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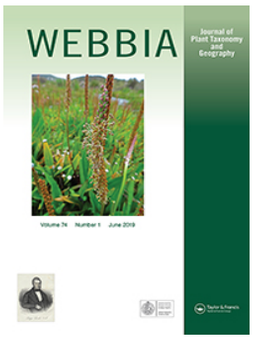

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ARTICLE

Germination capability of four charophyte populations (Characeae) from Mediterranean brackish ponds under warm experimental conditions

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ABSTRACT

The diaspore banks from two brackish ponds within the *Albufera de València* Natural Park (Spain) containing oospores of *Chara hispida*, *C. aspera*, *C. canescens* and *Nitella hyalina* were analysed and their germination capability studied under controlled temperature conditions. The top layer of sediment from each meadow was collected and sequentially sieved to isolate the charophyte oospores, subsequently identified and counted under the microscope. The density of apparently viable fructifications varied largely within the studied ponds and within specific meadows. In general, but not always, the most abundant oospores belonged to the species forming the meadow. An additional set of surficial sediment samples were collected to perform germination trials in an indoor culture room under three temperature treatments (21, 25 and 29°C). For each species, 219–272 fructifications were allocated inside small perforated packages that were placed in Petri dishes (n = 3) and submerged for 40 days in plastic containers. Germination rates were low for the four species in all temperatures (0–7%), with no large differences detected amongst treatments. *C. aspera* was the first species to germinate and the one with the most germlings. Higher temperatures negatively affected its germination; the same trend was shown by *C. canescens*. Dense oospore banks and low germination rates have been previously described for charophytes from temporary habitats. However, it seems that the recovery of charophyte meadows after droughts will be hindered by a warmer climate. Further studies concerning the optimal conditions for germination are needed to understand the future capability of charophyte meadows to re-establish in Mediterranean ecosystems.

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Charophyceae; diaspore bank; germination; high temperature; oospores

Introduction

Charophytes, commonly termed stoneworts, are worldwide-distributed benthic macroalgae from the family Characeae (Order Charales, Class Charophyceae, Division Chlorophyta), able to form dense meadows in freshwater, brackish and saline waterbodies (Schneider et al. 2015). Charophytes constitute one of the most abundant submerged macrophytes from Mediterranean ponds, playing a key role in ecological processes and water quality maintenance, e.g. having a negative influence on phytoplankton densities and improving water transparency (Rodrigo et al. 2015). As for most organisms inhabiting unpredictable environments, charophytes produce dormant stages, called oospores, which allow the persistence of species in the sediment after drying events or other disturbances (Brock et al. 2003; Rodrigo et al. 2010). Under the current scenario of climate change, predictions point to more frequent and harsher droughts in the Mediterranean region (IPCC 2014). Therefore, certain shallow ecosystems may turn from permanent to temporary waterbodies (Álvarez-Cobelas et al. 2005), and the submerged communities will inevitably face desiccation. Under this situation, the persistence of charophyte assemblages in particular will rely on two main aspects: (1) the number of drought-resistant propagules in the sediment (oospore bank), and (2) the ability of these oospores to germinate under favourable conditions, that is, their viability (Brock and Casanova 1991; Calero et al. 2018).

Charophytes produce a high number of small propagules, in accordance to the size–number trade-off of propagule formation (Grillas et al. 1993; Bonis and Grillas 2002). The oospore banks are long-lived, and multiple overlapping generations can accumulate in the sediment without breaking their dormancy for years (Brock et al. 2003; Rodrigo et al. 2010). Thus, oospore banks act as reservoirs that buffer the reproductive failure caused by environmental variability (Bonis et al. 1995). However, below a threshold of oospore density, charophytes may be unable to establish dense stands of vegetation (Bonis and Grillas 2002). In this context, a reliable estimation of the diaspore bank and its composition will allow prediction of the recovery success of submerged vegetation and the initial composition and diversity of the community after environmental disturbances (Jonsson 1993).

The diaspore banks from submerged macrophytes are evaluated by direct counts of all intact propagules in the

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sediment, which may present different dormancy stages. The oospore germination consists of two processes, breaking the dormancy and, subsequently, the induction of germination (De Winton et al. 2000). In this sense, germination trials allow the expression of the 'active seed bank', by counting the sporelings emerging from a given area or volume of sediment during a given period of time (Bonis and Grillas 2002). Several germination trials are based on the distribution of a thick layer of fresh sediment in a pot or a container (De Winton et al. 2004; Rodrigo et al. 2010). However, the isolation of individual oospores and their placement inside filters (to allow the emergence of the protonema, but not the exit of the oospore) allow the accurate monitoring of the development of a known number of oospores and the calculation of germination rates under different environmental conditions (Matheson et al. 2005; Rodrigo and Alonso-Guillén 2013; Puche and Rodrigo 2015).

One of the main stressors related to climate change is warmer temperature (Christensen and Christensen 2007; Giorgi and Lionello 2008). Temperature increase positively affects growth in charophytes from shallow ecosystems (Rojo et al. 2015, 2017), although they might be exposed to higher temperatures during spring and summer. However, little is known about the effect of these warmer temperatures on the oospore germination of charophytes. In previous works, we studied the life cycle and the reproductive phenology of four charophyte species related to environmental conditions, particularly underwater temperature, in two interdunal ponds located on the Mediterranean coast (Calero and Rodrigo 2018; Calero et al. 2018). Now, the aims of this study are (1) to determine the density of the oospore bank from the sediment of these ponds, and (2) to investigate, in laboratory experiments, the potential negative effect of increased temperature on the germination rates of these four species.

Material and methods

Study area and species

The sediment samples were collected in two brackish ponds within the *Albufera de València* Natural Park (Spain) (Figure 1). Both sites were created in 2007 as shallow permanent waterbodies under the European Union-funded project Life Enebro (2004–2008), whose main aim was to restore the seashore dune front and its associated waterbodies, called dune slacks. Annual precipitation in the region is 475 mm (average from 1989 to 2010; AEMET 2012), heterogeneously distributed throughout the year and with a typical summer drought. Thus, these Mediterranean ponds suffer dropping water levels and rising salinity in this season due to low precipitation and high evapotranspiration.

Four charophyte species grew in the ponds, forming monospecific meadows: *Chara hispida* (*Ch*), *Chara aspera* (*Ca*), *Nitella hyalina* (*Nh*) in Llacuna Nova del Canyar (LNC pond hereafter), and *Chara canescens* (*Cc*) in the 'Mallada Canescens' (MC pond). The two ponds are small (<60 ha) but differ considerably in size, shape, depth and proximity to the sea (Figure 1). The main difference between the ponds, which determines the species composition, is that the MC pond has almost twice the salinity (4.1 ± 1.1 g L^{-1} in the period 2014–2016, average ± SD) registered in the LNC pond (2.0 ± 0.5 g L^{-1} in the same period). For more details about both ecosystems and the studied species see Calero et al. (2015), Calero and Rodrigo (2018) and Calero et al. (2018).

Sediment potential in each meadow

Three sediment cores (diameter 5.7 cm, length 15 cm, surface 0.01 m^2) were used to collect fresh sediment from each site in late March 2017 (Figure 2(a)). The samples were stored in dark conditions at 4°C for a period of 4–10 days, depending on the species. Only the 3-cm top layer of the sediment was analysed, since the oospore density declines drastically below 4 cm (Bonis and Grillas 2002). The sediment was sequentially sieved by using different pore size sieves (1000, 500, 200 μm) and running water (Figure 2(b)). Apparently viable fructifications, i.e. those oospores not deformed when picked with forceps (Rodrigo and Alonso-Guillén 2013), were isolated, identified and counted under a binocular microscope (Olympus SZ61, 67.5× maximum magnification; Figure 2(c)). Different types of fructifications of *C. hispida* observed in the sediment were considered for counting: oospores preserving the oogonial integument, oospores without it, and gyrogonites (Figure 2(d)). Oospore numbers are expressed as density (×10^3 oospores m^{-2}).

Oospore germination trials

Three water temperatures were chosen as treatments: 21, 25 and 29°C. The criterion was based on the spring temperatures registered in the shallow waters of the ponds (Figure 3; Calero et al. 2017) and considering the foreseen increase of temperature predicted by the IPCC (2014). The same surficial sediment described above was used as a source of oospores of the different species. After less than 24 h of the identification and isolation of the fructifications, groups of 22–30 oospores (Figure 2(e)) were selected and introduced in 4 × 4 cm packages made with 200-μm pore size Nytal filter (SEFAR NITEX; three replicates per species; a total of 219–272 oospores per species) (Figure 2(f)). Each package was placed in a Petri dish containing sediment from the corresponding meadow. The package was buried by spreading on the top a thin layer of

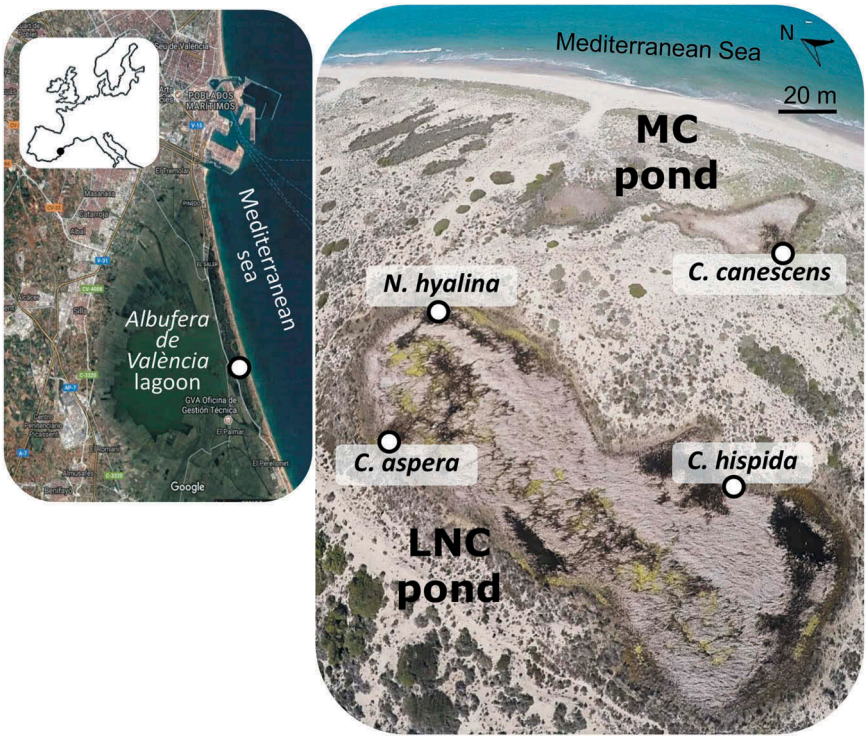

Figure 1. Location of the studied sites in the LNC and the MC ponds. The white dots indicate the sediment sampling sites. Aerial photograph taken by M. Lassalle with a kite.

the same sediment (approximately 0.5 cm), and commercial sand to avoid sediment resuspension (Figure 2(g)). All the sediment used in the experiment was autoclave sterilised (20 min, 121°C) to avoid the spontaneous germination of the seeds and the oospores contained within, as well as the potential competition with the tested oospores. Three Petri dishes per species and treatment were submerged in plastic containers with an 8-cm high water column (10-µm filtered water previously collected from the corresponding pond) (Figure 2(h)). The three species inhabiting the same pond (*C. hispida*, *C. aspera* and *N. hyalina*) were incubated in the same plastic containers. The experiment was performed in an indoor culture room at 20° C and artificial light (Sylvania Gro-Lux F58W tubes, 200–300 µmol photons m^{-2} s^{-1}, light:darkness 14:10 h), following the regular conditions used in the laboratory for charophyte cultures (Rojo et al. 2015, 2017). Higher water temperatures were achieved by means of aquarium heaters (Eheim 25W for 20–25 L).

Water conditions were monitored throughout the experiment. A probe provided with a data logger (Onset HOBO; UA-002 model) was set up in each container to measure underwater temperature and light radiation every half an hour. Salinity and pH were measured with portable field equipment (Multiline F/Set-3, WTW). To avoid a 'position effect' due to temperature and light heterogeneity, the position of the Petri dishes within the plastic containers were rotated every two days and the position of the plastic containers inside the culture room were rotated every four days. The containers were replenished every two days with deionised water to keep the water column and the salinity as constant as possible. After 38–44 days, when some germlings were already observed emerging from the sediment, the packages were dug up and opened. The number of germinated and damaged/broken oospores was counted; the length of the germlings was measured.

Statistical analyses

The normality and variance homogeneity criteria for the distribution of the number of germinated oospores, the number of broken oospores and the length of germlings were studied by the Shapiro Wilk and Levene tests, respectively. If the criteria were met, the means of the three variables in the three temperature treatments were compared by means of a one-way ANOVA per variable and per species, with *post-hoc* Tukey analyses; otherwise, non-parametric Kruskal–Wallis tests were used. Comparison of the water conditions between the temperature treatments during the germination trials was performed by means of one-way ANOVA for repeated measures. *Post-hoc* Tukey test was used for pairwise comparisons. Statistically significance was considered at $p < 0.05$.

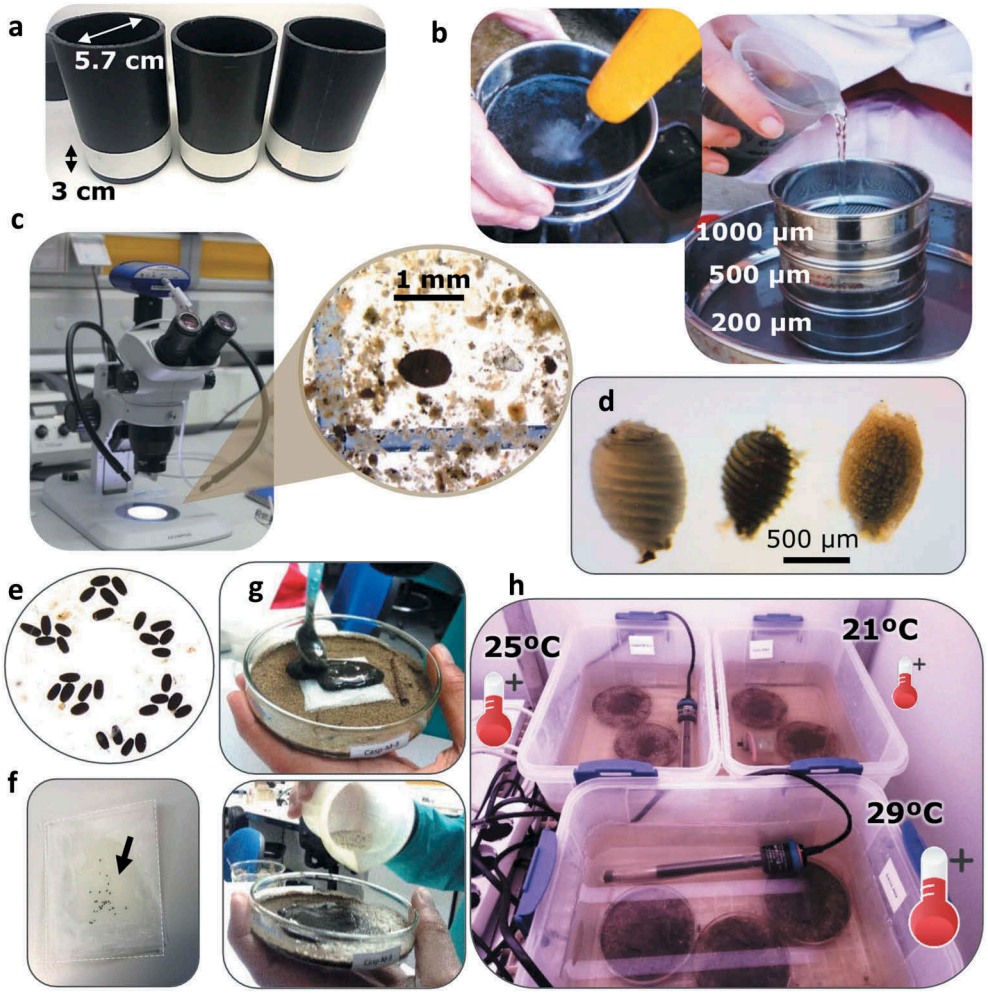

Figure 2. Outline of the procedures to obtain the oospores for density estimations and for the germination trials. (a) Sediment cores used to extract the sediment. (b) Sequential sieving of the fresh sediment by using different pore size sieves (1000, 500 and 200 µm). (c) Identification and counting of apparently viable fructifications. (d) The different types of fructifications of *Chara hispida* observed in the sediment and considered for counting (from left to right, in lateral view: an oospore preserving the oogonial integument, an oospore without it and a gyrogonite). (e) Thirty isolated oospores of *Chara canescens* ready to be placed inside the 200-µm pore size Nytal filter packages. (f) A 200-µm pore size Nytal filter package with the oospores inside (indicated with a black arrow). (g) The package is being buried in the sediment. (h) The incubation of *C. canescens* oospores under the three experimental temperatures in the culture room.

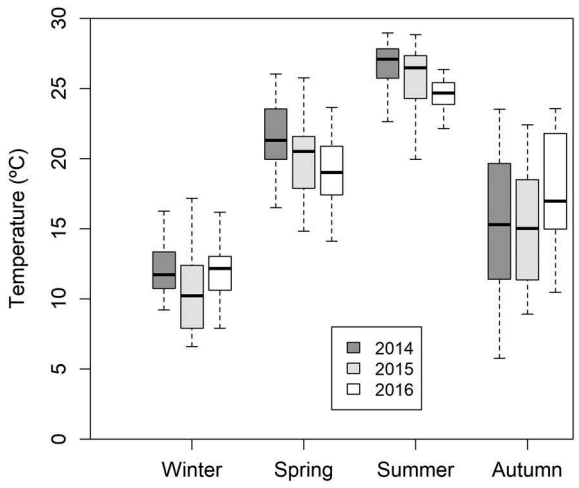

Figure 3. Box-plot of water daily mean temperature per season in a shallow site of the LNC pond for three consecutive years.

All statistical analyses were performed with the software IBM®SPSS® Statistics v22.0.0.0.

Results

Oospore density

In general, the total oospore bank in *Ch*, *Ca* and *Cc* meadows were quite dense, ranging from 5.2 to 53.7 × 10^3, from 34.5 to 55.5 × 10^3 and from 6.9 to 28.2 × 10^3 oospores m^{-2}, respectively. The oospore density of each species varied largely between the meadows (Figure 4(a)). The highest oospore density of each species was found at its own meadow, with values above 30 × 10^3 oospores m^{-2} in some replicates, except for *Nh* which reached higher densities where *Ch* and *Ca* grew, with maximum values of 16.1 and 23.3 × 10^3 oospores m^{-2}, respectively. For this species, in the absence of oospores

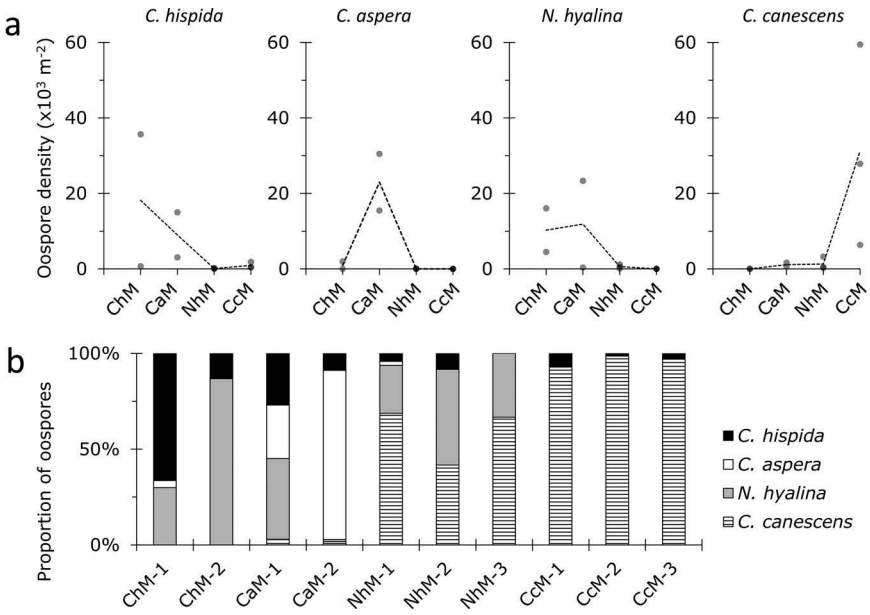

Figure 4. (a) Oospores density per species in the 3-cm top layer of the sediment cores taken at different charophyte meadows (M). The grey dots show each replicate (a darker colour indicates the overlapping of dots). The dashed lines connect the average value of each meadow. (b) Proportion of oospores per species in each sediment core (replicate). *Ch = Chara hispida; Ca = Chara aspera*; *Nh = Nitella hyalina*; *Cc = Chara canescens.*

from *Ch* and *Ca*, a density of 0.1–1.2 × 10^3 oospores m^{-2} was enough to establish the meadow.

In *Ch* and *Ca* meadows, a variable mix of oospores from all the studied species was found depending on the sample (Figure 4(b)). *Cc* oospores dominated the sediment bank at its own (93–99%) and *Nh* meadows (42–69%). *Nh* oospores dominated in meadows where it did not grow (87% in one replicate of the *Ch* meadow, and 42% in *Ca*). In *Nh* meadow, *Nh* and *Cc* oospores were mainly found.

Water conditions during the germination trials

The water pH at the beginning of the experiment was 8.0 for the LNC pond and 7.2 for the MC pond. Due to the imposed light:darkness cycle the water temperature fluctuated daily (Figure 5(a)), and this variation was sharper in the higher temperature treatments. In the case of the species from the LNC pond, the real average temperature during the experiment in the 21°C treatment was slightly below 21°C (Table 1). Underwater radiation changed during the light period between 10 and 30 µmol photons m^{-2} s^{-1} (Figure 5(b)). Initial salinity was slightly higher in the MC pond (2.1 g L^{-1} –4.1 mS cm^{-1} vs 1.7 g L^{-1} –3.6 mS cm^{-1} in the LNC pond). Due to the higher evaporation under the higher temperatures, the salinity achieved in the containers was significantly different between treatments (Table 1) (rep $ANOVA_{2,102}$ $F = 17.0$; $p < 0.001$ in the case of *Cc*; rep $ANOVA_{2,134}$ $F = 13.2$; $p < 0.001$ for the rest of the species), but only higher in the 25 and 29°C treatments than in the 21°C treatment.

Germination rates and germling length

Overall, the number of oospores that germinated over the experimental time was low, with rates lower than 10% in all cases (Figure 6(a)). *Ca* showed the highest proportion of germinating oospores, with higher rates in the lowest temperature (7.3 ± 2.5% in 21°C, average ± SE). For the rest of species, the highest germination rate was found at 25°C (2.2% in *Ch*,

Table 1. Summary of the water conditions achieved inside each plastic container during the germination trials (average ± standard deviation). Additionally, photosynthetic active radiation (PAR) supplied by the lamps in the incubation sites of each container is shown; it was measured with an atmospheric light meter (Li-Cor, LI-250 model). Ch-Ca-Nh container = where the species *Chara hispida*, *Chara aspera* and *Nitella hyalina* were incubated; Cc container = where *Chara canescens* oospores were incubated.

	21°C treatment		25°C treatment		29°C treatment		
	Temp. (°C)	Salinity (g L^{-1})	Temp.	Salinity (g L^{-1})	Temp.	Salinity (g L^{-1})	PAR (µmol photons m^{-2} s^{-1})
Ch-Ca-Nh container	20.2 ± 0.7	1.9 ± 0.2	24.4 ± 0.9	2.1 ± 0.2	28.3 ± 0.9	2.2 ± 0.4	298 ± 20
Cc container	21.0 ± 1.8	2.0 ± 0.2	24.8 ± 0.9	2.3 ± 0.3	28.3 ± 2.2	2.4 ± 0.5	206 ± 26

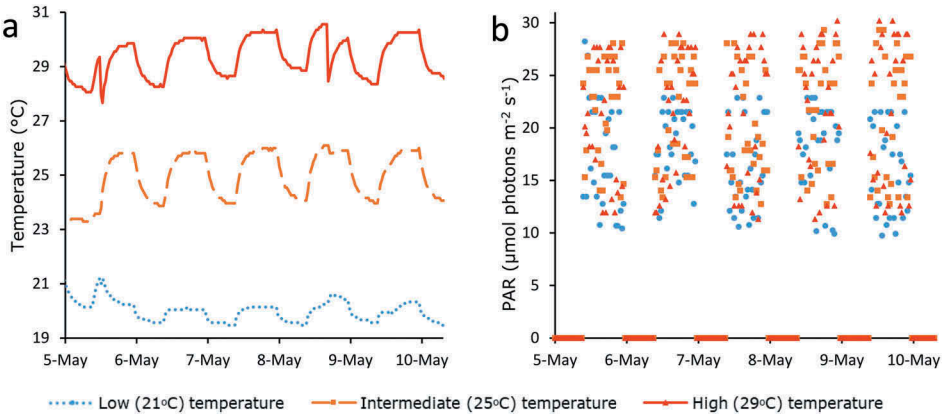

Figure 5. Daily fluctuations of (a) underwater temperature and (b) underwater light in the three treatments of the germination trials. As an example, both graphs only show 5-day data from the containers filled with water from the LNC pond. Light was measured in lux, but expressed as photosynthetically active radiation (PAR) after the calibration of the UA-002 sensor with a quantum sensor (Long et al. 2012).

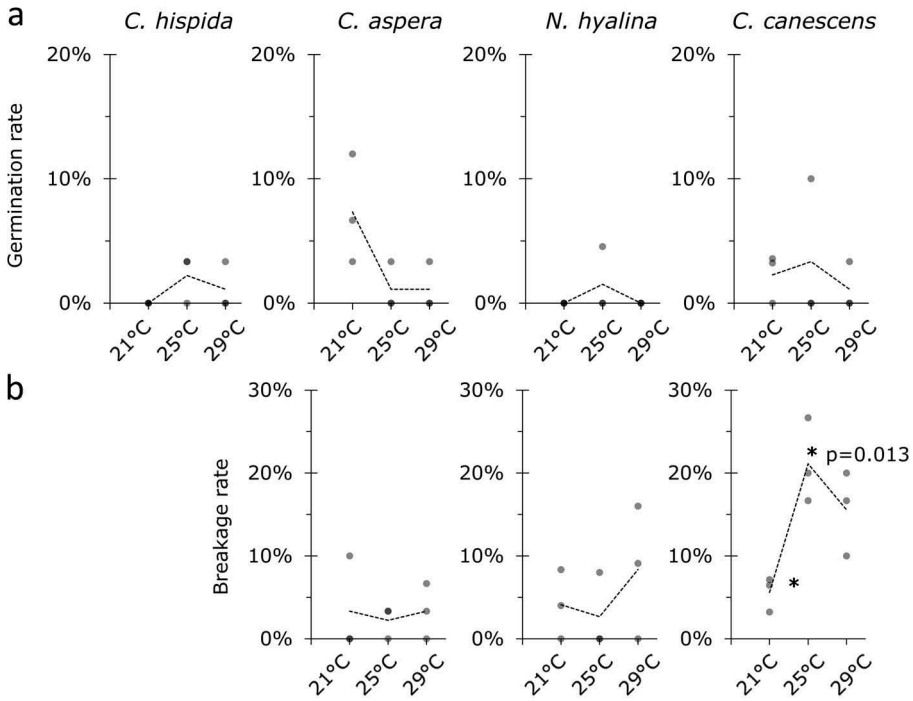

Figure 6. (a) Germination rates and (b) oospore breakage rates of the different charophyte species. The grey dots show each replicate (a darker colour indicates the overlapping of dots). The dashed lines connect the average value of each meadow.

3.3% in *Cc* and 1.5% in *Nh*). No significant differences were found in germination rates between treatments in any of the studied species ($p > 0.05$).

Since *Ca* was the first species to germinate (day 25), the longest germling was one of *Ca* in 21°C (13 mm; Figure 7). However, on average, *Cc* germlings growing at 21°C were the longest ones (9.6 ± 1.4 mm; Figure 7). The limited number of germinated oospores prevented the comparison of the germlings' length with statistical tests.

Oospore breakage rates

By the end of the experiment, some oospores of *Ca*, *Nh* and *Cc* were found damaged (literally broken). *Cc* showed the highest proportion (Figure 6(b)), with 5.6 ± 1.2% of broken oospores at 21°C, 15.6 ± 2.9% at 29°C and a significantly higher breakage rate under 25°C (21.1 ± 2.9%; $p = 0.013$). For the rest of the species, no significant differences were found between treatments ($p > 0.05$).

Discussion

High oospore densities

The oospore bank at the 3-cm top layer of sediment from the two studied ponds was quite dense in March 2017 (above 5×10^3 oospores m^{-2} in most replicates),

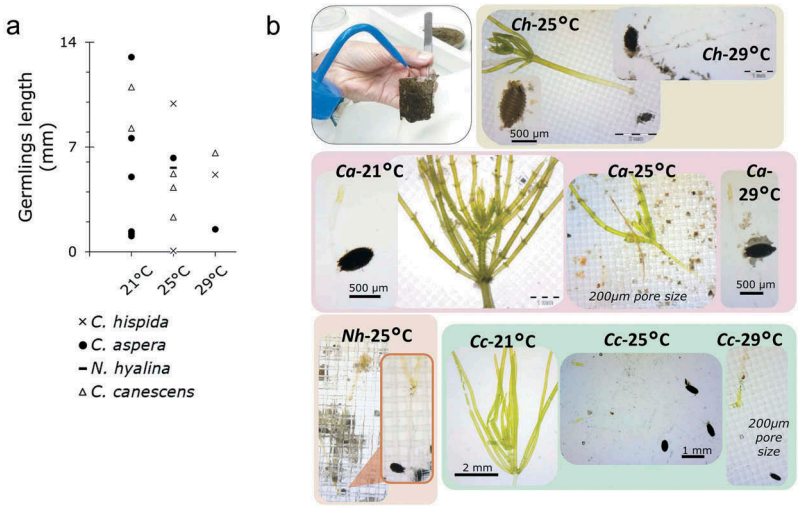

Figure 7. (a) Germling length. (b) A dug-out package and several germlings of the different charophyte species, indicating the temperature treatment in which they germinated. *Ch = Chara hispida; Ca = Chara aspera; Nh = Nitella hyalina; Cc = Chara canescens*.

showing values already recorded for temporary habitats (charophytes may reach values up to 1000 × 10^3 oospores m^{-2}; Bonis and Grillas 2002). Overall, it seems that charophytes need a high density of oospores to achieve a complete vegetation cover, particularly during colonisation of new habitats or after environmental disturbances (Van Den Berg et al. 2001). In this pond, our results indicate that a low density of oospores (0.1–1 × 10^3 oospores m^{-2}) was enough to establish the meadow of *N. hyalina*.

Within the studied ponds, the composition of the oospore bank varied locally, according to the high spatial variability described in other studies for charophytes (Bonis et al. 1995; Bonis and Grillas 2002). In the LNC and MC ponds, most of the variability in the oospore bank may be explained by the dominance of species in each area (i.e. which species formed each meadow). However, the abundance of charophyte shoots and the number of oospores in the sediment are not always necessarily related (Bonis and Grillas 2002; Van Onsem et al. 2018) because other processes may be involved. For example, the establishment of a *N. hyalina* meadow in the LNC pond occurred where the oospore density of this species was lower and only in the absence of oospores from *C. hispida* and *C. aspera*. This fact may be explained by the lower competitive capability of *Nitella* shoots (with a smaller size and a slow annual development) compared to other species (Baastrup-Spohr et al. 2015).

Low germination rates

The germination rates of the four studied populations were below 10%, close to the values found in other studies about charophytes (Van den Berg et al. 2001; Puche and Rodrigo 2015) and within the wide range described in the literature (Bonis and Grillas 2002; Sederias and Coleman 2007). Actually, the germination capability of the oospore bank is highly variable among species, sites and environmental conditions, and an accurate quantification requires the evaluation of the dormancy stage of oospores (Grillas et al. 1993; Bonis et al. 1995; Sederias and Coleman 2007; Holzhausen et al. 2018). The persistence of dormant propagules in the diaspore banks throughout favourable periods suggests that the cues for breaking dormancy are complex and diverse (Brock et al. 2003; Sederias and Coleman 20079), involving several interacting environmental variables and genetic factors. Particularly in variable and unpredictable habitats, such as the studied Mediterranean ponds, a high accumulation of oospores and low germination rates could be an adaptation to reduce the risk of population extinction during dry periods (Casanova and Brock 1996; Bonis and Grillas 2002).

Other reasons may explain the low germination rates found in this study, such as the duration of the experiment. In the germination trials performed by Casanova and Brock (1996), although 40 days was enough time in most of the cases, the stabilisation of the germination curves of some charophyte populations needed more days in some of the treatments. Additionally, the selection of 'apparently viable' oospores only based on their physical integrity may underestimate the viability evaluation. For charophytes, the diaspore viability has been traditionally determined by the 'crush test', based on the turgor and hardness of the oospore and the presence of inner starch after crushing it (Grillas et al. 1993; Bonis et al. 1995; Matheson et al. 2005; Sederias and Coleman 2007; Rodrigo et al. 2010). Holzhausen et al. (2018) recently applied the TTC test (triphenyltetrazolium chloride test), a method classically used for seeds of angiosperms, to test the enzymatic activity of charophyte

oospores and distinguish between 'still physiologically active' or 'definitely dead' oospores. However, as far as we know, no information about the physiological activity can be obtained without affecting the oospore and preventing its use for further germination experiments to test the effect of environmental factors. For further studies, viability testing and germination trials should be performed in parallel with different sets of oospores to try to solve this problem. The storage conditions of the sediment and the handling procedure involving the isolation of individual oospores (Casanova and Brock 1996) may have also reduced their viability.

Overall, we obtained a very low number of germinated propagules in the experiments. The isolation of oospores from the sediment of the LNC and the MC ponds was really time-consuming, and the number of fructifications obtained for the trials was not high enough. Probably with more oospores and more replicates by treatment we would have achieved higher statistical reliability. Nevertheless, our results can draw some preliminary clues about the influence of high temperature in the germination of charophytes. For further studies, several methodological issues still need to be solved, such as the increase in water salinity throughout the experiment or the uncontrolled daily fluctuations of temperature related to the presence or absence of experimental light.

Species-specific differences

The studied species showed distinct germination and breakage responses to the temperature treatments, which indicates that each species may present specific optimal conditions for germination (Casanova and Brock 1996). The oospores of *N. hyalina* showed the lowest success of germination, which explains the scarce representation of this species in the LNC pond in comparison to the other two species (Calero et al. 2015). *C. aspera* and *C. canescens* were the most reactive species to the experimental conditions: they germinated first and grew faster in all treatments. This response is consistent with their short life cycle and their annual character (Calero and Rodrigo 2018; Calero et al. 2018). Overall, the oospore bank and the germination success of different charophyte species do not necessarily mirror their frequency in the vegetation, but rather reflect different life-form strategies and environmental preferences (Brock and Casanova 1991; Grillas et al. 1993; Bonis et al. 1995).

No oospores of *C. hispida* were found broken by the end of the experiment. This might be related to the larger size of the oospores of this species in comparison to the rest (approximately 740 µm in length × 530 µm in width for *C. hispida*, 560 × 330 µm for *C. aspera*, 390 × 220 µm for *C. canescens* and 320 × 230 µm for *N. hyalina* from the studied ponds; unpublished data from the authors), which must confer more resistance to environmental conditions (Holzhausen et al. 2015). From our data, we cannot point out a clear reason causing the breakage of the *C. aspera* and *N. hyalina* oospores, since *C. canescens* was the only species to show statistically significant differences between temperature treatments, showing a high rate of oospore breakage after the exposure to 25°C. Additionally, a higher degree of calcification – which does not occur in *C. canescens* or *N. hyalina*– may have also improved the resistance of the *C. hispida* and *C. aspera* fructifications.

Negative effect of warm temperatures on germination

Although experimental higher temperature positively affects the growth of charophytes (Rojo et al. 2015, 2017), we conclude that high temperatures, meaning values reaching 25–29°C, do not favour oospore germination (no significant effect was found). These high temperatures can be easily reached during spring and summer in shallow systems such as the studied interdunal ponds (see Figure 3). It seems that the oospores of *C. canescens* were the most affected by the warmer temperatures: the lowest germination was found under the 29°C treatment, the longest germlings were recorded under the 21°C treatment and they were also the most fragile, with up to 15.6–21.1% of broken oospores by the end of the experiment under the 25 and 29°C treatments. It should be noted that these high water temperatures concurred with an increase in salinity conditions (see Table 1), despite the addition of deionised water to compensate evapotranspiration. Both factors (temperature and salinity) could be synergistically affecting the oospores from this experiment, in a similar way as expected for future climate change scenarios (Rojo et al. 2017). These negative effects are particularly worrying for *C. canescens*, because this species does not produce vegetative propagules for hibernation (such as *C. aspera* which produces bulbils; Blindow et al. 2003) and its annual recruitment mainly depends on the germination of oospores (Calero and Rodrigo 2018).

Conservation implications

During the restoration process of degraded and deteriorated aquatic ecosystems, revegetation is particularly essential to recover previous water quality conditions (Rodrigo et al. 2015; Blindow et al. 2016). Despite the low germination rates of charophytes, the high oospore densities may assure successful recovery of the meadows. For example, according to our results, more than 1000 germlings of *C. aspera* or *C. canescens* could emerge from each m^2 of the sediment from the studied ponds. However, the recovery of charophyte meadows seems

to be hindered by a warming climate. In the Mediterranean area, if temperatures rise rapidly during spring, as predicted (Christensen and Christensen 2007; Giorgi and Lionello 2008), the optimal conditions for germination and establishment of each species could be restricted to a short time period, affecting the phenology of charophytes. Moreover, species-specific germination capabilities may lead to a shift in the composition of charophyte communities. Other factors related to the increase in temperature, such as salinity enhancement, could also affect negatively the germination of certain species (Puche and Rodrigo 2015), clearly favouring the more halophile ones.

Further studies about the diaspore bank potential and the germination of oospores under different environmental conditions are needed to understand the capability of charophyte meadows to re-establish, and for better predictions of the future situation of shallow Mediterranean ecosystems. With this article, we hope to encourage other researchers to work towards overcoming all the methodological issues that prevent us from unravelling the germination capability of oospores in response to interacting environmental factors, such as temperature, light and salinity.

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