Water ecological requirements of Characeae taxa in eastern Spain

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Abstract

Presence of Characeae taxa is limited by the existence of clear and oligotrophic waters. Some other chemical water parameters can also influence the distribution of taxa, which can thus be used as ecological bioindicators. The area of eastern Spain contains a high diversity of water basins in both coastal and inland habitats that allow the study of ecological gradients. This work aimed to identify the most relevant and significant water chemical parameters that determine the distribution of Characeae taxa, and establish their optima and tolerance ranges for each parameter in eastern Spain. Ninety-six records corresponding to unpublished old and recent samplings of the presence of 17 taxa belonging to the genera *Chara*, *Nitella*, *Tolypella* and *Lamprothamnium* were related to water parameters that included salinity, pH, electric conductivity, total water hardness, alkalinity, concentrations of Na^+, Mg^{2+}, Ca^{2+}, Cl^-, and SO_4^{2-}, and Mg^{2+}/Ca^{2+} ratio. Principal Component Analysis showed that salinity was the major factor that determined the distribution of Characeae taxa, followed by concentration of Mg^{2+}, Mg^{2+}/Ca^{2+} ratio and alkalinity. When previously published records from the same area were added, non-parametric tests showed significant differences among taxa only for salinity, water hardness, and Mg^{2+}/Ca^{2+} ratio. These statistical analyses, along with optima and tolerance ranges for each parameter showed that Characeae taxa, especially *Lamprothamnium papulosum* and *Tolypella* spp., could be used as bioindicators in eastern Spain, although their ecological differentiation is not clear in many cases.

Keywords: biogeography; bioindication; charophyte; ecological gradient; water quality

Introduction

The family Characeae (Charophyceae *sensu stricto*) is one of the five major lineages that compose Charophyte green algae, which are the most closely organisms related to land plants (Karol *et al.*, 2001). It comprises more than 200 extant species grouped in six genera: *Chara*, *Lamprothamnium*, *Nitellopsis*, *Lychnothamnus*, *Nitella*, and *Tolypella*. Characeae are distributed in all the continents, and include both extant and fossil specimens (Schneider *et al.*, 2015; Schubert *et al.*, 2018).

The Characeae, together with some vascular macrophytes, constitute the main plant biodiversity of underwater meadows in lagoons with lentic, fresh and brackish waters. These meadows usually have low phytoplankton production that results in very clear water. They provide shelter from predators and a place of

development for the juvenile stages of fauna, thus regulating the balance at the base of the trophic pyramid (Barbosa *et al.*, 2021). They also play an important role in colonizing new habitats and stabilizing sediments (Sleith *et al.*, 2018).

Unlike vascular macrophytes, most Characeae require certain levels of water physical parameters, such as transparency that is directly related to seabed substrate (muddy or sandy), water depth (Torn *et al.*, 2015), altitude and waterbody size (Rey-Boissezon and Auderset-Joye, 2015), while other traits are less required or non-determinant, such as the water temperature (Wood, 1952). Water regimes that may include drying wetlands in summer also appear to determine the distribution of species. Some chemical parameters of the water also limit the presence of most Characeae, which cannot survive to high levels of eutrophication that are associated with the high concentrations of organic matter (Barbosa *et al.*, 2021; Bonilla *et al.*, 2023). Eutrophication is the main cause of the proliferation of cyanobacteria in the water. These cyanobacterial blooms may result in a pronounced day - night fluctuations of solved O_2 because of differential algal photosynthesis and respiration rates during the day and a decrease in transparency (Blindow, 2000). Human activities, such as agriculture and construction of irrigation canals in lakes, have led to a higher nutrient influx in wetlands that has been causing the progressive decline of most Characeae (Blindow, 2000; Cirujano *et al.*, 2008; Steinhardt and Selig, 2011).

Besides the general requirement of transparent and oligotrophic waters, the different Characeae species have differential tolerances to other water parameters, such as pH (Wood, 1952; Schubert *et al.*, 2018), water hardness (Blindow, 2000), alkalinity (Torn *et al.*, 2015), and especially salinity (Brock and Lane, 1983).

High pH values can cause an increase in the contents of carbonates and bicarbonates in the water and can also affect plant cell formation and photosynthesis rates. Most Characeae species develop in waters with high pH values, although optimal ranges of pH vary among species partly because of their relative ability to take up or export ions (Beilby *et al.*, 2022). On the other hand, most Characeae species prefer waters with high alkalinity. Most Charophytes are able to use soluble bicarbonates as a source of carbon dioxide during photosynthesis, releasing OH^- ions and consequently increasing pH. This ability depends on the species, i.e. *Chara aculeolata* Kützing, *Chara fragilis* Desv. in Loisel., *Chara hispida* L. and *Chara subspinosa* Ruprecht efficiently use bicarbonate in photosynthesis (Sand-Jensen *et al.*, 2018), while *Chara braunii* Gmelin can use bicarbonates as a carbon source in short time periods but relies on other carbon acquisition mechanisms in the long term (Heise *et al.*, 2023).

Charophytes also play a significant role in water calcium cycling and in its immobilization in sediments because the use of bicarbonates as a source of carbon in photosynthesis leads to the formation of insoluble calcium carbonate when calcium is present in the water, thus preventing an excessive pH rise. Charophytes accumulate calcium as calcite encrustation in their tissues, although the amount of calcium accumulation depends on the species thallus morphology (Apolinarska *et al.*, 2011). In several species such as *Chara fibrosa* Agardh ex Bruzelius the high concentrations of calcium cause retardation in shoot elongation and reduce the chlorophyll content (Asaeda *et al.*, 2014). High concentrations of magnesium can also lead to a precipitation of aragonite instead of calcite, and a ratio of Mg^{2+}/Ca^{2+} below or above 2 has been shown to determine whether precipitation of calcite or aragonite occurs (Apolinarska *et al.*, 2011). In *Chara*, the presence of magnesium reduces the calcification of Charophytes and slightly decreases the stress caused by the high abundance of calcium (Asaeda *et al.*, 2014).

Finally, Characeae species form a continuum from very salt sensitive to very salt tolerant species (Beilby, 2015). Salt content hinders the growth of aquatic vegetation because of its physiological effects such as the ion toxicity (mainly Na^+) or the difficulties in extracting water from the surrounding medium (Hart *et al.,* 1991). All the salt-tolerant Characeae taxa possess, as an outstanding feature, ability to completely regulate cell turgor pressure through adjusting of cell size and K^+, Cl^-, Na^+ and sucrose concentrations in the cell vacuoles (Beilby, 2015). In the Mediterranean region, salinity and water regime appear to be closely linked because of the human management of marshes (Grillas *et al.*, 1993).

The Iberian Peninsula contains a wide variety of water basins which display different hydromorphological and ecological patterns, especially in coastal marshes of eastern Spain. This area is characterized by the presence of rivers that carry water permanently, such as Mijares, Turia and Júcar, several ravines that receive the seasonal rainfall of autumn and spring, and coastal marshes that occupy the plains on the seashore. In eastern Spain, most records and chorological studies of Characeae taxa have been performed in the last decades of the XXth century. In some cases, ecological data were provided, but mainly as range values that lacked means or individual records (Boira and Carretero, 1985; Carretero, 1993), and as far as we know no ecological gradients were analyzed.

The aim of the present work is to analyze the ecological gradients of chemical parameters of the water where different species of Characeae were established in eastern Spain. The questions we addressed are: (i) What are the most relevant water parameters that determine the distribution of Characeae in eastern Spain? (ii) Are there significant differences in these parameters among taxa? (iii) Which are the optima and tolerance ranges of these water parameters for each Characeae taxon that may allow their use as bioindicators in eastern Spain?

Materials and Methods

Sampling of Characeae and taxonomic adscription

The localities studied were selected from previous field works that focused on the chorology of Characeae in eastern Spain (Boira and Carretero, 1985; Carretero, 1993; Rodrigo and Alonso-Guillén, 2016).

Sampled locations included varied ecological habitats, especially related with salinity gradients, which were colonized by different Characeae taxa (Figure 1). Coastal areas with strong marine influence were located from Peñíscola in north Castellón to Pego in north Alicante, covering circa 400 km of littoral. In the same latitudinal interval, post littoral areas subjected to prolonged annual flooding caused by continental water flows (rivers and aquifers) and with little marine intrusion (marshes and paddy fields) were also sampled. Finally, small inland lagoons, ponds and ditches with permanent, fresh and oligohaline waters were included. In the coastal locations, water came from post-dune lagoons and from evacuation channels of the irrigated crop areas. In the inland locations, water flows coming from the Júcar and Turia rivers, drainage ditches of anthropic origin, basins that received irrigation surpluses (2-5 m in diameter and 1-1.5 deep) and small lagoons determined the differences in water chemistry and the distribution of species.

Characeae specimens and water samples were mostly collected during the years 1982, 1983 and 1984. During these years, mean temperature in eastern Spain (Valencian Community) was 15.3 °C, 15.6 °C, and 14.6 °C respectively, while annual rainfall was 631 l m^{-3}, 308 l m^{-3}, and 405 l m^{-3} respectively (AEMET, 2023). In 2022, mean temperature in eastern Spain was 16.9 °C and annual rainfall was 687 l m^{-3}. Then, the locations were revisited and we could not find most of the recorded Characeae because of habitat losses and lower water quality. However, we were able to find some new locations in the Albufera Natural Park, close to the city of Valencia.

In all the sampling years, collection of Characeae taxa and water samples were carried out in June, July, and August, when algae reached their maximum development. For a longer-term conservation, Characeae specimens were kept alive in aquaria on a sandy substrate using deionized water and oxygen pumps until their complete identification, while water samples were kept at -4 °C until they were analyzed in the laboratory.

The taxonomic adscription of taxa follows Cirujano *et al.* (2008), who performed an identification key of the Iberian Charophytes included in the project "Flora Ibérica" (*http://www.floraiberica.es*).
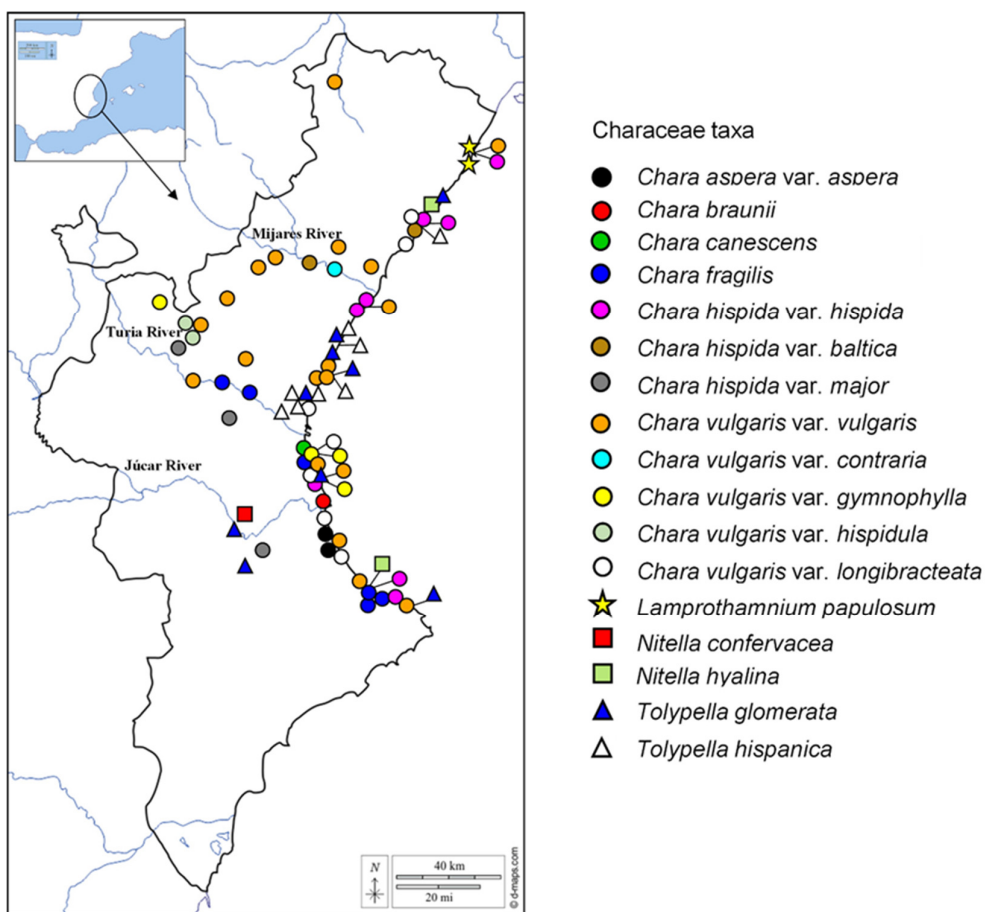
Figure 1. Location areas of the sampled Characeae populations in coastal, inland pools and lagoons in eastern Spain (provinces of Castellón, Valencia and Alicante)

(Source of the map: *https://d-maps.com/carte.php?num_car=14387&lang=es*)

Measurement of water ecological parameters

For all the samples, water pH, electric conductivity (EC) in mS cm^{-1} and salinity (mg l^{-1}) were determined *in situ* using a multiparametric probe (HANNA-Mod HI 9828).

For the samples collected from 1982 to 1984, concentration of the cations Na$^+$ (meq l^{-1}), Ca^{2+} (meq l^{-1}), Mg^{2+} (meq l^{-1}), and anions Cl$^-$ (g l^{-1}), SO$_4^{2-}$ (g l^{-1}), and HCO$_3^-$ (meq l^{-1}) was estimated following the Spanish official laboratory procedures (MAPA, 1984). Briefly, cations were measured using a flame photometer (Corning Clinical Flame Photometer 410C), Cl$^-$ using a chloride analyzer (Corning Chloride analyzer), SO$_4^{2-}$ by precipitation of BaSO$_4$ after adding BaCl$_2$; and HCO$_3^-$ by colorimetric methods.

For the samples collected in 2022, the same model of multiparametric probe was used *in situ*. In the laboratory, most of the water parameters were estimated following the standard methods of Baird and Bridgewater (2017). Briefly, concentration of the cations Na$^+$ and Ca^{2+} were measured using a flame photometer, while Mg^{2+} was estimated by the EDTA titrimetric method. Concentration of Cl$^-$ was measured by the argentimetric method and SO$_4^{2-}$ by the turbidimetric method. Finally, concentration of HCO$_3^-$ was estimated by colorimetric methods. Total hardness of the water was calculated as the sum of the concentrations of Ca^{2+} and Mg^{2+}. The ratio Mg^{2+}/Ca^{2+} was also calculated by dividing the corresponding concentrations following Schubert *et al*. (2018).

Statistical analyses
All the statistical analyses were performed using different packages of the statistical software R v.4.2.2.

Correlation among water parameters
The correlation between pairs of water parameters was estimated with the Pearson coefficient using the package "stats".

Relationship between presence of Characeae taxa and water parameters
A Principal Component analysis (PCA) was carried out to examine interrelations among the chemical water parameters, identify the most important ones, and visualize the groupings of ecologically related taxa. Since registered data contained some missing values, the package "missMDA" (v.1.18) (Josse and Husson, 2016) was used to replace them with plausible values by considering the similarities between water samples and the relationship between water parameters. Then, PCA was performed using the package "FactoMineR" (v.2.8) (Lê *et al.*, 2008). Two-dimensional graphs were created using the package "Factoextra" (v.1.0.7) (Kassambara and Mundt, 2020).

Differences in water parameters among Characeae taxa
For each group where parameters appeared very highly correlated (Pearson correlation coefficient > 0.9), we selected the one that was more widely recorded in the literature to check significant differences among taxa. To increase the number of samples, we added to the study the records of single pairs of parameter and presence of taxa (we did not consider the data that included only averages), which were already performed previously in the studied area (Table 1). Only taxa that had at least three independent measurements in a given parameter were analyzed. For each parameter, the Levene's test and the Shapiro-Wilk test were used to check equal variances ($p > 0.05$) of data and normal distributions ($p > 0.05$) of residuals of the ANOVA model, respectively. In our case, ANOVA residuals were not normal in all the tested parameters. Consequently, the nonparametric Kruskal-Wallis test was used to check significant differences among data instead of ANOVA. In the cases where significant differences were found, pair-wise Dunnett's tests were carried out. These statistical analyses were performed using the packages "dplyr" (v.1.1.2) (Whickham *et al.*, 2023), "car" (v.3.1-2) (Fox and Weisberg, 2019), and "dunn.test" in R.

Table 1. Source of records of pairs water parameter / presence of Characeae taxa in eastern Spain used to estimate significant differences among taxa and the optimum values and tolerance ranges of each parameter for each taxon. For each source of information, location of Characeae records and measured water parameters are provided. N: number of single data, EC: Electric conductivity

Reference	Study location	N	water parameters
Own results	Valencian Community (Castellón, Valencia and Alicante)	96	pH, EC, salinity, HCO_3^-, Na^+, Cl^-, SO_4^{2-}, Ca^{2+}, Mg^{2+}
Rodrigo and Alonso-Guillén, 2016	Albufera Natural Park (NP) (Valencia)	37	Salinity, pH
Carretero, 1993	Valencia province	9	pH, EC, Cl^-, SO_4^{2-}, Ca^{2+}, Mg^{2+}
Puche and Rodrigo, 2015	Albufera NP – Tancat de la Pipa	2	EC, salinity, HCO_3^-, Na^+, Cl^-, SO_4^{2-}, Ca^{2+}, Mg^{2+}
Romo *et al.*, 2007	Xeresa lake (Valencia)	6	pH, EC
Calero *et al.*, 2015	Albufera NP – Llacuna Nova del Canyar	4	pH, EC, HCO_3^-, Na^+, Cl^-, SO_4^{2-}, Ca^{2+}, Mg^{2+}
Puche *et al.*, 2018	Almenara – Quartons spring	2	pH, EC

Optimum values and tolerance ranges for water parameters

Sensitivity species to each water parameter was assessed using package Optimos Prime (v.0.1.2) (Sathicq *et al*., 2020). The package calculates the optimum value and tolerance ranges by weighted averaging, using the number of occurrences to adjust the tolerance assigned to each taxon following Potapova and Charles (2003).

Results

Sampling of Characeae and taxonomic diversity

A total of 17 taxa corresponding to the genera *Chara*, *Nitella*, *Tolypella* and *Lamprothamnium* were recognized in the studied areas (Table 2, Figure 1).

Table 2. Characeae taxa and locations where they were found in eastern Spain (N – number of samples)

Taxa	N	Locations	Year
Chara aspera var. *aspera*	2	Seasonally flooded grasslands of Xeresa (39°01'02"N 0°12'03"W) and Tavernes de la Valldigna (39°03'35"N 0°12'21"W)	1983
Chara braunii	1	Paddy field in Cullera (39°11'33"N 0°15'42"W)	1984
Chara canescens	1	Interdunal brackish pond in La Albufera Natural Park (39°20'44"N 0°18'50"W)	2022
Chara fragilis	5	Pond with permanent water in Oliva - Molinell (38°53'19"N 0°3'28"W), and irrigation channels in Llíria – Casinos (39°40'7"N 0°40'40"W) and Silla (39°21'10"N 0°23'29"W)	1983, 2022
Chara hispida var. *baltica*	2	Pond in Torreblanca – Ribera de Cabanes (40°10'58"N 0°10'43"E), Mijares river (39°54'43"N 0°0'53"W)	1982, 1984
Chara hispida var. *hispida*	12	Ponds and irrigation channels in Oliva – Molinell (38°54'4"N 0°4'2"W), Ponds in Ribera de Cabanes – Torreblanca (40°11'31"N 0°12'38"E), Nules – Burriana (39°52'4"N 0°6'27"W), and La Albufera (39°19'42"N 0°21'12"W), salt marsh drainage channel in Peñíscola (40°22'29"N 0°24'12"E)	1982, 1984
Chara hispida var. *major*	3	River in Chulilla – la Llosa (39°39'28"N 0°53'42"W), irrigation channels in Canals (38°56'59"N 0°35'35"W) and Chiva (39°28'38"N 0°42'45"W)	1983
Chara vulgaris var. *contraria*	1	Mijares river (39°55'24"N 0°2'30"W)	1984
Chara vulgaris var. *gymnophylla*	4	Paddy fields in Albal (39°24'5"N 0°23'37"W), El Palmar (39°18'47"N 0°19'17"W) and Sollana (39°16'40"N 0°21'32"W), stream in La Yesa – Alpuente (39°52'59"N 1°0'42"W)	1983, 1984
Chara vulgaris var. *hispidula*	2	Streams in Higueruelas (39°47'6"N 0°51'45"W), and Villar del Arzobispo – Domeño (39°39'24"N 0°40'29"W)	1982
Chara vulgaris var. *longibracteata*	7	Paddy fields in Catarroja (39°23'9"N 0°22'11"W) and Cullera (39°12'4"N 0°14'48"W), irrigation channels in Puebla de Farnals (39°34'15"N 0°17'5"W) and Albal (39°24'12"N 0°23'37"W), ponds in Gandía – Xeresa (39°0'37"N 0°11'22"W), Oropesa, and Torreblanca (40°10'38"N 0°10'49"E)	1981, 1982, 1983, 2022
Chara vulgaris var. *vulgaris*	26	Irrigation channels, ponds, paddy fields, flooded fields, streams in Canet d'en Berenguer (39°42'46"N 0°12'24"W), El Puig (39°35'21"N 0°16'35"W), Pego, Oliva – Molinell (38°54'09"N 0°4'3"W), Gandía (38°59'34"N 0°10'43"W), Nules (39°45'29"N 0°10'42"W), Higueruelas (39°46'03"N 0°51'3"W), Sagunto (39°37'15"N 0°15'36"W), Bejís (39°54'24"N 0°42'35"W), Bugarra (39°36'17"N 0°46'51"W), Gestalgar (39°36'10"N 0°50'12"W), Puebla de Tornesa (40°05'45"N 0°0'13"E), Fanzara (40°1'27"N 0°19'14"W), Montanejos (40°4'11"N 0°31'43"W), Cirat (40°3'16"N 0°27'25"W), Silla (39°20'53"N 0°22'56"W), la Mata, Peñíscola (40°22'36"N 0°24'6"E)	1982, 1983, 1984, 2022
Lamprothamnium papulosum	2	Salt marsh drainage channel in Peñíscola (40°22'26"N 0°24'13"E)	1984
Nitella confervacea	1	Lagoon in Navarrés (39°08'5"N 0°39'35"W)	1983
Nitella hyalina	2	Irrigation channel in Ribera de Cabanes (40°10'24"N 0°10'3"E), pond with permanent water in Oliva - Molinell (38°52'19"N 0°3'1"W)	1982, 1983
Tolypella glomerata	14	Irrigation channels in Pego (38°51'28"N 0°1'53"W), flooded saltmarshes in Ribera de Cabanes – Torreblanca (40°11'39"N 0°12'30"E) and Sagunto, flooded fields in Chilches, Sagunto and El Puig (39°37'18"N 0°15'47"W), pond in Navarrés (39°8'5"N 0°39'35"W), river in Xàtiva – Genovés (38°59'0"N 0°30'56"W), and paddy fields in la Albufera (39°18'27"N 0°21'40"W)	1982, 1983
Tolypella hispanica	11	Flooded fields in Almenara, Chilches, El Puig, Sagunto and la Puebla de Farnals (39°39'37"N 0°15'31"W), flooded saltmarsh in Ribera de Cabanes and Sagunto (40°10'41"N 0°11'2"E), pond in El Puig (39°34'5"N 0°17'02"W)	1983, 1984

The most common taxon was *Chara vulgaris* var. *vulgaris* L. that was present in both coastal and inland water basins. *Tolypella glomerata* (Desv.) Leonhardi, *Tolypella hispanica* Nordst. ex T.F. Allen., *Chara hispida* var. *hispida* L. and *Chara vulgaris* var. *longibracteata* (Kütz.) J. Groves and Bull.-Webst. were also common in coastal locations. By contrast, *Chara aspera* var. *aspera* Dethard ex Will, *Chara braunii*, *Chara canescens* Desv. and Loisel. in Loisel., *Lamprothamnium papulosum* (Wallr.) J. Groves and *Nitella hyalina* (DC) C. Agardh. were rarely found in coastal areas of eastern Spain, while *Chara hispida* var. *baltica* (Bruzelius) R.D. Wood and var. major (Hartm.) R.D. Wood, *Chara vulgaris* var. *contraria* (A. Braun ex Kütz.) Moore and var. *hispidula* (A. Braun) Moore, *Nitella confervacea* (Bréb.) A. Braun ex Leonhardi and *N. hyalina* were also rare in inland water basins.

Water ecological parameters

All the water samples showed high levels of transparency. Values of all the measured water parameters for each taxon are shown in supplementary material 1. A very high correlation (Pearson coefficient > 0.9) was found among the parameters related to salinity, corresponding to EC, salinity and concentrations of Na^+, Cl^-, and SO_4^{2-}. A very high correlation was also found between parameters related to water hardness: $Ca^{2+}+Mg^{2+}$ and Ca^{2+} on the one hand, and $Ca^{2+}+Mg^{2+}$ and Mg^{2+} on the other (supplementary material 2). From each of these three groups of highly correlated parameters, we selected salinity and concentrations of Ca^{2+} and Mg^{2+} respectively. As a whole, pH, salinity, HCO_3^-, Ca^{2+}, Mg^{2+} and Mg^{2+}/Ca^{2+} were selected for further analyses of differences among taxa.

The water of all the sampled localities was neutral to alkaline, with the pH ranging from 6.95 (in the coastal locality of Canet d'en Berenguer, Valencia) to 10.1 (in a flooded field near the coast in El Puig, Valencia), with a mean of 7.9 ± 0.5. The salinity was very variable, ranging from 307 mg l^{-1} of salt in a lagoon of Navarrés (inland Valencia) to 26767 mg l^{-1} in the saltmarshes of Peñiscola (Castellón), with a mean of 2546 ± 5366 mg l^{-1}. Alkalinity was medium to high: concentration of HCO_3^- ranged from 1.4 meq l^{-1} in a paddy field near the coast in El Saler (Valencia) to 16.2 meq l^{-1} in an irrigation channel in Chiva (inland Valencia), with a mean of 5.4 ± 3.1 meq l^{-1}. Sampled water basins also showed a high-water hardness. The concentration of Ca^{2+} varied from 3.3 meq l^{-1} in a paddy field from El Saler to 56.1 meq l^{-1} in a flooded field near the coast of Sagunto (Valencia), with a mean of 15.7 ± 13.8 meq l^{-1}. The concentration of Mg^{2+} ranged from 1.2 meq l^{-1} in a channel near the coast in Molinell to 83.5 meq l^{-1} in the same location where the highest concentration of Ca^{2+} was recorded, with a mean of 15.7 ± 17.9 meq l^{-1}. Finally, the Mg^{2+}/Ca^{2+} ratio ranged from 0.07 in Molinell, where the lowest value of concentration of Mg^{2+} was recorded, to 4.6 in a flooded saltmarsh of Ribera de Cabanes (Castellón). However, excepting this exceptionally high value that may be due to anthropogenic activities, all the remainder values of Mg^{2+}/Ca^{2+} ratios were below 2, with a mean of 1.0 ± 0.7.

Relationship between presence of Characeae taxa and water parameters

The three first components of the PCA accounted for 66,5%, 13.4% and 9.3% of the total variance, respectively (the cumulative variance was 89.2%, Figure 2). In agreement with the Pearson correlation coefficients (supplementary material 2), the parameters related to salinity (EC, salinity, and concentrations of SO_4^{2-}, Na^+ and Cl^-) appeared highly correlated among them and also to the parameters related to water hardness (concentrations of Ca^{2+}, Mg^{2+} and $Ca^{2+}+Mg^{2+}$). Salinity was the most important ecological variable related with the distribution of the 17 recorded taxa, and contributed 62.6% as a whole to the first principal component, while water hardness contributed only for 34.2% (supplementary material 3, Figure 2A). In contrast, concentration of HCO_3^- and Mg^{2+}/Ca^{2+} ratio were the parameters that most contributed to principal component 2 (44.0% and 38.8% respectively), and pH parameter contributed mostly to the principal component 3 (44.0%).

According to the first principal component, individuals of *Lamprothamnium papulosum* were clearly separated from other taxa (Figure 2B and C). According to the second principal component, although

individuals of the different taxa appeared highly intermingled, *L. papulosum* and *C. hispida* var. *hispida* required waters characterized by a lower alkalinity and Mg^{2+}/Ca^{2+} ratio while the opposite occurred mainly with *C. hispida* var. *major*, *C. vulgaris* var. *hispidula* and *T. hispanica*. The third principal component showed no clear grouping of individuals (supplementary material 4).
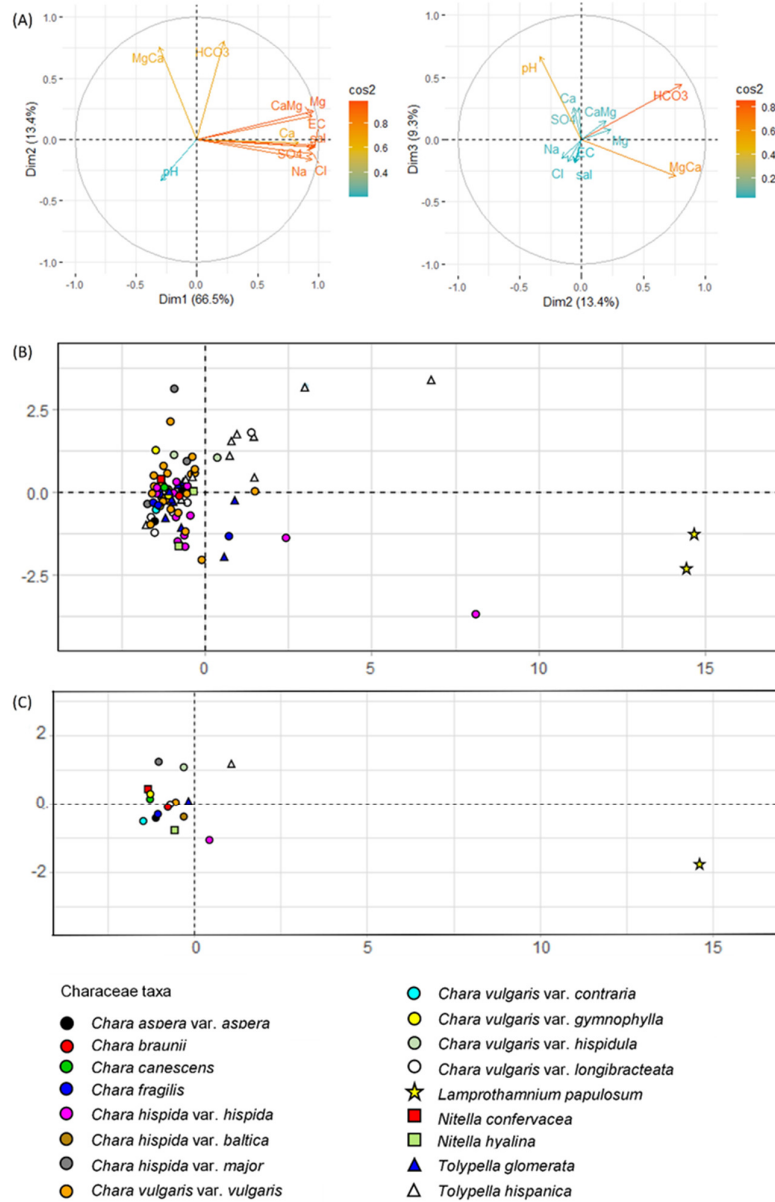

Figure 2. PCA of the 17 Characeae taxa growing in eastern Spain using the water parameters; (A) Contribution of each parameter to the three first components. Colors and vector length correspond to squared cosines that express the contribution level of a given parameter to a component (Abdi and Williams, 2010); (B) Grouping of individuals according to component 1 (x-axis, 66.54% of the total variation) and component 2 (y-axis, 13.43%); (C) Centroids corresponding to the individuals of each taxon according to components 1 and 2

A new PCA was performed without the individuals of *L. papulosum* to better analyze the relationships between the remainder Characeae taxa and the water parameters of the locations where they developed. In this case, the three first components of the PCA accounted for 61.9%, 14.6% and 10.5% of the total variance, respectively (the cumulative variance was 87.0%). While salinity parameters appeared highly correlated, concentrations of Ca^+ and Mg^+ were less correlated. Salinity was again the most important ecological variable related with the distribution of the Characeae taxa and contributed for 60.8% as a whole to the first principal component, while concentration of Mg^+ contributed for 11.8% (supplementary material 3, Figure 3A). In contrast, Mg^{2+}/Ca^{2+} ratio and concentration of HCO_3^- were the parameters that most contributed to principal component 2 (41.9% and 32.4% respectively), while none of the parameters contributed significantly to component 3 (Figure 3A).

According to the first principal component, individuals of *Tolypella hispanica*, *T. glomerata*, *Chara hispida* var. *hispida* and var. *baltica* were slightly separated from the rest, and developed in more saline waters, although many individuals of these taxa appeared intermingled with those of the remainder taxa (Figure 3B and C). Like the previous PCA, although many individuals of different taxa were intermingled along the second principal component, *C. hispida* var. *hispida* appeared in waters with lower alkalinity and Mg^{2+}/Ca^{2+} ratio while the opposite occurred with *C. hispida* var. *major*, *C. vulgaris* var. *hispidula* and var. *gymnophylla*, and *T. hispanica* (Figure 3B and C).

Differences in water parameters among Characeae taxa

The results of the PCA using our 97 taxon / water sample data pairs were highly concordant with the results of Kruskal-Wallis tests performed by adding data from previously published analyses of the same area, which also included *Chara vulgaris* var. *inconnexa* (T.F. Allen) R.D. Wood and *Chara connivens* Salzm. ex A. Braun. The water parameters that resulted in significant differences among taxa were salinity, concentrations of Ca^{2+} and Mg^{2+}, and Mg^{2+}/Ca^{2+} ratio (Table 3). In contrast, differences among taxa regarding concentration of HCO_3^- and pH were non-significant. However, these results have to be interpreted with caution, as some taxa could not be included in the Kruskal-Wallis tests because of their rarity in the studied area and the presence of some missing values.

Table 3. Results of the Levene's test for homogeneity of variance, Shapiro-Wilk normality test, and Kruskal Wallis chi squared test for the measured water parameters that were not highly correlated

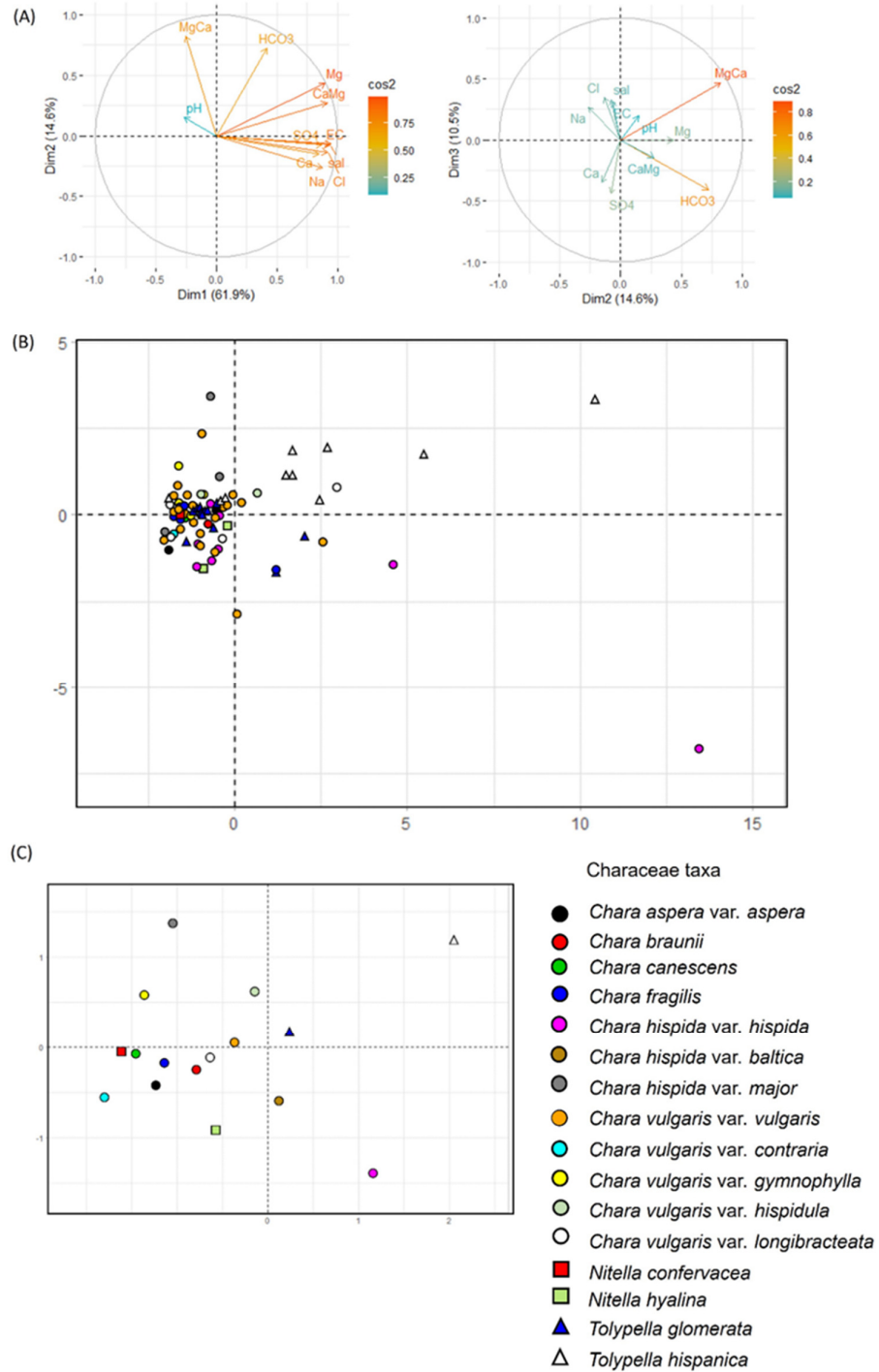| Water parameter	Levene's test			Shapiro-Wilk test		Kruskal – Wallis test		
	df	F value	Pr(>F)	W	p-value	χ^2	p-value	df
pH	12	1.424	0.165	0.972	0.010	16.547	12	0.167
Salinity	11	2.652	0.005	0.688	<0.001	34.832	11	<0.001
HCO_3^-	9	1.256	0.283	0.940	0.005	7.345	9	0.601
Ca^{2+}	6	0.885	0.513	0.757	<0.001	22.905	6	<0.001
Mg^{2+}	6	0.731	0.627	0.735	<0.001	20.526	6	0.002
Mg^{2+}/Ca^{2+}	6	0.445	0.845	0.699	<0.001	17.309	6	0.008

Figure 3. PCA of the Characeae taxa growing in eastern Spain (excepting *L. papulosum*) using the water parameters; (A) Contribution of each parameter to the three first components. Color and vector length correspond to squared cosines that express the contribution level of a given parameter to a component (Abdi and Williams, 2010); (B) Grouping of individuals according to components 1 (x-axis, 61.9% of the total variation) and 2 (y-axis, 14.6%); (C) Centroids corresponding to the individuals of each taxon according to components 1 and 2

Optimum values and tolerance ranges for water parameters

Although differences in pH among taxa were non-significant, we could observe some slight differences among genera (Table 4, Figure 4A). *Nitella* species were found in less basic waters (pH below 8), while *Tolypella* species were observed in more basic waters although they covered a wide range of pH (from 7.2 to 10.8). Like *Nitella* spp., *Chara* taxa and *L. papulosum* were found in waters with pH ranging from 6.95 to 8.9, depending on the species. *Chara vulgaris* var. *hispidula* and var. *inconnexa*, *C. connivens*, *C. braunii*, *C. canescens* and *L. papulosum* were found in water basins with ph lower than 8, while *Chara vulgaris* var. *gymnophylla* was found in waters with pH higher than 7.9. According to the non-significant differences among taxa regarding alkalinity, there were no clear differential values among genera either, and tolerance of taxa was highly dependent on the variable number of records (Figure 4B).

In relation with water hardness, *T. glomerata*, *N. hyalina* and *C. hispida* var. *hispida* were found in water basins with significant higher concentrations of Ca^{2+} (above 18 meq l^{-1} on average) than those in which *C. fragilis* and *C. vulgaris* var. *longibracteata* (below 8 meq l^{-1}) were observed (Table 4). Other species developed in waters with more extreme Ca^{2+} concentrations, although the very low number of records hinders an accurate interpretation of results (Figure 4C). However, locations where the highest concentrations of Ca^{2+} were recorded also displayed high levels of Mg^{2+} that could decrease the stress caused by the excessive presence of calcium. Thus, both species of *Tolypella* were found in locations with significantly higher concentrations of Mg^{2+} (above 19 meq l^{-1}) than *C. fragilis* and *C. vulgaris* var. *longibracteata* locations (below 6 meq l^{-1}). Requirements of water hardness for other *Chara* taxa and *Nitella* species varied distinctly (Figure 4D, supplementary material 5). The Mg^{2+}/Ca^{2+} ratio was the most discriminative parameter between genera. Both studied *Tolypella* species developed in water with significant higher ratios (above 1.3) than *Nitella* and some *Chara* species (i.e. *C. hispida* var. *hispida*) (below 0.7) (Table 4). However, *Tolypella* showed a wide tolerance range and some *Chara* species showed intermediate values and variable tolerance ranges (Figure 4E).

Table 4. Mean and standard deviation for the measured water parameters that were not highly correlated. Letters correspond to significant differences according to Dunnett's tests among those taxa that included at least three independent measurements. Salinity was measured in mg l^{-1}, and concentration of ions is expressed in meq l^{-1}

| Taxa	pH	Salinity		HCO3-	Ca2+		Mg2+		Mg2+/Ca2+	
C. aspera var. *aspera*	8.11 ± 0.62	1240 ± 500	cde	4.62 ± 2.26	53.5		48.3		0.90	
C. braunii	7.65	1050.6		3.30	8.92		9.2		1.03	
C. canescens	7.65 ± 0.31	2265 ± 2454		4.78	4.40		5.12		1.16	
C. connivens	7.41 ± 0.13	-		-	-		-		-	
C. fragilis	7.96 ± 0.61	828 ± 710	e	4.35 ± 1.42	3.84 ± 0.56	d	4.38 ± 0.44	c	1.15 ± 0.08	ab
C. hispida var. *hispida*	7.89 ± 0.45	3007 ± 3390	abc	4.12 ± 2.07	18.80 ± 12.50	ab	12.80 ± 15.10	bc	0.62 ± 0.49	c
C. hispida var. *baltica*	7.94 ± 0.63	2045 ± 1658	bcd	3.6	14.50		18.00		1.24	
C. hispida var. *major*	7.94 ± 0.30	1220 ± 674	bcde	9.17 ± 6.72	-		-		-	
C. vulgaris var. *vulgaris*	7.90 ± 0.43	1593 ± 1749	de	6.00 ± 2.93	15.00 ± 15.60	bc	13.50 ± 20.30	bc	0.87 ± 0.40	bc
C. vulgaris var. *contraria*	7.75 ± 0.21	1759 ± 1755		3.6	-		-		-	
C. vulgaris var. *gymnophylla*	8.29 ± 0.40	840 ± 403e	de	6.90 ± 2.44	-		-		-	
C. vulgaris var. *hispidula*	7.40 ± 0.14	1840 ± 962		-	13.60 ± 10.75		12.60 ± 7.07		1.05 ± 0.31	
C. vulgaris var. *longibracteata*	7.98 ± 0.39	1360 ± 1398	de	4.34 ± 2.53	7.04 ± 2.54	cd	5.74 ± 2.61	c	0.87 ± 0.44	abc
L. papulosum	7.72 ± 0.24	33478 ± 13484	a	4.75 ± 1.34	-		-		-	
N. confervacea	7.2	480		6.6	3.86		3.49		0.90	
N. hyalina	7.62 ± 0.29	1735 ± 426	abcd	4.01 ± 1.69	26.60 ± 23.40	ab	19.60 ± 25.10	abc	0.58 ± 0.40	c
T. glomerata	8.30 ± 0.84	2271 ± 2365	bcd	5.18 ± 4.13	13.00 ± 5.92	abc	15.80 ± 11.70	ab	1.32 ± 1.12	ab
T. hispanica	8.60 ± 1.29	3417 ± 3242	ab	6.30 ± 5.79	23.80 ± 14.90	a	34.90 ± 22.60	a	1.66 ± 1.21	a
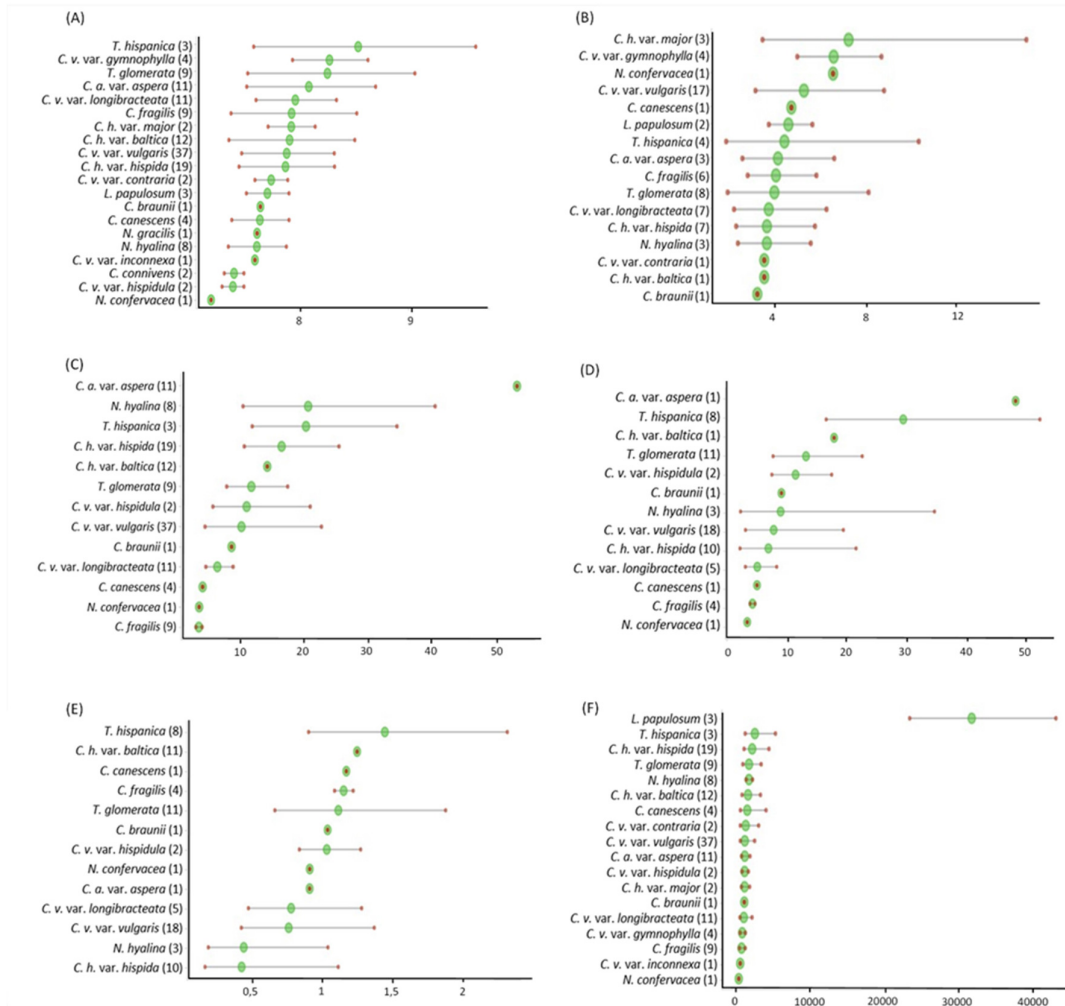
Figure 4. Optimum (green dot) and tolerance ranges (grey lines) of the Characeae taxa of the genera *Chara*, *Lamprothamnium*, *Nitella* and *Tolypella* found in eastern Spain to different chemical water parameters. (A) pH; (B) HCO_3^- concentration (meq l^{-1}); (C) Ca^{2+} concentration (meq l^{-1}); (D) Mg^{2+} concentration (meq l^{-1}); (E) Mg^{2+}/Ca^{2+} ratio; (F) salinity (mg l^{-1}). Numbers in brackets correspond to number of samples taken in different localities. Abbreviated names are: *C. a.* for *Chara aspera*, *C. v.* for *Chara vulgaris*, and *C. h.* for *Chara hispida*. Diagrams were created using the package "Optimos Prime" in R

In agreement with the PCA, *L. papulosum* appeared to be the most tolerant species to high levels of salinity beyond 40 g l^{-1} (Table 4, Supplementary Material 1). This species was also able to develop in lower salinities (up to 25 g l^{-1}) and was the species that displayed the widest tolerance range (Figure 4F). *Tolypella* species, *Chara hispida* var. *hispida* and var. *baltica*, *N. hyalina* and *C. canescens* formed an intermediate group with optimum of salinity ranging from 1,7 to 3.4 g l^{-1} on average. All the subspecies of *C. vulgaris*, *C. aspera* var. *aspera*, *C. hispida* var. *major*, *C. braunii*, *C. fragilis* and *N. confervacea* displayed a narrow range of salinity tolerance in eastern Spain and were optimally found in waters with salinity below 1,8 g l^{-1}.

Discussion

Diversity of Characeae in eastern Spain

In this work, 17 taxa were reported out of the 32 taxa cited in the studied region since 1910 (BDBCV, 2023). Including the data of previous studies, number of taxa represented is 19. Nowadays, we could not find most of the records that were cited in the eighties, especially in the coastal areas. Out of the four taxa that are considered vulnerable due to habitat loss (*Chara vulgaris* var. *hispidula*, *Lamprothamnium papulosum*, *Nitella confervacea* and *N. hyalina*) (Cirujano *et al.*, 2008), we could only find *Lamprothamnium papulosum* in Peñíscola. This decline in Characeae populations along time has been well documented in eastern Spain in the Albufera Natural Park. There, ten species that constituted extensive underwater meadows at the end of the 70s were recognized through the study of the oospores in the sediments, having declined since then (Rodrigo *et al.*, 2009). This Characeae biodiversity loss is mainly due to anthropic pressure and eutrophication that includes excessive inputs of sewage water and a reduction of historical inputs of river waters (Puche and Rodrigo, 2015). As most Characeae species inhabit oligotrophic waters, they are among the first submerged macroalgae to disappear during the eutrophication process (Barbosa *et al.*, 2021). From 2007 to 2010, some Characeae populations were able to recover in the Albufera Natural Park, but only in the most conserved locations and in small areas. Sixteen taxa were recently identified in places on ephemeral water such as paddy fields, channels and interdunal ponds, and on permanent waters such as constructed wetlands and a few springs (Rodrigo and Alonso-Guillén, 2016). In the rest of the lake, with an area of about 2.200 ha, no Characeae taxa persisted (Rodrigo *et al.*, 2009). Nowadays, besides eutrophication, salinization is one of the main threats of Valencian wetlands because of lower underwater supply, marine introgression, and evaporation (Puche and Rodrigo, 2015). Furthermore, Mediterranean wetlands are expected to be especially vulnerable to climate change because projection in this region points towards a 2 °C rising temperature, especially in the summer, coupled with an increase of 10-40% of drying (Zittis *et al.*, 2019), which could lead to an even higher salinity. This increase in salinity is expected to cause a decreased elongation of the main shoots, a reduction in weight in charophytes (Puche and Rodrigo, 2015), and a reduction in their fertility (Blindow *et al.*, 2003). In addition, alien invasive species have been negatively impacting the environment of Mediterranean wetlands because of fierce competition with native species, often causing their disappearance and modifying the function and structure of their habitats (Merino and Donat, 2011; Mayoral *et al.*, 2018).

The fact that most Characeae taxa have declined in the studied area highlights the importance and usefulness of previous unpublished records, as they can be used to analyze ecological gradients, predict and monitor the responses of aquatic systems to global change, and aid environmental restoration at a local scale.

Relationship between water parameters and distribution of Characeae taxa in eastern Spain

The high alkalinity and water hardness found in eastern Spain are the results of the fact that in this region, stream water generally derives from calcareous rocks. However, although concentrations of Ca^{2+} and Mg^{2+} tend to correlate with pH, conductivity and bicarbonate, this was not the case here. This is supported by the fact that relationships among surface water parameters are different in different water systems because of extraneous inputs and physical variations (Saalidong *et al.*, 2022).

Our results support the existence of environmental gradients mainly related to salinity and concentration of Mg^{2+} and Mg^{2+}/Ca^{2+} ratio that influence the distribution of charophytes. Both pH and alkalinity showed absence of a significant effect on their ecological distribution. However, our results disagree with other local studies that recorded other charophyte taxa, highlighting the importance of biogeographic history and the need of studying aquatic ecosystems in small environmental gradients (i.e. Leibold *et al.*, 2010). Charophytes were distributed in Chile mainly according to pH and alkalinity (Schubert *et al.*, 2018), in Pakistan essentially according to pH and salinity (Khuram *et al.*, 2021), and in northeastern USA according to Ca^{2+} concentration and salinity (Sleith *et al.*, 2018).

Salinity was approved to be the major water parameter that determined the distribution of Characeae in eastern Spain. Salinity, along with hydrological regime, was also identified as the main factor controlling the abundance of the different Characeae species in other Mediterranean areas, such as in southeastern France (Grillas, 1990) and in southern Spain (Moreno *et al.*, 2001; Espinar *et al.*, 2002). Similarly, salinity was also demonstrated as the major distinguishable factor in different ecosystems other than the Mediterranean ones, such as Australia (Brock and Lane, 1983) and the Baltic Sea (Schubert and Blindow, 2003). *Lamprothamnium papulosum* was the unique resistant species to the highest levels of salinity, above 24 g.l⁻¹, with a relatively wide range of tolerance. This result agrees with García and Chivas (2004), who also found that different species of *Lamprothamnium* genus are good indicators of salinity in Australia. There, *Lamprothamnium* species appeared in non-marine lakes, with salinity ranging from 3 to 70 g l⁻¹. This wide range of tolerance may occur because *Lamprothamnium* species develop in lakes with both permanent and ephemeral waters, where salinity fluctuates along the year, being slightly saline in wet winters and autumns to hypersaline in summer (Brock and Lane, 1983). These conditions occur above all in Mediterranean coastal wetlands, where the summer is hot and dry. The physiological mechanisms that allow *L. papulosum* to tolerate varying salinities during different developmental stages (e.g. vegetative and reproductive phases) involve the total maintenance of cell turgor pressure through changes in the cell osmolality (Wichmann and Kirst, 1989).

A second group of Characeae species appeared to be moderately tolerant to salinity. It included *Tolypella glomerata*, *T. hispanica*, *Chara hispida* var. *hispida* and var. *baltica* and *C. canescens*. This result agrees with other previous studies. For instance, when the Albufera Natural Park was covered by brackish waters, *L. papulosum*, *C. aspera*, *C. hispida* var. *baltica* and *T. hispanica* developed as shown by the presence of charophyte fructifications present in the lagoon sediments (Rodrigo *et al.,* 2009). In addition, these taxa generally appeared to be moderately tolerant to salinity in other locations too, such as the Baltic Sea (*C. hispida* var. *baltica* and *C. canescens*; Blindow, 2000), southeastern France (*T. glomerata* and *C. canescens*, Grillas *et al.*, 1993), and Estonia (*C. hispida* var. *baltica* and *C. canescens*; Torn *et al.*, 2015). In agreement with our results, it has been shown that species of *Tolypella* are able to respond to salinity changes by complete turgor regulation (Winter *et al.*, 1996). Accordingly, *Tolypella hispanica* has already been recorded as halophytic in the Iberian Peninsula (Cirujano *et al.*, 2013). Conversely, *Tolypella glomerata* has been shown to be euryhaline in the Mediterranean wetlands, where salinity can fluctuate greatly depending on seasonal rainfall (Moreno *et al.*, 2001). For instance, in the Camargue, this species could tolerate up to 6 g l⁻¹ without any decrease in plant biomass (Grillas, 1990 and Grillas *et al.*, 1993). However, this species has only been found in freshwaters in other ecosystems such as the Baltic Sea (Blindow, 2000). *Tolypella* spp. and *Chara hispida* var. *hispida* are able to regulate cell turgor pressure by accumulating K⁺ anions within cells (Blindow *et al.*, 2003), while *C. canescens* regulates turgor although incompletely (Winter and Kirst, 1991).

Finally, a third group of species was only observed in waters with lower salinity. It included *Nitella* spp. *Chara aspera* var. *aspera*, *C. braunii*, *C. fragilis*, *C. hispida* var. *major* and all the varieties of *C. vulgaris*. In agreement with our results, *N. hyalina* has been confirmed as stenohaline because it is unable to regulate turgor pressure (Puche and Rodrigo, 2015). *Nitella hyalina* was also found in the least saline and most flooded habitats of the southwestern Spanish wetlands (Espinar *et al.*, 2002). Generally, in Chile *Nitella* species prefer also water with lower conductivity than *Chara* species (Schubert *et al.*, 2018). *Chara braunii* and/or *C. fragilis* have also been recorded in freshwaters in the United States (Wood, 1952), in the Baltic Sea (Blindow, 2000) in Pakistan (Khuram *et al.*, 2021), and in southeastern France (Grillas, 1990; Grillas *et al.*, 1993). Although in Australia, in Germany, and in southeastern Spain *C. fragilis* was found in habitats with both fresh and saline waters (Brock and Lane, 1983; Moreno *et al.*, 2001; Becker *et al.*, 2016). While, both *C. aspera* and *C. vulgaris* were able to regulate cell turgor in higher salinity although incompletely (Winter and Kirst, 1990 and 1992), and have been revealed as euryhaline in many locations (Grillas, 1990, Moreno *et al.*, 2001; Blindow, 2000; Blindow *et al.*,

2003; Becker *et al.*, 2016). In fact, although *C. aspera* and *C. vulgaris* were found generally in freshwaters, in some locations of our study areas (such as Pego) these species grew also in waters with up to 9 g l^{-1} of salinity. This result has been related in both species to the existence of genetic differences among populations due to their reproductive isolation that cause phenotypical and physiological variations (Grillas *et al.*, 1993; Blindow *et al.*, 2003). More recently, it has been demonstrated that genetic differentiation also exists among populations of *C. vulgaris* from Iran even if morphological differences did not appear (Noedoost *et al.*, 2015). Similarly, Schneider *et al.* (2016) demonstrated that while genetically related Characeae taxa may show considerable phenotypic plasticity, in some groups taxa that are phenotypically similar showed a high genetic diversity. The phenotypic plasticity may also include physiological processes. *Chara polyacantha* A. Braun and *Chara rudis* A. Braun displayed differential morphological and physiological responses to variable environmental conditions (Pukacz *et al.*, 2014). Regarding response and tolerance to salinity, complex physiological processes including the synthesis and accumulation of various substances that increase the cellular osmotic value and induction of cell ultrastructural changes have been identified in Characeae (Holzinger and Pichrtová, 2016). The differential tolerance to salinity among populations has been demonstrated in laboratory conditions in *C. aspera* (Blindow *et al.*, 2003), suggesting that these differences are more related to genetic variations than to environmental inductions. Populations of eastern Spain may thus be non-tolerant to moderate salinities in most of the locations.

The concentration of Mg^2 and the Mg^{2+}/Ca^{2+} ratio were the main decisive factors for the distribution of Characeae taxa following water salinity parameter in eastern Spain. Most previous studies did not consider the Mg^{2+}/Ca^{2+} ratio when analyzing water. However, when this parameter was included, it appeared to be not determinant (Schubert *et al.*, 2018). A high concentration of Mg^{2+} can have different effects. On the one hand, Mg^{2+} has been shown to interact with the processes of Ca^{2+} accumulation and precipitation, but not vice versa (Gomes and Asaeda, 2010), while a higher ratio of Mg^{2+}/Ca^{2+} could cause a reduction of tissues calcification (Apolinarska *et al.*, 2011). Conversely, at high concentrations, Mg^{2+} is able to maintain a high percentage of water-soluble phosphorus that is available to plants. Besides, a high concentration of Ca^{2+} leads to the co-precipitation of unsoluble phosphate with calcium carbonate. Thus, Mg^{2+}/Ca^{2+} ratio may affect phosphorus nutrient sink ability of charophytes (Gomes and Asaeda, 2010). Another effect of a high Mg^{2+}/Ca^{2+} ratio is that it significantly affects the composition of encrustation in Characeae species, which can be polymineral (Apolinarska *et al.*, 2011). All these effects can differentially modify the growth of different charophyte taxa. Our results showed that in Ribera de Cabanes and Torreblanca, where Mg^{2+}/Ca^{2+} ratio was above 2, only *Tolypella* species were reported. Also, both *T. glomerata* and *T. hispanica* were found to grow in waters with significantly higher concentrations of both Ca^{2+} and Mg^{2+} than those where *Chara* taxa grow, especially *C. fragilis* and *C. vulgaris* var. *longibracteata*. However, previous works that studied the influence of Ca^{2+} and Mg^{2+} on the distribution of Characeae species showed contrasting results. Asaeda *et al.* (2007) observed that *Nitella hyalina* and *Chara fibrosa* var. *fibrosa* grew in water basins with a Mg^{2+} concentration approximately four times greater than Ca^{2+} one, in Myall Lake, Australia. In *Nitella*, availability of Ca^{2+} and Mg^{2+} affected the morphological appearance of plants: both elements aided shoot elongation, but Mg^{2+} did it at a higher rate than the equivalent levels of Ca^{2+} (Gomes and Asaeda, 2010). In the Baltic Sea, *C. braunii* and *C. fragilis* have been found in soft and hard waters while *C. aspera* was further observed in calcium-rich freshwater (Blindow, 2000).

pH parameter had not a significant effect on Characeae taxa diversity in eastern Spain. However, some previous studies reported that pH is one of the main factors that determine the presence of a given Characeae taxon (i.e. Wood, 1952 in the United States). This contrasting pH effect could be related to the homogeneous geology of the area of eastern Spain characterized by the predominance of limestones leading to a basic pH with small differences among locations. However, a slight pH gradient influenced the presence of *Nitella*, *Chara* and *Tolypella* genera and arranged their distribution from lower to higher pH values respectively. Likewise, Schubert *et al.* (2018) and Becker *et al.* (2016), have cited presence of *Chara* spp. in water basins with higher

pH than *Nitella* spp. and *Tolypella* spp. Conversely, this research also confirmed that some *Chara* species grew in the range of *Nitella* pH values (below 8), like *C. braunii* (Wood, 1952; Schubert *et al.*, 2018) and *C. canescens* (Wood, 1952).

In eastern Spain, all the Characeae taxa were found in waters with high alkalinity (above 1.4 meq l^{-1}). This result agrees with previous studies performed in other locations, such as in Estonia, where most freshwater *Chara* species also were revealed to prefer water alkalinity superior to 1.3 meq l^{-1} (Torn *et al.*, 2015). In spite of this general high alkalinity requirement, we could not find any significant difference among taxa. Previous studies performed in other locations are not conclusive either. For instance, in Chile, only *Chara* species developed with alkalinities higher than 1 meq l^{-1}, while *Nitella* species and *C. braunii* were able to grow in less alkaline waters (Schubert *et al.*, 2018). However, in Doñana national Park (South Spain), the opposite occurred, and the abundance of *Nitella hyalina* appeared to be linearly and positively related with the bicarbonate content of the water column (Espinar *et al.*, 2002).

Conclusions

Water chemical parameters related to salinity, followed by Mg^{2+}/Ca^{2+} ratio and Mg^{2+} and Ca^{2+} concentrations, were the most relevant parameters that determined the distribution of Characeae in eastern Spain. Significant differences in the values of these parameters existed among Characeae taxa, which allow their use as bioindicators. However, certain taxa were not easily ecologically differentiated, excepting *Lamprothamnium papulosum* and maybe *Tolypella* spp., in which some overlap appeared among individuals. The results observed may be limited to a local scale, as varying ecological gradients have been identified in other locations that highlight the importance of biogeography in Characeae bioindication. The differences in ecological gradients of different areas may be partly due to genetic differences among populations, and open a window into future studies that may relate genetical, ecological, and physiological features of Characeae taxa.

Authors' Contributions

Conceptualization: HB and MF; Data curation: HB and BS; Formal analysis: MF; Investigation: HB, MF and BS; Methodology: HB and BS; Project administration: MF; Resources: HB and BS; Software: MF; Supervision: MF; Validation: MF; Visualization: HB, MF and BS; Writing – original draft: MF; Writing – review & Editing: MF and BS.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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