Limnetica, 34 (2): 349-364 (2015). DOI: 10.23818/limn.34.27
 ISSN: 0213-8409

Increased water salinity negatively affects charophytes from a spring created within the Albufera de València Natural Park

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Received: 16/12/2014 Accepted: 14/04/2015

ABSTRACT

Increased water salinity negatively affects charophytes from a spring created within the Albufera de València Natural Park

In 2007, a rice field was transformed into a zone that simulates the different habitats which were typical before the environmental crisis that the current *Albufera de València* Natural Park experienced in the 70's, in order to increase the species and habitat richness. One of these recreated habitats was a spring –a pond fed by groundwater. The spring was spontaneously covered with charophytes shortly after being flooded, and a community dominated by *Chara hispida* and *Nitella hyalina* remained, which were responsible for the maintenance of the clear-water state the spring exhibited. However, in recent years, there has been a sharp reduction in charophyte coverage and biomass. The values of some variables, which could have influenced this reduction, changed substantially over time. One of these variables was water salinity, which almost tripled in less than four years. In this study, the effects of increased salinity on the growth of charophytes and on the germination of fructifications (oospores and gyrogonites) were analysed to unravel whether a change in this variable could explain at least a part of the observed reduction of charophyte stands. Laboratory experiments were performed by applying two treatments, based on the use of water with different salinity levels, to the charophyte cultures (water collected from the spring in 2009, "Lower salinity", and in 2013, "Higher salinity"). The increase in salinity caused both a decrease in the elongation of the charophyte's main shoots and a reduction in weight in the higher salinity treatment. The decrease in growth was more pronounced in the more stenohaline species, *N. hyalina*. The stoichiometric composition of charophytes was also affected, depending on the salinity conditions and the species. At the end of the experiment, the means of percentage of carbon, nitrogen and phosphorus were significantly lower under the "Higher salinity" treatment only for *N. hyalina*. Although there were no statistically significant differences in fructification germination with the salinity treatments, qualitative differences in germling size were observed. Without rejecting other factors that might have negatively affected, in a synergistic manner or individually, the development of the charophytes in the spring, it seemed that the increase in salinity was one of the involved factors.

Key words: Characean algae, *Chara hispida*, *Nitella hyalina*, growth, germination, stoichiometry.

RESUMEN

El incremento de salinidad afecta negativamente los carófitos en un* ullal *artificial del Parque Natural de l'Albufera de València

En 2007 se comenzó a transformar un arrozal del Parc Natural de l'Albufera de València *en un área que pretendía simular los diferentes hábitats acuáticos característicos de la zona antes de la crisis ambiental de los años setenta, para incrementar la riqueza de especies y hábitats. Uno de estos hábitats recreados fue una surgencia de agua subterránea, lo que localmente se conoce como un* ullal. *Los fondos de dicho* ullal *se recubrieron espontáneamente de carófitos al poco de ser inundados, quedando la comunidad de macrófitos dominada por* Chara hispida *y* Nitella hyalina*, responsables del mantenimiento del estado de aguas claras que presentó el* ullal. *Sin embargo, en los últimos años se ha producido una fuerte regresión de la cobertura y biomasa de los carófitos. Los valores de diversas variables, que podrían haber influido sobre esta regresión, han cambiado sustancialmente a lo largo del tiempo; una de ellas es la salinidad del agua, que se ha casi triplicado en tan solo 4 años. Aquí se analiza el efecto del incremento de salinidad sobre el crecimiento de los carófitos y sobre la germinación de oósporas y girogonitos, para averiguar si este cambio en salinidad podría explicar parte de la reducción observada en el*

desarrollo de las praderas. Mediante experimentos de laboratorio aplicando dos tratamientos con diferente salinidad (agua del ullal *tomada en 2009, de menor salinidad, y en 2013, de mayor salinidad, para los cultivos de carófitos), se ha comprobado que el aumento de salinidad provoca una disminución en la elongación del eje principal de los carófitos, y que el peso final de los especímenes que crecen a mayor salinidad es menor. La disminución del crecimiento es más acusada en la especie más estenohalina,* N. hyalina. *La composición estequiométrica también se ve afectada en* N. hyalina*: al final del experimento, el porcentaje medio de C, N y P fue significativamente menor en el tratamiento de mayor salinidad. Sin embargo, no se han encontrado diferencias estadísticamente significativas en la germinación de las fructificaciones con el aumento de salinidad, pero sí cualitativas en el tamaño de los germinados. Sin descartar otros factores, que de forma sinérgica o individualmente, podrían haber afectado negativamente el desarrollo de los carófitos en el* ullal*, parece ser que el incremento de salinidad es una de esas causas.*

Palabras clave: *Caráceas,* Chara hispida*,* Nitella hyalina*, crecimiento, germinación, estequiometría.*

INTRODUCTION

Charophytes (Characeae family) are a group of submerged macrophytes (Coops, 2002) that can grow in a high diversity of aquatic habitats, from shallow and seasonal water bodies to deeper and permanent water bodies (Cirujano *et al*., 2008). Charophytes are considered to be pioneer species that establish themselves in newly created habitats where they may occur in dense meadows, reaching an even higher biomass than that of the vascular plants under particular conditions (Blindow & Schütte, 2007). Many studies reported the important roles these macroalgae played in the maintenance of clear water states in aquatic ecosystems (Van Donk & Van de Bund, 2002) through direct effects on phytoplankton and periphyton via resource competition (Van Donk *et al*., 1993), through the release of allelopathic substances (Wium-Andersen *et al*., 1982; Jasser, 1995; Rojo *et al*., 2013) and by preventing the re-suspension of sediment particles (Barko & James, 1998). This is why charophytes may play an essential role in restoration projects on anthropically affected aquatic habitats (Van den Berg *et al*., 1998; Alonso-Guillén, 2011; Rodrigo & Alonso-Guillén, 2013).

The Albufera de València Natural Park (hereafter AVNP) is made up of the largest littoral lagoon in the Iberian Peninsula, as well as other types of small aquatic ecosystems (e.g., natural springs of groundwater, small interdunal ponds, rice fields, etc.). Since the end of the 1960s (20th century), the area has been negatively affected by anthropic pressure (Dafauce, 1975; Soria, 2006). The main lagoon was affected by excessive inputs of sewage water and also by a reduction of historical inputs from the Xúquer River. The result was its transformation from a clear water lagoon, with the bottom covered by aquatic vegetation (mainly charophytes), to a hypertrophic turbid water system dominated by phytoplankton and filamentous algae and the total disappearance of submerged macrophytes (Romo *et al*., 2005; Soria & Vicente, 2002; Rodrigo *et al*., 2009). In 2007, the Water Authorities (*Confederació Hidrogràfica del Xúquer*) recreated the aquatic ecosystems within the AVNP by transforming 40 hectares of former rice fields in the northern part of the lagoon (these rice fields are called Tancat de la Pipa) into an area that mimicked the different aquatic habitats that the AVNP possessed in the past. Constructed wetlands and phytodepuration were completed to improve the water quality of the inputs to the main lagoon. After these transformations, Tancat de la Pipa currently consists of two shallow small lagoons, four areas of constructed wetlands planted with emergent vegetation and an artificial spring (Rodrigo *et al*., 2013). In 2009, this area was declared a Reserve Zone within the AVNP. The artificial spring was fed by oligohaline groundwater by means of an artesian borehole. When it was created, the well produced abundant water, and the bottom of the basin was quickly covered by charophyte meadows of the species *Chara*

hispida Linnaeus 1753, *C. vulgaris* Linnaeus 1753, *C. baltica* Bruzelius 1824 and *Nitella hyalina* (De Candolle) C. Agardh 1824 (Rodrigo *et al.*, 2015) that originated from the germination of oospores and gyrogonites (calcified oospores) from the sediment from the past. However, since 2011, a large reduction in the biomass and coverage of the charophyte meadows was observed (Rodrigo *et al.*, 2015). Several limnological variables were monitored since the flooding of the basin, which allowed significant changes in the values of some of these variables to be detected: (i) a reduction in the groundwater supply; (ii) an increase in nutrient concentrations (N and P); (iii) a reduction in water transparency; (iv) an increase in salinity; and (v) an increase in the predation pressure from herbivorous birds (Rodrigo *et al.*, 2015). All of these factors might have had a negative effect, individually or synergistically, on the spring's charophyte meadows. Therefore, the study of these factors, one by one and combined, is necessary to understand why the decline in charophytes occurred and how to implement restoration actions (Rodrigo *et al.*, 2015). One of the factors cited above is the increase in water salinity. The artificial spring water changed from approximately 1.5 g/l (conductivity ~3000 µS/cm) in the spring of 2009 to 4 g/l ~ 7200 µS/cm) measured at the end of summer 2012 (Rodrigo *et al.*, 2015), which represented a 2.6-fold increase. For this reason, this study focused on the increase in salinity to verify whether this increase explained, at least partially, the reduction in the charophyte meadows of *C. hispida* and *N. hyalina*, the species that remained dominant in the artificial spring.

The different charophyte species differentiate from each other in their salinity tolerance range, among other aspects. Euryhaline charophytes, such as *C. hispida* or *C. vulgaris*, may tolerate a wide range of salinity because they can regulate their turgor pressure by means of the accumulation of K^+ ions within cells (Blindow *et al.*, 2003). Stenohaline charophytes, such as *N. hyalina*, cannot regulate their turgor pressure and, consequently, are more sensitive to salinity increases. However, very high salinity may turn into a stress factor limiting the growth of the euryhaline species (Blindow & Schütte, 2007). Water salinity can also affect the way in which the same species of charophyte grow in different habitats: from single tussocks in brackish water to forming dense vegetation mats in freshwater (Blindow *et al.*, 2009). Moreover, as stated above, sediment charophyte fructifications (oospores and gyrogonites) represent

Table 1. Values of the chemical variables in the experimental waters (2009: "Lower salinity" treatment; 2013: "Higher salinity" treatment) and ratio 2013/2009 waters for these variables. *Valores de las variables químicas en las aguas experimentales (2009: Tratamiento "Baja salinidad; 2013: Tratamiento "Alta salinidad") y proporción de estos valores en el agua de 2013 respecto a la de 2009.*

Variable	2009 water	2013 water	2013/2009 ratio
Conductivity (mS/cm)	4.5	9.1	2.0
Salinity (g/l)	2.3	5.1	2.2
Bicarbonate alkalinity (mg/l)	126	172	1.4
Total alkalinity (as $CaCO_3$) (mg/l)	127	173	1.4
Carbonate (CO_3) (mg/l)	< 12	< 12	1.0
Chloride (mg/l)	1050	2160	2.1
Sulphate (mg/l)	186	656	3.5
Dissolved calcium (mg/l)	243	345	1.4
Dissolved magnesium (mg/l)	103	237	2.3
Dissolved sodium (mg/l)	443	1110	2.5
Soluble phosphorus (phosphate and orthophosphate) (mg/l)	0.01		
Nitrate (mg/l)	1		

key remains in the formation of charophyte populations (Bonis & Grillas, 2002). They are resistant propagules that can be dormant for years (at least 60 years; Rodrigo *et al*., 2010) and may germinate when the appropriate conditions are met (Silvertown, 1988; Casanova & Brock, 1996). One of the factors influencing germination may also be salinity (Riddin & Adams, 2009); hence, this may affect the recruitment of new individuals to existing populations. The main hypothesis of this study was that, as the increase in salinity affected the turgor pressure in algae and plants, the charophyte growth would be reduced. It was also expected that this would affect *N. hyalina* to a greater degree than *C. hispida*. Furthermore, it was expected that the recruitment of new individuals from the germination of sediment fructifications would be reduced with the increase in salinity. Therefore, we hypothesized that this double negative effect of salinity increase (on growth and on recruitment) might be one of the causes of the decline in the charophyte populations monitored in the Tancat de la Pipa's spring. Two laboratory experiments were performed to test these hypotheses. In the first experiment, the effect of the salinity increase on the growth, share of carbonate incrustation, chlorophyll concentration and stoichiometric composition (C:N:P) of *C. hispida* and *N. hyalina* individuals from the Tancat de la Pipa were tested by exposing charophyte cultures to two salinity treatments. The second experiment studied the effect of the increase in salinity on the germination of oospores buried in sediment covered by water from the two treatments.

MATERIAL AND METHODS

Origin of charophytes and sampling

C. hispida individuals were taken from three different sites in the Tancat de la Pipa's artificial spring in 2013 (Fig. 1). This artificial spring is located within the AVNP (UTM 30S 728418 4360728) (Rodrigo *et al*., 2015). It has a surface area of 4 ha, a mean depth of 0.9 m and a maximum depth of 2.2 m. The basin was flooded with oligohaline water from an artesian well located in the north-west part of the basin. The flow was initially 4 l/s until 2012, when the well stopped flowing. During that year, the well was rebuilt, and for several months, the flow was totally restored. However, after several months, the flow stopped again. The water salinity of the spring when this study was performed was 5.1 g/l, mainly due to chlorides (2.2 g/l) and sodium (1.1 g/l). The bicarbonate concentration was 0.17 g/l (Table 1). Charophytes were taken using a boat with the help of a hook. Charophytes were kept in plastic bags and immediately transported in a cooler to the laboratory to initiate pre-experimental cultures. *N. hyalina* individuals also came from the spring, but the specimens for the pre-experimental cultures were taken from cultures kept in our culture room (where all of the species found in the Tancat de la Pipa spring are maintained), as no *N. hyalina* populations were found in the artificial spring in 2013.

Experimental setup

Experiment I: Effect of salinity on charophyte growth

For the preparation of the pre-experimental charophyte cultures, apical parts (the top most 3-4 branchlet whorls) were cut from the origin specimens, and five of these parts were equidistantly planted in small plastic pots (65 ml), each one containing a mixture of sterilized sediment collected from the Tancat de la Pipa's spring and commercial sand (2:1, v:v) (for more culturing details, see Rubio *et al*., 2015). The pre-experimental cultures were set up in transparent plastic aquaria (16 cm in diameter, 25 cm high, 4.5 l capacity), with five pots in each aquarium. There were two aquaria for each species. One aquarium for each species was filled with water taken from the spring in 2009 (treatment named "Lower salinity"), which was kept in the laboratory refrigerator (in sealed bottles at 4 °C, in darkness). The other aquarium for each species was filled with water taken from the spring in 2013 (treatment named "Higher salinity"), and it was a mixture of the water taken

from the three sites where *C. hispida* was sampled (Fig. 1). The 2009 and 2013 water samples were both filtered through Whatman GF/F glass fibre filters to remove most of the organisms and other particulate matter before filling the aquaria. We were aware that the properties (in variables, such as the microbial community, organic matter, etc.) of the water collected in 2009 might have slightly changed while in storage. However, the simple dilution of 2013 water with distilled water to decrease salinity would not match the ionic proportions of the salinity components from the 2009 water sample. Faced with this trade-off, we considered that it would be preferable to use the 2009 water, considering that the effect of salinity on charophytes was going to be tested. The number of charophyte individuals was 25 (5 per pot × 5 pots per aquarium) per treatment and species. The pre-experimental cultures were allowed to grow for 20 days in the culture room at 20 °C and under a 12:12 h light-darkness cycle. A photosynthetically active radiation of 130 μmol m^{-2} s^{-1} at the water surface was provided from above by F58 W/T8 Sylvania fluorescent tubes.

The experimental cultures were prepared by taking individuals of the same length from the pre-experimental cultures. The procedure of planting, culturing and treatments was repeated. This time, there were 4 pots in each aquarium (20 replicates: 5 individuals per pot × 4 pots per aquarium) in each treatment ("Lower salinity" and "Higher salinity") and species (*C. hispida* and *N. hyalina*). All of the pots and individuals were labelled for further recognition. On this occasion, the initial length of each planted individual was measured. Three individuals from those remaining in the pre-experimental cultures were randomly chosen and used to determine the initial dry weight. The charophytes were rinsed to eliminate epiphytes and were introduced into a desiccation heater at 70 °C for 24 h. A high precision (0.1 mg) Sartorius BP121S balance was used to weigh the dry charophytes.

After 25 days of culture, the total length of the main axis was measured by means of a plastic ruler that was gently introduced in the aquaria. After 33 days of culture, two pots were randomly chosen for each treatment and species, and all of the specimens were pulled out. The total length of the main axis, dry weight and carbonate incrustation weight were measured. Chlorophyll concentration in the charophyte tissues was also determined. After 48 days of culture, the same variables were measured in the charophytes from the two remaining pots of each treatment and species.

The growth rate for the total length and dry weight was calculated as ((length or $\text{weight}_{t=\text{final}}$ − length or $\text{weight}_{t=0}$)/length or $\text{weight}_{t=0}$)/$\text{time}_{\text{in days}}$. The carbonate incrustation weight was determined as the difference in weight before and

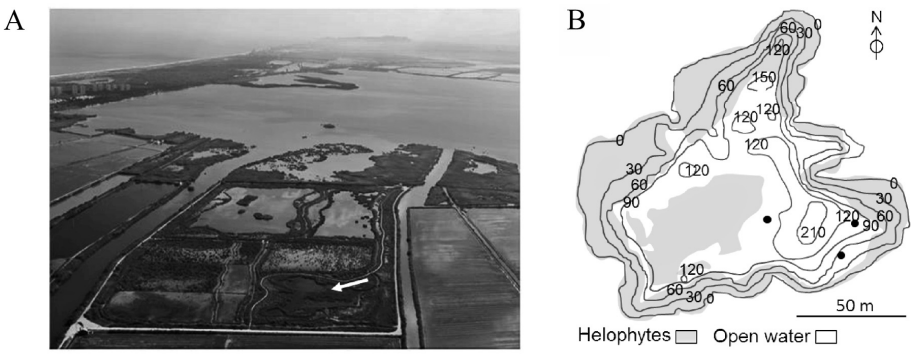

Figure 1. A: Aerial photograph of the Tancat de la Pipa (*Albufera de València Natural Park*) by *Confederació Hidrogràfica del Xúquer*. The arrow indicates the spring. B: Bathymetric map of the spring. Sampling sites for collection of charophytes are indicated. *A: Fotografía aérea (*Confederació Hidrogràfica del Xúquer*) del Tancat de la Pipa (*Parc Natural de l'Albufera de València*) donde se señala el* ullal *(flecha). B: Mapa batimétrico del* ullal *donde se indican los lugares de recolección de los carófitos.*

after treating the dry charophytes with an HCl solution to remove the external carbonate, according to the protocol of Kufel *et al*. (2013). The charophytes free from carbonate were used to determine the stoichiometric composition (C:N:P) of the organic matter. Percentages of C and N were calculated using a Perkin-Elmer CHN/O-2400 autoanalyser. P content was analysed using inductively coupled spectrometry (ICS) after the digestion of samples with nitric and perchloric acids (Sparks, 1996). Chlorophyll *a* and *b* concentrations were extracted from the apical parts of the macrophytes (upper 0.5-1 cm) using acetone (80 %). Apices were weighed (fresh weight after gently pressing the plants with drying paper) and introduced into test tubes containing an extractant solution. The tubes were then sonicated for 15 min (ELMAsonic S30H) to disrupt cell walls and then placed in a freezer (−20 °C) in darkness. After 24 h, the tubes were centrifuged, and the spectral absorption of the supernatant was measured (HitachiU-2001 spectrophotometer) at 470 nm, 630 nm, 645 nm and 665 nm. The Lichtenthaler's (1987) formulas were applied to obtain μg Chlor./g WW.

Experiment II: Effect of salinity on oospores-gyrogonites

C. hispida oospores and gyrogonites buried in sediments were obtained by sieving the fresh sediment through 1000 μm and 500 μm pore size sieves. Apparently viable fructifications were collected using a binocular and forceps. The apparent viability was checked following Rodrigo *et al*. (2010). A total of 360 fructifications we-

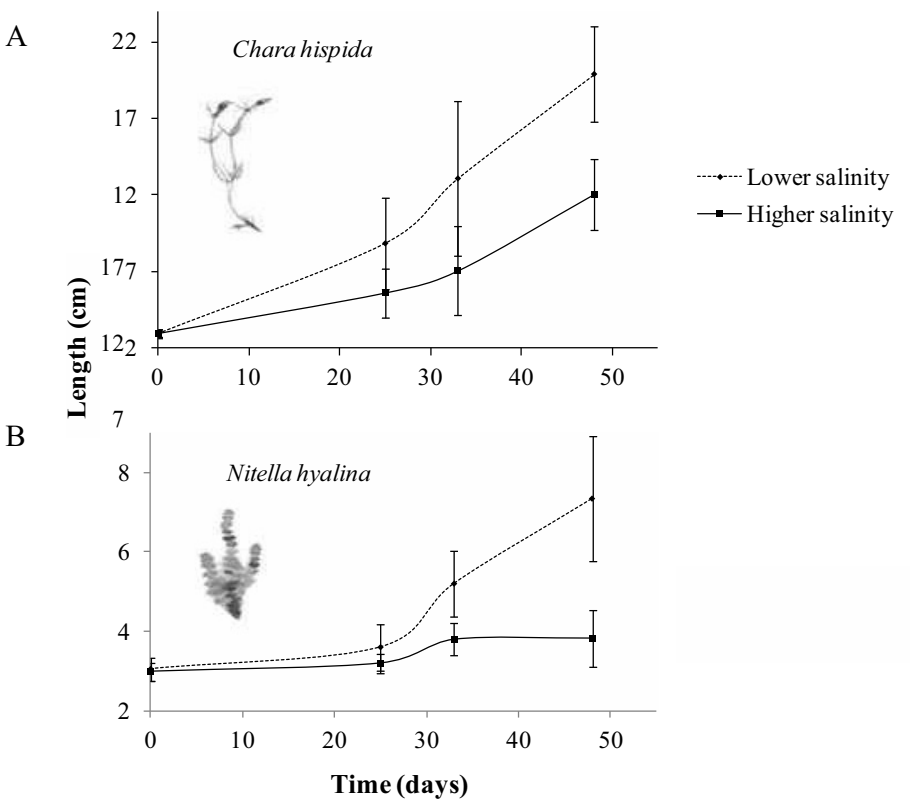

Figure 2. Time variation of the main axis length in *C. hispida* and *N. hyalina* individuals under the two experimental treatments. Vertical bars indicate the standard errors (n = 20 in t_0 and t_{25}; n = 10 in t_{33} and t_{48}). *Variación a lo largo del tiempo de la longitud del eje principal de los individuos de* Chara hispida *y* Nitella hyalina *en los dos tratamientos experimentales. Las barras verticales representan el error típico (*n = *20 en* t_0 *y* t_{25}*;* n = *10 en* t_{33} *y* t_{48}*).*

re collected and distributed in twelve 4×4 cm packages (30 fructifications in each one) made with 200 μm pore size Nytal filters (to allow the emergence of the protonema), following the protocol described in Alonso-Guillén (2011). Six of these packages were buried in Tancat de la Pipa sediment contained in six Petri dishes. The Petri dishes were submerged in plastic containers that were filled with 2009 water ("Lower salinity" treatment). The six remaining packages were used for the other treatment with 2013 water ("Higher salinity" treatment). After 30 days (when germlings were already observed emerging from the sediment), the 12 packages were dug up and opened, and the number of germinated fructifications were counted.

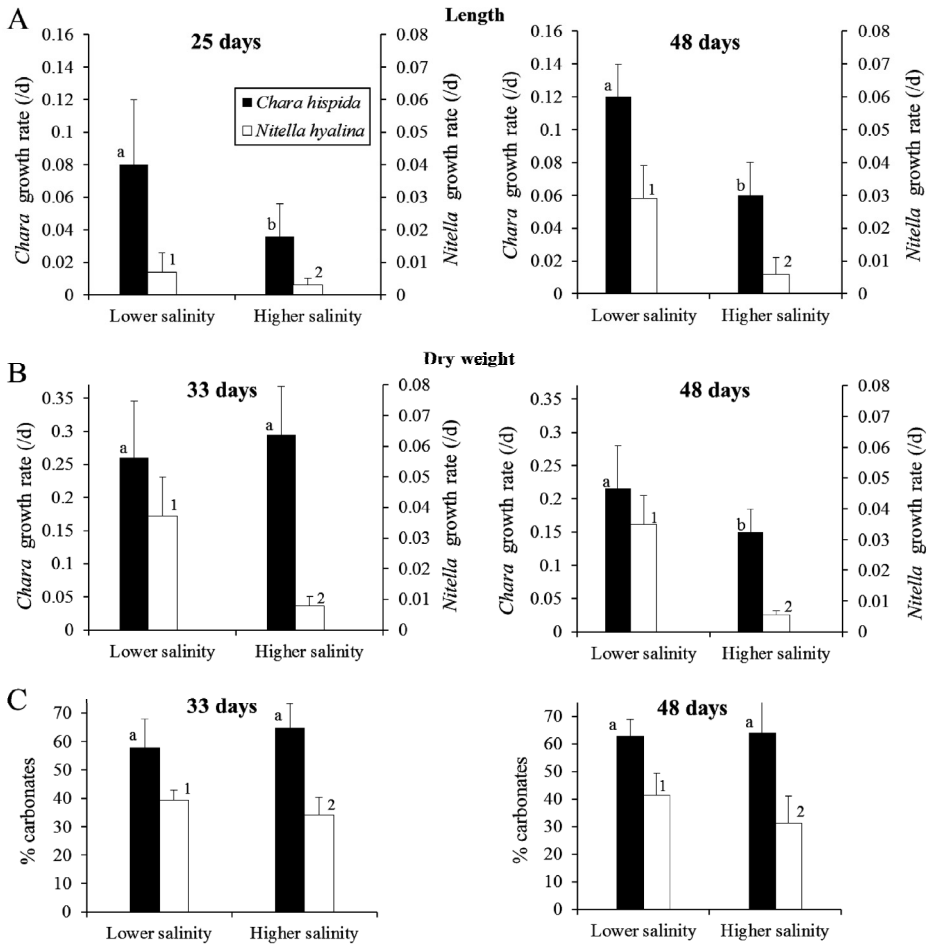

Figure 3. Mean growth rates (/d) based on variation of the main axis length (A) and total dry weight (B) in *C. hispida* and *N. hyalina* after 25 (left) and 48 (right) days of the experiment. C: Mean percentage of the carbonate incrustations represented from the total dry weight after 33 (left) and 48 (right) days of the experiment. Vertical bars show the standard errors (n = 20 in t_{25} and n = 10 in t_{48} in A; n = 10 in B and C). The letters and numbers above the bars indicate if there were statistically significant differences between the treatments in *C. hispida* and *N. hyalina*, respectively (two "a" or "1": there was no difference; "a" and "b" or "1" and "2": there was a difference). *Tasas de crecimiento medias (/d) basadas en la variación de la longitud del eje principal (A) y del peso seco total (B) en* Chara hispida *y* Nitella hyalina *a los 25 (izquierda) y 48 (derecha) días del experimento. C: Porcentaje medio que representa el carbonato de las incrustaciones respecto del peso seco total a los 33 (izquierda) y 48 (derecha) días de experimento. Las barras verticales indican el error típico* (n = *20 en* t_{25} *y* n = *10 en* t_{48} *en A;* n = *10 en B y C). Las letras y los números sobre las barras indican si hay diferencies estadísticamente significativas entre tratamientos en* C. hispida *y* N. hyalina, *respectivamente (dos "a" o "1": no hay diferencias; "a" y "b" ó "1" y "2": sí hay diferencias).*

Water analyses

The concentrations of the main ions contained in the 2009 and 2013 water samples from the Tancat de la Pipa's spring were analysed. The alkalinity of bicarbonate and carbonate, total alkalinity (expressed as $CaCO_3$) and carbonate (CO_3) were analysed using mass spectrometry (SM 2320-B/05 and SM 4500-CO2-D/05). Chloride and sulphate were analysed using ionic chromatography-mass spectrometry (PI-LTL-6.191). Dissolved calcium, magnesium and sodium were analysed using ionic chromatography-mass spectrometry (PI-LTL-6.223). Conductivity and salinity were measured using a WTW®probe. The nitrate and phosphate concentrations were analysed following the APHA (1991) methodology. Once the nutrient concentrations of both water samples were known, a solution of nitrate was used to equal the concentrations in the water of both treatments (Table 1). Phosphate concentrations were initially the same in the 2009 and 2013 water samples.

Statistical methods

The normality in the distribution of the variables was checked by the Kolmogorov-Smirnov tests, and the homoscedasticity was checked by the Levene test. When both conditions were met, one-way analysis of variance (ANOVA) tests were used to compare the means of the different variables among the treatments for each charophyte species. The initial conditions in the length of the main axis, dry weight and chlorophyll concentrations were also checked. For the growth experiment, several ANOVA tests were carried out to detect possible differences in the

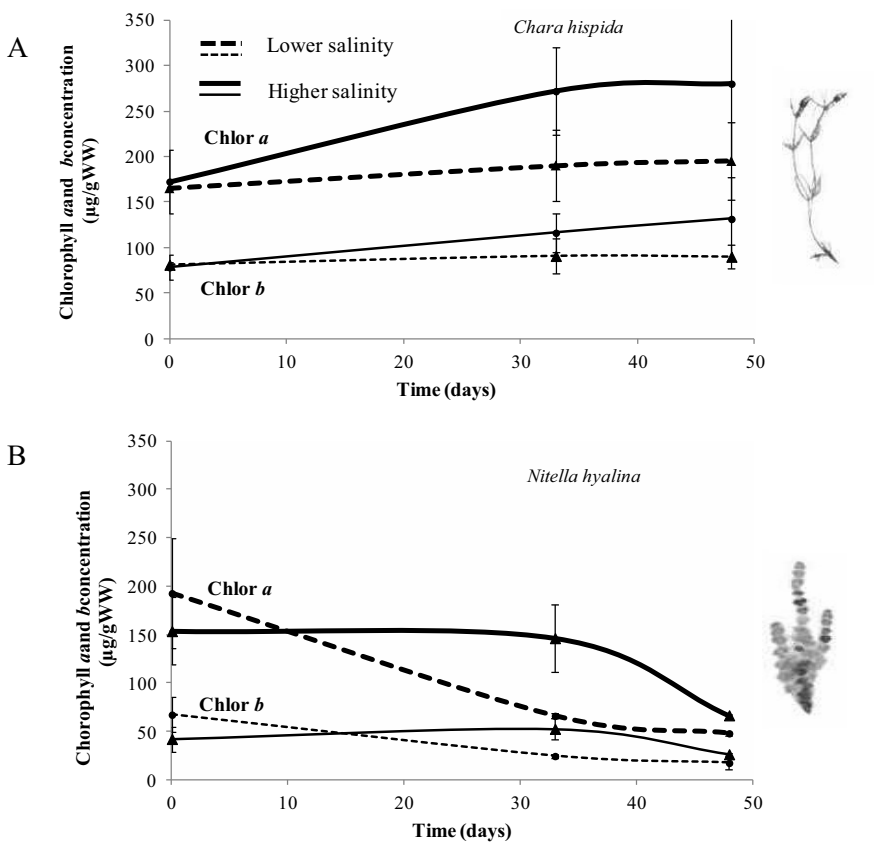

Figure 4. Chlorophyll concentration (µg/gWW) at 0, 33 and 48 days of the experiment for *C. hispida* (A) and *N. hyalina* (B). Vertical bars indicate the standard errors (n = 3). *Concentración de clorofila a y b (µg/gPF) en distintos momentos experimentales (0, 33 y 48 días) para* Chara hispida *(A) y* Nitella hyalina *(B). Las barras verticales indican el error típico (*n = *3).*

means of the different variables at 25, 33 and 48 days of the experiment. The mean stoichiometric composition (C, N and P) between the initial time and final time (48 days) was also compared by means of ANOVA tests. For the germination experiment, a one-way ANOVA test was used to compare the possible differences in the mean fructification germination percentage between treatments. For all of the analyses, significant differences were considered when $p < 0.05$. All of the statistical analyses were performed using SPSS v22.0.0 software.

RESULTS

Experiment I: Effect of salinity on charophyte growth

In the "Lower salinity" treatment, the initial mean (± standard deviation) charophyte length was 2.9 ± 0.2 cm for *C. hispida* and 3.1 ± 0.3 cm for *N. hyalina*. In the "Higher salinity" treatment, it was 3.0 ± 0.2 cm for both *C. hispida* and *N. hyalina* (Fig. 2), and there were no statistically significant differences in length among the treatments in any of the species. After 25 days of cultivation, there were statistically significant differences in the total length of both species when comparing the treatments (*C. hispida*: 19.9 ± 3.1 cm in the "Lower salinity" treatment and 12.1 ± 2.3 cm in the "Higher salinity" treatment; *N. hyalina*: 7.3 ± 1.6 cm in the "Lower salinity" treatment and 3.8 ± 0.7 cm in the "Higher salinity" treatment), and the differences persisted until the end of the experiment. Consequently, growth rates also showed significant differences ($F = 17.5$, $p < 0.001$ for *C. hispida*; $F = 9.0$, $p = 0.005$ for *N. hyalina* at 25 days and $F = 33.3$, $p < 0.001$ for *C. hispida*; $F = 35.8$, $p < 0.001$ for *N. hyalina* at 33 days; Fig. 3A). Growth rates based on variation of length were

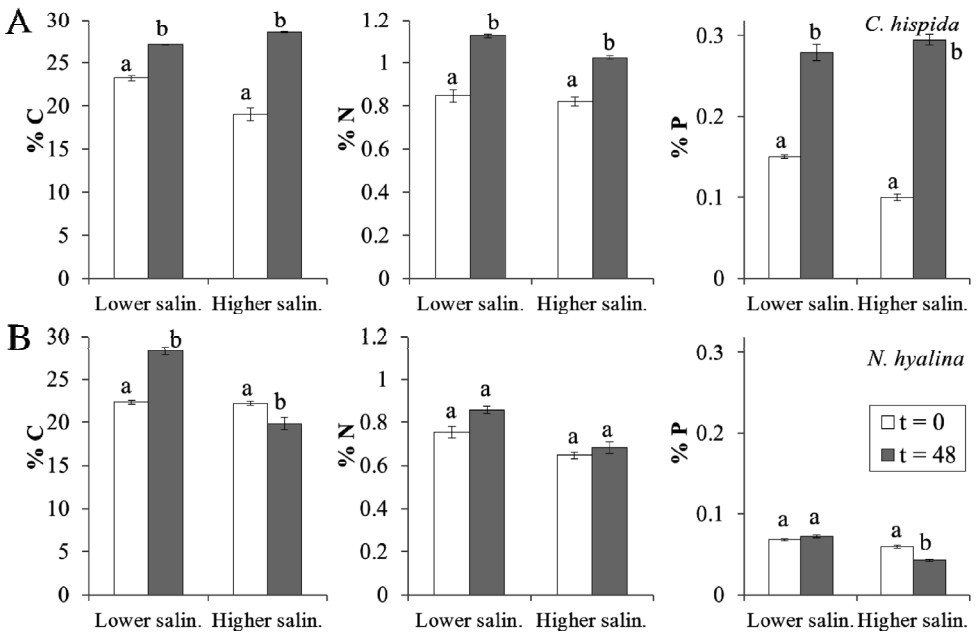

Figure 5. Stoichiometric composition of *C. hispida* (A) and *N. hyalina* (B) under the two treatments "Lower salinity" and "Higher salinity" at the start and end of the experiment. Vertical bars indicate the standard errors ($n = 3$). The letters indicate if there were statistically significant differences between the start and the end of the experiment in each treatment (two "a": there was no difference; "a" and "b": there was a difference). *Composición estequiométrica de* Chara hispida *(A) y de* Nitella hyalina *(B) en los dos tratamientos aplicados "Baja salinidad" y "Elevada salinidad" al principio y al final del experimento. Las barras verticales muestran el error típico* (n = *3). Las letras indican si hay diferencies estadísticamente significativas entre el tiempo inicial y final de cada tratamiento (dos "a": no hay diferencias; "a" y "b": sí hay diferencias).*

remarkably higher for *C. hispida* than for *N. hyalina*. *N. hyalina* did not grow in total length after 33 days of cultivation.

There were no significant differences in the total dry weight in $t = 0$ among the treatments for both species. In *C. hispida,* significant differences among treatments were not found until 48 days of cultivation ($F = 7.2$; $p = 0.016$), when higher weights were recorded under the "Lower salinity" treatment. In *N. hyalina,* significant differences occurred at 33 days ($F = 35.2$; $p < 0.001$) and 48 days ($F = 63.5$; $p < 0.001$). At both times, the growth rate was higher in the "Lower salinity" treatment (Fig. 3B).

C. hispida individuals did not show significant differences in the share of incrustation carbonate referred to total weight either at 33 or 48 days of incubation. Instead, *N. hyalina* specimens had a higher percentage under the "Lower salinity" treatment for both 33 and 48 days ($F = 5.1$; $p = 0.04$) (Fig. 3C).

There were no significant differences in the initial chlorophyll concentrations among the treatments for both species. No significant differences were found in *C. hispida* in any of the further studied time periods (33 and 48 days) among the treatments for chlorophyll *a* or *b*. In *N. hyalina*, the chlorophyll *a* concentration was significantly higher at 33 and 48 days of the experiment in the "Higher salinity" treatment ($F = 15.5$, $p = 0.017$ and $F = 164.2$, $p = 0.001$, respectively) (Fig. 4). The chlor. *a*/chlor. *b* ratio was significantly different among the treatments only at 33 days in *C. hispida* ($F = 235.8$, $p < 0.001$).

Initial molar C:N:P was 400:12:1 for *C. hispida* and 907:24:1 for *N. hyalina*. In *C. hispida*, the percentage of C, N and P significantly increased at the end of the experiment (48 days), but there were no statistically significant differences at the final time among the treatments. In *N. hyalina*, the percentage of organic C increased at the end of the experiment in the "Lower salinity" treatment and decreased in the "Higher salinity" treatment, as was the case of % P. At the end of the experiment, all percentage means were significantly lower under the "Higher salinity" treatment ($p < 0.001$ for % C, $p = 0.003$ for % N, $p < 0.001$ for % P) (Fig. 5).

Experiment II: Effect of salinity on germination of oospores-gyrogonites

The first germlings were observed emerging from the sediment 25 - 30 days after flooding. Many germlings were larger in the "Lower salinity" treatment (Fig. 6A), and the percentage of fruc-

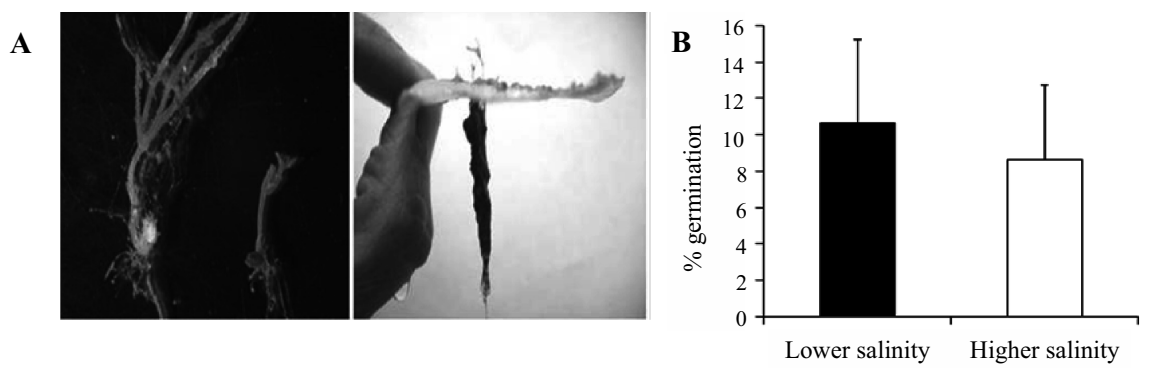

Figure 6. A: Photograph of two germlings from two *C. hispida* oospores (left: germling from an oospore buried in sediment flooded by "Lower salinity" treatment water; right: a germling from the "Higher salinity" treatment water). Details of one of the packages containing the oospores used for the germination experiment: the germlings can be observed above the filter and the rhizoidal system below. B: Means of the germination percentage of oospores-gyrogonites of *C. hispida* in each treatment. Vertical bars indicate the standard errors ($n = 6$). *Fotografía de dos germinados a partir de dos oósporas de* C. hispida *(a la izquierda: plántula germinada a partir de una oóspora enterrada en sedimento cubierto por agua del tratamiento "Baja salinidad"; a la derecha: lo mismo pero para el tratamiento "Elevada salinidad"). Detalle de uno de los sobres desde donde germinaban las oósporas: en la parte superior se observan las plántulas y en la parte inferior se aprecia el sistema rizoidal. B: Media del porcentaje de germinación para las oósporas-girogonitos de* C. hispida *en cada tratamiento. Las barras verticales muestran el error típico (*n = *6).*

tification germination was slightly higher in this treatment (Fig. 6B); however, this difference was not statistically significant among the treatments.

DISCUSSION

Charophytes are highly dependent on the physical and chemical features of the water where they live (Coops, 2002; Van Donk & Van de Bund, 2002), and the different species have distinct tolerance ranges to salinity (Blindow *et al*., 2003). Indeed, some studies stressed salinity as a key factor for the distribution and survival of charophytes (Grillas, 1990; Grillas *et al*., 1993; Soulié-Märsche, 2008). The oligohaline or subhaline (Hammer, 1986) waters of the Tancat de la Pipa's spring underwent a relevant increase in salinity over a period of almost four years (Rodrigo *et al*., 2015), related to the underwater supply and the continuous evaporation of the spring. In some Mediterranean shallow water bodies, an increment of salinity of 10 g/l in a week was observed and related to this effect (Soulié-Märsche, 2008). The difference in salinity (quantitative and qualitatively) was accurately simulated in this experimental study thanks to the possibility of using original spring water from 2009. Therefore, *C. hispida* and *N. hyalina* were subjected to, for example, a two-fold chloride and sodium concentration between treatments, or to a 3.5-fold higher sulphate concentration. The increase in salinity negatively affected the growth of both *C. hispida* and *N. hyalina*. Some charophyte species, such as *C. hispida,* face the changes in environmental salinity within a particular range by means of turgor pressure regulation. This does not happen in more stenohaline species, such as *N. hyalina*, or, at least, not so efficiently (Blindow *et al*., 2003). The increase in water salinity causes the cell turgor pressure to decrease. This turgor pressure is the engine for cell expansion, and if it is affected, individual growth will decrease (Winter & Kirst, 1990). Moreover, the maintenance of the turgor pressure requires energetic costs (Blindow & Schütte, 2007), and this is also detrimental for individual growth. Our results supported these statements. The different salinity in the "Lower salinity" treatment (2009 water) when compared to the "Higher salinity" treatment (2013 water), the most relevant difference among them, caused significantly lower growth in the charophytes of both species in the "Higher salinity" treatment. At the end of the experiment, *C. hispida* individuals grown under "Higher salinity" were 39 % shorter and 27 % lighter than those grown under "Lower salinity." *N. hyalina* individuals were 47 % shorter and 55 % lighter. The increase in salinity affected the elongation of charophytes and also the total biomass, and the *N. hyalina* specimens were more susceptible than the *C. hispida* ones. Using the dry weight, it is possible to figure out whether the charophytes are investing not only in growth in length but also in lateral growth (e.g., producing ramifications or modifying cell thickness). Winter & Kirst (1990) demonstrated that a salinity increment interrupted internodal cell elongation before affecting the cell division rate. Thus, although the individuals did not grow in length, the cells could continue dividing and increasing in thickness. In our case, the specimens of both species had a significant lower weight under "Higher salinity" at the end of the experiment, and the growth rates based on this variable were also lower under this treatment.

Charophytes inhabiting alkaline waters precipitate calcium carbonate in the form of incrustations covering the tissues, due to the photosynthetic activity which modifies the carbonic acid-carbonate equilibria in the surrounding water. *C. hispida* was described as a species that was much more highly incrusted with calcium carbonate than *N. hyalina* (Cirujano *et al*., 2008), and this was also observed during the annual cycle study of the incrustations of these species in the Tancat de la Pipa's spring (Rodrigo *et al*., 2015). The results obtained in the present study agreed with these observations. For a particular species, variations in the photosynthetic activity in the same aquatic environment might be reflected in changes in the percentage that the calcium carbonate incrustations represent in the total weight. Moreover, the calcium bicarbonate concentration (the main carbonate chemical form in the spring waters due to pH values always being higher than 8; Rodrigo *et al*., 2015) increased by 1.4-

fold from 2009 to 2013. However, in the case of *C. hispida*, no significant differences in the percentage of incrustations were found to be related to the length of the experiment, nor between the treatments. Therefore, whether the photosynthetic activity varied or not, we affirmed that the salinity increment did not affect the formation of carbonate incrustations for this species. *N. hyalina* individuals did not significantly vary in the percentage of incrustation weight with time, but there was a statistically significant reduced share of carbonate incrustations under "Higher salinity." This fact may be interpreted, with caution, as the increased salinity might be affecting the photosynthetic activity in *N. hyalina*.

Chlorophyll concentrations in photosynthetic organisms can be constitutively different among species and can also vary within a particular species for different reasons (e.g., adaptation to different radiation conditions, different photosynthetic rates, etc.; Schagerl & Pichler, 2000). Moreover, variations in the chlor. *a*/*b* ratio may indicate changes in the light harvesting complexes, because chlorophyll *b* is an accessory light harvesting pigment (Schneider *et al*., 2006). *C. hispida* individuals in "Higher salinity" tended to increase the normalized chlorophyll *a* during the first 33 days of experimentation. If we consider that an increase in the chlorophyll *a* concentration is positively correlated to a rise in photosynthetic rates, the results found in this study agree with Winter & Kirst's (1990) observations. These authors noted that the photosynthetic rate for *Chara aspera* was higher in water with 5-10 g/l than in water with lower salinity. The lack of variation in chlorophyll *a* concentration at the end of the experimental period might be because the organisms were affected by both the salinity and the appearance of epiphyte algae growing on their tissues, causing a reduction in light availability (Rodrigo *et al*., 2013). Consequently, charophytes would be investing more energy in maintaining their turgor pressure and developing defence mechanisms against epiphytes, rather than investing it in producing photosynthetic pigments. This was evident to the naked-eye when observing the pale colour of the apical parts at the end of the experiment. In *N. hyalina,* a decrease in the concentrations of both chlorophyll *a* and *b* was observed from 33 days of the experiment onwards, and there were almost no differences among the treatments. The growth of epiphytic algae on *N. hyalina* was also evident, and it was likely that the photosynthetic activity could also be reduced by loss of light availability due to this circumstance. The absence of variation in the chlor. *a*/*b* ratios during most of the experiment for both species indicated a lack of changes in the light harvesting capacity involving chlorophyll *b* (Schneider *et al*., 2006).

Variations in carbon fixation in charophytes may also affect the assimilation of other elements and, therefore, cause shifts in the stoichiometric composition of these primary producers (Li *et al*., 2013). The identity and size of the plant, as well as the growth rate differences, can produce large variations in N and P (Ågren, 2004). Thus, the initial stoichiometric composition was different for *C. hispida* and *N. hyalina*, and it was more modified in the case of *N. hyalina* under "Higher salinity," which could be related to the higher stress undergone by this species under these conditions.

Overall, the oospore-gyrogonite germination rates in charophytes are low (Rodrigo *et al*., 2010). This fact fits well with the results of this study, where the rates were approximately 10 %. There were no statistically significant differences in the germination rates among the water salinity treatments. However, it was observed that germlings produced under the "Lower salinity" treatment were qualitatively larger than those under "Higher salinity." This fact supports the results obtained in the growth experiment: higher salinity negatively affected growth. Therefore, we can suggest that an increase in water salinity, by itself, did not affect the germination rates of *C. hispida* fructification buried in a common sediment. Some authors proposed factors affecting charophyte oospore germination, such as the anoxic conditions of the substrate (Forsberg, 1965), environmental temperature, light (Carr & Ross, 1963) or endogenous control mechanisms (Bonis & Lepart, 1994), and the features of the sediment are surely more important for germination than those of the water above the sediment.

CONCLUSIONS AND FINAL REMARKS

From the results obtained in this study, we can conclude (i) that a 2.2-fold salinity increment negatively affected the growth rate (considered as both length and biomass increase) of freshwater charophytes that can also live in oligohaline waters; (ii) that, moreover, the negative effect was more evident in stenohaline species, which did not possess enough efficient mechanisms to face salinity shifts, such as *N. hyalina*, when compared to more salinity-tolerant species, such as *C. hispida;* and (iii) that an increase in water salinity did not seem to affect the germination rates of fructifications buried in the same sediment, but it affected the germling growth once the small plants made contact with the water. We can suggest, therefore, that the salinity increase registered in the Tancat de la Pipa's spring waters might be one of the causes that have participated in the regression of the charophyte meadows observed in this aquatic ecosystem. Although it is true that the shift in salinity in the spring was gradual over a relatively long period, which would have allowed the organisms of the different species to acclimatize to the increase in solutes in water, it is also evident from our experiments that the vegetative growth of both charophyte species was negatively affected. The *C. hispida* individuals were collected from the spring in 2013, when the water already had higher salinity levels; therefore, they would already be acclimatized to the current water features. However, when they were cultivated in lower salinity water (the "Lower salinity" treatment, 2009 water), the growth rates were considerably higher than in the 2013 water. Hence, it was demonstrated that the salinity increase supposed an obstacle to the growth of these charophyte species.

In this study, the effects of salinity on charophytes were analysed, keeping other variables constant. However, in the Tancat de la Pipa's spring, several variables (both physical-chemical and biological) have changed simultaneously; therefore, it would be necessary to test whether synergy exists between the several variables. For example, Steinhardt & Selig (2011) proved that *C. contraria* germination rates decreased more when salinity increased if sediment resuspension occurred at the same time, rather than only when salinity increased. The effect, as a whole, of all the variables that varied in the study site over recent years should be addressed in further studies to properly address the restoration of charophytes, which represent key organisms in the establishment and maintenance of clear water phases in aquatic environments (Coops, 2002; Kufel & Kufel, 2002; Blindow *et al*., 2002; Rodrigo *et al*., 2015).

ACKNOWLEDGEMENTS

The authors wish to thank to Fidel Rubio, María Carramiñana, Matilde Segura, Sara Calero and Dr. Carmen Rojo (Integrative Ecology Laboratory, ICBiBE, UV), the members of the Evolutionary Ecology Laboratory (ICBiBE, UV) and the staff of the Reserve Area Tancat de la Pipa in the AVNP for their laboratory and field assistance. Stoichiometric analyses were performed at the laboratory of Dr. Miguel Álvarez Cobelas (CSIC). English was edited by Daniel Sheerin (Online English S.C.).

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