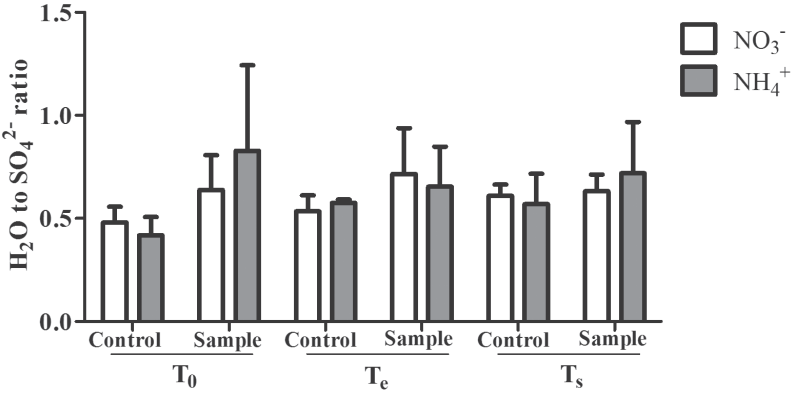

Fig. 3. FTIR absorbance ratios of the water (1632 cm^{-1}; D. Sali, personal communication) and sulfate (1145 cm^{-1}; Smith, 1999) peaks in the different experimental conditions. The control crystals were obtained form sterile media. The bars above the label "sample" refer to the crystal obtained from *Dunaliella salina* growth medium collected in early exponential growth phase (T_0), middle exponential growth phase (T_e) and stationary growth phase (T_s). The error bars represent the standard deviations (n = 3).

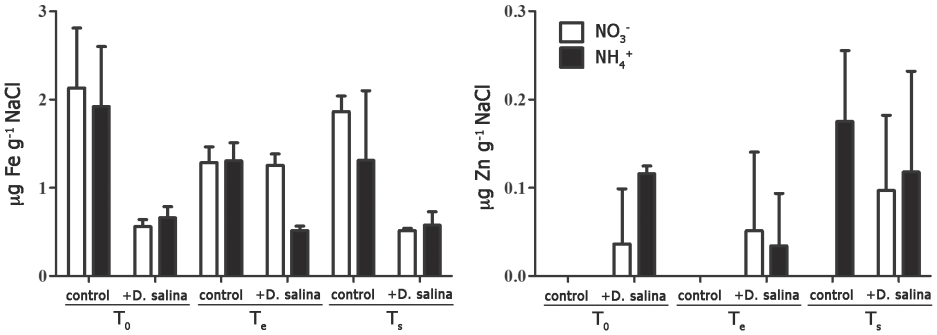

Fig. 4. Fe and Zn contents of salt crystal generated from either sterile media (control) or from media in which *Dunaliella salina* had been grown (+*D. salina*). Both for the control and the growth media, the N source was either NO_3^- (□) or NH_4^+ (■). The data are shown as means of three biological replicates. The error bars represent the standard deviations (n = 3).

Structure of NaCl crystals

The observation under the optical microscope indicated that the medium from *Dunaliella salina* cultures, upon crystallization, produced fewer and bigger crystals than the control medium (Fig. 5).

X-ray diffraction analysis

The position of the peaks corresponding to the plane orders 200 and 220 was not affected by the presence of the algae (Fig. 6). Instead, differences were observed in the size of the constitutive crystallites evaluated according to the Debye-Scherrer's law: the average size of the crystallites was smaller in the presence of *Dunaliella salina* (Fig. 7). The measurements were repeated several times, but no statistics is shown, since the structure of the crystallite is by definition invariable in a given crystal (Franz *et al.,* 1994).

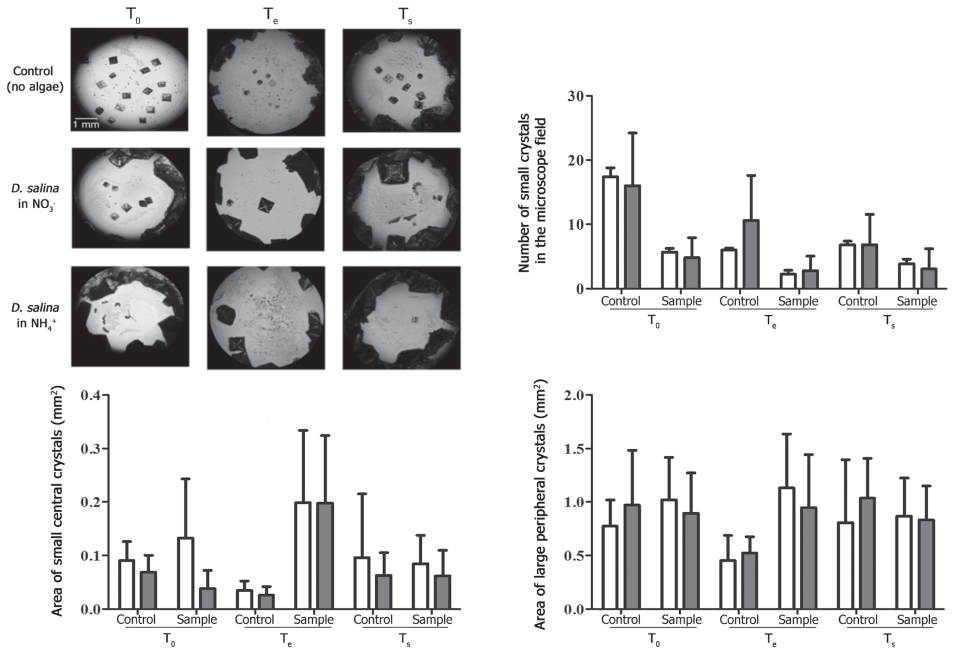

Fig. 5. The photographs shown in the top left panel were obtained with a stereoscopic microscope (5X); they depict the salt crystals generated in the presence and absence of *Dunaliella salina* cells, in media containing either NO_3^- or NH_4^+ as the sole N source. Only the controls for the medium containing NO_3^- are shown since no obvious differences were observed as a function of the N source. The bar graphs show the number and the surface area of the small crystal found in the central area of the microscope field and the surface area of big crystals at the periphery of the microscope field. The crystals were obtained from media in which *D. salin*a had been growing (samples) and from sterile media (controls). For the cultures, the medium was sampled at different growth phases (T_0 = early exponential phase, T_e = middle exponential phase, T_s = stationary phase). The control crystals were obtained from sterile media incubated under the same conditions as the growth media for the same time. The error bars represent the standard deviations ($n \geq 10$).

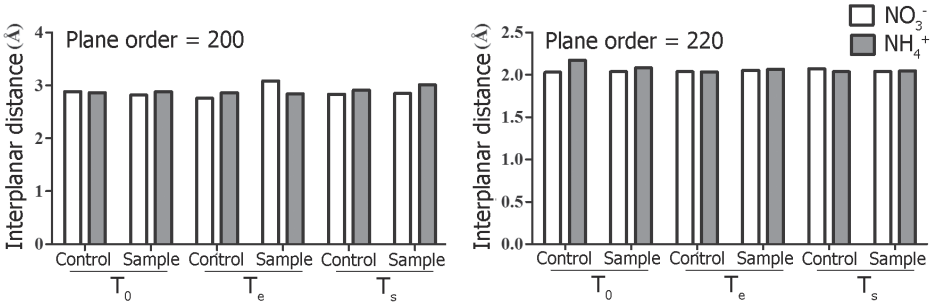

Fig. 6. Interplanar distance for the plane orders 200 and 220, as determined by X-ray diffraction in the different experimental conditions. The control crystals were obtained form sterile media. The bars above the label "sample" refer to the crystal obtained from *Dunaliella salina* growth medium collected in early exponential growth phase (T_0), middle exponential growth phase (T_e) and stationary growth phase (T_s). Errors are less than 5%.

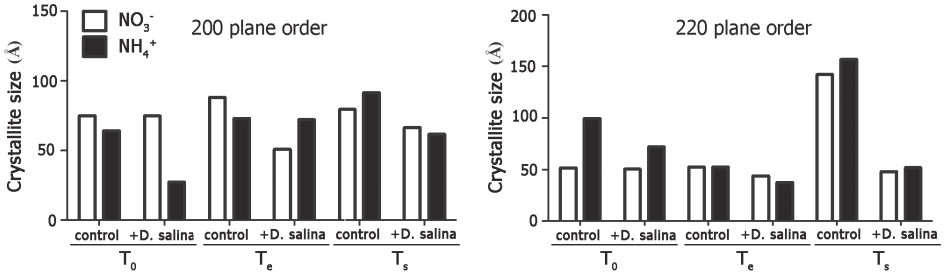

Fig. 7. Dimension of crystallites for NaCl crystals obtained from sterile media or from *Dunaliella salina* culture media. Both for the control and the growth media, the N source was either NO_3^- (□) or NH_4^+ (■). The crystallite sizes were derived from the Debye-Sherrer's law for 200 (left panel) and 220 (right panel) plane orders. Since the crystallite dimension are physical entity that do not allow statistical variation within the same crystal, no indication of variance is provided.

DISCUSSION

Our study mostly aimed at investigating the impact of the presence of *Dunaliella. salina* on salt crystals. In addition, we characterized the responses of *D. salina* to changes of NaCl external concentrations and N chemical form to verify whether and to what extent the physiological status of the algae (inferred from compositional and variable fluorescence analyses) was related to the impact of the alga on the NaCl crystals.

Cell responses to different salinities and N-sources

The changes of growth rate as a function of salinity showed a step-wise pattern, with similar growth rates for cells acclimated to 0.3 and 1.0 M NaCl and 2-fold lower growth rates at 2 and 3 M NaCl (Fig. 1). A similar pattern (although in reverse) was observed for the cell volume (Fig. 2). These responses were independent from the chemical form in which N was supplied. The energetic demand of glycerol synthesis (Oren, 1999) may limit growth above a certain salinity threshold. However, if this was the case, growth rate should decrease with salinity, above that threshold; in our experiment this did not happen and we are thus prone to exclude a growth limitation imposed by glycerol metabolism (at least in the salinity range between 2 and 3 M NaCl). Further experiments are required to clarify this point.

The relationship between salinity and cell volume was only partially connected to osmotic effects (Fig. 2): the largest differences of cell volume appeared to be linked to growth rates, whereas only modest (and not always significant) variations were observed for cultures with equal growth rates acclimated to different salinities.

Our data suggest that cells acclimated to different salinities and N-sources maintained similar antenna size (the chl *a*/chl *b* ratios were similar in all conditions) and photosystem II quantum yield (F_v/F_m was mostly independent of salinity) (Tab. 1). The homeostasis of these parameters is usually associated with salt tolerance (Stepien & Johnson, 2009; Mateos-Naranjo *et al.*, 2010). For instance, the Fv/Fm of the salt-tolerant angiosperm *Thellungellia halophila* is not affected by prolonged exposure to high salinity, whereas the same is not true for

Arabidopsis thaliana, a NON salt-tolerant relative of *T. halophila* (Stepien & Johnson, 2009). Interestingly, *D. salina*, as opposite to *Thellungelia* and similarly to *Arabidospis*, showed conspicuously higher non-photochemical quenching when grown at 3 M NaCl then at lower salinities (Tab. 1). Non-photochemical quenching is associated with regulated heat dissipation from the photosynthetic apparatus, which is often important to prevent reactive oxygen species formation and photodamage (Essemine *et al.*, 2012; Stepien & Johnson, 2009; Kanazawa & Kramer, 2002; Maxwell & Johnson, 2000; also see Ihnken *et al.* (2011) for a thorough discussion of NPQ in *Dunaliella*, although *D. tertiolecta* and not *D. salina*). In the work by Stepien and Johnson cited above, the increased NPQ in *Arabidopsis* exposed to high salinity was mostly attributed to high-energy-state quenching and electron transport to O_2, with a smaller contribution of NaCl dependent photoinhibition. The lack of a salinity-dependent NPQ increase in *Thellungellia* was instead interpreted as the consequence of a down-regulation of electron transfer from PSII to oxygen mediated by plastid terminal oxidase (PTOX) (Stepien & Johnson, 2009). In *D. salina*, PTOX was found to play a rather minor role in electron transport even under excess energy (Einali *et al.*, 2013) and this may explain the elevated NPQ that we observed when this organism was cultured at very high (3 M) NaCl.

An increase of NaCl concentration from 2 to 3 M, at 30°C, would cause a change in Henry's constant that would lead to a 20% lower CO_2 concentration in water. We hypothesize that the smaller size of the electron sink represented by CO_2 fixation increased the need for non-photochemical energy dissipation. It should however be said that the impact of lower CO_2 solubility may be somewhat mitigated by the activation of a CO_2 concentrating mechanism (Giordano *et al.*, 2005; Liska *et al.*, 2004; Giordano & Bowes, 1997; Fisher *et al.*, 1996). When N was supplied as NH_4^+ rather than as NO_3^-, non-photochemical quenching (both as NPQ and qN) was higher. We propose that this was due to the necessity for alternative energy dissipation pathways, when NO_3^- reduction does not drain electrons from the system. The higher non-photochemical quenching in the presence of NH_4^+ could also be due to a partial uncoupling action of the high concentration of NH_4^+ in our growth medium, which may further activate the xanthophyll cycle in the already challenged high NaCl-grown cells (their transthylakoidal pH gradient may be decreased by the high ATP demand for glycerol metabolism and ions pumping; e.g. Pick, 1992 and reference therein).

Growth salinity profoundly altered cell organic composition. In general, cells grown at higher salinity were enriched in protein. The physiological bases of this are not obvious. Alkalyal *et al.* (2010) reported that when *Dunaliella salina* was transferred from 2.5 M NaCl to 3.4 M NaCl, the largest increase of Expressed Sequence Tags (EST) concerned sequences for proteins involved in photosynthesis (35.7% of the EST with assigned functions), primary metabolism (13.8%) and especially protein synthesis (35.7%, 80.3% of which encoded ribosomal proteins). Liska *et al.* (2004) confirmed that a large number of proteins are involved in *D. salina* response to high salinity, most of them synthesized *ex-novo* when the cells are challenged with a hyperosmotic shock (also see Chen & Jiang, 2009 and Ramos *et al.*, 2011 and references therein). Although quantitative proteomic data for high NaCl-acclimated cells are not available (to the best of our knowledge), our data (and others; e.g. Mishra *et al.*, 2008) suggest that the greater dimensions of the expressed proteome is a characteristic of cells acclimated to growth at high salinity as of cells subject to hyperosmotic shock.

In cells cultured at 3 M NaCl, cell composition was also affected by the N source (Tab. 2). When NH_4^+ was the sole N-source, the carbohydrate and lipid

pools were appreciably larger than at lower NaCl concentrations. Under our conditions, N is unlikely to be limiting and limitation is most probably due to the energy (light) or carbon supply. If energy is limiting, the use of a cheaper N source (NH_4^+ instead of NO_3^-) may allow the build up of C storage pools (N is harder to store) and thus an increase in the carbohydrate and lipid storage (e.g. Palmucci *et al.*, 2011; Giordano & Bowes, 1997). In the presence of NH_4^+, the carbohydrate to lipid ratio was the same at all salinities, in all growth phases (Tab. 2); in NO_3^-, instead, cells growing exponentially at 3 M NaCl showed a clear preference for C allocation to the cheaper carbohydrate pool than to the more expensive lipid pool (Raven, 2005; Montechiaro *et al.*, 2006; Montechiaro & Giordano, 2010); this again points towards a lower energy availability when NO_3^- was the N source, which however becomes relevant only under high salinity, possibly because of the energy demand for osmotic control.

The provision of NH_4^+ as the sole N source may allow the cells to allocate more C also to glycerol synthesis (which in the FTIR data would be included in the carbohydrate pool). This would contrast with what was observed by Giordano & Bowes (1997), who found higher glycerol content in NO_3^--grown *D. salina* cells than in their NH_4^+-grown counterparts. It should however be considered that Giordano and Bowes (1997) used much higher N concentration (with a potentially much greater NH_4^+ toxicity). Furthermore, in this study, NO_3^--grown cells showed higher cell quota of K, Cl and Ca; this may be the symptom of a greater contribution of these ions to intracellular (especially cytosolic; Ginzburg *et al.*, 1985) osmolarity. Alternatively, this time in agreement with Giordano and Bowes (1997), the higher ionic contribution to osmotic equilibrium in NO_3^--grown cells may be the consequence of a greater C allocation to starch in NH_4^+-grown cells, which may reduce the osmotic volume and make ions accumulation less necessary.

High salinity favored the accumulation of P, Fe and Zn in the cells, regardless of the N source. A greater demand for P may be associated with glycerol metabolism (Li *et al.*, 2012). An enhanced Fe uptake capacity at higher salinity has been demonstrated by Fisher *et al.* (1997; 1998), although the function of the increased Fe cell quota remains to be clarified. The high Fe cell quotas measured in the course of this study (cf. Ho *et al.*, 2003) confirmed the tendency of *D. salina* to accumulate Fe and probably reflected the higher Fe availability in the growth media, which was similar to that in saltwork crystallizers (Davis, 2000). The accumulation of Zn is likely related to the necessity of cells to increase carbonic anhydrase activity (of which Zn is the metal cofactor) to cope with the lower CO_2 solubility at high salinity (Liska *et al.*, 2004; Giordano & Bowes, 1997; Fisher *et al.*, 1996).

NaCl crystal quality is affected by the presence of the algae

The NaCl crystals were bigger (Fig. 5) but constituted by smaller building blocks (crystallite, Fig. 7), if they were produced from media in which the algae had been growing rather than from sterile media. Since these changes were unequivocally associated with the presence of the algae and since *Dunaliella salina* is known to release organic matter (Giordano *et al.*, 1994), we suggest that the decrease of crystallite size is due the intercalation of organic matter of algal origin that interferes par with the growth of crystallites, limiting their mean size, limiting their mean size. It is noticeable that, although the rate of organic release by *D. salina* cells is greater when the algae are cultured in the presence of NH_4^+ rather than of NO_3^- (Giordano *et al.*, 1994), the chemical form in which N was

supplied to the cultures had no influence on the dimension of crystallites (Fig. 7). This may simply be due to the fact that the incremental release of organic matter in the presence of NH_4^+ is in excess to the amount required to impact crystallite size.

The FTIR spectra of crystals obtained from spent culture media suggest that the presence of the alga increased the (bound) water content of the crystals; this supports the reports of hollow crystals with water inclusions produced in crystallizers where *D. salina* is abundant (Davis & Giordano, 1995). This fact has substantial economical implication, since a higher water content of the crystals is likely to have repercussions on the dehydration process and affect the production cost of NaCl (Davis, 2009).

The presence of *D. salina* alters the amount of metal contaminants in the salt. In our culture conditions, *D. salina* accumulated Fe intracellularly (see above) and, in the process, depleted the medium (and therefore the salt) of this element. As opposite to Fe, the amount of Zn in the crystals increased appreciably when the alga was present. It can be estimated that at least one per cent of the Zn contained in *D. salina* cells was released into the medium, over the duration of the experiment. No Zn was measured in the crystals when no alga was present in the medium from which the crystals were obtained. The Zn in the salt is most probably a consequence of the secretion of extracellular Zn-carbonic anhydrase (CA) by *D. salina* (Giordano & Bowes, 1997). The CO_2 concentrating mechanism (Giordano *et al.*, 2005) of this organism makes great use of extracellular CA (Giordano & Bowes, 1997). Especially at high salinity, when CO_2 solubility is lower, the amount of CA secreted is likely to be very large high. Since each CA molecule contains one atom of Zn, this may lead to a substantial Zn enrichment of the salt.

CONCLUSION

Both salinity and the chemical form in which N was supplied to *Dunaliella salina* cultures significantly affected the physiology and the composition of the algal cells. Some of the physiological features of the algae (e.g. organic release, Fe uptake and Zn release with extracellular carbonic anhydrase; Giordano *et al.*, 1994) had a direct influence on salt quality.

The presence of *Dunaliella salina* affected the structure of NaCl crystal by increasing the overall size of the NaCl crystals and by reducing the size of their constitutive units (crystallites). We propose that this is due to the intercalation of organic matter at the margins of crystallites. The amount of Fe (which was lower) and Zn (which was higher) in the crystals was also dependent on the presence of the alga.

Our results point towards a potentially great impact of *Dunaliella salina* on the quality of NaCl crystal and thus on the necessity for a careful management of the biological community of saltworks.

FTIR and TXRF spectroscopy, for their low running costs, rapidity and easy sample preparation, are appropriate techniques to monitor the impact of the biological community on NaCl crystals, in solar saltworks.

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