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Charophyceae and polyphenols: the influence of global change stressors

Abstract

Global change is an environmental problem on a world-wide basis. Climate warming, increased eutrophication, enhanced ultraviolet radiation (UVR) penetration and their combined effects endanger aquatic ecosystems around the world. Submerged macrophytes (among them charophytes, Charophyceae) are subjected to these changes. These organisms can respond by varying their production of polyphenolic compounds with different roles: allelopathic capacity, protection against stress factors, etc. In this study, we performed two laboratory experiments to analyse the response of charophytes in the production of polyphenols under different scenarios. In the first experiment, *Chara hispida* and *Chara vulgaris* from two different Spanish populations (Somolinos lake and Quartons spring) were subjected to two levels of temperature and nitrate concentrations (Exp. I-TxN). In the second experiment, *Chara hispida* from two different Spanish populations (Somolinos lake and Llacuna del Dossel pond) were exposed or not exposed to UVR (Exp. II-UVR). In both cases, polyphenol identification and quantification were carried out by ultra-high-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS) and different extraction protocols were applied to optimize the methods for charophytes. Exp. I-TxN results showed that (i) the production of polyphenols was species-specific (due to its basic ecological features) and population-specific (related to the adaptability to environmental changes), (ii) the increased temperature promoted the increase in the content of polyphenols, and conversely, (iii) there was no evidence that different nitrate concentrations affect the production of the analysed substances. Exp. II-UVR results showed that (i) the polyphenol concentration (mainly gallic acid) was higher when *Chara hispida* was cultivated with UVR, and (ii) the population from the coastal wetland was the most sensitive. Despite the methodological difficulties in obtaining polyphenols in Charophyceae, this study demonstrates that their production is affected by global change factors and opens future prospects to forecast which polyphenols will have a greater concentration based on the phylogeny (species), conditions of origin (intraspecific variability due to local adaptation) and foreseeable changes in the environment.

Keywords: Charophytes, *Chara hispida, Chara vulgaris,* temperature, nitrate, UV radiation, allelochemicals, protectors

1 Introduction

Environmental factors related to global change, such as enhanced warming, increased ultraviolet radiation (UVR), eutrophication, or drought (IPCC 2021; EEA 2023), receive currently increased attention because they can interact with the biodiversity and the functioning of all type of aquatic ecosystems (SALA et al. 2000; JACKSON et al. 2016). For example, the concomitant effect of high temperatures and low rainfall, particularly in shallow water bodies or small lakes may cause a loss of water with an increase in the concentration of nutrients, such as overabundant agricultural nitrate and salts (GIORGI & LIONELLO 2008; JEPPESEN et al. 2011). Furthermore, this reduction of the water column height will enable greater amounts of UVR to penetrate into the water, sometimes reaching the bottom of these systems (RUBIO et al. 2015), where the charophytes (green macroalgae from the family Characeae, Order Charales, Class Charophyceae, Division Chlorophyta) develop. They are of key relevance in aquatic habitats all over the world (BLINDOW et al. 2014; SCHNEIDER et al. 2015), and are a key group of benthic primary producers to predict the effects of global change on the functioning and the structure of inland aquatic ecosystems (RODRIGO et al. 2010; PEŁECHATA et al. 2015; TORN et al. 2019, BRZOZOWSKI et al. 2022).

Polyphenolic compounds are secondary metabolites synthesized by primary producers and characterized by the presence of one or several aromatic rings per molecule with one or more hydroxyl groups (QUIDEAU et al. 2011). According to their chemical structure, polyphenols are classified in (i) non-flavonoid compounds (i. e. stilbenes and phenolic acids, which are also divided into derivatives of the cinammic acid –e. g. caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid-, and derivatives of the benzoic acid –e. g. benzoic acid, gallic acid, protocatechuic acid, vanillic acid, benzaldehyde, methylfurfural, protocatechuic aldehyde, vanillin-) and (ii) flavonoid compounds (subdivided into anthocyanins, flavonols –e. g. astragalin, isoquercetin, kaempferol, quercetin, quercitrin- and catechins –e. g. catechin, epicatechin gallate, epicatechin, etc.-) (SINGLA et al. 2019). These substances have several roles in the organisms, mainly allelopathy against other primary producers such as microalgae or other organisms in aquatic ecosystems (ZHU et al. 2010; GAO et al. 2011; VIVEROS-LEGORRETA et al. 2022), but also to prevent oxidative stress (HOLZINGER & PICHRTOVÁ 2016), for example, to prevent the negative effects caused by UVR, like other UV-absorbing compounds as the mycosporine-like amino acids (MAAs). Polyphenolic composition is species-specific in macroalgae (PAVIA et al. 2003; PARYS et al. 2007) and, probably, it is also conditioned by the adaptation of the light environment of origin (RUBIO et al. 2015), in the way that populations from shallow ecosystems receiving more UVR increase more readily their production of polyphenols against enhanced UVR (GROSS 2003a, b; GROSS et al. 2007).

However, information about the production of polyphenolic substances in Charophyceae is very scarce, especially considering their response to changes in factors related to global change. The present study aimed to assess the interactive effects of increased temperature, enhanced nitrate concentrations, UVR presence and the origin of the species, on the production of polyphenolic compounds by charophytes. Two separate bifactorial-designed laboratory experiments were performed: (i) *Chara hispida* and *C. vulgaris* taken from two contrasting environments and exposed to different conditions of temperature and nitrate concentrations, and (ii) *C. hispida* collected from two different habitats and exposed to different radiation conditions (UVR absence/presence). As a standardized protocol to extract and determine polyphenols

in Charophyceae is lacking, several methodological procedures were attempted to optimize the measurements.

2 Material and Methods

2.1 Temperature x Nitrate experiment (Exp. I-TxN)

Two charophyte species were chosen: *Chara hispida* L. 1753 and *Chara vulgaris* L. 1753. The specimens were collected from Somolinos lake (Sierra de Ayllón Protected Area, 1270 m, 41°15004″ N, 3°03054″ W, central Spain), which is an oligotrophic, moderately deep (7 m maximum depth) mountain lake in a cold climate. The other site was the Quartons spring (Almenara, Castellón, 0 m, 39°45016″ N, 0°11027″ W, eastern Spain), which is a meso-eutrophic shallow (1 m maximum depth) water body surrounded by agriculture fields, fed by ground water and located in a warmer climate (PUCHE et al. 2018) (Fig. 1). These species were chosen because they are cosmopolitan, widely distributed and can be found in quite different environments.

The experiment had a two-factor design: (i) temperature (two levels, 20 °C and 24 °C), and (ii) nitrate concentration (two levels, the concentration found in the origin environment –lower nitrate hereafter– and twice this value –higher nitrate hereafter–). Six replicates for each condition and origin were set (a total of 96 specimens were used).

Apical parts of each species were planted in small pots (one individual per pot). Each pot was placed in a 1 l container containing dechlorinated tap water. These pots were placed inside 8 rectangular containers also containing water (Fig. 2A). All specimens were acclimated for one week to the experimental conditions (specified above) in a culture room with controlled light and temperature. Nitrate concentrations for Somolinos lake were 9 mg l^{-1} and 18 mg l^{-1} and for Quartons spring 30 mg l^{-1} and 60 mg l^{-1}. A concentrated standard solution with $NaNO_3$ was prepared and the necessary volume of this standard was added to each container to achieve the desired experimental concentrations. These values were chosen according to the nitrate concentrations measured in each habitat. The low temperature treatment (20 °C) corresponded to the value set up in the culture room. The higher temperature condition (24 °C) was achieved using aquarium heaters (Eheim Jäger 25 W for 20 l) located inside the rectangular containers (for more details of the experiment see PUCHE et al. 2018). The experiment lasted for 3 weeks.

Three individuals of each condition were randomly chosen for polyphenol analyses. First, the above-ground part of these specimens was taken and located on drying paper to remove the external water. Later, each one was placed inside paper envelopes and then lyophilized at -77 °C, 150 mTorr for 48 h (Lyophilizer VirTis Sentry 2.0, SP Scientific; Warminster, PA, USA). Lyophilised material was weighted by means of a precision scale (Kern PLJ 600-3NM; Balingen, Germany).

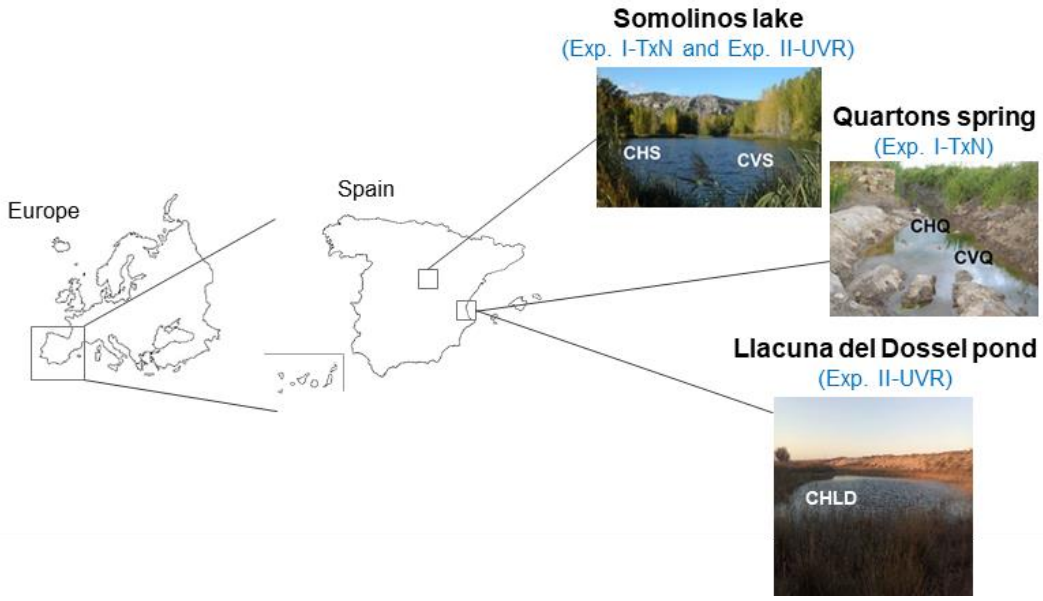

Fig. 1 Location of the sites used as the origin of *Chara hispida* and *Chara vulgaris* (Exp. I-TxN) and *Chara hispida* (Exp. II-UVR). The location of the meadows of each population is shown in the photographs (CH: *Chara hispida*, CV: *Chara vulgaris*, S: Somolinos lake, Q: Quartons spring, LD: Llacuna del Dossel pond).

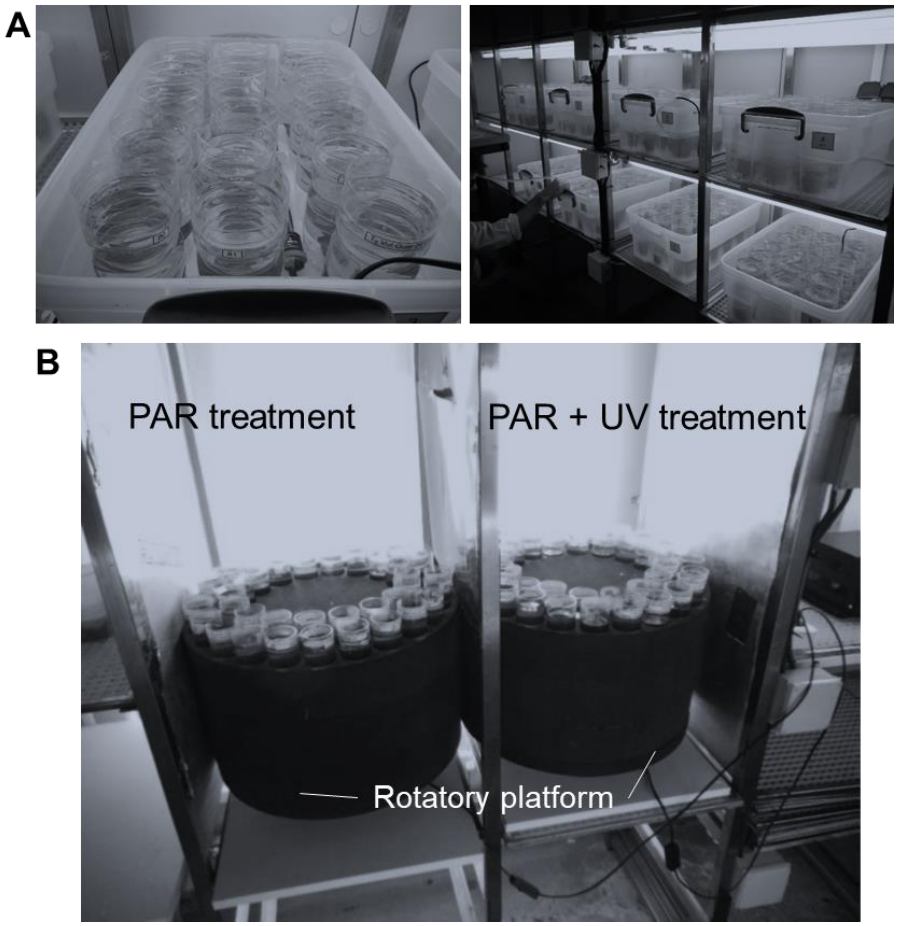

Fig. 2 Scheme of the experimental design of A) Exp. I-TxN and B) Exp. II-UVR (PAR: photosynthetically active radiation).

This study addressed the determination of 20 polyphenolic substances (Tab. 1) based on the previous study performed by ROJO et al. (2013a). Five standards were used (Sigma-Aldrich, Madrid, Spain, >99.9 % purity index; indicated in Tab. 1) for quantitative determination. A primary solution (1000 µg ml^{-1} in methanol) was prepared from each standard, followed by the creation of a composite standard by mixing all polyphenols.

Tab. 1 List of analysed polyphenols in Exp. I-TxN. The compounds with available standards are indicated in bold font.

Compound group	Compound	Empirical formula	CAS number	Molecular weight (g/mol)
NON-FLAVONOIDS				
CINAMMIC ACID	Caffeic acid	$C_9H_8O_4$	331-39-5	180.16
	Ferulic acid	$C_{10}H_{10}O_4$	537-98-4	194.18
	p-coumaric acid	$C_9H_8O_3$	501-98-4	164.16
BENZOIC ACID	Benzoic acid	$C_7H_6O_2$	65-85-0	122.12
	Gallic acid	$C_7H_6O_5$	149-91-7	170.12
	Protocatechuic acid	$C_7H_6O_4$	99-50-3	154.12
	Methylfurfural	$C_6H_6O_2$	620-02-0	110.11
	Protocatechuic aldehyde	$C_7H_6O_3$	139-85-5	138.12
	Vanillin	$C_8H_8O_3$	121-33-5	152.15
FLAVONOIDS				
FLAVONS	Astragalin	$C_{21}H_{20}O_{11}$	480-10-4	448.37
	Isoquercetin	$C_{21}H_{20}O_{12}$	482-35-9	464.38
	Quercetin	$C_{15}H_{10}O_7$	117-39-5	302.24
	Quercitrin	$C_{21}H_{20}O_{11}$	522-12-3	448.38
CATECHINS	**Catechin**	$C_{15}H_{14}O_6$	154-23-4	290.27
	Epigallocatechin gallate	$C_{22}H_{18}O_{11}$	989-51-5	458.37

Previously to the analyses of the experimental samples, three extraction methods were assayed using as testing material C. vulgaris from a stock culture: (i) Liquid-liquid extraction method (LLE) in which around 0.15 g of lyophilized sample was introduced in a 15-ml Falcon tube with 3 mL of n-hexane (to remove pigments such as chlorophylls and other compounds of non-polar nature), the samples were homogenized during 3 minutes (Homogenizer T 25 digital ULTRA-TURRAX®), 1 ml of 70:30 v/v MeOH-H2O was added and centrifuged at 3,000 rpm for 15 minutes (Centrifuge Eppendorf 5810 R; Hamburg, Germany), the organic phase was discarded, and 0.5 ml of the hydroalcoholic extract was transferred to an amber vial (Análisis Vínicos S. L. Tomelloso, Spain) with polypropylene cap (Agilent Technologies, USA); (ii) Solid

phase extraction method (SPE) in which samples were vacuum passed through STRATA™ Phenomenex cartridges previously pre-conditioned with 6 ml of methanol and 6 ml of deionized water. The elution of polyphenols was made using two alternative solutions: 6 ml of methanol and 6 ml of 2 % (v/v) ammonium hydroxide in methanol (each cartridge was used only once), the tubes were evaporated at 40 °C in dry air flow (approximately for 2 h) and were resuspended in 1 ml of 0.25 % (v/v) acetic acid in deionized water, later they were immersed in an ultrasonic bath (Elmasonic S120/H, Sigen, Germany) during 4 minutes and transferred to vials to be injected in the LC-MS/MS (see below); (iii) Acid hydrolysis method, which consisted in adding 5 ml of 2 N HCl to the extracted samples (and kept at 4 °C for 24 h) before starting the procedure with the SPE method to liberate most of the polyphenols from the vegetal matrix (the tubes were centrifuged for 15 min, the samples filtered and pour in 250 ml flask containing deionized water and brought to SPE procedure). The LLE was the method yielding the best results (not shown), therefore, this was chosen to treat the samples from the Exp. I-TxN.

2.2 Ultraviolet radiation experiment (Exp. II-UVR)

Only *C. hispida* was used, from two origins. One of the origins was Somolinos lake (see above) and the second was an interdunal pond called “Llacuna del Dossel” (Cullera, Valencia, 0 m, 39º12′30″ N; 0º14′5″ W, eastern Spain; hereafter Llacuna del Dossel pond) (Fig. 1), a shallow water body (60 cm maximum depth) located at sea level within the Albufera de Valencia Natural Park. The reason to change this water body is because the Quartons spring was dry when we went for sampling. The “Origin” treatment was established by using *C. hispida* individuals from Somolinos lake and from Llacuna del Dossel pond. The UVR treatment had two levels: (i) absence of UVR, the charophytes were cultivated only with photosynthetically active radiation (PAR) emitted by high-pressure sodium lamps (53 W m^{-2}) and (ii) presence of UVR (the same PAR + UVA: 1.2 W m^{-2} and UVB: 0.2 W m^{-2}). Therefore, the four conditions were stablished. Twenty individuals of *C. hispida* were cultivated under the conditions of each treatment (a total of 80 individuals). The individuals were planted in small pots and introduced into cylindrical methacrylate beakers (30 cm high; 5 cm diameter) filled with dechlorinated tap water. Then all beakers were put in a perforated rotatory structure to ensure that they received identical amounts of UVR (Fig. 2B). The cultures had a mean water temperature of 20.1 ± 0.1 °C, mean conductivity of 1083 ± 9 µS cm^{-1}, mean pH of 9.1 ± 0.1, mean dissolved oxygen of 17.5 ± 0.9 mg l^{-1}, with saturation percentages higher than 200 %. The experiment lasted for four weeks (including one week of acclimatization). Since the individual charophyte biomass was small, all specimens of each treatment were pooled together and from these samples the polyphenols were extracted, measuring each sample twice in the chromatograph. The same polyphenol standards as in the previous experiment were used, plus quercetin 3-rhamnoside. The LLE method described above was used.

With the samples of this experiment, the analytical method for polyphenol determination was improved by (i) choosing different amounts of sample to be processed (0.05 versus 0.1 g), (ii) adding or not adding 1 ml of n-hexane to eliminate any compound which could interfere in the determination of polyphenols, and (iii) using different percentages of the solvent MeOH-H_2O (70:30 v/v versus 50:50 versus 30:70). Five milligrams of each polyphenol standard per kg dry weight of charophyte were added in these tests.

2.3 Analytical procedure

In both experiments, the analytical method was validated following a within-laboratory protocol. Specificity/selectivity, linearity, recovery, precision, detection and quantification limits, and quality control were studied. The polyphenol analyses were performed in an Agilent 6410 (Waldbronn, Germany) triple quadrupole (QQQ) mass spectrometer equipped with electrospray ionization (ESI) in combination with an ultra-high performance chromatographic instrument (HP1200 LC series), equipped with an injector, a degasser, a quaternary pump, and a column oven. The instrumental parameters are shown in Tab. 2A. The separation was made using a Kinetex C18 column from Phenomenex (Torrance, California, USA). The mobile phase consisted of a gradient of: (a) 0.25 % CH_3COOH with Milli-Q water and (b) MeOH. Before being placed in the chromatograph, both phases were degasified in an Elmasonic S120/H ultrasonic bath (Sigen, Germany) for 15 min. Each injection had a duration of 35 min, and an equilibration time of 15 min to recover and stabilize the column conditions before the next injection. The determination was performed using a negative ionization mode, and the optimal conditions for the ESI source are described in Tab. 2B. The polyphenols with no available standards were measured qualitatively. The estimation of the amount of each polyphenol was based on the area of the peak corresponding to the transition precursor ion→product ion, considering 100 % the area of the highest peak in all samples and calculating the percentage that the other peaks would represent. Some of the identified polyphenols showed well-defined peaks in the same transition but at different retention times, thus, they were considered structural isomers. Qualitative and quantitative data were processed using the *MassHunter Workstation* software from A GL Sciences (Tokyo, Japan). The reagents provider was VWR (Barcelona, Spain): methanol was HyperSolve Chromanorm® Reagent Pharmacopoeia European para HPLC-Gradient, adequate for UPLC/UHPLC, n-hexan was from Analab Normapur®; hydrochloric acid from EMPARTA® (37 %); acetic acid 96% from AnalaR NORMAPUR; deionized water was produced by a Milli-Q SP Reagent Water System of Millipore (Bedford, MA, USA).

2.4 Statistical analyses

Regarding Exp. I-TxN, two-way ANOVAs were carried out (after checking compliance with the assumptions of normality and homoscedasticity) to analyse the effect of temperature and nitrate concentration on the polyphenol production (taking into account the studied species and populations). The level of significance was set for all statistical analyses to a $p < 0.05$. All analyses were performed with the SPSS Statistics-24 software (IBM Corp., Chicago, IL, EEUU).

Tab. 2 Description of (A) the conditions for the liquid chromatography (LC), and (B) the electrospray ionization (ESI) source.

A. LC CONDITIONS	
Analytical column	Kinetex XB-C18: 50 × 2.1 mm, 1.7 µm size particle, 100 A pore size, Phenomenex
Column temperature	30 °C
Injected volume	10 µl
Mobil phase	(a) Milli-Q water with 0.25% acetic acid and (b) methanol
Flow speed	0.2-0.3 ml min^{-1}
Linear gradient	0 min (5 % of b); 22 min (60 % of b); 25 min (95 % of b); 35 min (0.5 % of b), and a further conditioning cycle to the initial conditions (balance time = 15 min)
B. ESI SOURCE CONDITIONS	
Ionization features and source	MS/MS carried out in SRM (*selected reaction monitoring*) mode with electrospray ionization (ESI) in negative mode
Gas temperature	350 °C
Gas flow	11 l min^{-1}
Nebulizer	25 psi
Capillary tension	400 V
Conus voltage	1.27 µA

3 Results

3.1 Temperature x Nitrate experiment (Exp. I-TxN)

Gallic acid was the only polyphenol that could be identified quantitatively in the samples but only with the transition 169 → 125 (not the 169 → 79); therefore, we will consider the obtained signals as a structural isomer of the gallic acid. The concentration of gallic acid was the highest in *C. hispida* from Somolinos lake (Fig. 3) with more than 40 mg/kg DW. The concentration of this polyphenol was significantly higher in the coastal population under warmer temperature (Fig. 3A). However, gallic acid concentrations did not differ significantly among nitrate treatments in any of the populations of both studied species (Fig. 3B).

Protocatechuic acid (a major metabolite of anthocyanin) (Fig. 4A) and caffeic acid (Fig. 4B) were the polyphenols that could be analysed qualitatively (together with the gallic acid, with five peaks at different retention times).

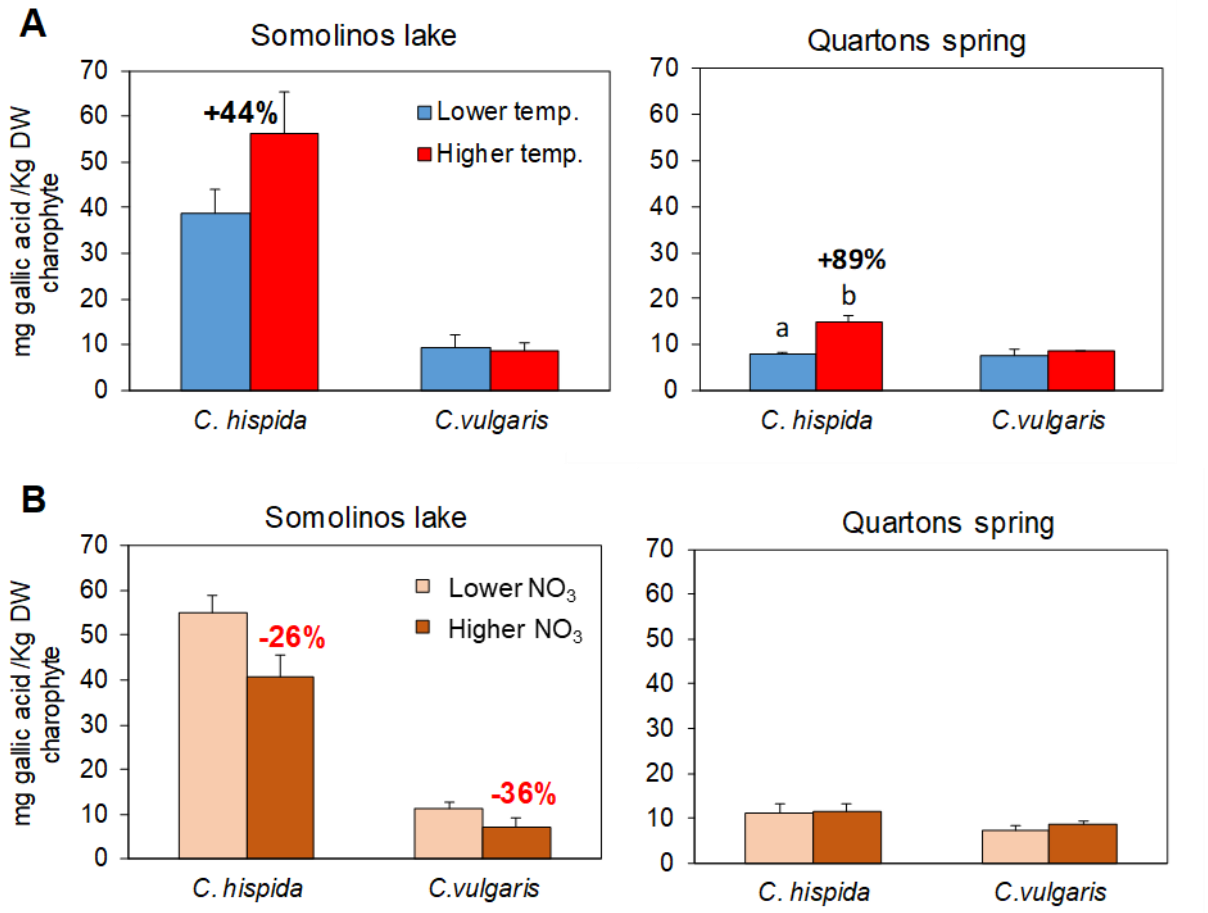

Fig. 3 Mean concentrations of a structural isomer of gallic acid in each of the four experimental conditions (A: separated by temperature treatment; B: separated by nitrate concentration treatment) for *Chara hispida* and *C. vulgaris* from Somolinos lake and Quartons spring. The percentages of change between the levels of the treatments are represented in the graphs. The thin bars show standard errors.

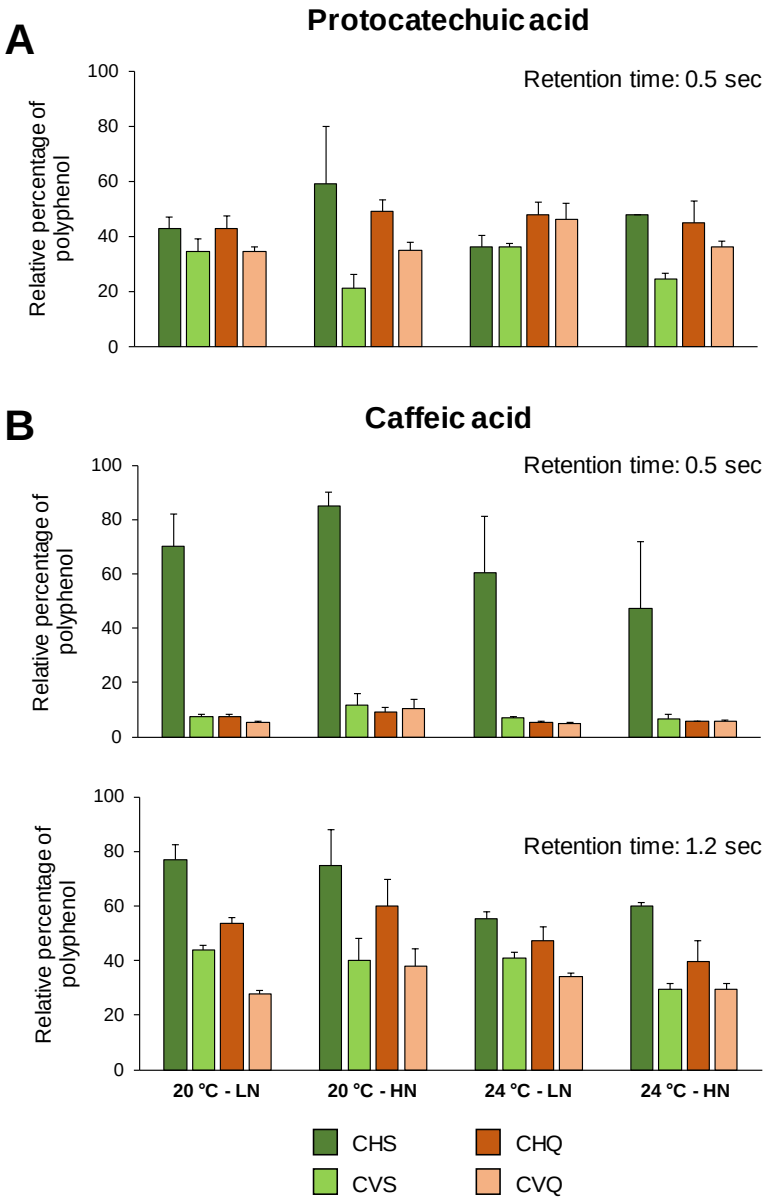

Fig. 4 Relative percentage of (A) protocatechuic acid, and (B) caffeic acid in *Chara hispida* (CH) and *C. vulgaris* (CV) from Somolinos lake (S) and Quartons spring (Q) in the different experimental conditions. LN: lower nitrate concentration, HN: higher nitrate concentration. The thin bars show standard errors.

Considering all samples independently of their origin and species, only the concentration of caffeic acid was statistically lower at 24 °C (Fig. 5A). Neither the nitrate concentration nor its interaction with temperature had significant effects on the relative percentage of the analysed polyphenols. Comparing the species independently of the population origin and temperature and nitrate treatments (Fig. 5B), the percentage of both caffeic and protocatechuic acids were significantly higher in *C. hispida* than in *C. vulgaris*. When comparing the origin of the populations independently of the temperature and nitrate treatments, the percentage of caffeic acid was higher in charophytes from Somolinos lake than in charophytes from Quartons spring (Fig. 5C).

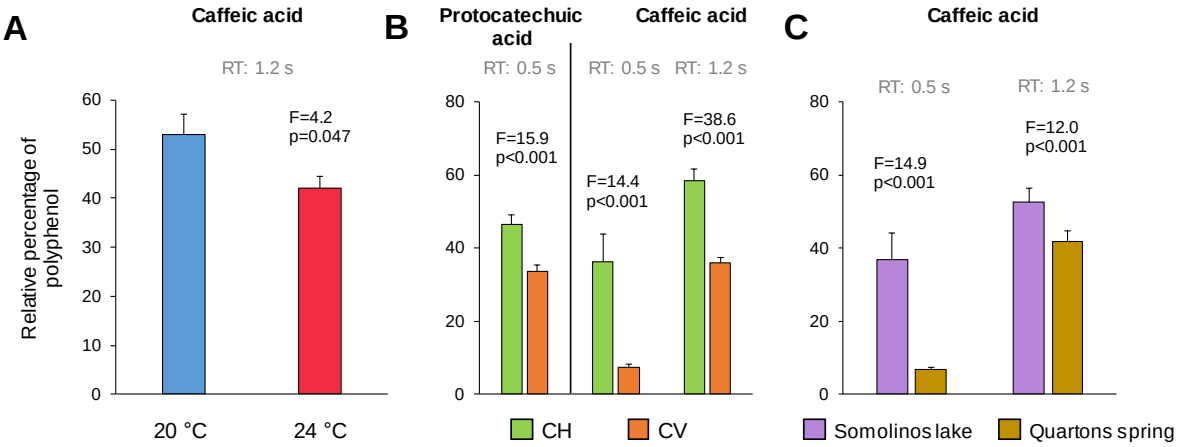

Fig. 5 Mean values of (A) the percentage of caffeic acid in the charophytes grown at the two temperatures, (B) the percentage of protocatechuic and caffeic acids in *Chara hispida* (CH) and *C. vulgaris* (CV) (the vertical line separates the two compounds), and (C) the percentage of caffeic acid in the charophytes from lake Somolinos and from Quartons spring. RT: retention time. In each graph, the ANOVA F-statistic and p-value are shown. The thin bars show standard errors.

3.2 Ultraviolet radiation experiment (Exp. II-UVR)

3.2.1 Optimization and validation of the analytical procedure

The extraction with 0.1 g of lyophilized charophyte sample (instead of 0.05 g) yielded better results in the obtaining of catechin, epigallocatechin gallate, and quercetin 3-rhamnoside (Fig. 6A). No differences were found with the obtaining of gallic acid. Quercetin was not obtained in any case. The extraction using or not n-hexane was similar for gallic acid and quercetin 3-rhamnoside (Fig. 6B). In the case of the catechin and epigallocatechin gallate, the use of n-hexane improved the extraction of these polyphenols (Fig. 6B). Regarding the different proportions of methanol-water as a solvent, the 30:70 ratio (v/v) produced the lowest concentrations of catechin, and quercetin 3-rhamnoside (Fig. 6C); instead, the 70:30 proportion yield the highest concentration of these polyphenols. The epigallocatechin gallate was only detected when the 70:30 ratio was used, but for gallic acid, this ratio caused the lowest recovery. As a consequence of these results, the chosen proportion to treat the experimental samples was 70:30 v/v methanol-water (described in the Material and Methods section).

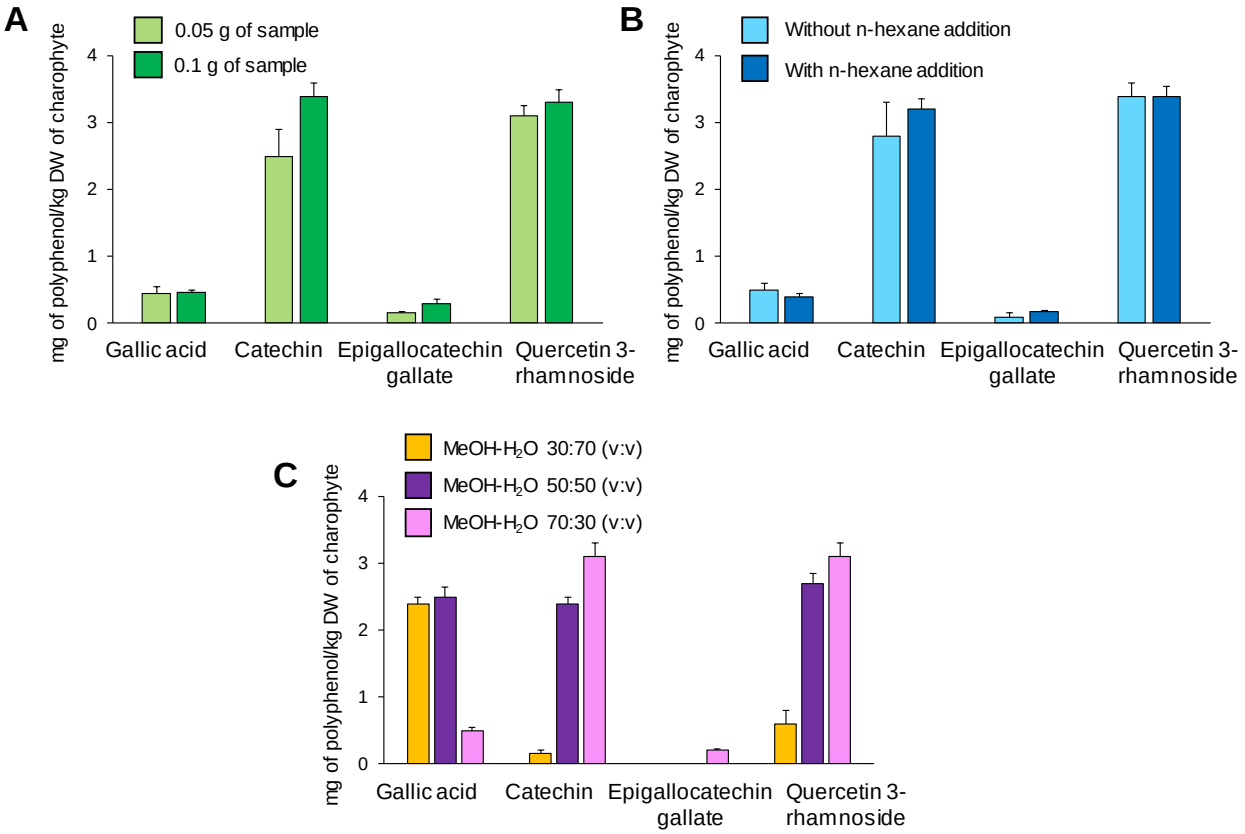

Fig. 6 Mean values of the polyphenol concentrations obtained using (A) two different amounts of charophyte sample, (B) without and with n-hexane addition, and (C) trying different proportions of methanol-water. The thin bars show standard errors.

3.2.2. Polyphenol identification and quantification

Only two polyphenol compounds were obtained in sufficient amounts to be quantified: gallic acid and quercetin 3-rhamnoside. The first showed higher concentrations in the *C. hispida* population from Llacuna del Dossel pond in both conditions (PAR and UVR) (Fig. 7A). In both populations the concentration was higher in the UVR treatment, this increase being more pronounced in the Llacuna del Dossel pond population (40 % versus 29 %) (Fig. 7A). The quercetin 3-rhamnoside concentrations were much lower than that of gallic acid and did not vary either with the origin of population or with the light conditions (Fig. 7B). This is a one-replicate-situation because the samples were pooled due to low biomass of individual plants.

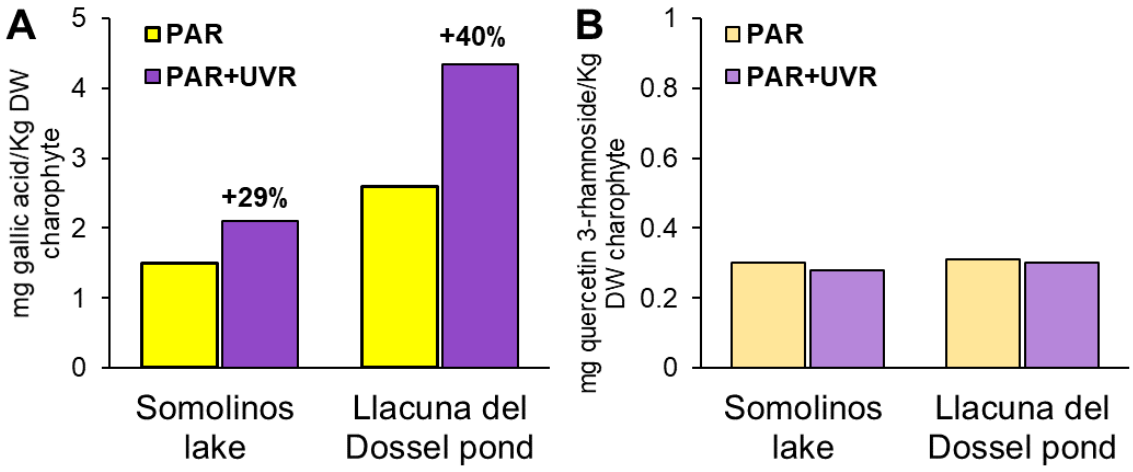

Fig. 7 Polyphenol concentrations in *Chara hispida* from Somolinos lake and Llacuna del Dossel pond under PAR (photosynthetically active radiation) only and PAR+ ultraviolet radiation (UVR). Gallic acid (A) and quercetin 3-rhamnoside (B) concentrations (notice the difference of scale in the y-axis). The percentage of increase between treatments is indicated. No variability bars because the samples were pooled due to low biomass of individual specimens.

4 Discussion

4.1 Difficulties in extraction and quantification of polyphenols from charophytes

There exists a wide list of bibliography focusing on polyphenol extraction methods in food and vegetal-origin drinks (ROBINS 2003; CAPRIOTTI et al. 2014). However, studies about polyphenol determination in macroalgae are scarce. Furthermore, most of the investigations using algae in polyphenol studies are based on the antioxidant properties of these compounds related to human health (O'SULLIVAN et al. 2011; HEFFERNAN et al. 2015). Up to now, there is no standard method that can be used to extract all polyphenols or their structural isomers, but different methods provide different results. Another difficulty is that polyphenols can appear in their conjugated form, in which one or more sugar remains linked to hydroxyl groups or to the aromatic ring or associated with other compounds (QUIDEAU et al. 2011). Moreover, there are polyphenols easily extractable which are soluble in aqueous-organic solvents, and others that have high-molecular weights or are linked to the vegetal matrix which can be retained in the extraction residuals (SAURA-CALIXTO et al. 2007).

Most studies dealing with polyphenol extraction are based on the use of mixtures of water with organic solvents at acid conditions to achieve the highest extraction efficiency and higher stability of the easily oxidable compounds. Here, to concentrate the samples and eliminate the non-desirable substances which might interfere with the analyses, the SPE (solid phase extraction) method (ROBINS 2003; MULDERIJ et al. 2007) was assayed. Considering the possibility that polyphenols were retained in the STRATA™ cartridge, two different elution methods were assayed (only methanol and ammonium hydroxide-basified methanol). It was expected that the last method would facilitate the elution of polyphenols from the cartridge, however, there were no differences in the intensity of the obtained peaks with the different solvents. Then, it can be considered that the loss of polyphenols might be due to their water solubility, not being retained in the solid phase. Another cause of the loss of intensity is that, independently of the type of used solvent, the extraction of polyphenol was incomplete

and a certain amount of polyphenols was retained in the extraction residuals. The polyphenolic substances identified in this study are low-molecular-weight compounds easily solubilized in organic solvents. The Charophyceae probably possess non-extractable polyphenolic compounds that remain retained in the water-organic extraction waste or linked to the vegetal material, causing an underestimation of polyphenols (SAURA-CALIXTO et al. 2007). To study this possible issue, the charophytes were subjected to acid hydrolysis to eliminate the link of non-extractable polyphenols to the vegetal matrix. However, the peak intensity neither improved. Acid hydrolysis might improve the extraction yield but it also could degrade extractable polyphenols (JU & HOWARD 2003). Moreover, although polyphenols have different structures and molecular weights, their chemical properties are extremely similar (PARYS et al. 2007). Consequently, the determination of these compounds in an individual way is very complex and, in some cases, it is even not feasible (RODRÍGUEZ-BERMALDO DE QUIRÓS et al. 2010). This is why the polyphenol determination during the first experiment was based on percentages of each structural isomer per individual.

As stated above, despite the use of different methods to improve polyphenol determinations in both experiments, there were some difficulties in the quantification of polyphenols from charophytes. Therefore, the data in absolute values have to be considered with caution. However, since the different samples were treated in the same way, the response of the species in the production of polyphenol compounds facing different environmental scenarios can be relatively compared.

4.2 Influence of temperature and nitrate

The production of potentially toxic substances in macrophytes can differ according to the species (due to its phylogenetic position) or the origin (due to the adaptation and/or acclimation capacity facing environmental changes). This study has confirmed that species of the *Chara* genus can produce polyphenols as already suggested by ROJO et al. (2013a), particularly gallic and caffeic acids. Gallic acid has been previously detected in macrophytes such as *Myriophyllum spicatum* (NAKAI et al. 2010), in freshwater algae such as *Cladophora glomerata* (KORZENIOWSKA et al. 2020; NUTAUTAITE et al. 2022), and among charophytes, only in *Zygogonium* (Zygnematophyceae, the same ZCC clade as Charophyceae -DE VRIES et al. 2016; HOLZINGER & PICHRTOVÁ 2016-). Therefore, our study represents the second time that gallic acid has been reported for charophyte species, in this occasion two members of the Characeae family. Caffeic acid has been reported as a polyphenol produced by the macrophytes *Hydrilla verticillata* and *Vallisneria spiralis* (GAO et al. 2011).

The content of polyphenol compounds was different in each of the studied species. Although *C. hispida* and *C. vulgaris* belong to the same monophyletic group, they present morphological, physiological and ecological differences (RODRIGO et al. 2015). *C. vulgaris* is a small species, a good and rapid colonizer, with high growth rates, while *C. hispida* is a more robust species from more stable environments with a higher potential competition with other primary producers (with which they compete allelopathically) (ROJO et al. 2013a, b). This may explain the high contents of gallic acid and caffeic acid in *C. hispida* from Somolinos lake. Biotic and abiotic factors (e. g. competition for resources, temperature, light regime, nutrient load, etc.) can affect the production of allelochemicals (GROSS 2003a, b; GROSS et al. 2007; PAKDEL et al. 2013). Nutrient availability and light regime may directly influence the synthesis and regulation of the secondary metabolism at an enzymatic level, which could explain the variability

in the response of the different species. However, most studies dealing with this topic are based on higher plants (KORICHEVA et al. 1998; SMOLDERS et al. 2000) and few on submerged macroalgae (DALE 1985; BAUER et al. 2009). Moreover, it is considered that submerged macrophytes have lower concentrations of phenolic compounds than emergent and floating-leaved plants due to lower exposure to UVR, more pronounced nutrient limitations and lower vulnerability to herbivory according to SMOLDERS et al. (2000). In our study, under the same light intensity and without interspecific interactions, it was possible to demonstrate whether the nutrients affect the production of polyphenols in the two charophyte species. The tested nutrient here, nitrate, seems not to be responsible for changes in the phenolic production. In contrast to mycosporine-like aminoacids (MAAs), polyphenolic substances do not contain nitrogen, which makes sense that their production does not vary with nitrate availability. Increased temperature seems to enhance the production of gallic acid only in *C. hispida* and not in *C. vulgaris*.

4.3 Influence of ultraviolet radiation

UVR can not only affect the photosynthesis in aquatic primary producers, such as plants and charophytes, but also activate photoprotective mechanisms such as the synthesis of UV-absorbing compounds, like MAAs and polyphenolic substances (XIONG 2001; RUBIO et al. 2015). Both are water-soluble and natural UV-screen compounds because of their aromatic groups. In our experiment, the production of secondary metabolites such as polyphenols was influenced by both UVR and the origin of the population. As a response to UVR as a stress factor, *C. hispida* produced more gallic acid which might diminish the oxidative stress. However, the production of quercetin 3-rhamnoside was so small that the variation was minimal under UVR. As stated above, gallic acid has been previously found in other macrophytes, mainly in phanerogams (ZHU et al. 2010), relating it to its allelopathic potential against cyanobacteria. Our study suggests that gallic acid can also have a UVR-protective effect.

C. hispida from Llacuna del Dossel pond showed a higher concentration of polyphenol compounds. Nevertheless, the results of this experiment emerge from just one replicate per treatment (pooled samples) and should be interpreted with caution. Despite this, it is expected that populations living in a shallow habitat, such as Llacuna del Dossel pond, therefore more exposed to UVR fluctuations, may have a quicker and stronger response to the production of polyphenols than the population from the deeper waterbodies, such as Somolinos lake. As a response mechanism to light changes, charophytes can also exhibit morphological variations (SCHNEIDER et al. 2006). Plasticity to face these changing conditions seems to depend on local adaptation, and is higher in species or populations inhabiting shallower environments (RUBIO et al. 2015).

4.4 Concluding remarks

Despite the methodological difficulties for polyphenol determination in Charophyceae posed during our investigation, this study opens prospects to search for these compounds in this important component of aquatic ecosystem meadows as allelopathic substances and/or oxidative stress protectors, and to find out whether they respond to global change stressors, both individually and interactively. Our study has

demonstrated that two species of Charophyceae (*C. hispida* and *C. vulgaris*) produce polyphenol compounds (mainly gallic acid and caffeic acid). Facing a global change scenario, increased temperature might play a more important role in the production of polyphenols than increased eutrophication. It has been proven that the polyphenol production pattern is not only species-dependent but also population origin-dependent, and distinct for the different measured compounds.

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