The presence of the green alga *Dunaliella salina* in crstallyzer ponds of salinas can appreciably affect the quality of NaCl crystals

Mario Giordano*, Filippo Bargnesi, Simona Ratti

Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, via Brecce Bianche, 60131 Ancona, Italy;

Corresponding author: Mario Giordano, m.giordano@univpm.it.

Running title: Dunaliella and NaCl crystals

ABSTRACT *Dunaliella salina* is a halotolerant green microalga that inhabits the crystallizer ponds of salt works. Its cells are known to release organic matter and their presence has been associated with a lower quality of the NaCl crystals. The mechanistic connection between the release of organic matter and the low quality of crystals in the presence of *Dunaliella salina* is however missing. In this work we investigated the structure of the salt crystals in the presence and absence of *D. salina*, in the attempt to ascertain whether indeed the NaCl crystals were affected by the alga and, if this was the case, what was the molecular interaction between the organic matter released by *D. salina* and the NaCl. This paper is derived from the paper Giordano et al. 2014 Cryptogamie-Algologie 35 (3): 285-302, which the reader is invited to read for further details and additional data.

Key words: cell composition, FTIR spectroscopy, NaCl, saltworks, X-ray diffraction.

1. INTRODUCTION

Dunaliella salina is a common inhabitant of salt crystallizing ponds of solar saltworks (Davis and Giordano, 1996). Its presence in these basins is the result of inadequate control in the nutrient fluxes upstream, although it is often considered inevitable. In the past, Giordano et al. (1994) demonstrated that live cells of *D. salina* release recent photosynthate to the external medium. The same authors also showed that the rate and amount of C released was affected by the N source and pCO_2. Thus, poor biological management of saltworks (and thus increased amount of N, especially as NH_4^+) and global climate changes may increase the amount of C in the crystallizers and thus decrease the value of salt and the revenue of salt producers.

Deleterious effects of organic matter for salt production in ponds of low and intermediate salinity are well documented, and management procedures to control these substances are widely practised (e.g., Davis, 1978, 1990, 1993; De Medeiros Rocha and Camara, 1993; Sammy, 1983). However, the impact of the organic matter that originates in the concentrating ponds of highest salinity and in the crystallizers has received little attention.

The market value of NaCl depends, at least to some extent, on the characteristics of the salt crystals (whether they are hollow, solid, large or small) and by the amount of contaminants they contain. Premium prices are paid for large solid crystals (Butts, 1977) and for salt with contaminants not exceeding 0.03 to 0.05 % Ca, 0.02 to 0.04 % Mg, 0.11 to 0.16 % SO_4^{2-}, and 0.01 to 0.02 % insoluble matter (Davis, 1990; although different local regulation may change the acceptable levels of contaminations). Furthermore, the liquid drainage from vehicles loaded with freshly harvested small and hollow salt crystals precipitated from mucilaginous brine may significantly increase losses in the course of transport from the crystallizers to the washing facility, and may damage the transport roads. Also the cost of washing salt crystals that are hollow and heavily contaminated, whether the contaminants are organic or inorganic, increases appreciably, due to the need for more effective machine, higher energy demand, increased maintenance and, not trivially, greater losses of salt during the process. Treatments to reduce contamination (e.g. use of caustic soda and centrifuges to remove magnesium from the crystals) my further increase the cost of salt production.

In this work we used state of the art techniques to determine if and how the organic matter released by *Dunaliella* affects the quality of salt crystal. The present paper is a brief compendium of our results, which will be published in a more complete form in a forthcoming paper in an international journal.

2. MATERIALS AND METHODS - Analyses of cells

2.1. Cultures

Batch cultures of the green alga *D. salina* were grown axenically in 250 mL Erlenmeyer flasks containing 150 mL of AMCONA medium (Table 1), with either NH_4^+ or NO_3^- as the N-source and at a NaCl concentration of 3 M. Cultures were maintained at 20°C,

under a continuous photon flux density (PFD) of 100 µmol photons·m^{-1}·s^{-1}, provided by cool white fluorescent tubes. The algae were allowed to grow at the above conditions for at least 4 generations, prior to any measurement. At that point, the cells were re-inoculated in the same medium, at an initial density of $2 \cdot 10^6$ cells mL^{-1}. The growth was followed by daily counts with an automatic cell counter (CASY TT, Innovatis AG, Reutlingen, Germany): the measurements reported in this paper were obtained after 14 days since the inoculum, when the cultures were in stationary growth phase.

2.2. Contaminant determination

Crystal contaminants were determined by Fourier Transform InfraRed spectroscopy (FTIR, Giordano et al. 2001) and Total reflection X-ray fluorescence (TXRF). In both cases a drop of medium was allowed to dry on a sample holder (silicon for FTIR, crystal for TXRF measurements) and then analysed. The analyses were conducted with a S2 Picofox TXRF spectrometer (Bruker AXS Microanalysis GmbH, Berlin, Germany; Bruker, 2008); in order to reduce scattering and make the sample layer thinner and more homogeneous, the samples were resuspended in polyvinyl alcohol (0.3 g L^{-1}) in a 9:1 volumetric ratio (v/v). Ga was used as internal standard. For FTIR measurements, a Tensor 27 FTIR spectrometer (Bruker Optik GmbH, Ettlingen, Germany) was used as described in Palmucci et al. (2011).

2.3. Crystal size

Two crystal dimensional classes were defined, based on the fact that upon crystallization, two groups of obviously different dimensions were observed. The crystallization was allowed to occur at room temperature on microscope slides, after the cells were separated from the medium by centrifugation. The number of crystals of each class was estimated under a stereoscopic microscope.

Medium without *Dunaliella* was used as a control for all measurements.

2.4. Crystal microstructure

The growth medium and the medium without algal cells (control) were dried. The NaCl crystals that formed were used for the measurements. The samples were irradiated with X-rays (λ = 1.54 Å), generated by a 1.6 Kw X-ray source (Philips PW1830, Philips, Almelo, The Netherlands) equipped with a Germanium monocromator. The deflection patterns of the beam were recorded on a photographic film and the diffraction image was use to describe the crystal structure according to Bragg's law:

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

where λ is the wavelength, d is the distance between two planes of atoms laying on the same geometrical space, Θ is the angle between the incident radiation and the

plane, *n* is a natural positive number (Bragg et al., 1913). A tricosane standard of known cells parameters was used as reference to identify the Θ value.

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

$$\tau = \frac{K \cdot \lambda}{\beta \cdot \cos\Theta}$$

Where τ is the mean size of the ordered crystalline domains, K is the shape factor (0.9) and β is the width of the peaks at half maximum intensity. The main peak present in the diffractogram (plane order 220) was used in this study.

2.5. Statistics

The data were expressed as the mean ± standard deviation of measurements obtained from at least three distinct cultures. The statistical significance of differences of means was determined by analysis of variance (ANOVA) and Tukey's post-hoc test. The level of significance was set at 95%.

3. RESULTS

3.1. Contaminants

The presence of *D. salina* determined an increase of the Zn content in the salt crystals and an even larger decrease of the Fe content (Table 2).

The analysis of FTIR spectra suggest that the crystal formed in the presence of *D. salina* contained larger amount of bound water than crystals formed in the absence of the alga.

Hints of absorption by amide and carbohydrate groups were detected in the FTIR data. However, the signal was too low for its difference from the control to be statistically confirmed.

3.2. Crystal structure

Under the stereoscopic microscope, the NaCl contained in medium obtained from *D. salina* cultures crystallized generating a higher proportion of large crystals. This was true regardless of the N source (see fig. 1 in Giordano et al. 2014).

X-ray diffractometry showed that the microstructure of the crystals was not affected by the presence of *Dunaliella* (data not shown). However, the crystallites were significantly smaller in the presence of the algae (Fig. 1).

4. CONCLUSIONS

The presence of *D. salina* clearly affects NaCl crystals both in terms of their structure and of their contaminants:

1. The organoleptic properties of the salt are most likely affected by the modified Fe and Zn content.
2. At the resolution of a steroscopic microscope, the crystal formed from *D. salina* growth medium were overall larger than those obtained from the control medium.
3. The crystallites were instead smaller in the presence of *D. salina*. The smaller size of the crystallites unequivocally indicates that exogenous substances interfered with the expansion of the crystalline reticulum, when the algal cells were present in the medium.
4. The medium in which *D. salina* had been growing generated crystal that contained more bound water, which is suggestive of the presence of cavities.

ACKNOWLEDGEMENTS

We wish to thank Prof. Paolo Mariani (Università Politecnica delle Marche) for his assistance for X-ray diffractometry.

Prof. Giordano thanks the “Assemble” project for facilitating part of this work.

REFERENCES

1. Butts D.S. (1977), Solar evaporation chemistry of Great Salt Lake brines. In: D.C. Greer (Ed)
2. Desertic Terminal Lakes. *Proceedings of the International Conference on Desertic Terminal Lakes*, Weber State College, Utah Water Research Laboratory, Utah State University, Logan, pp. 125-129.
3. Davis J.S. (1978), Biological communities in a nutrient enriched salina. *Aquatic Botany*, 4, 23-42.
4. Davis, J. S. (1990), Biological management for the production of salt from seawater. *Introduction to applied phycology.* SPB Academic Publishing, The Hague, 479-488.
5. Davis J.S. (1993), Biological management for problem solving and biological concepts for a new generation of solar saltworks. *Seventh Symposium on Salt*, 1, 611-616.
6. Davis J. and Giordano M. (1996), Biological and physical events involved in the origin, effects, and control of organic matter in solar saltworks. *International Journal of Salt Lake Research*, 4, 335-347
7. De Medeiros Rocha R. and Camara M. R. (1993), Prediction, monitoring and management of detrimental algal blooms on solar salt production in north-east Brazil. In *Proceedings of the Seventh Symposium on Salt, 6-9 April 1992* (pp. 657-660).
8. Franz H., Ciuchi F., Di Nicola G., De Morais M.M. and Mariani P. (1994), Unusual lytropic polymorphism of deoxyguanosine-5'-monphosphate: X-ray diffraction analysis of the correlation between self-assembling and phase behavior. *Physical Review E*, 50, 395-402.
9. Giordano M., Davis J.S., and Bowes G. (1994), Organic carbon release by *Dunaliella salina* (Chlorophyta) under different growth conditions of CO2, nitrogen, and salinity. *Journal of Phycology*, 30, 249-257.
10. Giordano M., Bargnesi F., Mariani P., Ratti S. (2014). Dunaliella salina (Chlorophyceae) affects the quality of NaCl crystals. Cryptogamie, Algologie, 35(3):285-302.
11. Giordano M., Kansiz M., Heraud P., Beardall J., Wood B. and McNaughton D. (2001), Fourier transform infrared spectroscopy as a novel tool to investigate changes in intracellular macromolecular pools in the marine microalga *Chaetoceros muellerii* (bacillariophyceae). *Journal of Phycology*, 37, 271–279.
12. Palmucci M., Ratti S. and Giordano M. (2011), Ecological and evolutionary implications of carbon allocation in marine phytoplankton as a function of nitrogen availability: a Fourier transform infrared spectroscopy approach. *Journal of Phycology*, 47, 313-323.
13. Sammy N. (1983), Biological systems in North-Western Australian solar fields. *Sixth International Symposium of Salt*, 1, 207-215.

Table 1. Recipe of the growth medium "Artificial Multi- purpose Complement for the Nutrition of Algae" (AMCONA).

Medium component	Final concentration
NaCl	363 mM
Na_2SO_4	25 mM
KCl	8.04 mM
$NaHCO_3$	2.07 mM
KBr	725 mM
H_3BO_3	372 mM
NaF	65.7 mM
$MgCl_2$	41.2 mM
$CaCl_2$	9.14 mM
$SrCl_2$	82 mM
$NaNO_3$	549 mM
NaH_2PO_4	21 mM
Na_2SiO_3	205 mM
$CuSO_4$	40 µM
Metal mix I	
$FeCl_3$	6.56 µM
Na_2EDTA	6.56 µM
Metal mix II	
$ZnSO_4$	254 nM
$CoSO_4$	5.69 nM
$MnSO_4$	2.42 µM
Na_2MoO_4	6.1 nM

Na_2SeO_3	1 nM
$NiCl_2$	6.3 nM
Na_2EDTA	8.29 µM
Vitamine	
Tiamine-HCL	297 nM
Biotine	4.09 nM
Vitamine B_{12}	1.47 nM

Table 2. Variation in the abundance of elements in NaCl crystals produced from medium in which *Dunaliella* was growing relative to the control medium. A "+" indicates that the abundance of an element in the NaCl crystal is stimulated by the presence of *D. salina*; a decrease is indicated by "-", no change is indicated with "=". The variations are significant with $p < 0.05$ (n = 3)

Element	Variation
P	=
S	=
Cl	=
K	=
Ca	=
Mg	=
Fe	-
Cu	=
Cr	=
Zn	+
Br	=
Sr	=
Pb	=

FIGURE LEGEND

Figure 1. The figure depicts typical measurements of crystallite size in NaCl crystal produced from medium in which *D. salina* had been growing for 14 days and from medium without algae. A single reading is representative of a very large number of crystallites. For theoretical reasons (Franz et al, 1994), although the measurements were conducted on triplicate cultures and various instrumental replicates, the standard deviation is usually not calculated. The data are derived from Giordano et al. 2014.