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Reuse of SWRO brine for the production of carotenoids from *Dunaliella salina* and removal of macronutrients

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ABSTRACT

This research determines the advantages of the use of brine from reverse osmosis seawater desalination processes as hypersaline culture media to grow *Dunaliella salina*. In this work, we evaluated the growth of a new strain of *D. salina* (*Ds. Janubiense*-ITC5.105) cultured in residual brine and in artificial hypersaline media compared with the productivity of a well-established strain of this species (*Ds. BCA421*-ITC5.003). Parameters such as concentration of chlorophylls a and b and total carotenoids accumulated by the two strains throughout the cultivation course were established. The results show maximum biomass yield of $26.44\,g\,m^{-2}\,d^{-1}$ for *Ds. BCA421*-ITC5.003 grown in a controlled media with similar salinities to that of desalination brine; and maximum carotenoids productions of $20.93\,mg\,m^{-2}\,d^{-1}$ for *Ds. Janubiense*-ITC5.105 grown in brine from seawater desalination processes. This SWRO brine would be an ideal medium to grow this kind of species at low cost in areas with seawater desalination plants. Moreover, the growth of *D. salina* offers a high rate of nutrient fixation, such as NO_3^- and PO_4^{3-}, of up to 99 and 71%, respectively. Due to the fact that desalination brine contains certain amounts of these nutrients in its composition, its use as culture media requires a lower reagent expense. The cultivation also favors the complete removal of these ions from brine, thus improving the quality of the final disposal.

Keywords: Dunaliella salina; Brine; SWRO; Carotenoids; Reuse; Nutrients

1. Introduction

Nowadays, microalgae are becoming increasingly interesting for research studies and industries. One of them, the halophilic green biflagellate microalga *Dunaliella salina* has been recognized as an efficient source of carotenoids [1]. Its main commercial application is the production of β-carotene, which was developed for the first time by Western Biotechnology Ltd. and Betatene Ltd. in Australia in 1986, and more recently by other companies [2]. β-Carotene is an increasingly demanded pigment with wide market applications: as food coloring agent (its most important application); as provitamin A (retinol) in food and animal feed; as cosmetic additive or in multivitamin preparations; and as a health product popular for its antioxidant content [3]. The facilities for the cultivation of *D. salina* must be located in areas with maximum solar radiation, minimum clouds, a warm climate, and a high availability of hypersaline water

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[4]. These cultivations are based on an autotrophic growth in a medium with nutrients, and carbon dioxide as the source of carbon [5]. The cost of the cultivation medium (hypersaline water) for *Dunaliella* is a one-third of the total cost of intensive production of *D. salina* biomass [6].

D. salina in hypersaline conditions (1.5 M to 3 or 4 M NaCl) suffers a cellular growth decline that causes the carbon flow to increase the production of glycerol and plastidic isoprenoids [7]. Under these high-salinity conditions, glycerol acts in the cytoplasm as a solute that is capable to keep membrane and protein integrity. Cellular responses to saline stress are regulatory and seem to depend on a series of mechanisms linked to the modification of the equilibrium of abscisic acid. Moreover, several studies support that the increase in NaCl concentration in the medium causes an increase in β-carotene [8,9].

The global production of *D. salina* is estimated to be around 1200 tons per year. Its cultivation takes place in open basins, with no or scarce process control, which is the conventional method for the commercial production of *Dunaliella*. β-Carotene production plants that follow this method are located currently in the USA, Israel, China, and Australia [3].

Furthermore, most fresh water supplied in the Canary Islands comes from desalination processes. Nowadays, the capacity installed in the Canaries is of 526,000 $m^3 d^{-1}$ [10] and 65% of this production comes from reverse osmosis processes [11]. The conversion used in reverse osmosis plants depends on the type of feedwater; generally, for seawater, which is the usual case in the Canary Islands, it ranges from 35 to 50% [12]. Taking into account this conversion, brine production in the Canary Islands is about 368,200–526,000 $m^3 d^{-1}$. This amount of brine provides a halophilic medium that could offer the optimal characteristics to be used for cultivation means. On the one hand, costs generated from the creation of an artificial saline environment where microalgae are currently grown are saved. This is especially relevant for those places where large cultivation areas are required. On the other hand, brine disposal would be minimized in those places where marine communities, which are highly sensitive to salinity fluctuations, could be harmed. This is the case of seagrasses such as *Posidonia oceanica* and *Cymodocea nodosa*, among others [13,14].

2. Materials and methods

2.1. Microalgae identification

Two *D. salina* strains from the collection of the Instituto Tecnológico de Canarias (ITC) were used. The *Ds. BCA421*-ITC5.003, isolated in saltworks located in the southeastern part of Gran Canaria in 1992 [8] and *Ds. Janubiense*-ITC5.105, from the Salinas de Janubiense saltworks located in the island of Lanzarote [15]. Both strains were scaled up in growth chambers in the following conditions: a constant temperature of 25 ˚C, agitation by air bubbling with air enriched with 3% (v/v) CO_2, and continuous illumination with an average irradiation of 200 μmol $m^{-2} s^{-1}$. The aim of the scaled-up cultivation is to reach an inoculum density and volume that guarantees the cultivation viability and the preadaptation of algae to the experimental conditions.

2.2. Growth conditions

The studies were carried out in the ITC facilities, located in Santa Lucía de Tirajana municipality, in Gran Canaria Island, Spain, latitude 27˚48´51.28´´N and longitude 15˚25´24.97´´O. The brine from reverse osmosis desalination process is taken from the desalination plant situated 150 m from the facility, which supplies the population in the area with fresh water. The typical composition of this brine is shown in Table 1. The experiments and monitoring were carried out during a period of a year, between February 2009 and March 2010, distinguishing two seasonal periods: one in summer and another one in winter, which were defined according to the water temperature.

The experimental conditions corresponded to those found in the most widespread cultivation systems for this species, open raceway-type tanks [16]. These

Table 1
Composition of *SWRO brine* and *Seawater* media

Parameters (mg L^{-1})	Brine media	Seawater (control) media
pH	7.70	6.40
Conductivity (μS cm)	91,400	88,500
Chloride	35,322.50	38,340.00
Sulfate	5,510.15	179.50
Silica	43.50	<1
Calcium	1,052.95	67.20
Magnesium	2,233.30	35.10
Sodium	18,715.70	23,730.40
Potassium	884.75	29.10
Nitrate	12.40	85.70
Nitrite	0.05	<0.05
Iron	0.10	3.47
Ammonium	0.15	0.20
Carbonate	5.00	<5
Bicarbonate	277.05	11.30

raceways, also known as flow-through systems, consist of long channels in a circuit, where water is forced to flow through mechanical agitation. In this study, raceways of 3 m^2 of surface, double circulation channel, and a total volume of 250 L were used. A continuous water flow of 40 cm s^{-1} was kept.

Three experimental conditions were established and an artificial hypersaline environment was used as control, composed of seawater and centrifuged marine salt to reach a salinity of 70 g L^{-1} TDS. Two strains were cultivated in this medium, one inoculated with the *Ds. BCA421*-ITC5.003 stock, and designated *Control-D 421*, and the other, *Control-D. Janubiense*, inoculated with the *Ds. Janubiense*-ITC5.105 stock. The third experiment, designated *Brine-D. Janubiense*, consisted in the cultivation of *Ds. Janubiense*-ITC5.105 in a medium consisting of a 70 g L^{-1} SWRO brine from the desalination facilities in the southeast of Gran Canaria.

The f/2 medium [17] was used as nutrient source in all experimental conditions. It includes food qualityreagents (CODEX) with a NO_3^- concentration of 75 mg L^{-1} and PO_4^{3-} of 3.4 mg L^{-1}. A continuous flow of 0.1 L min^{-1} of CO_2 was injected by means of porcelain micro sprinklers. The initial cellular density for both strains under study was set to 1.5×10^5 cells mL^{-1}.

2.3. Determination of cellular density and pigments

The temperature and pH of the cultivation media were measured daily and at the same time, together with the cellular density (using direct count with Thoma cameras) and the content in pigments (total amount of chlorophyll a and b and total carotenoids). The samples for the measurement of pigments were prepared through a cold extraction with methanol on a concentrated biomass sample by means of centrifugation, at 5,000*g*, during 7 min in an Allegra X-22 Series Beckman Coulter centrifuge. A spectrophotometric analysis was then conducted at wavelengths of 470, 653, and 666 nm, according to the formula described by Wellburn, shown in Table 2 [18]. Water samples were also taken to analyze the contents in nutrients (NO_3^- and PO_4^{3-}) by means of a spectrophotometry (220–275 nm). In the case of NO_3^-, the analysis was taken on water samples which had been centrifuged and prefiltered at 0.2 μm to eliminate most particulate organic matter [19]; in the case of PO_4^{3-}, a colorimetric analysis according to the blue ascorbic acid method was applied, using Systea Micromac 1000 automatic analyzer. The loss of water by evaporation, estimated by the increase in the salinity of the cultivation media, was replenished daily by adding fresh water.

Table 2
Wellburn equations for Cl_a, Cl_b, and C_{x+c} in methanol given by UV absorbance data at 470, 653, and 666 nm

Pigments	Equations
Chlorophyll a (Cl_a)	$Cl_a = 15.65 \cdot A_{666} - 7.34 \cdot A_{653}$
Chlorophyll b (Cl_b)	$Cl_b = 27.05 \cdot A_{653} - 11.21 \cdot A_{666}$
Carotenoids (C_{x+c})	$Cl_{x+c} = (1000 \cdot A_{473} - 2.86 \cdot Cl_a - 129.2 \cdot Cl_b)/221$

The cultivation cycles were conducted until a phase of stationary growth was reached. The research was reproduced in two seasonal periods, winter–spring (between the months of January and April) and summer–autumn (between the months of September and October) in order to determine possible seasonal variations in the growth cycle.

Dry weight yields were carried out on lyophilized biomass, where the ash content had been previously estimated.

3. Results

The temperature was 21.81 ± 2.65°C during the winter period and 29.53 ± 1.69°C during the summer period, and the pH values were between 7.30 ± 0.16 and 7.92 ± 0.34 during the sampling period in winter and summer, respectively. The brine used had a salinity (NaCl) of 0.9 ± 0.13 M.

The three different experimental cultures show a very similar evolution at all times, as can be observed in Fig. 1. After a short acclimatization period of about four to five days, its growth increases reaching its maximum cell density in the following five or six days. It can be observed that, during the winter period, the species *Control-D. 421* show an increased growth. However, during the summer period *Brine-D. Janubiense* is the experimental culture that grows most and best, although the yields are inferior to those obtained during the winter period.

Even though *Brine-D. Janubiense* has a smaller biomass growth, as observed in Fig. 1, the results plotted in Fig. 2a show that, grown in brine, it presents a higher concentration of total carotenoids both in summer and in winter. Even the same strain grown as control (*Control-D. Janubiense*) produces higher concentrations of total carotenoids than those obtained by *Control-D.421*. In average, carotenoids concentrations of up to 0.98 and 1.26 mg L^{-1} d^{-1} have been reached for the periods of winter and summer, respectively, in *Brine-D. Janubiense* cultivations.

Regarding the obtained yields for microalgae biomass, Fig. 2b shows that the highest values are

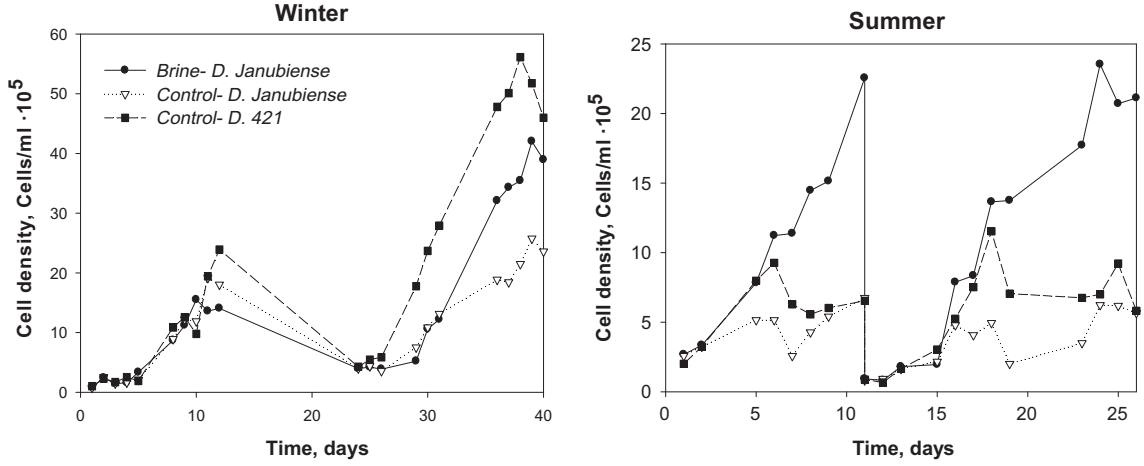

Fig. 1. Concentration evolution of the different *Dunaliella salina* strains, measured by cell density in cells/mL $\times 10^5$, in winter and summer.

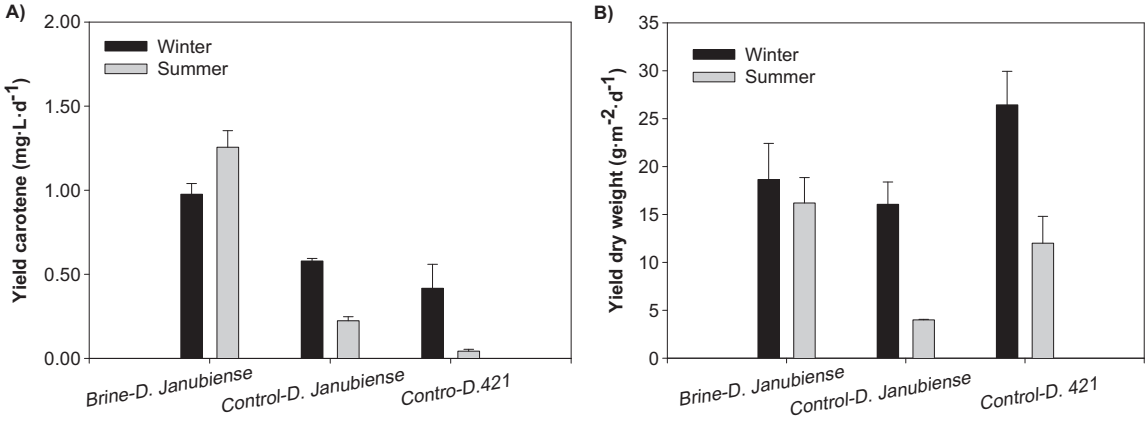

Fig. 2. (a) Concentration of total carotenoids yielded per day in the different cultivations studied. (b) Estimation of the biomass obtained at the end of the cultivation cycles for the different *Dunaliella* strains.

achieved during the winter cycle with maximum values of 26.44 g m^{-2} d^{-1} for *Control-D. 421*, though significantly high values are also obtained for *Brine-D. Janubiense* and control, such as 18.65 and 16.06 g m^{-2} d^{-1}. Even though the maximum biomass productivities are found during the winter, it should be highlighted that the best yield during the summer period, regarding the biomass attainment, was obtained from *Brine-D. Janubiense*, with a value of 16.20 g m^{-2} d^{-1}.

The ratio chlorophyll a/b shows a clearly differentiated evolution, as can be seen in Fig. 3a. It shows a progressive increase in winter of the lower values for *Brine-D. Janubiense* and the highest value for *Control-D. 421*. During the summer this development is not that clear, as can be seen in the graph, since the values for the three species are relatively lower. Even in the case of *Control-D. Janubiense* the value was lower than that observed in winter. The ratio Cl_a/Cl_b indicates more stress at higher Cl_b concentrations.

In Fig. 4, the nutrient assimilation rate (NO_3^- and PO_4^{3-}) for the species *Brine-D. Janubiense* is shown. Here, we can observe a very important assimilation of NO_3^-, corresponding to a 79 and 98% in winter and summer, respectively. However, the values related to the PO_4^{3-} assimilation are slightly lower, of about 13 and 70% in winter and summer, respectively.

The most relevant data obtained during the cultivation year are shown in Table 3. We can see that

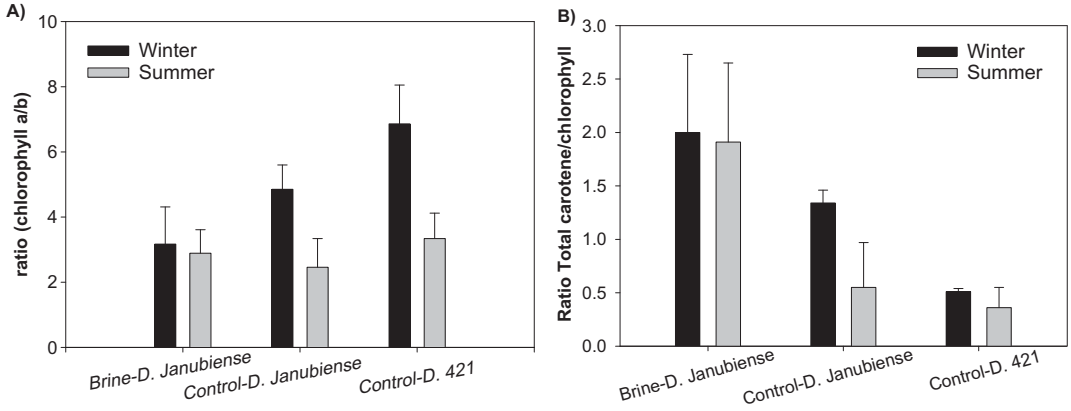

Fig. 3. (a) Ratio of chlorophyll a/b as a measure of stress levels for the cultivations studied. (b) Ratio of carotene/chlorophylls as a measure of stress levels for the cultivations studied.

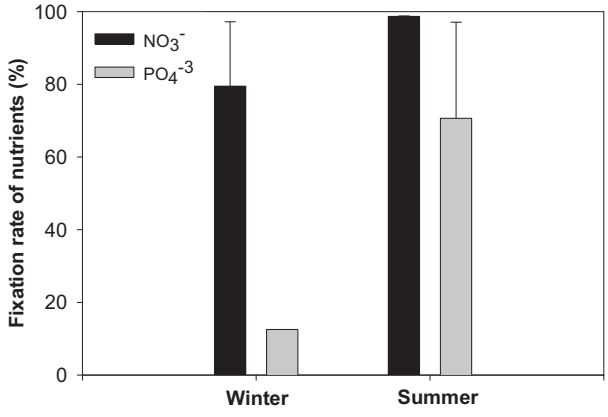

Fig. 4. Fixation of nutrients (NO_3^- and PO_4^{3-}) for *Brine-D. Janubiense* in winter and summer.

the most significant values are those yielded by *Brine-D. Janubiense*, which especially shows relatively high values in the content of total carotenoids, much higher than those obtained by the other strains studied.

4. Discussion

Clear fluctuations in the cellular growth curve can be observed both during the winter and summer cycles, highlighting a clear reduction of the cellular growth rate and maximum densities in the summer cycle in the control cultivations. This reduction observed in the cellular growth in summer is less pronounced for the cultivation in brine (see Fig. 1). This confirms that brine is an ideal media for the cultivation of these species. This phenomenon could indicate a differentiated cellular adaptation pattern in this stock, supported by factors related to brine composition (higher organic charge, differentiated ionic balances, etc.), as has already been reported for other stress conditions in similar environments [16,20,21]. In spite of the decrease in the cellular growth of *Control-D. Janubiense*, this reduction was lower than the one observed in the stock *Control-D. 421* (used as biological control).

The variations between the productivity observed in the winter and summer cycles reproduce the

Table 3
Summary of the main values obtained during the cultivation in raceways

	Brine-D. Janubiense		*Control-D. Janubiense*		*Control-D. 421*	
	Winter	Summer	Winter	Summer	Winter	Summer
T (℃)	22.10 ± 2.31	29.80 ± 1.75	21.99 ± 2.32	29.70 ± 1.4	21.37 ± 2.18	29.20 ± 1.8
pH	7.07 ± 0.34	7.78 ± 0.43	7.55 ± 0.53	8.68 ± 0.64	7.29 ± 0.67	7.32 ± 0.73
Ash free dry weight ($g\,L^{-1}$)	0.03 ± 0.013	0.04 ± 0.00	0.03 ± 0.005	0.01 ± 0.00	0.04 ± 0.02	0.01 ± 0.00
Chlorophyll a ($mg\,L^{-1}$)	0.46 ± 0.18	0.44 ± 0.26	0.36 ± 0.09	0.13 ± 0.10	0.62 ± 0.22	0.12 ± 0.05
Chlorophyll b ($mg\,L^{-1}$)	0.12 ± 0.03	0.15 ± 0.05	0.10 ± 0.02	0.04 ± 0.03	0.12 ± 0.03	0.07 ± 0.05
Total carotenoids ($mg\,L^{-1}$)	0.98 ± 0.06	1.26 ± 0.60	0.58 ± 0.02	0.22 ± 0.02	0.42 ± 0.12	0.04 ± 0.01
NO_3^- (% fixation)	98.87	96.12	98.36	43.13	97.76	100
PO_4^{2-} (% fixation)	44.20	87.54	31.08	96.63	39.77	98.46

pattern described before, including a decrease in dry weight yield per surface and time in the summer cycle. The temperature, together with the variables related to the increase of incident radiation and evaporation rate, can be at the root of this phenomenon. This decrease in the biomass yield was only apparent in the case of *Brine-D. Janubiense*.

The carotenoid production yield shows a similar variation pattern between the winter and summer cycles, except in the case of *Control-D. Janubiense*. Here, an increase in the carotenoid cultivation yield is observed in the summer cycle. A higher carotenoid cellular synthesis capacity would be one of the key elements to explain the response of *Ds. Janubiense*-ITC5.105 during the summer cycle. A higher carotenoid concentration would act as a photoprotection element given an increase in the incident radiation; moreover, the carotenoid cellular concentration can be related to a higher osmotolerance capacity against salinity changes due to an increase in the evaporation rate [22,23]. High carotenoid concentrations are related to higher cellular glycerol contents, a basic osmoregulator in the case of the genus *Dunaliella*. These results could suggest the presence of components in brine that would act as promoters of cellular adaptation responses before temperature increases and related phenomena. In previous research, there has been a mention to possible analogous of phytohormones or stimulants [24–25], though most probably this response is related to the possible organic component in brine or the presence of bacteria. In any case, these statements would require a deeper study.

The highest biomass yield (dry weight) is observed in the winter cycle in the control stock (*Control-D. 421*). In general in all experimental conditions, a productivity equivalent to the one described in previous research is observed [3,26–28]. The highest carotenoid productivity is, however, observed in *Brine-D. Janubiense* during the summer cycle, (see Fig. 3b). This yield $1.256\,mg\,L^{-1}$ ($0.08\,g\,m^{-2}\,d^{-1}$) is higher to that described in the bibliography (between 0.1 and $0.05\,g\,m^{-2}\,d^{-1}$).

The highest carotenoid yield corresponds to straim *Ds. Janubiense*-ITC5.105 both in control condition as in brine, during the winter and the summer cycles. This enables the carotenoid production by *D. salina* cultivation in SWRO brine.

The Cl_a/Cl_b quotient is considered as a good cellular stress indicator. Thus, the lowest values are related to cellular adaptation strategies for stress conditions. These results (in the winter cycle) reveal a situation of higher stress both in *Brine-D. Janubiense* and *Control-D. Janubiense* in comparison with *Control-D. 421*. Although these last differences could have a genotypical origin, it would not be the case in brine cultivations, which is something we should relate to the cultivation matrix, brine.

In previous researches [31], cellular stress responses related to *D. salina* strains in brine have been confirmed. These stress responses were not detected in conventional biotrials mesocosm studies with microalgae exposed to brine or products related to the treatment of membranes. However, outdoor cultivations form a high-tension environment where latent ecotoxic phenomena not revealed in conventional biotrials (mesocosm studies) can be produced.

These results can be a real indicator of the presence in brine of components or derivative substances to treat and clean membranes (both organic and inorganic), that could positively affect microalgae, stimulating them to produce carotenoids. SWRO brine composition would result in an increase of the carotenoid productive capacity of *Dunaliella*, since carotene synthesis is maximum during stress conditions.

In the summer cycle, these stress-related cellular behavior patterns are not that obvious and no significant appreciations in the Cl_a/Cl_b quotient are observed between the brine and control strains. However, the carotene/chlorophyll quotient does show similar patterns to those observed in the winter cycle studies, which includes a higher stress indicator in brine cultivations, see Fig. 3a.

A predominant effect of the temperature on the growth matrix during the summer cycles cannot be dismissed.

Furthermore, *Dunaliella* shows a high-macronutrient fixation capacity (purification capacity) showing an average reduction of macronutrient contents (NO_3^-) in brine of 79–99% of the fertilized medium (brine macronutrients + fertilization). In the case of phosphates (PO_4^{3-}), this fixation rate is lower, between 12 and 71%, which would indicate the need of optimizing the cultivation medium. One of the main brine-related pollutants, especially in confined disposal environments, despite the increase in salinity, is the incorporation of macronutrients to the environment, enhancing eutrophisation processes. In this sense, the case of disposal in the Dead Sea [29–30] is a good description. The interposition of *D. salina* cultivations would act as an efficient means of macronutrient biopurification in brine. The PO_4^{3-} fixation has been related to the cellular incorporation of orthophosphoric-based membrane treatment reagent degradation compounds.

5. Conclusions

SWRO brine is an ideal medium for the growth of species such as *D. salina*, which is of high biotechnological value. The results show an effect produced due to the adaptation of microalgae to this type of environment. This results in high-carotenoid production rates in comparison with the commonly used cultivation media. Although the way in which this type of brine stimulates carotenoid production in *D. salina* has not been described yet, it does open up the possibility of using this kind of cultivations as an alternative, especially in those areas where there is high-brine production through desalination processes due to the lack of fresh water. Furthermore, the great intraspecific variability of *D. salina* allows the development of ambitious selection programs of strains better adapted to growing in this type of culture medium.

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