Submerged macrophyte meadows are critically endangered by current global change, and this is even more evident in shallow waterbodies from the Mediterranean region. This thesis deeps into the role played by charophytes (a group of submerged macrophytes) in these ecosystems within a global change context. Through a multiscale experimental approach, not only the effects of global change-related factors on charophytes themselves but also on the aquatic community linked to them, and on some aspects of the ecosystem functioning are examined.

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PhD Thesis

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Programa de Doctorat en Biodiversitat i Biologia Evolutiva

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CERTIFY that **Mr. Eric Puche Franqueza** has carried out under our direction and with the greatest use, the research work included in this doctoral thesis, and entitled: "**Submerged macrophytes as key players in aquatic ecosystems under global change: a multiscale experimental approach**", to obtain the degree of Doctor in Biodiversity.

And for the record, in compliance with current legislation, we issue this certificate in València on October 16, 2020.

*CERTIFIQUEN que el **Sr. Eric Puche Franqueza** ha realitzat sota la nostra direcció i amb el major aprofitament, el treball d'investigació recollit en aquesta tesi doctoral, i que du per títol: "**Els macròfits submergits com a elements clau en ecosistemes aquàtics sota el canvi global: una aproximació experimental multiescala**", per a optar al grau de Doctor en Biodiversitat.*

I per deixar-ne constància, en compliment de la legislació vigent, expedim el present cerificat en València a 16 d'octubre de 2020.



María A. Rodrigo Alacreu **Carmen Rojo García-Morato** **Eric Puche Franqueza**

«Però això és una altra història i haurà de ser contada en una altra ocasió»

"But that's another story and shall be told another time"

Michael Ende

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Abstract

Current global change is imposing alterations in the ecosystems worldwide through interactive changes in main environmental factors (*e.g.* temperature, nutrient concentration and ultraviolet radiation). Freshwater ecosystems are highly vulnerable to these changes and, specifically in the Mediterranean region, the situation is worse since the majority of them are shallow, exposed to environmental and anthropic disturbances. The meadows of submerged macrophytes, and particularly, charophytes, are a conspicuous element of these systems with a crucial role for their functioning. They provide habitat for both planktonic and benthic organisms and maintain water quality by limiting phytoplankton growth, reducing nutrient loading and preventing sediment resuspension. However, these meadows are declining critically due to current global change and this thesis addresses the performance of submerged macrophytes and the foreseeable impacts in the ecosystems they inhabit in the context of a changing world. The main aims were i) to investigate the specific and infraspecific responses of charophytes facing the interactive effects of global change-related factors, ii) to elucidate the propagation of these effects through the meadow-associated biological community, emphasizing the relevance of non-trophic relationships, and iii) to disentagle the role of charophytes in the functioning of Mediterranean shallow lakes facing the foreseeable changes and focusing on the sediment microbial community. These goals were addressed through microcosm experiments with a common garden approach with coastal and high-mountain populations of two charophyte species, laboratory mesocosms simulating macrophyte-dominated shallow systems and field in-lagoon mesocosms with macrophytes meadows in a coastal ecosystem. We found both species- and population-specific patterns in the response of charophytes to concomitant environmental changes regarding growth, morphologic and metabolic variables. The coastal populations came up as those with the greatest phenotypic plasticity to overcome the expected environmental changes. On a community scale, through a

network approach, a charophytes-zooplanktonic herbivores tandem emerged as crucially important for the structure of the aquatic community. Furthermore, contrasting configurations (phytoplankton and macrophyte-dominated) were achieved by subjecting the communities to ultraviolet radiation and warming scenarios, respectively. Transferring this approach to natural ecosystems allowed the emergence of different patterns of benthic-pelagic coupling between ponds and lakes. Finally, we assessed how charophytes meadows influence the sediment microbial community by favouring denitrification, thus, impacting on the functioning of aquatic ecosystems. This thesis has contributed to depict the complex puzzle of shallow freshwater ecosystems placing charophytes meadows as a central piece in their structure and functioning within the current global change context.

Resum en extens[1]

Els ecosistemes aquàtics mediterranis i els macròfits submergits sota el canvi global

*El canvi global està imposant serioses i ràpides alteracions en els ecosistemes arreu del món, causant, entre d'altres, fragmentació d'hàbitats, eutrofització de l'aigua, acidificació, invasions biològiques i, en última instància, la pèrdua de biodiversitat, així com dels serveis proveïts pels ecosistemes. Aquests efectes vénen donats per canvis simultanis en els principals factors ambientals relacionats amb el canvi global (*i.e. *temperatura, concentració de nutrients i radiació ultraviolada, RUV). Els sistemes aquàtics continentals estan exposats a tots aquests factors i són considerats com molt vulnerables al canvi global. A la regió mediterrània, on hi ha un fort impacte antròpic (*e.g. *la forta pressió urbanística i l'agricultura intensiva que s'hi practica), s'espera que l'impacte del canvi global siga encara més notori. A més, en aquesta regió semiàrida, els ecosistemes aquàtics són, majoritàriament, llacs petits i somers, cosa que els fa més vulnerables front a les pertorbacions ambientals i antròpiques lligades al canvi global. Les previsions climàtiques per a aquesta regió per a finals de segle estimen un increment de la temperatura mitjana anual de 4-5°C junt a una dràstica disminució de les precipitacions. Açò conduirà a una disminució de la fondària de la columna d'aigua dels sistemes aquàtics i a alteracions en els règims hidrològics, afavorint l'increment de la concentració de nutrients en aquests sistemes ja de per si eutrofitzats, i alhora facilitarà que la RUV penetre més profundament, arribant inclús al fons d'aquests sistemes.*

D'entre els organismes que componen les comunitats aquàtiques, els macròfits submergits, i concretament, els caròfits, són uns dels més conspicus en els sistemes aquàtics mediterranis. Les praderes que formen aquests organismes tenen capacitat de modificar físicament el seu entorn, incrementant la diversitat d'hàbitats i contribuint al flux de recursos, per la qual cosa se'ls considera com a enginyers de l'ecosistema. Així, aquestes praderes serveixen com a embornal de nutrients i estan fortament lligades a la comunitat microbiana del sediment influint en els cicles biogeoquímics,

[1]*Aquest és un resum en extens sense referències taules ni figures on es resumeix la temática global de la tesi, s'estableixen els objectius, es repasa la metodologia emprada i es presenten i discuteixen els principals resultats. Finalment, es mostren les consideracions finals i conclusions.*

eviten la terbolesa de l'aigua estabilitzant el sediment amb el seu sistema rizoïdal, competeixen amb el fitoplàncton pels nutrients, amb qui estableixen també interaccions al·lelopàtiques, i serveixen de refugi i suport vital per a tota una sèrie d'organismes tant planctònics com bentònics. Malgrat aquestes funcions importants, les praderes de caròfits estan disminuint críticament en les últimes dècades degut a múltiples causes, i agreujat pel canvi global. Aquests organismes són sensibles a canvis ambientals, tant a curt com a llarg termini, cosa que els fa uns potents sentinelles dels efectes del canvi global sobre els sistemes aquàtics. Encara que s'han dut a terme estudis que investiguen els efectes independents de diversos factors ambientals sobre els macròfits submergits, en els últims anys hi ha una crida cap a estudis que aborden els efectes interactius dels factors ambientals relacionats amb el canvi global sobre aquests organismes com aproximació més realista del que està passant a la natura.

*A més, aquests impactes sobre els macròfits submergits tindran repercussions en la comunitat biològica lligada a ells, i per tant en el funcionament dels ecosistemes que habiten. Com hem vist, en aquestes comunitats aquàtiques s'estableixen tota una sèrie de relacions tròfiques i no-tròfiques que s'haurien de considerar en els models ecològics per tal de comprendre millor com respondran aquests sistemes al canvi global i com els efectes sobre un element clau, com els caròfits es propagaran a través d'aquesta xarxa multi-interacció. En aquest sentit, s'ha definit el paper de les espècies fundacionals com aquelles que centralitzen les interaccions no-tròfiques del sistema, que se situen a la base de la xarxa ecològica (*i.e. *productors primaris) i que dominen en biomassa. D'aquesta forma, és d'esperar que els caròfits complisquen aquest paper en els sistemes aquàtics. No obstant, se sap poc sobre com els efectes ambientals sobre els caròfits, així com sobre la resta d'organismes aquàtics, afectaran a les interconnexions que mantenen l'estructura d'aquestes comunitats. En aquesta tesi aprofitem açò, establint un model ecològic que considera els diversos tipus d'interaccions que s'hi donen en les comunitats aquàtiques d'un sistema somer i sotmetem a les comunitats a diversos escenaris de canvi global.*

Objectius de la tesi

*Amb aquesta tesi es tracta de dilucidar la funció realitzada pels caròfits en els ecosistemes aquàtics mediterranis sota un context de canvi global. A través d'una aproximació experimental amb escales de complexitat consecutives (*i.e. *poblacions, estructura de la comunitat i funcionament de l'ecosistema) s'han avaluat no només els efectes de factors relacionats amb el canvi global sobre els caròfits, sinó també sobre les comunitats aquàtiques lligades a aquests. D'aquesta manera, els objectius principals que es plantegen són:*

O1. *Investigar la resposta dels caròfits a nivell específic i infraspecífic front als efectes interactius d'uns dels principals factors de canvi global (*i.e. *concentració de nitrat, temperatura i RUV).*

O2. *Analitzar la propagació d'aquests efectes a través de la comunitat biològica lligada a les praderes de caròfits, emfatitzant la rellevància de les interaccions no-tròfiques.*

O3. *Discernir la implicació dels caròfits en alguns aspectes del funcionament dels llacs somers mediterranis fronts als canvis ambientals esperats.*

Metodologia: una aproximació experimental multiescala

La tesi es divideix en tres nivells de complexitat respecte a les praderes de caròfits: (i) ecologia dels organismes, poblacions i infra-poblacions, (ii) ecologia de les interaccions i (iii) ecologia funcional. Cadascun d'aquests nivells s'aborda des d'una escala experimental diferent (experiments de microcosmos, experiments de mesocosmos al laboratori i experiments de mesocosmos al camp).

*Per tal d'analitzar la resposta dels caròfits front a canvis ambientals a nivell d'organisme, poblacional i infra-poblacional, es va treballar amb poblacions de dues espècies cosmopolites de caròfit (*Chara hispida *L. i* Chara vulgaris *L.) procedents de dos sistemes amb característiques limnològiques clarament diferents (una llacuna costera i un llac d'alta muntanya). En els diferents experiments a escala de microcosmos*

*realitzats en el laboratori, aquestes poblacions foren sotmeses a canvis realistes i simultanis en diferents factors ambientals, com la concentració de nitrogen, la temperatura i la RUV. Les respostes d'aquestes poblacions a curt termini (els experiments tingueren una duració d'entre 15 i 26 dies) foren estudiades en base a variables referents al creixement (*e.g. *taxa de creixement), la morfologia (*e.g. *elongació de l'eix principal, ramificació lateral, distància internodal), el metabolisme (*e.g. *concentració de clorofil·les, producció de compostos d'absorció de RUV, activitat nitrat-reductasa, taxa de respiració) i la composició estequiomètrica. Per tal d'evitar l'efecte pseudorèplica, cadascun dels individus va ser sotmès a les condicions experimentals de forma aïllada i, a més, la posició que ocupaven les rèpliques dins la cambra de cultiu fou canviada periòdicament per tal d'evitar l'efecte posició. A més, les condicions experimentals desitjades en cada experiment foren també controlades i corregides periòdicament per tal d'evitar distorsions en les respostes observades. En tots els experiments, els individus van passar per un període d'aclimatació (pre-experimental) previ a l'inici del període experimental. Així mateix, a l'inici de l'experiment es van escollir rèpliques a l'atzar per tal d'obtenir mesures de les variables en temps inicial i poder comparar els seus valors amb els mesurats en les rèpliques restants a temps final.*

Concretament, en el Capítol 1 aquestes poblacions de caròfits foren sotmeses, en un disseny de jardí comú, a una sèrie de concentracions de nitrat en l'aigua (arribant a un màxim de 50 mg N-NO_3/L) per tal d'avaluar el llindar de tolerància d'aquests organismes front a l'eutrofització de l'aigua en referència als compostos nitrogenats. Aquests experiments es van dur a terme, per una banda amb els individus (rèpliques de cada població estudiada) flotant en l'aigua, sense estar units al sediment (situació menys realista, però necessària per tal de comprovar l'efecte del nitrat sobre els caròfits sense interferència de cap altre compost de nitrogen) i per altra banda, plantats en un sediment homogeni per a totes les poblacions (situació més realista on, a banda del nitrat, també entren en joc altres fonts de nitrogen presents al sediment).

En el Capítol 2, els individus d'aquestes poblacions foren sotmesos, en un experiment factorial, a dos nivells de concentració de nitrat a l'aigua (un dels nivells, anomenat Baix nitrat, corresponia a la concentració més baixa del lloc d'origen de cada població durant el període vegetatiu, mentre que l'altre nivell, anomenat Elevat nitrat, suposava un increment del doble d'aquesta concentració) i dos nivells de temperatura (20 i 24°C), representant, així, un escenari realista d'escalfament i eutrofització en els sistemes d'on procedien les poblacions estudiades. El Capítol 3 engloba dos experiments en els que es van sotmetre individus d'aquestes poblacions de caròfits a un increment de la RUV junt a un escalfament o un augment de la concentració de nitrat. Els nivells testats en aquests factors foren: RUV (presència/absència), temperatura (23 i 27°C) i concentració de nitrat (Baix nitrat, corresponent a la concentració més baixa durant el període vegetatiu en l'ecosistema d'alta muntanya, i Elevat nitrat, corresponent a un increment de deu vegades aquesta concentració). Per a aquests experiments, els individus de caròfit foren plantats i col·locats dins d'uns cilindres de metacrilat que deixaven passar la RUV i la radiació fotosintèticament activa (RFA) que procedia d'uns tubs especials (tubs de RUV-A, RUV-B i tubs de vapor de sodi a alta pressió) situats a la part superior del muntatge de laboratori. Les dosis de radiació foren mesurades amb un espectroradiòmetre en diferents punts de la columna d'aigua on creixien els caròfits.

Respecte a l'estudi de la propagació dels efectes ambientals sobre els caròfits a través de la comunitat aquàtica associada a ells, es van establir uns sistemes experimentals en la planta d'aquaris del Servei Central de Suport a la Investigació Experimental de la Universitat de València, que simulaven un ecosistema aquàtic somer amb praderes de caròfits en un experiment de mesocosmos. Aquests sistemes corresponien a uns tancs (mesocosmos) de 170 L de capacitat (0,75 m de llargària x 0,48 m d'amplària x 0,47 m de columna d'aigua). El fons d'aquests tancs fou cobert amb una mescla de sediment artificial, grava i sediment natural provinent d'una llacuna costanera. En aquest sediment i en una meitat de cada tanc es van plantar caròfits de l'espècie C. hispida *procedents de la mateixa llacuna que el sediment, per*

tal que s'establira una pradera uniforme en aquesta meitat del tanc. Posteriorment, els tancs foren emplenats amb aigua de l'aixeta junt amb un inòcul d'aigua de la llacuna. D'aquesta manera es va aconseguir que s'establira tota una comunitat planctònica i bentònica associada a la pradera de caròfits. Els ambients considerats en cada tanc foren: pelàgic, corresponent als organismes planctònics en la meitat del mesocosmos sense pradera de caròfits, entre-pradera, corresponent als organismes planctònics de l'aigua lliure dins de la pradera de caròfits, i bentònic, corresponent als caròfits mateixa així com als organismes que vivien sobre la seua superfície. En total es van establir 12 mesocosmos que foren les rèpliques de l'experiment. Aquestes foren sotmeses, en quadruplicats, a tres escenaris de canvi global amb la temperatura i la RUV com a factors assajats: un escenari de RUV (consistia en una dosi de RUV que s'afegia a la RFA que rebien els mesocosmos i una temperatura de l'aigua de 22°C), un escenari d'escalfament (amb un increment de temperatura de 4°C, per tant 26°C a l'aigua i soles RFA com a radiació) i un escenari control sense escalfament ni dosi de RUV. L'experiment va durar dos mesos. Al primer mes i al final de l'experiment, cadascun dels ambients establerts en cada mesoscom va ser mostrejat respecte a productors primaris (fitoplàncton, fitobentos, cianobacteris i caròfits) i consumidors (bacteris heteròtrofs, zooplàncton, zoobentos i gasteròpodes). Els organismes planctònics foren identificats i recomptats mitjançant microscòpia invertida, a partir de mostres d'aigua degudament filtrades i fixades. Per als organismes bentònics, es van raspar individus de caròfits per tal de recollir els organismes que vivien sobre la seua superfície. Amb açò, es va calcular la densitat de cada taxó, referenciat al volum d'aigua (per al cas dels organismes planctònics) o al pes sec de caròfit (per al cas dels organismes bentònics). Posteriorment, aquestes densitats foren convertides a biomassa de carboni amb les fórmules establertes per a aquests tipus d'organismes. La biomassa de caròfits en cada mesocosm fou mesurada al final de l'experiment i a través de fotografies zenitals fetes a cada mesocosm es va poder establir una relació biomassa-àrea ocupada de pradera, per tal d'extrapolar la biomassa de caròfits a la meitat de l'experiment.

*Amb aquestes comunitats biològiques, es va construir la xarxa multi-interacció (*i.e. *considerant interaccions tròfiques i no-tròfiques entre els seus elements) que es presenta en el Capítol 4, establint una sèrie de criteris taxonòmics i funcionals per a definir els nodes i les connexions entre aquests. Aquesta xarxa fou analitzada atenent a l'estructura global (amb paràmetres, com per exemple, la connectància, la modularitat o l'aniuament), així com respecte al paper jugat pels diferents nodes (per exemple, aplicant diferents índexs de centralitat que mesuren la importància topològica de cada node, o analitzant el paper que juguen els nodes per a connectar els diferent mòduls funcionals que s'estableixen en la xarxa). En el Capítol 5, s'avaluen els canvis que es donaren en les comunitats aquàtiques dels mesocosmos experimentals i com aquests canvis es projecten en l'estructura de la xarxa multi-interacció. A més, es van calcular uns índexs de resistència i resiliència comparant els canvis produïts en la biomassa en carboni dels nodes entre els escenaris pertorbats i l'escenari control. En el Capítol 6, basat en les comunitats aquàtiques de l'experiment de mesocosmos, es van definir les xarxes purament tròfiques (només considerant les interaccions tròfiques) i es van comparar amb les xarxes multi-interacció mitjançant una sèrie d'índexs mesoescala. Aquests índexs topològics tenen en compte les relacions entre nodes de fins a un nombre determinat de passos (*i.e. *no sols considerant els veïns directes d'un node, sinó també els nodes amb els que interaccionen indirectament). D'aquesta forma, es va aprofundir en la rellevància de les interaccions no-tròfiques sobre el paper que juguen els diferents elements de les comunitats aquàtiques, i com les condicions ambientals modulen els canvis deguts a aquest tipus d'interaccions. En el Capítol 7, s'utilitzen els coneixements apresos en els anteriors capítols respecte a les xarxes ecològiques d'aquests sistemes per tal de comparar l'acoblament d'hàbitats entre llacunes i llacs amb praderes de caròfits. Les comunitats planctòniques i bentòniques dels sistemes estudiats van ser mostrejades seguint els mateixos protocols que en els capítols anteriors. Així mateix, es van aplicar els mateixos criteris per a la definició dels nodes funcionals. En aquest capítol, a més, es va fer un anàlisi centrat en la composició taxonòmica (*i.e. *diversitat, riquesa, dominància) i es va estudiar la*

redundància funcional de cada node definit. Aquesta aproximació més taxonòmica es va unir a l'aproximació funcional proveïda per la construcció de les xarxes multi-interacció dels sistemes estudiats.

*Per últim, el Capítol 8, correspon a un experiment amb mesocosmos al camp (*i.e. *limnocorrals) en una llacuna costanera sobre l'efecte interactiu de les praderes de caròfits i la RUV en la comunitat microbiana del sediment d'aquest sistema. Aquests limnocorrals (un total de 12) consistien en uns tancaments (0,25 m^2) ancorats al sediment de la llacuna, amb les parets fetes de malla plàstica que permetia el pas de l'aigua i de microorganismes però prevenia l'impacte d'altres organismes (*e.g. *carrancs, peixos, aus aquàtiques). La part superior dels limnocorrals estava coberta per uns plàstics especials que filtraven diferencialment la RUV per tal d'establir les condicions experimentals desitjades. Sis dels limnocorrals foren coberts amb un plàstic que filtrava a la meitat la RUV ambiental i els altres sis es cobriren amb un plàstic que no filtrava pràcticament res de RUV. A més, en sis dels limnocorrals es van plantar individus de* C. hispida *procedents d'una llacuna costanera propera, mentre que els altres sis es van deixar sense caròfits. Les condicions experimentals es van tractar d'uniformitzar entre les rèpliques d'un mateix tractament i les variables ambientals (*e.g. *temperatura de l'aigua, pH, conductivitat, nitrògen i fosfor total, dosis de radiació) es van mesurar de forma periòdica per tal de detectar anomalies. L'experiment va durar dos mesos. En cadascun dels limnocorrals es van col·lectar testimonis de sediment a l'inici i al final de l'experiment. En aquests testimonis es va separar una capa de sediment superficial i una de sub-superficial. El material corresponent a aquestes dues capes de sediment es va destinar a: i) l'estimació de la densitat bacteriana, ii) l'anàlisi de la composició taxonòmica de la comunitat bacteriana, iii) l'anàlisi de l'abundància i composició de microalgues i cianobacteris (sols en la capa superficial), i iv) l'anàlisi de la composició estequiomètrica del sediment.*

Principals resultats i discussió

En aquesta tesi s'ha demostrat que, darrere de la resposta de les poblacions de caròfit als canvis en els principals factors de canvi global, estan involucrades tant la filogènia (respostes específiques d'espècie) com l'adaptació a l'ambient local que habiten les poblacions (respostes específiques de població). Açò tindrà conseqüències en quant a la distribució geogràfica d'aquests organismes en els ecosistemes aquàtics, establint unes poblacions guanyadores amb suficient capacitat de superar les pertorbacions, en detriment d'altres que no seran capaços de fer-hi front.

Respecte a l'eutrofització per compostos nitrogenats, hem descobert que els caròfits tenen un límit de tolerància elevat front a la concentració de nitrat a l'aigua. D'acord amb els nostres resultats, el nitrat per se *no és tòxic per al metabolisme i el creixement d'aquests organismes, inclús en concentracions molt més elevades que les considerades com a perjudicials en treballs anteriors. Per tant, açò ens ha permès discernir que les raons ecològiques, i no tant les fisiològiques, lligades a l'augment de nutrients en l'aigua (com per exemple l'explosió en el creixement del fitoplàncton) podrien ser les causes del declivi de les praderes de caròfits en els ecosistemes aquàtics. A més, hem observat diferències específiques d'espècie en la resposta dels caròfits a l'eutrofització. Les poblacions de* C. vulgaris *mostraren una major capacitat de incorporació de nitrogen quan més nitrat hi havia a l'aigua, que les de* C. hispida*, reforçant així el caràcter pioner atribuït a* C. vulgaris*. A més, hem pogut confirmar que les poblacions costaneres d'aquesta espècie són les millor adaptades a les concentracions de nitrat més elevades, remarcant així l'efecte de l'ambient local en la resposta d'aquests organismes a les pertorbacions.*

La temperatura és altre dels principals factors de canvi global. Les poblacions de caròfits que cohabiten en un mateix sistema presenten respostes diferents front a l'escalfament. De nou, les poblacions de C. vulgaris *(tant de costa com de muntanya) mostraren una gran plasticitat fenotípica, sent les més afavorides per l'increment de temperatura. Quan aquest escalfament es va acompanyar d'un augment en la*

concentració de nutrients, l'efecte interactiu d'aquests factors va ser evident en quant a l'assimilació i l'acumulació de nitrogen en els teixits dels caròfits. Les poblacions costeres demostraren una major capacitat d'emmagatzemar nitrogen als seus teixits quan més nitrat hi havia a l'aigua, i aquest fet es va veure afavorit per l'augment de la temperatura. Aquest resultat demostra que les poblacions dels ambients més variables són les més reactives front a un escenari realista d'escalfament i eutrofització dels sistemes aquàtics.

*La RUV també està fortament lligada al canvi global i és considerada com a perjudicial per als organismes aquàtics en general, degut als danys genètics que provoca, entre d'altres. En els ecosistemes aquàtics, la reducció de l'altura de la columna d'aigua, degut a les alteracions hidrològiques provocades pel canvi global, fa que la RUV puga penetrar inclús fins al fons d'aquests sistemes, impactant sobre els organismes lligats al sediment, com els caròfits. A més, l'increment de les dosis de RUV ve acompanyat d'un increment de la concentració de nutrients i de la temperatura. No obstant això, aquests efectes interactius han sigut poc estudiats en macròfits submergits. Els resultats d'aquesta tesi remarquen que l'increment de la temperatura mitiga de forma més eficient que l'increment de la concentració de nutrients els efectes deleteris de l'augment de RUV sobre els caròfits. A nivell molecular (*e.g. *respecte a la producció de compostos d'absorció de RUV) no s'observen diferències entre les poblacions de caròfit estudiades. Aquest fet pot ser degut a que aquests processos es deuen a mecanismes més conservadors d'adaptació cel·lular front a l'estrès. A més, se suggereix un compromís entre la producció d'aquest tipus de molècules i el creixement dels caròfits, ja que l'escalfament va afavorir el creixement d'aquests organismes però va previndre de la producció de les molècules protectores front a la RUV. Probablement, sota aquest escenari, els caròfits opten per mecanismes de fotoreparació de l'ADN que són energèticament menys costosos. A pesar d'aquesta uniformitat en la resposta a nivell molecular de les poblacions de caròfit estudiades, a nivell morfològic sí que es va observar una major plasticitat fenotípica de les poblacions costaneres respecte a les de*

*muntanya. D'aquesta forma, es revela una major capacitat protectora-restauradora front a un escenari d'increment de RUV, escalfament i eutrofització de les poblacions d'ambients més variables (*i.e. *poblacions costaneres) front a aquelles d'ambients més estables (*i.e. *poblacions de muntanya).*

Respecte a l'estudi dels efectes ambientals sobre la comunitat aquàtica associada a les praderes de caròfit, s'ha aplicat una aproximació de xarxa considerant, de forma novedosa per a aquest tipus de sistema, interaccions tròfiques i no-tròfiques. Així, els caròfits foren el node més central en quant a que fou el millor connectat amb la resta d'elements de la xarxa. Els caròfits són els principals contribuents d'interaccions no-tròfiques del sistema i, per tant, són candidats potencials a exercir-hi un paper fundacional. A més, els herbívors zooplanctònics emergiren com a importants connectors entre l'ambient planctònic i bentònic. Açò probablement és degut a la seua elevada mobilitat i el seu ampli espectre de dieta que inclou tant organismes planctònics com bentònics. Combinant aquests resultats, se suggereix un tàndem macròfits-herbívors estructural- i funcionalment important en els ecosistemes aquàtics. De fet, davant la simulació d'una pertorbació que impacta aquests elements, l'estructura completa de la xarxa es veu afectada. El dany en els caròfits afecta principalment a la comunitat bentònica, però també al plàncton lligat a pradera. Per la seua banda, quan els herbívors es veuen perjudicats, els ambients planctònics i bentònics es veuen més aïllats per la pèrdua d'aquesta funció de pont que exerceixen aquests organismes en la comunitat.

En sotmetre aquesta comunitat experimental a escenaris de canvi global, es va poder comprovar que, sota un increment de la RUV, principalment els mixòtrofs i els bacteris heteròtrofs es veuen afavorits en detriment dels caròfits, els herbívors i els carnívors zooplanctònics. Açò apunta a la prevalència del bucle microbià davant d'aquest escenari. No obstant, front a un escenari d'escalfament, els caròfits assolixen la major biomassa i els herbívors i diatomees associats a les seues praderes també es veuen afavorits. Així, l'estructura de la xarxa ecològica es veu afectada i

s'aconsegueixen dues configuracions diferents: una dominància del plàncton sota l'escenari d'increment de la RUV i una dominància dels caròfits sota l'escenari d'escalfament. Aquestes configuracions recorden als estats alternatius definits per als sistemes somers i evidencien el paper fonamental de les praderes de macròfits en el seu assoliment.

A més, respecte a la importància que té la inclusió de les relacions no-tròfiques en els models ecològics dels sistemes aquàtics, es va poder demostrar que la influència estructural dels nodes en aquests canvia dràsticament quan aquest tipus d'interacció és incorporat a les xarxes purament tròfiques. Açò posa en evidència la sobreestimació de les cascades tròfiques a costa de l'emmascarament de la importància estructural d'altres elements que, malgrat no participar en la xarxa com a font d'aliment, realitzen una important tasca en el funcionament de l'ecosistema (com per exemple els macròfits submergits i les algues filamentoses). A més, en incorporar les relacions no-tròfiques, l'hàbitat bentònic (on es condensen aquestes interaccions) emergeix com a crucial per a l'ecosistema. Amb aquests resultats, es recolça la idea de certs autors que demanen la consideració de l'hàbitat bentònic així com les connexions plàncton-bentos per tal d'assolir una visió més realista i menys esbiaixada del funcionament dels ecosistemes aquàtics, especialment en el context de canvi global.

Transferint aquests coneixements respecte a les xarxes ecològiques a sistemes aquàtics naturals, s'han pogut observar diferents patrons d'acoblament entre hàbitats en llacunes i llacs amb praderes de caròfits. En analitzar les comunitats d'aquests sistemes morfomètricament diferenciats, es va observar un acoblament bentònic-pelàgic en les llacunes que no es donava en els llacs. Taxonòmicament, les llacunes mostraven un major grau de barreja entre el bentos i el plàncton. No obstant, en la xarxa ecològica emergiren tres mòduls funcionals diferenciats (un bucle microbià, una cadena tròfica planctònica i una bentònica) que estaven conectats per elements com els caròfits, els mixòtrofs i els herbívors associats a la pradera. En canvi, en els llacs van sorgir dos mòduls clarament aïllats (plàncton i bentos). Aquests resultats impliquen que

en els llacs, la important producció primària bentònica quedaria desconnectada del flux de matèria i energia del sistema. No obstant això, els nodes bentònics dels llacs mostraren la major redundància funcional, i açò podria minimitzar l'efecte de la pèrdua d'espècies bentòniques sobre la integritat de la comunitat.

Continuant amb el desenvolupament anterior i anant un pas més enllà, a través de l'experiment amb limnocorrals, es va poder avaluar l'efecte de les praderes de caròfits i la RUV sobre la comunitat microbiana del sediment en una llacuna costanera. Aquesta comunitat està reconeguda com a una part fonamental dels cicles biogeoquímics dels sistemes aquàtics, i per tant, del seu funcionament. Els resultats d'aquesta tesi revelen l'elevada diversitat que presenta la comunitat microbiana del sediment d'aquests sistemes. La RUV va afectar negativament la biomassa i riquesa dels microorganismes (tant microalgues com bacteris) que composen el biofilm perifític, encara que aquest efecte deleteri es va veure minimitzat per la presència de praderes de caròfit. Aquestes praderes van afavorir el creixement de bacteris desnitrificants, la qual cosa és beneficiosa per a reduir la càrrega en nitrogen d'aquests sistemes freqüentment eutrofitzats, principalment per nitrogen. Amb açò, es recolzen els resultats anteriors respecte a la rellevància de l'hàbitat bentònic i els efectes del canvi global sobre aquest.

En aquesta tesi, s'ha representat un trencaclosques complex en el qual les praderes de caròfits són una peça central que acullen els principals elements connectors del sistema, proveeixen d'hàbitat a un ampli rang d'organismes, afavoreixen la presència de productors primaris no tòxics i fàcilment comestibles, contribueixen amb carboni i nitrogen i promouen la desnitrificació. Així, estan enormement involucrats en el funcionament dels ecosistemes aquàtics, subjugant la seua resposta front als canvis ambientals. Donada la posició central dels caròfits en els sistemes que habiten, i basant-se en els resultats oferits en aquesta tesi, les portes per a futures investigacions respecte a les praderes d'aquests organismes romanen obertes. Com a exemple, algunes d'aquestes investigacions futures haurien d'abordar, a una escala regional o inclús continental, la resposta dels caròfits a canvis simultanis en factors ambientals.

Altres estudis haurien d'incorporar el pes de les interaccions en les xarxes multi-interacció per tal d'aconseguir models que permeten quantificar, d'una forma més realista, la transferència energètica en els ecosistemes aquàtics. Per tant, es requereix trobar una moneda comuna per a mesurar les interaccions tròfiques i no-tròfiques. A través de tots aquests avanços, seria possible aprofundir en els mecanismes que propicien la resposta dels vulnerables ecosistemes aquàtics sota el canvi global i promoure una gestió que afavorisca les praderes de macròfits submergits.

Consideracions finals i conclusions

1. *Les respostes dels caròfits, com a organismes i com a poblacions, front als canvis ambientals assajats són degudes tant a la filogènia com a l'adaptació a l'ambient local que habiten.*
2. *Els caròfits tenen una elevada tolerància a la concentració de nitrat en l'aigua. Per tant, el nitrat,* per se*, no és tòxic per a aquests organismes i cal atribuir el declivi de les praderes en sistemes eutrofitzats a raons ecològiques derivades d'aquest increment de nutrients.*
3. *Els organismes de les poblacions costaneres (especialment els de l'espècie* Chara vulgaris*) són aquells que presenten una major plasticitat fenotípica i tenen una major capacitat de reaccionar i superar les pertorbacions relacionades amb el canvi global com l'escalfament de l'aigua, l'eutrofització i els seus efectes interactius.*
4. *Els efectes deleteris de la RUV en les poblacions de caròfits es veuen minimitzats principalment per l'increment de la temperatura. Aquesta millora és més evident en les poblacions costaneres, per tant, queda demostrada la major capacitat de resposta d'aquestes poblacions en comparació amb els seus homòlegs d'alta muntanya.*
5. *Aquestes respostes amb patrons específics d'espècie i de població comprometran la distribució d'aquests organismes en els ecosistemes aquàtics*

*continentals, establint un conjunt de poblacions guanyadores (*i.e. *poblacions costaneres) en detriment d'altres poblacions perdedores (*i.e. *poblacions de muntanya).*

6. *La incorporació d'interaccions no-tròfiques en l'estudi dels ecosistemes aquàtics dominats per praderes de caròfits és crucial per a establir models ecològics més realistes que permeten una millor comprensió del funcionament d'aquests sistemes.*

7. *En la xarxa multi-interacció experimental ací estudiada, el node dels caròfits va ser el millor connectat amb la resta d'elements. Aquests organismes poden ser considerats com a espècies fundacionals degut a que centralitzen les interaccions no-tròfiques, són la base d'aquestes xarxes (*i.e. *són productors primaris) i dominen en biomassa.*

8. *Els grans herbívors zooplanctònics lligats a les praderes van emergir com a connectors eficients entre els mòduls funcionals de la xarxa.*

9. *El tàndem caròfits-herbívors és crucial per a l'estructura i funció d'aquests sistemes.*

10. *Quan les comunitats aquàtiques són sotmeses a escenaris de canvi global, s'assoleixen dues configuracions clarament diferents: dominància del fitoplàncton sota un escenari d'increment de la RUV i dominància dels caròfits sota un escenari d'escalfament. L'actuació de les praderes de macròfits submergits és fonamental per a aconseguir aquestes configuracions.*

11. *L'aplicació de l'aproximació de xarxa en sistemes naturals condueix a l'aparició de patrons diferents d'acoblament d'hàbitats entre llacunes i llacs amb praderes de macròfits. L'acoblament bentònic-pelàgic ocorre en llacunes mentre que en llacs, els mòduls funcionals romanen desconnectats.*

12. *La presència de praderes de macròfits protegeix la comunitat microbiana del sediment dels efectes nocius de la RUV i promou el creixement de bacteris*

desnitrificants. Aquest fet és beneficiós per a reduir la càrrega interna dels eutrofitzats ecosistemes somers mediterranis.

13. *Combinar el coneixement sobre l'ecologia dels caròfits amb el referent a les implicacions a nivell de comunitat en un context de canvi global ha permès acostar-se a la complexitat dels sistemes aquàtics mediterranis i a comprendre millor la seua resposta front a les pertorbacions ambientals a les quals estan exposats.*

|GENERAL INTRODUCTION|

Freshwater ecosystems under the global change context: complexity over complexity

Global change is imposing notorious and rapid alterations in the ecosystems around the world causing, among other effects, habitat fragmentation, water eutrophication, acidification, biological invasions and, ultimately, the loss of biodiversity as well as the provided ecosystem services (Sala *et al.* 2000, Steffen *et al.* 2004, Visconti *et al.* 2015). It should be noted that the incidence of global change is expected to vary depending on the different regions of the planet (IPCC 2014). This fact confers a geographic complexity that has to be taken into account to predict the responses of ecosystems facing the foreseeable environmental changes. Furthermore, the global change effects are driven by a complexity of environmental factors acting simultaneously (*e.g.* temperature, ultraviolet radiation (UVR), nutrients and salts concentration) and induced by anthropogenic impacts (Heino *et al.* 2009, Zhang *et al.* 2017). Therefore, to assess how global change is affecting ecosystems, it is also crucial to attend to the likely interactions (*e.g.* synergisms and antagonisms) occurring between these factors (Breitburg *et al.* 1998, Christensen *et al.* 2006, Carrillo *et al.* 2008, Lindenmayer *et al.* 2010). Freshwater ecosystems are exposed to all these stressors and are considered as very vulnerable to global change (Winder and Schindler 2004, Ormerod *et al.* 2010, Angeler *et al.* 2014, Jackson *et al.* 2016). These ecosystems house an intrinsic structural and functional complexity with different habitats (both planktonic and benthic) coupled with each other through matter and energy flows among their elements (Lodge *et al.* 1998, Tokeshi and Arakaki 2012). Thus, the study of freshwater ecosystems in a global change context supposes an issue of complexity over complexity that must be tackled to better predict the future of these valuable ecosystems (Woodward 2009).

In the Mediterranean region, all these different levels of complexity come together even more notably, putting it in a priority place in the study of global change (Beklioglu *et al.* 2007, Giorgi and Lionello 2008). It is a semi-arid region in which the majority of

waterbodies are shallow and small lakes, considered as the most vulnerable to environmental changes (Álvarez-Cobelas *et al.* 2006, Parcerisas *et al.* 2012). Climate forecasts for this region by the end of the century include an increase in the average annual temperature of 4-5°C together with a drastic reduction in rainfall (Fig. 1; Christensen *et al.* 2007, Giorgi and Lionello 2008, IPCC 2014). This combination will lead a decrease in the water column and changes in hydrological regimes triggering a higher water concentration of nutrients and salts and allowing the UVR to penetrate deeper (even to the bottom of these shallow systems; Fig. 1; Mariotti *et al.* 2008, Lelieveld *et al.* 2012, Rubio *et al.* 2015). These environmental changes are combined with anthropogenic impacts such as urbanization, sewage disposal and the massive use of fertilizers (such as nitrate) in intensive agriculture that have increased dramatically the internal loading of these ecosystems, making eutrophication one of its main threats (Fig. 1; Beklioglu *et al.* 2007, Rodrigo *et al.* 2013).

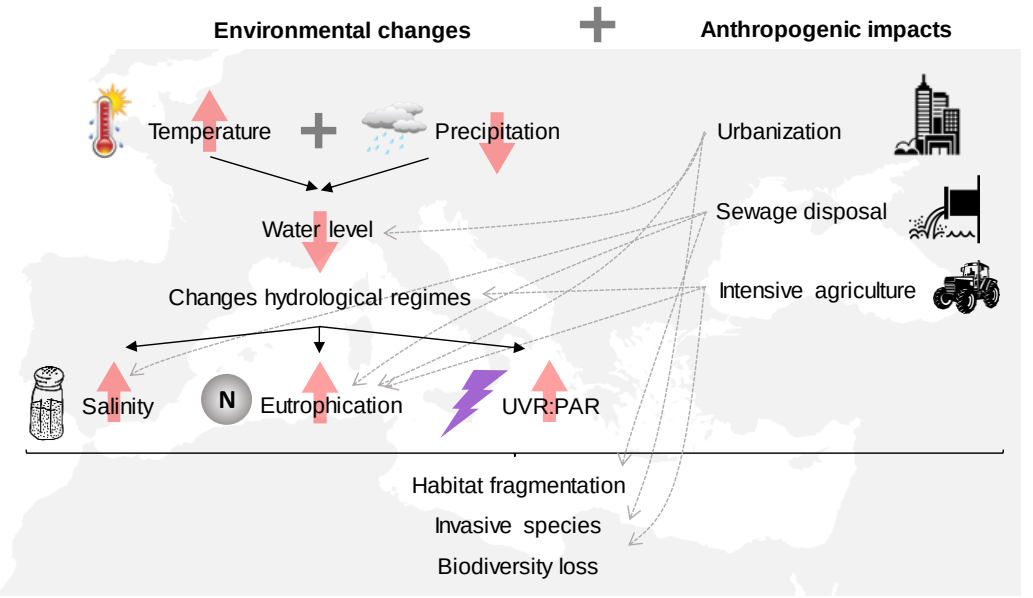


Fig. 1. Diagram showing the relationships between the foreseeable environmental changes and the major anthropogenic impacts in the Mediterranean region as well as their effects on the structure and biodiversity of freshwater ecosystems.

Under these highly variable and, in many cases, unpredictable conditions, the responses of the organisms inhabiting these ecosystems occur at a morphological, metabolic and even phenological level in order to persist and ensure the survival of future generations (Rodrigo *et al.* 2010, Ortells *et al.* 2014, Franch-Gras *et al.* 2019). The production of diapausing eggs by zooplankton (García-Roger *et al.* 2006, 2008, Carmona *et al.* 2009) or the production of drought-resistant diaspores by plants (Brock *et al.* 2003, Rodrigo and Alonso-Guillén 2013) are common strategies that allow to stablish banks in the sediment for future recovery of communities after a disturbance. But other types of responses have also been studied: behavioural, such as the avoidance of surface waters by crustacean zooplankton in response to an increase of UVR (Alonso *et al.* 2004); metabolic, such as the production of photoprotective compounds and the activation of repair mechanisms by primary producers under changes in the light environment (Banaszak 2003, Carrillo *et al.* 2008, Rojo *et al.* 2012); or phenological, like the variation in the timing of life events of submerged macrophytes facing changes in temperature, water depth and salinity (Calero *et al.* 2017a). Therefore, this thesis will address the study of Mediterranean freshwater ecosystems in a context of global change, combining experimental simulations and field work on natural communities, considering concomitant environmental factors as well as the functional diversity of these ecosystems.

Submerged macrophytes meadows: a central piece in the freshwater puzzle

Submerged macrophytes are one of the main primary producers in freshwater ecosystems around the world, although they have become seriously impaired in recent decades (Sand-Jensen *et al.* 2001, Rodrigo *et al.* 2010, Zhang *et al.* 2017). Among them, charophytes (green macroalgae from the Family Characeae, Class Charophyceae, Division Chlorophyta) are the group with the greatest presence and diversity in Mediterranean aquatic ecosystems (Cirujano *et al.* 2008). These organisms structure aquatic communities by forming dense meadows that can modify their physical surroundings, increasing habitat diversity and influencing the flow of resources, thus

they are considered as ecosystem engineers (Crain and Bertness 2006). In the shallow lakes and ponds typically found in the Mediterranean region, charophytes can dominate the entire water column (Sand-Jensen and Borum 1991, Rodrigo *et al.* 2015) and may be even more effective in maintaining water clarity, due to their potential persistence all year round resulting better competitors for nutrients and light than microalgae (Álvarez-Cobelas *et al.* 2005). In several studies, this structuring role has been reviewed (Scheffer 1993, Jeppesen *et al.* 1998, Blindow *et al.* 2002, Van Donk and van de Bund 2002), considering the different functions played by these organisms (Fig. 2). Submerged macrophytes in general, and charophytes in particular, act as a sink of nutrients (reducing the internal loading of aquatic ecosystems) and are tightly connected with the sediment microbial community below their meadows thus, influencing nutrients dynamics and biogeochemical cycles (Barko and James 1998, Rodrigo *et al.* 2007, Baveye 2019). Moreover, they stabilize the sediment with their rhizoidal system, preventing its resuspension and, therefore, reducing the water turbidity (Van Donk and van de Bund 2002). They also provide with vital support to the periphytic community living on their surface (Vadeboncoeur and Steinman 2002, Rojo *et al.* 2017a) and act as a refuge for planktonic organisms against their predators (Hampton *et al.* 2000, Rodrigo *et al.* 2015). Additionally, submerged macrophytes stablish allelopathic interactions with other primary producers (*e.g.* microalgae and cyanobacteria; Van Donk and van de Bund 2002, Rojo *et al.* 2013a, b) and serve as a food source for macroorganisms such as aquatic gastropods, herbivorous fish and waterbirds (Bakker *et al.* 2016, Wood *et al.* 2017).

Furthermore, submerged macrophytes (such as charophytes) serve as gauges of the ecological integrity of aquatic ecosystems due to their sensitivity to both long- and short-term changes in environmental factors (Lacoul and Freedman 2006, Hossain *et al.* 2017). In this vein, Schneider *et al.* (2006) assessed changes in charophytes morphology towards more flattened structures (by means of changes in the orientation and elongation of branches) under an increase in photosynthetically active

radiation. Other studies have reported increases in the growth and changes in the stoichiometry of charophytes under warmer conditions and eutrophication (Rojo *et al.* 2015, 2020) as well as the damage caused by salinity increases (Puche and Rodrigo 2015, Rojo *et al.* 2015). Moreover, Rubio *et al.* (2015) demonstrated, through a short-term experiment, the higher production of UV-absorbing compounds by charophytes under increasing UVR conditions. In the field, charophytes showed different phenological patterns depending on species- and even population-specific responses to environmental factors (Calero *et al.* 2017a, b). However, attempts to delve into the interacting effects of concomitant changes in environmental factors over submerged macrophytes are still scarce (Kosten *et al.* 2009, Cross *et al.* 2015, Rojo *et al.* 2017b). In fact, in the last years, several studies call for the assessment of the interaction between environmental factors to get more realistic predictions and interpretations of the effects of global change over freshwater ecosystems (Carrillo *et al.* 2008, Jackson *et al.* 2016, Rojo *et al.* 2017b, Villar-Argaiz *et al.* 2018), as it has been done for years in other types of ecosystems such as those marine (Gao *et al.* 2012, White *et al.* 2018) and terrestrial (Shaver *et al.* 2000, Wu *et al.* 2011). In response to these calls, in this thesis we intend to investigate the interactive effects of various factors related to global change (*i.e.* temperature, nitrate concentration and UVR) on charophytes meadows as well as on their associated community.

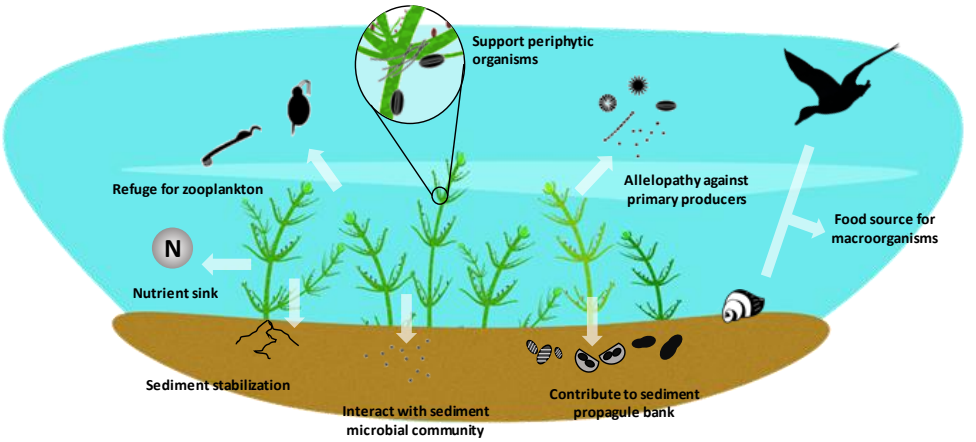


Fig. 2. Illustration depicting the main functions of submerged macrophytes on the nutrient dynamics, trophic structure and ecological interactions in freshwater ecosystems.

The vulnerability of submerged macrophytes to environmental changes potentially spreads throughout the aquatic community since, as previously commented, these organisms establish a myriad of both trophic and non-trophic connections with the different biological elements that make up freshwater ecosystems, occupying a central position in their gear (Carpenter and Lodge 1986, Hilt and Gross 2008, Rodrigo *et al.* 2015). In fact, for several years, these biotic interactions centralized by submerged macrophytes meadows with both macroorganisms (*e.g.* fish and invertebrates) and microorganisms (*e.g.* phyto-, zooplankton and bacteria) have been emphasized (Brönmark and Vermaat 1998, Le Bagousse-Pinguet *et al.* 2012, Zhao *et al.* 2013). In this sense, more recently Borst *et al.* (2018) and Ellison (2019) have described the foundation species (*e.g.* grasslands, trees in forests or sponges in corals) as those that i) dominate the system in terms of biomass, ii) are basal species (*i.e.* primary producers) and iii) compile the majority of non-trophic interactions in the system. Undoubtedly, charophytes meadows are a potential candidate to fill this function in shallow aquatic systems. However, little is known about how the differential responses of submerged macrophytes, as well as that of the rest of organisms in freshwater ecosystems facing environmental changes, will affect the interconnections of aquatic communities and, thus, the feedbacks maintaining the structure and function of these systems (Capon *et al.* 2015, Su *et al.* 2019). Therefore, a step forward in understanding the response of these ecosystems to current global change is to implement complex ecological models that contemplate this multi-interaction network in the context of environmental changes (Benton *et al.* 2007, Woodward *et al.* 2010, Spivak *et al.* 2010). In this thesis, we develop an ecological model merging trophic and non-trophic connections that depict the interrelation between charophytes meadows and their associated community and we put it in a global change context. Therefore, we expect to improve the understanding of the complex interactions occurring in the aquatic communities of freshwater ecosystems, in which submerged macrophytes meadows are central.

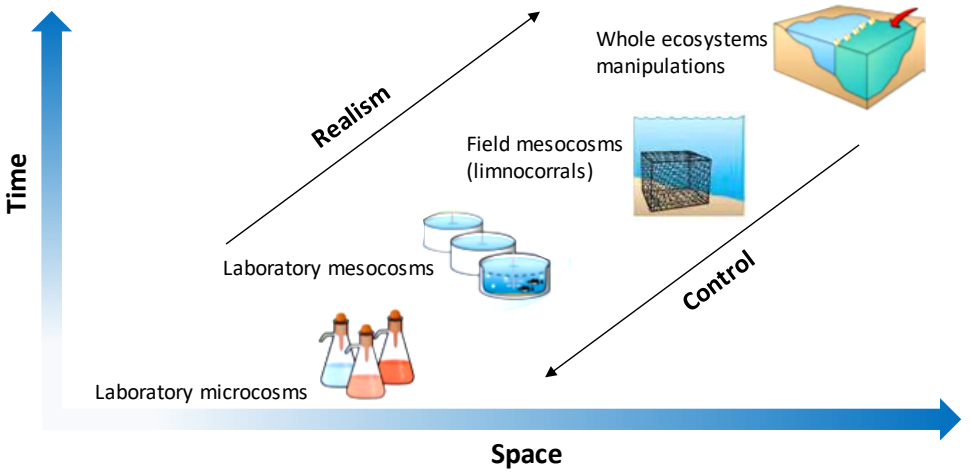
A multiscale experimental approach: unravelling the puzzle

Several decades ago, insightful research that considers a range of different organizational scales regarding the study of ecological systems was already advocated (Levin 1992). In fact, ecosystems do not have a single characteristic scale (Carpenter 1996) and the disturbances to which they are subjected affect different levels of organization (organisms or individual, population and community levels, and functional-ecosystem level; Woodward *et al.* 2010). This idea is transferred to experimentation in ecology with different types of experiments that address different scales of complexity answering diverse questions (Petersen *et al.* 2009). These experiments differ, mainly, in terms of the replicability, control and realism they provide. Generally, a rule of thumb is that the more realism, the less control and replicability (Fig. 3A). Thus, at the simplest end are microcosm-scale laboratory experiments, in which, generally, environmental components are related to the physiology, metabolic state and/or the growth of study organisms (at the individual- or even population-level) in small flasks or recipients (Fig. 3B; Beyers and Odum 1993). The mesocosm-scale experiments are in the next step of complexity. These experiments gain in realism with respect to those of microcosm since they include a greater biological complexity, with different trophic levels at the same time (*e.g.* laboratory mesocosms), and the whole community under complex environmental conditions (*e.g.* field mesocosms or limnocorrals) both allowing to test the response at the community level to global change (Fig. 3B; Stewart *et al.* 2013). At the other end (greatest complexity) are the whole-ecosystem experiments in which experimental manipulation of one or several biotic or abiotic factors in an ecosystem is conducted (Fig. 3B; Carpenter *et al.* 1995). Specifically, for freshwater ecosystems, these different-scale experiments are being used since 1970s (*e.g.* Gerhart and Likens 1975, Sarnelle 1997, Ahn and Mitsch 2002). However, the implementation of the global change perspective in these experiments is more recent and mainly focused on the effect of warming (*e.g.* Petchey 2000, Meerhoff *et al.* 2007, Yvon-Durocher *et al.* 2010),

eutrophication (*e.g.* Spivak *et al.* 2010), light quality (Carrillo *et al.* 2002) or even their interaction (*e.g.* McKee *et al.* 2003, Feuchtmayr *et al.* 2010, Netten *et al.* 2010, Carrillo *et al.* 2017).

The debate about the appropriateness of smaller-scale experiments for extrapolating results to the real world and about the almost unmanageable complexity that large-scale experiments entail has been raging for decades (Benton *et al.* 2007). Some authors firmly defend the whole-ecosystem experiments, arguing that micro- and mesocosm experiments are unrealistic simplifications with limited relevance to natural ecosystems (Carpenter 1996, Schindler 1998, Haag and Matschonat 2001). However, more recently, Benton *et al.* (2007) reviewed the supports for small-scale experiments as a very useful approach to deal with complex and intractable global problems, such as the response of ecosystems to current global change. These experiments offer a mechanistic perspective that allows the understanding of ecological processes behind the observed responses of the elements making up the ecosystems and provide the mathematical or computations models with the necessary biological understanding in which their assumptions are based (Benton *et al.* 2007). Other authors also support these ideas and attempt to extrapolate findings from mesocosm experiments to natural ecosystems (Kemp *et al.* 2001, Smith *et al.* 2005, Stewart *et al.* 2013). In fact, Woodward *et al.* 2009 advocate for the experimental context in the study of food webs by benefiting of the replicability offered by mesocosms to assess certain food-web properties. These authors give also some examples regarding the suitability of combining experimental studies with field work regarding freshwater ecosystems (*e.g.* Weidman *et al.* 2011). Taking advantage of this perspective, in this thesis we addressed a multiscale experimental approach that allowed to study the effects of global change in freshwater ecosystems dominated by charophyte meadows at different organizational levels, from individual-population level to community level and finally, inferring the impact on the ecosystem functioning.

A



B

	Type of experiment	Scale	Methods	Advantages	Disadvantages	Examples
	Laboratory microcosms	0.001-1 m^3	No attempt made to simulate an ecosystem	Allow highly controlled study of individual processes	Extrapolation to ecosystem context can be difficult	Temperature x Salinity on charophytes (Rojo et al. 2015) Salinity on charophytes (Puche and Rodrigo 2015)
	Laboratory mesocosms	0.01-100 m^3	Walls isolate organisms and processes. Chemical and physical environment is maintained through technology.	*(Both for laboratory and field mesocosms)* Very high degree of control over internal biological and physical conditions. Convenient location allows continuous monitoring and manipulation. High degree of realism and replication possible	May be expensive to construct and maintain depending on the degree of environmental control. Isolation and artifacts may distort dynamics	Warming and herbivory on submerged macrophytes (Cao *et al.* 2014)
	Field mesocosms (limnocorrals)	1-100 m^3	Semipermeable enclosure allows water and small organisms to move in or out and excludes others			Multiple factors on the development of macrophyte meadows (Rodrigo *et al.* 2013) UVR x nutrient input on phytoplankton (Carrillo *et al.* 2017)
	Whole ecosystem manipulations in nature	Scale of small natural ecosystems	Manipulate one or more factors. Maintain both control and treatment ecosystems	High degree of realism	Expensive; difficult to control and replicate for large ecosystems with environmental gradients	Eutrophication on the loss of macrophytes in experimental ponds (Irvine *et al.* 1989)

Fig. 3. A) Conceptual diagram representing an idealised two-dimension experimental framework (time x space). As the scale of experiments increases from simple laboratory microcosms to complex whole ecosystem manipulations, greater realism (in the sense of the ability to reproduce key properties of natural systems) is achieved but control over experimental conditions declines, B) main characteristics of the different types of experiments. Modified from: Petersen *et al.* 2009.

Thesis objectives

The main goal of this thesis is to elucidate the role played by charophytes in freshwater ecosystems from the Mediterranean region in a context of global change. By means of an experimental approach at different and consecutive scales of complexity (*i.e.* organisms, populations, community structure and ecosystem functioning) not only the effects on of global change-related factors on charophytes themselves but also on the aquatic community linked to them are examined.

The specific objectives and sub-objectives derived from this main goal are:

O1. To investigate the response of charophytes at specific and infraspecific levels facing the interactive effects of main global change-related factors.

- **O1.1.** To unravel the maximum tolerance threshold for nitrate concentration in the water in the specimens from populations of two charophytes species coming from nitrate-rich and nitrate-poor waterbodies.
- **O1.2.** To estimate the effects of a concomitant increase in water nitrate concentration and temperature in two species of charophytes from two limnologically contrasted waterbodies.
- **O1.3.** To estimate the effects of increase in UVR doses together with an increase of water nitrate concentration or temperature on two species of charophytes from two limnologically contrasted waterbodies.

O2. To elucidate the propagation of these effects through the biological community associated with the charophyte meadows, emphasizing the relevance of non-trophic relationships.

- **O2.1.** To establish taxonomic and functional criteria for the construction of the multi-interaction ecological network (*i.e.* considering trophic and non-trophic interactions) that depict the community linked to the charophyte meadows.
- **O2.2.** To evaluate the impact of different global change-related scenarios on the structure and function of the community linked to charophyte meadows.
- **O2.3.** To delve into the structural roles played by the different biological elements making up these communities, as well as the relevance of non-trophic interactions established among them.
- **O2.4.** To apply the findings obtained in these experimental multi-interaction networks to aquatic communities in natural ecosystems of different typologies (*i.e*. ponds and lakes).

O3. To disentangle the role of macrophytes in some aspects of the ecosystem functioning facing the foreseeable changes in Mediterranean shallow lakes.

Thesis structure

The aforementioned objectives, related to three experimental scales (*i.e.* microcosm, mesocosm in the laboratory and mesocosm in the field), addressed in this thesis, lead to the division of the compendium of publications on which it is based (presented as chapters in this thesis), into three large blocks (Fig. 4). Block 1 comprises the manuscripts on the microcosm scale experiments (Chapters 1 to 3). In these experiments we used specimens of the charophytes *Chara hispida* and *Chara vulgaris* from a Mediterranean coastal shallow waterbody (Quartons Spring) and from a continental mountain lake (Lake Somolinos; Fig. 4) with a common garden approach. Block 2 includes the manuscripts derived from the experiment at the mesocosm scale (Chapters 4 to 6) with specimens of *C. hispida* from a Mediterranean coastal interdunal pond (Pond Llacuna del Dossel; Fig. 4) and applying a network approach. Furthermore, in Chapter 7 this network knowledge was used for assessing aquatic communities related to charophyte meadows in natural ecosystems (Fig. 4). Finally, Block 3 consists of a manuscript (Chapter 8) addressing a field experiment (*i.e.* limnocorrals) with specimens of *C. hispida* from the Pond Llacuna del Dossel planted in a shallow waterbody located in a coastal protected area; the target was the sediment microbial community and the sediment stoichiometry (Fig. 4). In the General discussion section, these three blocks are considered together, compiling and discussing the main results. Finally, the main conclusions are presented in a Final remarks and conclusions section.

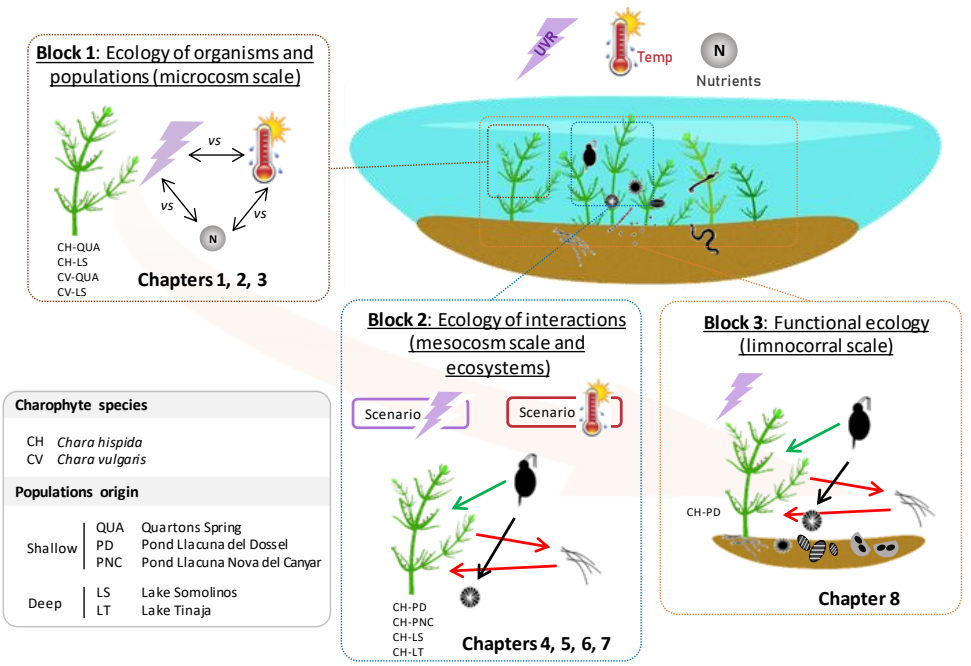


Fig. 4. Scheme of the general structure of this thesis in three blocks depending on ecological issues and their addressed experimental scale. In each of the blocks, the chapters that compose it are specified, and the target of study is represented: the charophytes themselves in the microcosm block, the interactions between the charophytes and the rest of the community organisms in the mesocosm and natural ecosystems block, and the effect of charophytes over the sediment community in the natural waterbody in the field mesocosm block. Likewise, the environmental conditions tested in each block are represented. The species and origin of charophytes used in each experimental scale are also specified in the legend to the left.

Six of the manuscripts presented in this thesis have been previously accepted and published in internationally indexed scientific journals. Those in Chapters 7 and 8 are submitted and ready for submission, respectively. In the thesis, the articles are presented maintaining the criteria of the journal where they have been published or submitted, although, to facilitate the reading, they have been edited like the rest of the text in the thesis. The content and aims of each of the manuscripts are summarized below:

Chapter 1: This manuscript delves into the nitrate tolerance threshold of two charophyte species (*C. hispida* and *C. vulgaris*) coming from two contrasting ecosystems in terms of nitrate concentration in the water, an oligotrophic deep

mountain lake and a mesotrophic shallow coastal waterbody. Specimens of these populations were subjected to a wide range of nitrate concentrations in two experiments (with free-floating or with planted individuals). Variables regarding growth, morphological architecture, stoichiometry and metabolism were measured.

Chapter 2: This publication focuses on the effect of a concomitant increase in nitrate concentration and in water temperature on the same charophyte populations as in manuscript in Chapter 1. The experimental design was factorial, subjecting individuals from each population to two levels of nitrate concentration and temperature. Changes in the growth, morphology, metabolism and stoichiometry were assessed, paying special attention to the interactive effects of the tested factors as well as to the species- and population-specific responses.

Chapter 3: This study encompasses two factorial experiments in which the interactive effects of the UVR together with an increase in water temperature or in the concentration of nutrients on the same populations of charophytes as for the previous manuscripts (Chapters 1 and 2) are addressed. Variables regarding growth, morphology, stoichiometry and metabolism related to the radiation (*i.e.* photosynthetic pigments and UV-absorbing compounds) were measured. These interactive effects were classified as additive, antagonistic or synergistic comparing them with a control condition.

Chapter 4: This manuscript establishes the taxonomic and functional criteria to construct the ecological network of an experimental simulation of a shallow ecosystem dominated by macrophytes. The functional nodes and the set of trophic and non-trophic interactions linking them were defined, resulting in a multi-interaction network. This network was analysed regarding its global structure and the roles played by its nodes. The effect of a simulated decrease in charophytes over the rest of the elements attending to the network structure was also studied.

Chapter 5: This publication combines the network perspective explained in Chapter 4 and an indoor-mesocosm experimentation with environmental scenarios (withtemperature and UVR as tested factors). The experimental aquatic communities

linked to charophytes meadows were subjected in replicates to the different scenarios. The carbon biomass represented by each node in the networks was assessed and the roles they played as well as their vulnerability to disturbances were analized. Finally, these results were gathered to explain the whole-community configurations attained under the environmental scenarios and to predict the performance of shallow freshwater ecosystems to the current global change.

Chapter 6: This manuscript focuses on the relevance of non-trophic interactions for the shallow freshwater ecosystems. Based on the aquatic communities from the mesocosm experiment of Chapter 5 the nodes roles between the multi-interaction networks of these communities (i.e. considering both trophic and non-trophic interactions) and the trophic network (i.e. considering only trophic interactions) were compared by means of several topological nodes indices. Furthermore, it was analysed how the environmental conditions can modulate the non-trophic effects in these networks.

Chapter 7: In this study, four natural ecosystems (two ponds and two lakes) with charophyte meadows were assessed through a model that combine the taxonomic composition of different habitats (both planktonic and benthic) with the multi-interaction perspective introduced in manuscripts of Chapters 4 to 6. This combination allowed to find differences in habitat coupling depending on the typology of the ecosystem and to define functional modules highly relevant in the response of aquatic ecosystems to disturbances.

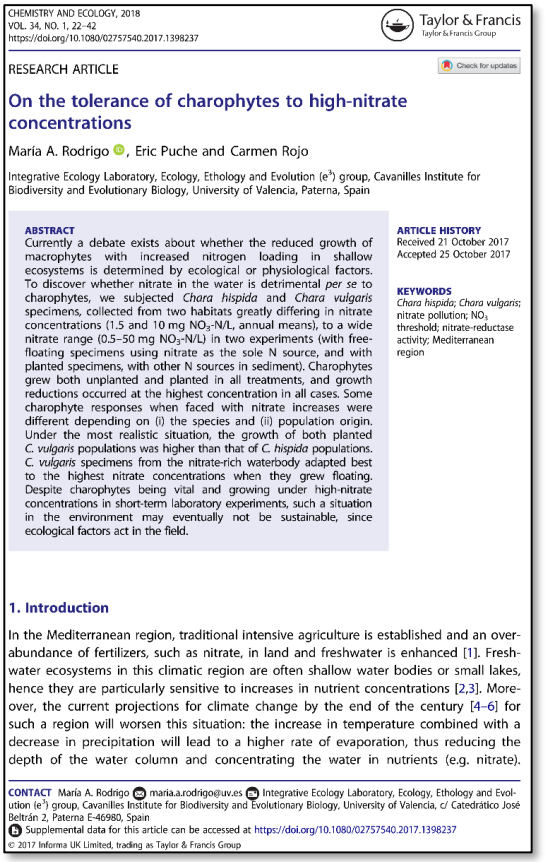
Chapter 8: This manuscript comprise a field experiment carried out in a nitrate-enriched coastal shallow pond located in a protected area. We used limnocorrals to perform a factorial design experiment with the presence/absence of charophytes meadows and natural/filtered UVR as tested factors. The main goal in this work was to assess how the sediment microbial community, which has a clear impact on the functioning of these ecosystems, is affected by sunlight UVR and how this effect could be modulated by the presence of charophytes meadows.

|BLOCK 1| ECOLOGY OF ORGANISMS, POPULATIONS AND INFRA-POPULATION LEVELS

(MICROCOSM SCALE)

| CHAPTER 1 |

On the tolerance of charophytes to high-nitrate concentrations



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RESEARCH ARTICLE

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On the tolerance of charophytes to high-nitrate concentrations

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ABSTRACT

Currently a debate exists about whether the reduced growth of macrophytes with increased nitrogen loading in shallow ecosystems is determined by ecological or physiological factors. To discover whether nitrate in the water is detrimental *per se* to charophytes, we subjected *Chara hispida* and *Chara vulgaris* specimens, collected from two habitats greatly differing in nitrate concentrations (1.5 and 10 mg NO_3-N/L, annual means), to a wide nitrate range (0.5–50 mg NO_3-N/L) in two experiments (with free-floating specimens using nitrate as the sole N source, and with planted specimens, with other N sources in sediment). Charophytes grew both unplanted and planted in all treatments, and growth reductions occurred at the highest concentration in all cases. Some charophyte responses when faced with nitrate increases were different depending on (i) the species and (ii) population origin. Under the most realistic situation, the growth of both planted *C. vulgaris* populations was higher than that of *C. hispida* populations. *C. vulgaris* specimens from the nitrate-rich waterbody adapted best to the highest nitrate concentrations when they grew floating. Despite charophytes being vital and growing under high-nitrate concentrations in short-term laboratory experiments, such a situation in the environment may eventually not be sustainable, since ecological factors act in the field.

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KEYWORDS
Chara hispida; *Chara vulgaris*; nitrate pollution; NO_3 threshold; nitrate-reductase activity; Mediterranean region

1. Introduction

In the Mediterranean region, traditional intensive agriculture is established and an over-abundance of fertilizers, such as nitrate, in land and freshwater is enhanced [1]. Freshwater ecosystems in this climatic region are often shallow water bodies or small lakes, hence they are particularly sensitive to increases in nutrient concentrations [2,3]. Moreover, the current projections for climate change by the end of the century [4–6] for such a region will worsen this situation: the increase in temperature combined with a decrease in precipitation will lead to a higher rate of evaporation, thus reducing the depth of the water column and concentrating the water in nutrients (e.g. nitrate).

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Abstract

Currently a debate exists about whether the reduced growth of macrophytes with increased nitrogen loading in shallow ecosystems is determined by ecological or physiological factors. To discover whether nitrate in the water is detrimental *per se* to charophytes, we subjected *Chara hispida* and *Chara vulgaris* specimens, collected from two habitats greatly differing in nitrate concentrations (1.5 and 10 mg NO_3-N/L, annual means), to a wide nitrate range (0.5–50 mg NO_3-N/L) in two experiments (with free-floating specimens using nitrate as the sole N source, and with planted specimens, with other N sources in sediment). Charophytes grew both unplanted and planted in all treatments, and growth reductions occurred at the highest concentration in all cases. Some charophyte responses when faced with nitrate increases were different depending on (i) the species and (ii) the population origin. Under the most realistic situation, the growth of both planted *C. vulgaris* populations was higher than that of *C. hispida* populations. *C. vulgaris* specimens from the nitrate-rich waterbody adapted best to the highest nitrate concentrations when they grew floating. Despite charophytes being vital and growing under high-nitrate concentrations in short-term laboratory experiments, such a situation in the environment may eventually not be sustainable, since ecological factors act in the field.

Keywords: *Chara hispida*; *Chara vulgaris*; nitrate pollution; NO_3 threshold; nitrate-reductase activity; Mediterranean region

Resum

Actualment existeix un debat sobre si la reducció del creixement dels macròfits degut a l'increment de la càrrega en nitrogen en ecosistemes aquàtics somers està determinada per factors ecològics o fisiològics. Per tal de descobrir si el nitrat en l'aigua es perjudicial per se *per als caròfits, nosaltres vam sotmetre espècimens de* Chara hispida *i* Chara vulgaris*, recol·lectats en dos hàbitats molt diferents respecte a la concentració de nitrat (1,5 i 10 mg NO_3-N/L, mitjana anual), a un ampli rang de nitrat (0,5-50 mg NO_3-N/L) en dos experiments (amb exemplars flotant lliures usant nitrat como a font de N única, i amb exemplars plantats, amb altres fonts de N al sediment). Els caròfits cresqueren tant flotant com plantats en tots els tractaments i les reduccions en el creixement van ocórrer sota la major concentració en tots els casos. Alguna de les respostes dels caròfits davant l'increment de la concentració de nitrat fou diferent depenent de (i) l'espècie i (ii) l'origen de la població. Sota la situació més realista, el creixement dels exemplars plantats d'ambdues poblacions de* C. vulgaris *fou major que el de les poblacions de* C. hispida*. Els espècimens de* C. vulgaris *de l'hàbitat ric en nitrat foren els que millor s'adaptaren a les majors concentracions de nitrat quan cresqueren flotant. Tot i que els caròfits mostraven un aspecte vital i cresqueren sota elevades concentracions de nitrat a curt termini en els experiments de laboratori, en la natura possiblement aquesta situació no siga sostenible, degut als factors ecològics que actuen al camp.*

Paraules clau: Chara hispida*; contaminació per nitrat; llindar de NO_3; activitat nitrat-reductasa; regió mediterrània*

1. Introduction

In the Mediterranean region, traditional intensive agriculture is established and an over-abundance of fertilizers, such as nitrate, in land and freshwater is enhanced [1]. Freshwater ecosystems in this climatic region are often shallow waterbodies or small lakes, hence they are particularly sensitive to increases in nutrient concentrations [2,3]. Moreover, the current projections for climate change by the end of the century [4–6] for such a region will worsen this situation: the increase in temperature combined with a decrease in precipitation will lead to a higher rate of evaporation, thus reducing the depth of the water column and concentrating the water in nutrients (*e.g.* nitrate). These rapid changes in the environment may affect the biodiversity and functioning of these ecosystems [7–10].

Currently there is a debate about whether the reduced growth of macrophytes with increased N loading in shallow ecosystems is determined by ecological or physiological factors [11,12]. With regard to charophytes, one important component of macrophyte flora in aquatic ecosystems, the elevated nitrate concentration has been described to be the strongest contraindication for the presence of charophytes in the wide range of waterbodies they typically inhabit [13]. From field data (62 sites in 124 waterbodies with over 400 site samples), and based on logistic regression, these authors predicted a transition from charophyte presence to absence in aquatic ecosystems at a concentration of approximately 2 mg NO_3-N/L. The experimental study with the species *Chara globularis*, whose growth was also progressively impaired above this concentration, supported their conclusion. However, we have evidence that *Chara hispida* and *Chara vulgaris* can live forming meadows with nitrate concentrations higher than 2 mg NO_3-N/L in waterbodies affected by seepage from agricultural runoff, as is the case of ponds and lakes in the Iberian Peninsula [14–17]. The confirmation of the different tolerances requires a greater effort in the study of the possible harmful, or toxic, effects of the nitrate excess on the charophytes. In fact, apart from Lambert and Davy's study [13], and the one performed by Simons *et al.* [18], we have not

encountered investigations dealing with nitrate concentration thresholds in other charophyte species.

Our aim is to contribute to the knowledge of the effect of different nitrate concentrations on the charophytes, including levels that represent a foreseeable increase in nitrate concentration due to the enhanced use of fertilizers in the territory, as well as at much higher concentrations, to unravel the maximum tolerance threshold. Our investigation is performed on two cosmopolitan charophyte species, which are also very common in the Mediterranean area [19]. Based on our previous knowledge cited above, our first hypothesis is that there are charophyte species that can grow well in much higher nitrate concentrations than those indicated by Lambert and Davy [13].

It is known from studies mainly on seaweeds, that macroalgae exhibit different strategies (related to uptake velocities and nitrogen –N– storing capacity in the cells) to use nitrate when it is in low concentrations and when, suddenly, it is abruptly available [20,21]. Moreover, the N uptake depends on different factors such as the metabolism (*e.g.* nitrate-reductase activity), the morphology and the tissue type of different macroalga species, as well as on their nutritional history, or the nutrients in their environment [21,22]. With these findings in mind, our second and third hypotheses are that the performance when faced with nitrate enhancement of different charophyte species will be different, and that such differences will be observed even within the same species, in populations from natural environments with highly different nitrate concentrations in the water. To test our three hypotheses, we perform nitrate enhancement experiments with *C. hispida* and *C. vulgaris* from two origin sites in Spain that differ, among other features, in their nitrate loading. The present study provides new insights into the nitrate tolerance of different charophyte species and populations which allow them to survive under high concentrations of this nutrient. We hope that deepening the understanding of the charophyte nitrate

threshold will help to lay the groundwork for charophyte conservation and restoration in vulnerable Mediterranean freshwater ecosystems.

2. Materials and methods

2.1 Population origin and culture

The specimens of *C. hispida* and *C. vulgaris* used in the experiments were collected from two Spanish sites: the Somolinos lake (Sierra de Ayllón Protected Area, Guadalajara, 1270 m a.s.l. 41°15'04"N 3°03'54"W) which is an oligotrophic, deep high mountain lake in a cold climate, and the Quartons spring (Almenara, Castellón, 0 m a.s.l. 39°45'16"N 0°11'27"W) which is a mesotrophic shallow waterbody in a warm climate (Table 1).

Table 1. Main limnological features (annual variation) of the sites the four charophyte populations come from.

		Origin site	
Variable		Somolinos lake	Quartons spring
Temperature (March-May)	°C	10 - 12	20 - 23
Conductivity	µS/cm	430 - 469	1892 - 2730
pH		8.0 - 8.5	7.1 - 7.8
Nitrate	mg NO_3-N/L	1.3 - 1.8	2.0 - 18.9
TN	mg N/L	1.4 - 2.0	2.2 - 19.7
TP	mg P/L	0.005 - 0.019	0.010 - 0.046

Note: The temperature range is for the vegetative growth period.

The harvested charophytes were transported from the field to the laboratory at the University of València. Plants were washed with dechlorinated tap water, and apical parts, plus a few nodes, were cut and planted in small pots containing a mixture of sand and sediment from the two origins. The pots were placed in containers filled with dechlorinated tap water and the charophytes began to grow [8]. These stock cultures were kept in an indoor culture room at a constant temperature (22°C) under artificial illumination provided by Sylvania Gro-Lux F58W tubes (100 µmol photons/m^2 s; light:darkness 13:11 h). These conditions have been tested as non-limiting to the growth of these charophytes [9,23].

2.2. Experimental setup

2.2.1. Experiment I: unplanted specimens

This experiment was designed to unravel the maximum nitrate threshold in the water that the studied charophytes can live under, without interference from any other N compound.

2.2.1.1. Pre-experimental part

The experimental design (Fig. S1A Supplementary material Chapter 1) consisted of growing individuals from the four populations (2 species × 2 origins) at different nitrate concentrations. The nitrate concentration treatment levels were 0.5, 1.5, 3.0, 7.5, 15.0, 30.0 and 50.0 mg NO_3-N/L (0.04–3.57 mM; 50.0 mg NO_3-N/L represents 221 mg NO_3/L, which is four times higher than the legal limits for nitrates established by the current Council Directive concerning the protection of waters against pollution caused by nitrates from agricultural sources -91/676/EEC-). Pre-experimental acclimatisation consisted of growing several individuals from each population under the different treatments for five days (Fig. S1A), which is time enough for the charophytes to grow and acclimatise to the new environment [8,23].

2.2.1.2. Experimental part

The shoot tips required for each treatment level were randomly selected and cut from the pre-experimental (acclimatisation) cultures to be used in the experiment (Fig. S1B). We used 5 replicates for each population and condition; therefore, for this design 140 individuals were necessary. Extra shoot tips, similar to those used for the experiment, were obtained from the pre-experimental cultures to determine the initial biomass of the specimens for each treatment group (biomass at t_0) (fresh weight, FW, and dry weight, DW). The specimens were gently pressed with drying paper, and the FW was determined using a Sartorius (BP121S) precision balance. After drying the specimens in an oven at 72°C for 24 h, the DW was determined.

The shoot tips were individually introduced into 250 mL plastic beakers (Fig. S1C) containing 200 mL of a nitrate solution of the above indicated concentrations. Only one specimen was placed in each beaker in order to avoid the pseudo-replication effect which can be caused by the common bucket effect [24]. The nitrate solutions were prepared by adding the necessary amount of sodium nitrate ($NaNO_3$, Merck, Germany) to dechlorinated tap water to achieve the desired concentrations. Hence Na^+ varied slightly between treatments (up to 82.1 mg Na/L or 3.6 mM). We did not expect interference between the higher salinity at the higher nitrate concentration treatments, since Barker *et al.* [25] demonstrated that salinity did not interact with the nitrate treatments in their mesocosms experiments with macrophytes. To allow the growth of the charophytes, phosphorus was added to each beaker at a final concentration mimicking oligo-mesotrophic conditions (0.01 mg PO_4-P/L -0.32 µM-) from a concentrated solution of potassium dihydrogen phosphate (KH_2PO_4, Merck, Germany). The N:P molar ratios in the water in the different treatments were 111, 332, 664, 1661, 3321, 6643 and 11071. All the beakers were placed on the shelves of the culture room, and the position of the beakers was carefully changed every two days in order to avoid a site effect (as seen in [26]). The charophytes received light from above with the specifications previously mentioned. The volume of 200 mL was maintained during the experimental period as well as the nitrate and phosphate concentrations (the water in each beaker was analysed for nitrate and phosphate concentrations and the corresponding nitrate and/or phosphate was added when necessary). Every two days, the pH, conductivity and oxygen concentration were measured in each beaker to detect abnormal values and to rectify them. Nitrite and ammonium were measured at the end of the experiment (t_f) to register the possible transformation of nitrate by chemical and/or biological activity. The experiment lasted eighteen days.

Radiation was measured by means of a Q 32010 Li-Cor quantum spherical sensor connected to a Li-Cor 250 meter. The water nitrate, nitrite, and ammonium concentrations were measured using standard methods [27]. Water pH, conductivity

and oxygen were measured by using portable measurement equipment (WTW®probes).

2.2.2. Experiment II: planted specimens

This experiment was designed to discover the response of the studied charophytes to several nitrate concentrations in water (up to 50 mg NO_3-N/L), in a more realistic situation: individuals were planted in sediments containing other N compounds. Since the number of variables (see Section 2.3 below) that were intended to be measured requires a certain amount of charophyte biomass, and as the aim of the experimental design was to avoid pseudo-replication, the availability of specimens/biomass was not large, hence two different trials were performed, with several variables being measured in the first trial (called hereafter Exp. IIa), and others in the second (Exp. IIb) (detailed information below).

2.2.2.1. Pre-experimental and experimental parts

The pre-experimental part for Exp. II was exactly the same as described for Exp. I. The experimental part consisted of individually planting shoot tips in small pots which contained the same substrate used in the stock cultures (Fig. S1D). A thin layer of washed commercial sand was distributed over the sediment to avoid nutrient diffusion from the sediment to the water (Fig. S1E). We used 4–5 replicates for each population and condition. Each pot was gently introduced into one tall plastic beaker (to avoid the pseudo-replication effect). Each beaker contained 1 L of the nitrate concentration solution of each treatment level for each individual. The nitrate solutions were prepared by adding the necessary amount of sodium nitrate to dechlorinated tap water to achieve the desired concentrations. No phosphorus was added to the water; we expected the charophytes to take up P from the sediment, as occurs in the stock cultures and other experiments in the laboratory [8,9]. All the beakers were placed on the shelves of the culture room (Fig. S1F) and the position of the beakers was carefully changed every two days in order to avoid a site effect. They also received light from

above with the same specifications described for Exp. I. The volume of 1 L was maintained during the experimental period as well as the nitrate concentration. Water pH, conductivity and oxygen concentration were also measured in each beaker. Ammonium and orthophosphate concentrations were also measured in the water at the end of the incubation period. The experiments lasted fifteen days (when most of the specimens had already reached the water surface).

The nitrate and ammonium concentrations were measured in the sediment initially and after the incubation period by the extraction method [27]. Approximately 10 g of dry sediment was treated with 50 mL of CaCl 0.01 M and an autoanalyzer was used. These measurements were made by the laboratory of the National Museum of Natural Sciences (CSIC, Madrid).

2.3. Measured variables in the charophytes

2.3.1. Growth rate and morphological architecture

These variables were measured in the three trials. When each experiment finished, the specimens dedicated to growth and morphology measurements were either taken from the beaker or carefully removed from their sediment pot (cutting the above-ground part) and immediately placed on a tray with a gridded background and water, to leave the charophyte as extended as possible, and then a picture was taken (Fig. S1G). The image analysis software ImageJ [28] was used in order to measure the morphological variables. Following this, final (t_f) FW and DW were determined.

The initial DW (in milligram) was subtracted from the final DW and normalised with the initial DW, thus obtaining the normalized dry weight (NDW), expressed as a percentage, which gives a measurement of the production by unit weight of each specimen. The relative growth rate (RGR, /d) was determined as '(ln final DW–ln initial DW)/t(days)' [29]. The morphological variables measured were the length of the main axis (LMA, in centimetre) and the number of lateral ramifications (R) and nodes. Calculated variables were final minus initial LMA, or variation in LMA (LMAV, in

centimetre) which can be used as a measurement of the absolute elongation. Moreover, to get an idea of changes in the shape or architectural complexity [8,9,30], we calculated the weight distribution, or robustness, as the final DW/LMA ratio (in milligram/centimetre), the inter-nodal distance (LMA:N in centimetre) and the number of ramifications per node (R/N).

2.3.2. Stoichiometric composition

At the end of experiments I and IIb (Fig. S1H), dry individuals from each population and treatment were crushed by means of an automatic tissue grinder (Tissuelyser II Qiagen), adding two small steel balls, and using two shaking series of 15 s at 4500 rpm. The balls were removed with the help of a magnetic bar. The samples were kept desiccated in plastic tubes until stoichiometric analyses were conducted. Total C and N were determined using a Perkin-Elmer CHN/O-2400 elemental autoanalyser. The measurements of C and N in replicate samples were within 5% of the coefficient of variation. The analyses were performed at the laboratory of the National Museum of Natural Sciences (CSIC, Madrid). The results are expressed as a % of the element in the biomass. Carbonate of the encrustations was not removed because the sample amount for stoichiometric analyses was small and we were mainly interested in the N acquisition.

2.3.3. Nitrate-reductase activity

At the end of experiments I and IIb, nitrate-reductase activity was measured (Fig. S1I) modifying the protocol described by Cabello-Passini *et al.* [31]. The apical parts of each specimen were cut, weighed to determine FW (approximately 0.1 g for the *C. hispida* specimens and 0.03–0.07 for *C. vulgaris*) and placed in a 1.5 mL microcentrifuge tube. To disintegrate the tissues, and facilitate the measurement of the nitrate-reductase activity, the specimens were ground by means of an automatic tissue grinder, adding one small steel ball and shaking in two series of 10 s at 3000 rpm. After removing the ball with a magnetic bar, 1.25 mL of assay buffer was added to each tube (N-free

dechlorinated tap water, pH 8.2, 2.25% (v/v) npropanol and 30 mM $NaNO_3$). The assay tubes were incubated in darkness in a water bath at 30°C for 1 h. After the incubation period, the tubes were incubated for 5 min at 95°C to denature charophyte enzymes and to liberate nitrite from the cells. Nitrite was determined after the samples had cooled to room temperature. The tubes were centrifuged for 10 min at 12000×*g* and 1 mL of the supernatant was reacted with 200 µL of a solution containing 1% (w/v) sulphanilamide in acidified distilled water and 0.02% (w/v) N-(1-naphthyl)-ethylenediaminedihydrochloride in distilled water. Two types of controls were used: a tube with only the assay buffer, and tubes containing charophytes plus the assay buffer (one for each population and treatment). The nitrite determination reagents were added to the first control tube and this was used as the blank in the spectrophotometer. No nitrite reagents were added to the other control tubes. All the controls were incubated in the same way as the samples. The absorbance of the samples and controls was determined at 543 nm. Fresh weight normalised $absorbance_{543}$ in the controls was subtracted from the fresh weight normalised $absorbance_{543}$ in the samples to correct the effects on the nitrite determination of the absorbance due to pigment presence in the analysed solution. The concentration of NO_2 was determined against a standard curve prepared with KNO_2. The results are expressed in nanomoles of nitrite per mg FW per hour.

2.3.4. Metabolic activity: net respiration rate

Immediately after the completion of Exp. IIa, the in vivo respiration rates were assessed (Fig. S1J) using an adaptation [8] of the Winkler method [32], based on changes in water dissolved oxygen (DO) concentration during short-term incubations due to the respiratory activity of charophytes. Three whole specimens (without rhizoidal systems) for each population and treatment were pulled out from the pots, rinsed (to remove possible epiphytes) and introduced into dark Winkler flasks (120 mL) containing the pertinent nitrate solution in which the charophyte had been growing for each treatment. A small magnetic bar was previously introduced into each flask.

Water DO concentration was measured in each flask before introducing the charophytes, placing the flasks on a magnetic stirrer to gently mix the water. An optical O_2 probe (Hach USA IntelliCAL™, LDO101) with a special adaptor for the flask mouth (which prevented oxygen exchange with the air) was used to measure the water DO concentrations (mg/L). Immediately after introducing the charophytes, the flasks were tightly closed, preventing the formation of air bubbles, and they were incubated in the culture room for 45 min. After the incubation time, the flasks were gently opened and the DO concentration was measured again following the same procedure as described above. The DO measurements were normalised using the DW of each charophyte (the DW was measured after the last oxygen measurement). The respiratory rate (RR) was calculated using the following formula:

Respiratory rate (mg O_2/g DW h) = (initial DO (mg/L) – final DO (mg/L)) × Flask volume (L)/(DW(g) × time (h))

2.4. Statistical analysis

Due to the low number of replicates, non-parametric tests were used to compare the distribution of data in each nitrate dose. Kruskal–Wallis χ^2 values were considered for multiple comparisons and Mann–Whitney *U* values with Monte Carlo probabilities for two-sample comparisons. When there were significant differences, the data series were subjected to polynomial fitting and the most statistically significant functions were chosen. Statistically significant differences were considered to be present at $p<.05$. All analyses were performed with IBM SPSS Statistics-22 software (IBM Corp, Armonk, NY).

3. Results

3.1. Changes in water and sediment after cultivation

Water pH, temperature and dissolved oxygen did not change significantly throughout Exp. I (unplanted charophytes; Table 2). Conductivity ranged from 954 to 1331 µS/cm with increased nitrate concentrations at t_0. This was 26% higher at t_f ($p<.001$). Nitrite appeared in the water in Exp. I (Fig. 1) ranging from averaged values of 0.02–0.1 mg

NO_2-N/L at t_f. The nitrite exhibited quite a similar pattern in all the charophyte populations: higher concentrations at the lowest and the highest nitrate concentrations. Nitrite concentration represented 12–18% of nitrate concentration at 0.5 mg NO_3-N/L and only 0.1–0.2% at 50 mg NO_3-N/L. Ammonium also appeared at t_f in all treatments (Table 2), and this represented 30–40% of nitrate concentration at 0.5 mg NO_3-N/L and only 0.3–0.5% from 15 mg NO_3-N/L of external nitrate. Orthophosphate concentrations were kept at t_f at the same values as at t_0. Only the treatments of *C. vulgaris* from Quartons presented a lower concentration (0.005 mg PO_4-P/L, mean value).

Water pH, temperature and dissolved oxygen from Exp. II did not vary either throughout the cultivation period (Table 2). Ammonium was also detected in the water at t_f (0.03–0.05 mg NH_4^+-N/L, with the maximum values corresponding to the 50 mg NO_3-N/L treatment). Orthophosphate concentrations at t_f were low (mean of 0.003 mg PO_4-P/L). Nitrate concentration in the sediment varied from 2.5 mg N/kg sed. at t_0 to lower values in the low-nitrate treatments, and to higher values in the high-nitrate treatments at t_f (Table 2). Ammonium in the sediment also changed from an initial concentration of 8.2 mg N/kg sed. to reduced values of around 5 mg N/kg sed. but no trend was observed with increasing water nitrate concentrations (Table 2).

3.2. Morphology and growth

When the charophytes were cultivated unplanted (Exp. I, Fig. 2A), the four populations grew under all nitrate concentration treatments. The RGR, based on dry weight, of the two populations of *C. hispida* grew in a similar way from 0.04 to 0.08 /d up to 3 mg NO_3-N/L, increasing to 0.12 /d up to 30 mg NO_3-N/L. The RGR of the population from Quartons decreased to the values of the lowest nitrate concentration at 50 mg NO_3-N/L. However, the growth pattern of both *C. vulgaris* populations was different; the RGR of *C. vulgaris* from Somolinos significantly reduced with the nitrate increase ($U_{0.5–50}$=0; $p_{Monte\ Carlo}$=0.029), whereas *C. vulgaris* from Quartons had higher rates with

increased nitrate concentrations ($U_{0.5\text{-}50}$=0; $p_{Monte\ Carlo}$=0.029) (Fig. 2A). The measurement of elongation (LMAV) was also statistically different under the different nitrate concentrations for each population (Fig. S2A Supplementary material Chapter 1) and resembled the pattern shown by the RGR based on dry weight. The number of new ramifications (Fig. S2B) was significantly enhanced under the highest concentrations only in *C. vulgaris* from Quartons ($U_{0.5–50}$=1; $p_{Monte\ Carlo}$=0.026). The measurement of robustness (DW/LMA) did not change significantly with the treatments in both *C. vulgaris* and *C. hispida* from Quartons.

Table 2. Values of physical and chemical variables in the water and the sediment at the beginning and the end of the experiments.

Variable	units	Time of the experiment t_0	t_f
EXP. I (UNPLANTED)			
Water			
Temperature	°C	22.0-22.2	
pH		8.2-8.4	
Conductivity	µS/cm	954-1331[a]	1414-1735[a]
Dissolved oxygen	mg/L	8.5-9.9	8.3-10.4
Ammonium	mgNH_4-N/L	-	0.10-0.25[b]
Orthophosphate	mgPO_4-N/L	0.010	0.005-0.009
EXP. II (PLANTED)			
Water			
Temperature	°C	22.0-22.2	
pH		8.4-8.5	
Conductivity	µS/cm	1076-1300[a]	
Dissolved oxygen	mg/L	13.5-14.9	
Ammonium	mgNH_4-N/L	-	0.03-0.05[a]
Orthophosphate	mgPO_4-P/L	-	0.002-0.004[b]
Sediment			
Nitrate	mgN/kg sed.	2.5	1.2-8.6[a]
Ammonium	mgN/kg sed.	8.2	4.5-5.2[b]

[a] The maximum values corresponded to the 50 mg NO_3-N/L treatment.
[b] No trend observed with increasing nitrate concentration.

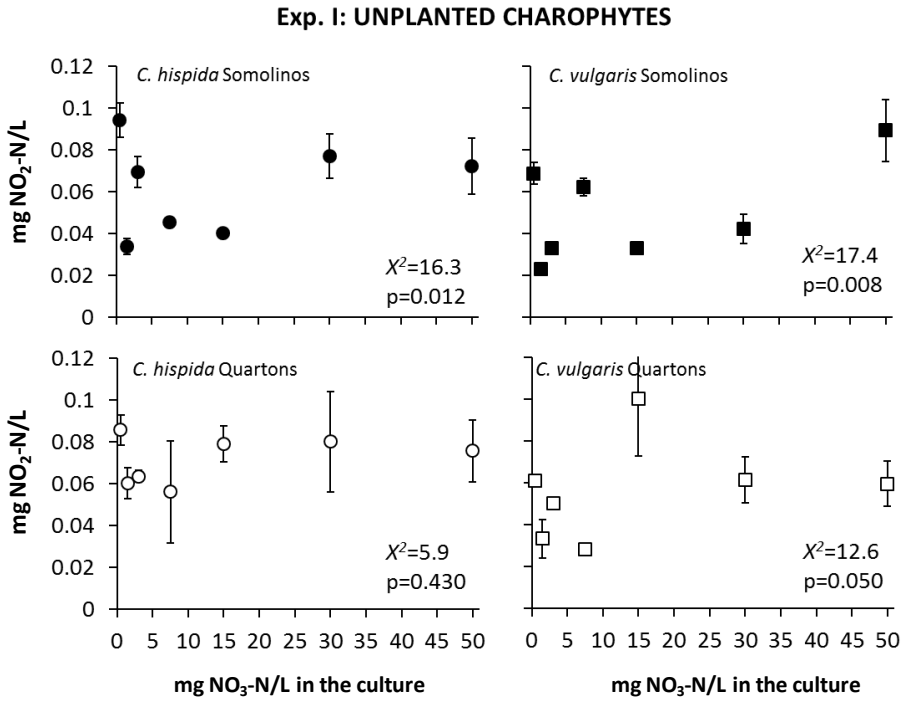

Fig. 1. Average values of final nitrite concentrations in the water of the beakers filled with the seven nitrate-dose solutions and the free-floating charophytes (Exp. I) of each of the four populations, *C. hispida* and *C. vulgaris* (from Somolinos lake and from Quartons spring). Bars show standard errors. Each graph shows the results of Kruskal–Wallis tests (χ^2 and probability) which compare the values in the seven nitrate doses.

When the charophytes were cultivated by planting in sediment (Exp. II, Fig. 2B) the four populations showed similar RGR patterns, with higher values (0.9–0.14 /d) at 0.5 mg NO_3-N/L, and slightly reduced values at intermediate concentrations (0.07–0.08 /d) which again increased up to 30 mg NO_3-N/L. Three out of the four populations showed a reduction in RGR at 50 mg NO_3-N/L in comparison to 0.5 mg NO_3-N/L ($U_{0.5–50}$=0; $p_{Monte\ Carlo}$=0.012 for *C. hispida* from Somolinos; $U_{0.5–50}$=1.5; $p_{Monte\ Carlo}$=0.028, $U_{0.5–50}$=1.5; $p_{Monte\ Carlo}$=0.028 for *C. hispida* and *C. vulgaris* from Quartons). Overall, no significant differences were found in other morphological variables such as robustness, the number of ramifications or elongation.

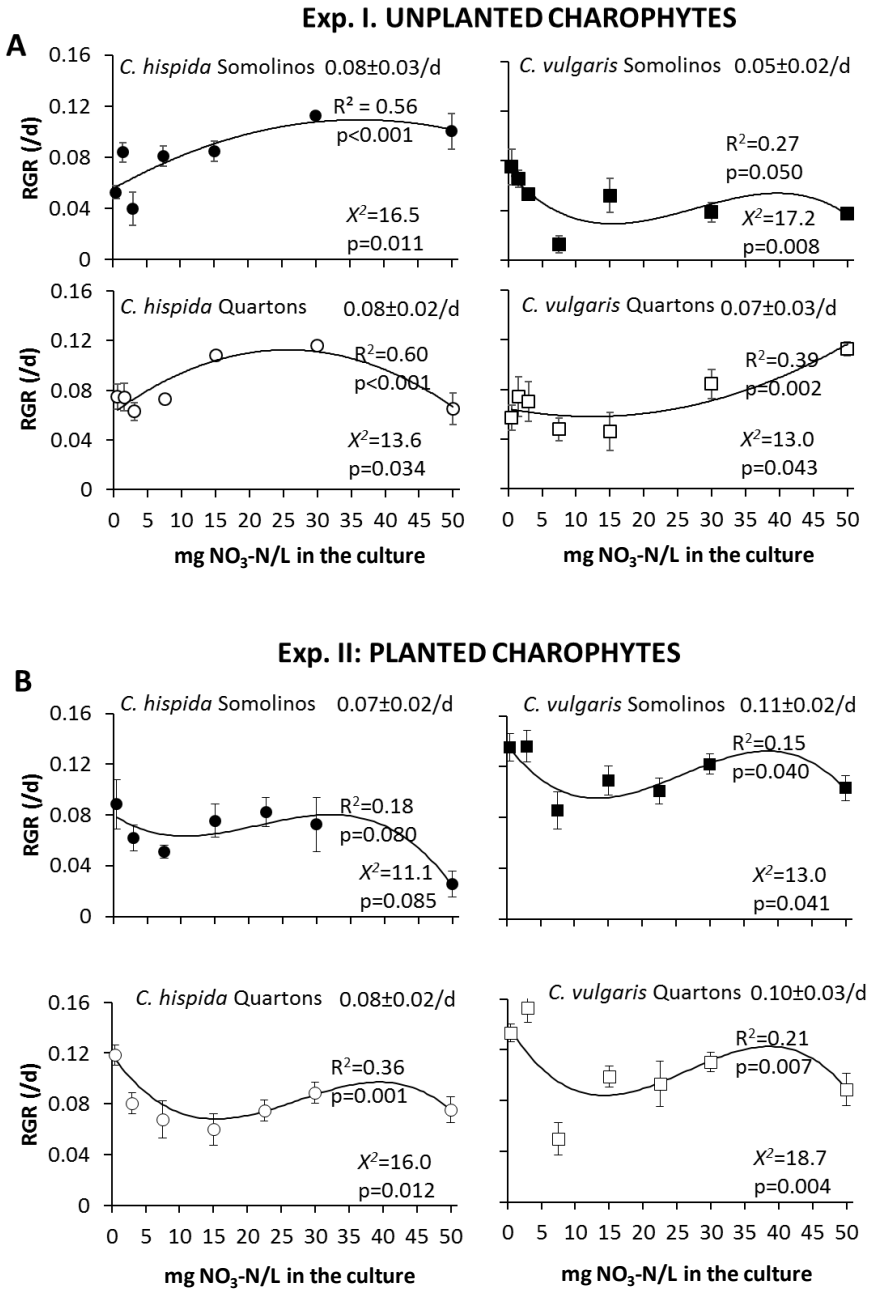

Fig. 2. Average values of the RGR for the four charophyte populations cultivated unplanted (A) and planted (B) under seven nitrate doses. Bars show standard errors (95% confidence intervals presented in Table S1 Supplementary material Chapter 1). Each graph shows the results of Kruskal–Wallis tests (χ^2 and probability) which compare the values in the seven nitrate doses, and R^2 and probabilities of the curve fittings (equations in Table S2 Supplementary material Chapter 1). Average values for all the doses±standard deviation are also indicated.

A lower elongation per day was evident comparing charophyte growth when grown free-floating or planted (Fig. 3). Most affected were the two populations of *C. vulgaris*, whose mean values of elongation were only 9% and 13% of what they could have elongated had they been planted (the specimens from Somolinos and Quartons, respectively). *C. hispida* specimens from both origins elongated up to 31–35% when grown free-floating compared to their growth when planted. In the latter, daily *C. vulgaris* elongation from both origins (0.8–1.0 cm/d) was higher than *C. hispida* elongation of (0.3–0.6 cm/d).

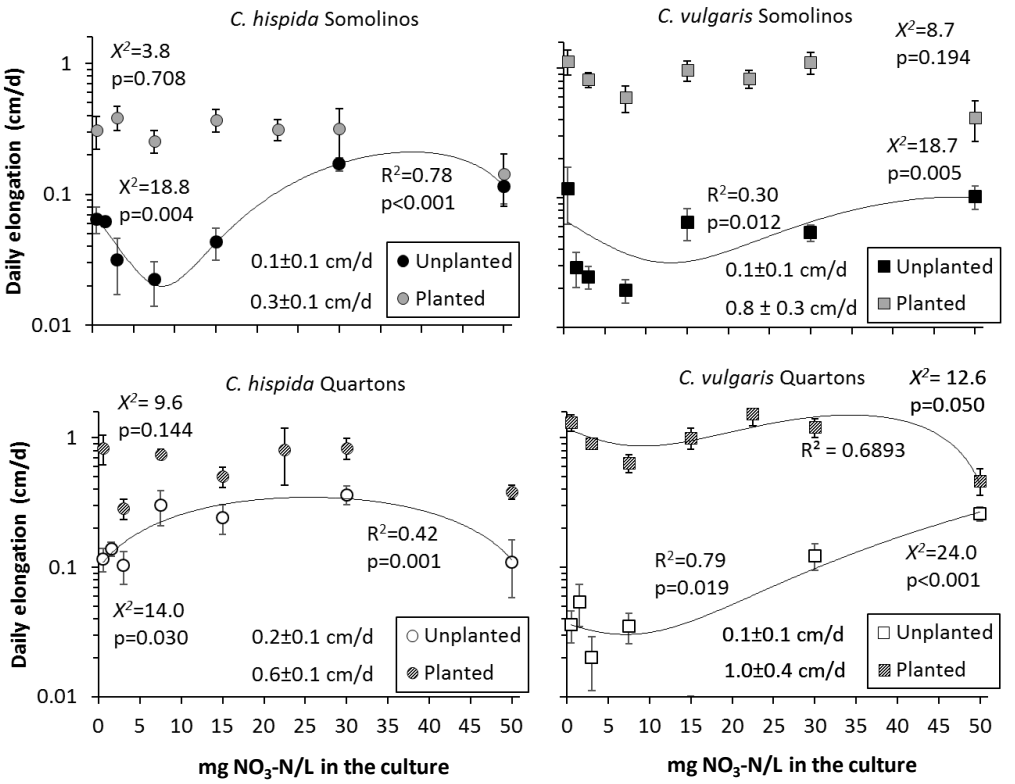

Fig. 3. Mean increased length per day of the four populations of charophytes in Exp. I (unplanted charophytes) and in Exp. II (planted charophytes). Bars show standard errors (95% confidence intervals presented in Table S1 Supplementary material Chapter 1). Each graph shows the results of Kruskal–Wallis tests (χ^2 and probability). R^2 and probabilities of the curve fittings (equations in Table S2 Supplementary material Chapter 1) are presented when there were significant differences among nitrate doses. Average values for all the doses±standard deviation are indicated.

3.3. Stoichiometric N composition

The %N in the biomass when charophytes grew unplanted (Exp. I, Fig. 4A) was significantly different among treatments in each of the four populations, and all populations presented a general similar pattern: an increase in %N in the charophyte biomass up to approximately 15 mg NO_3-N/L, and from this threshold the %N in the biomass decreased again at the highest nitrate concentrations (Fig. 4A). In the range of 0.5–3 mg NO_3-N/L, only the specimens from Somolinos showed a significant linear increase in %N in the cells with increasing nitrate in the water (%N=0.10 mg NO_3-N/L + 0.60, R^2=0.97; p<.001 for *C. hispida* and %N=0.21 mg NO_3-N/L + 0.71, R^2=0.98, p<.001 for *C. vulgaris*). Both *C. vulgaris* populations had a mean %N (1.2–1.4%) higher than those of *C. hispida* (0.8%).

When the charophytes grew planted in sediment with the same nitrate concentration gradient in the water, the %N in the biomass (Exp. IIb, Fig. 4B) was also significantly different among doses. Both *C. hispida* populations showed a similar response pattern, a linear decrease in %N with increasing nitrate concentrations in the water (at a negative rate of 0.003% of N for each milligram of NO_3-N in the water). *C. vulgaris* from both origins also showed a similar pattern amongst themselves, but an opposing one to the other species: an increase in %N in the biomass with increasing nitrate concentration in the water (at a rate of 0.003–0.004% of N for each milligram of NO_3-N in the water).

3.4. Nitrate-reductase activity

The nitrate-reductase (NR) activity when charophytes grew unplanted (Exp. I; Fig. 5) showed a distinctive peak at 3 mg NO_3-N/L in both species from Somolinos, with values near 0.7 nmol nitrite/mg FW h. In the rest of the treatments, NR activity was lower and slightly higher in *C. vulgaris*.

When charophytes grew planted (Exp. IIb; Fig. 5), the NR activity was very low in *C. hispida* from both origins (unfortunately *C. vulgaris* NR activity could not be analysed due to damage to samples), with no statistical differences among treatments in the specimens from Somolinos.

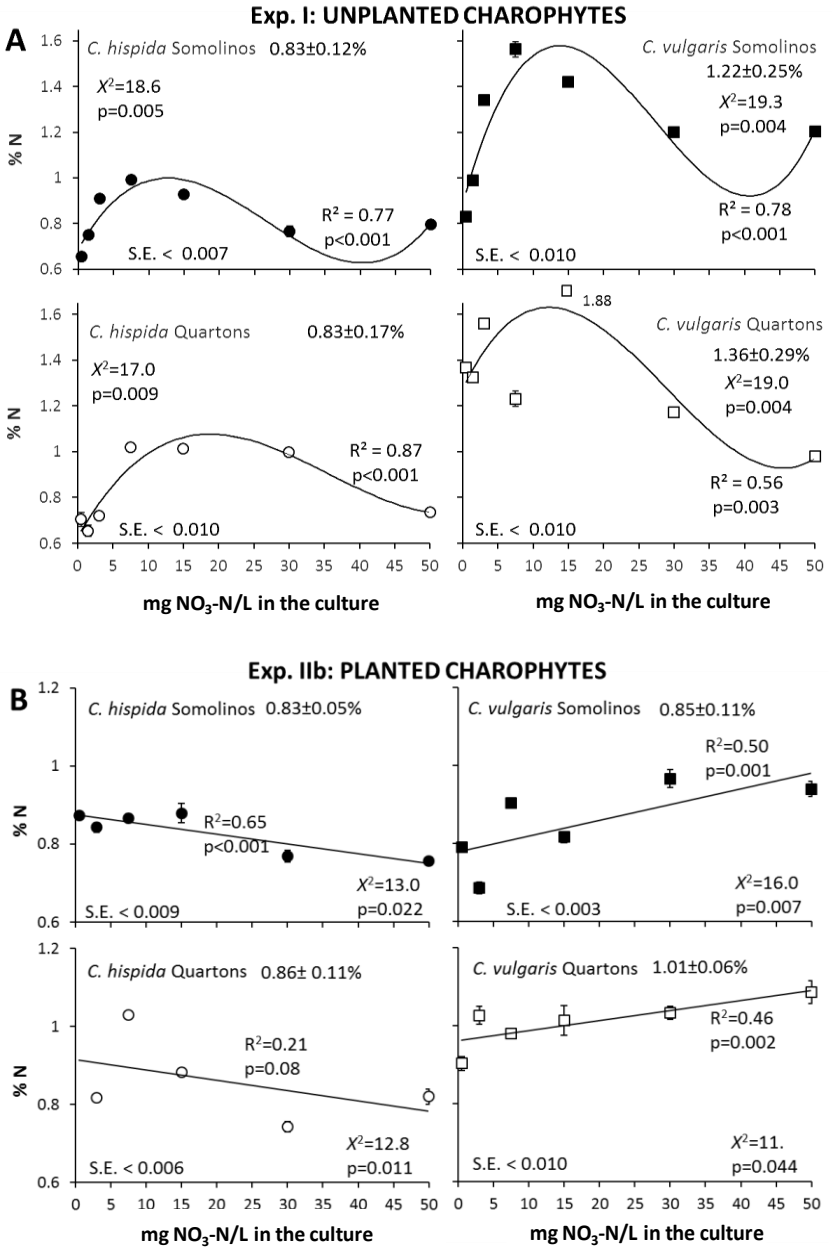

Fig. 4. Average values of percentage of nitrogen for the four charophyte populations cultivated under seven nitrate doses. A: Exp. I (unplanted charophytes), B: Experiment IIb (planted charophytes). Notice the difference in the y-scale between graphs in A and B. Bars show standard errors (S.E.) (95% confidence intervals presented in Table S1 Supplementary material Chapter 1). Each graph shows the results of Kruskal–Wallis tests (χ^2 and probability), R^2 and probabilities of the curve-linear fittings are presented. Average values for all the doses±standard deviation are indicated.

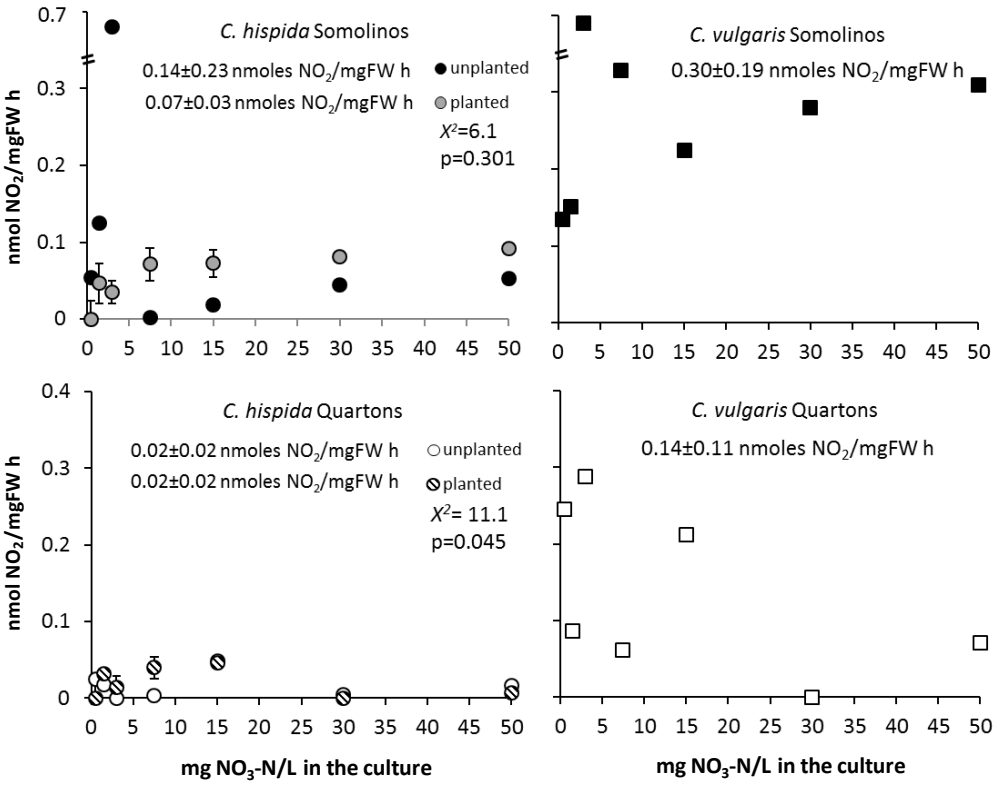

Fig. 5. Nitrate-reductase activity expressed as nanomol of nitrite per milligram fresh weight of charophyte per hour, cultivated under different nitrate concentrations in Exp. I (unplanted charophytes) and Exp. IIb (planted charophytes; bars show standard errors, 95% confidence intervals presented in Table S1 Supplementary material Chapter 1); results of Kruskal–Wallis tests (χ^2 and probability) are shown. Average values for all the doses±standard deviation are also indicated.

3.5. Metabolism (respiratory rate)

The respiratory rates calculated at the end of Exp. IIa (planted charophytes, Fig. 6) were in general higher in *C. vulgaris* from Somolinos. No statistical differences were found in the mean respiratory rates between treatments in this population, nor in *C. hispida* from the same origin. *C. hispida* from Quartons showed higher rates at the lowest and the highest nitrate concentrations assayed, while *C. vulgaris* exhibited the highest rates at nitrate concentrations in water higher than 15 mg NO_3-N/L.

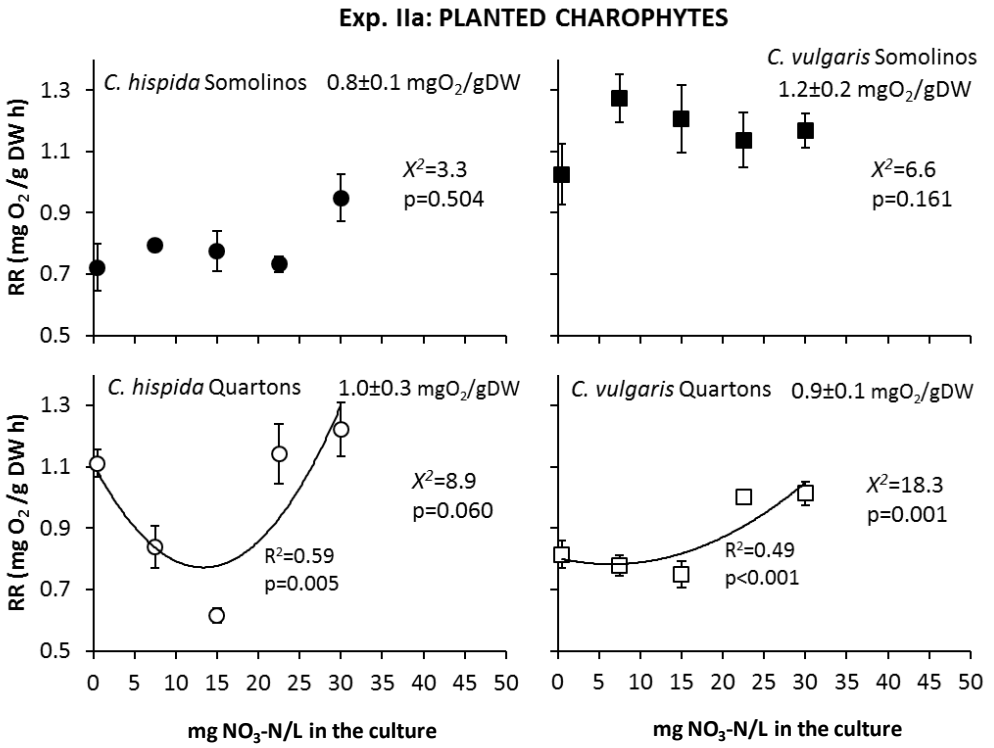

Fig. 6. Average values of respiration rates (RR) for the four charophyte populations cultivated under different nitrate concentrations in Exp. IIa (planted charophytes). Bars show standard errors (95% confidence intervals presented in Table S1 Supplementary material Chapter 1). Results of Kruskal–Wallis tests (χ^2 and probability), R^2 and probabilities of the curve fittings are presented when there were significant differences among nitrate doses. Average values for all the doses±standard deviation are also indicated.

4. Discussion

4.1. Charophyte growth and morphology

Our first hypothesis is verified. The four charophyte populations grew, both unplanted and planted, with mean RGR that were always higher than 0.04 /d under all treatments, even when the nitrate concentration was as high as 50 mg NO_3-N/L. However, growth reductions were observed at this highest concentration in all cases, with the exception of *C. vulgaris* from the high-nitrate waterbody when the specimens grew free-floating. In this case, growth was enhanced by the highest nitrate

concentrations not only in elongation of the main axis but also in the appearance of new lateral ramifications. As expected [33], the growth rate was higher when the specimens grew rooted in the substrate, the way they grow naturally, and with all the required elements available from the sediment. But to untangle the possible deleterious effect of nitrate on the growth of charophytes, it was necessary to grow them just in water, with a supply of nitrate and a phosphorus concentration resembling oligotrophic conditions. There were differences in growth rates and morphological variables (robustness, elongation, number of ramifications, etc.) with the increased nitrate concentrations when the charophytes grew unplanted, but these differences were not great. And, in some cases, the variability of the different replicates was high due to the plasticity of these organisms [34].

Our results with planted charophytes are totally different from those obtained by Lambert and Davy [13], who found the growth of *C. globularis* extremely sensitive to nitrate supply in a similar laboratory experiment also lasting 14 days, therefore being comparable to our one. These authors used much lower nitrate concentrations (up to 10 mg NO_3-N/L) and the nitrate concentration where the RGR was reduced by half (IC_{50}) was 5.6 mg NO_3-N/L for this species. Simons *et al.* [18] reported no reduction in stem-tip extension after 12 days at up to 4.6 mg NO_3-N/L in either *C. major* or *C. connivens*. Lambert [35] also recorded charophytes growing in the field at nitrate concentrations of 19 mg/L. In our case, the pattern of growth response to the nitrate dose was very similar in the four populations: lower rates registered in the intermediate nitrate concentrations. This striking fact needs further research. But also in all the cases, the RGR was reduced again when the nitrate concentrations were over 30 mg NO_3-N/L. It is well-known that the autecology of the distinct species of charophytes can be very different, also in terms of tolerance to stress caused by several factors. In fact, *C. hispida* and *C. vulgaris* are two of the species that best resist eutrophication and pollution [36], and *C. vulgaris*, in particular, appears of interest for developing phytoremediation strategies [37]. We already had evidence that these two

species can live in waterbodies with high-nitrate concentrations [15–17]. However, we did not know the maximum threshold such species would be able to resist. Our experiments demonstrate that, at least in short-term laboratory conditions, both *C. hispida* and *C. vulgaris* can grow healthily under up to 30 mg NO_3-N/L.

4.2. Nitrogen in the charophyte biomass

The experiment with unplanted charophytes, where nitrate was the sole source of N for growth, showed similar general patterns of N content in the charophyte biomass with increasing nitrate concentrations for the four populations. Namely, is an increase in %N in the charophyte biomass until approximately 15 mg NO_3-N/L and a decrease at higher concentrations. The processes of nitrate transport and reduction in *Chara* cells is a delicate balance between influx and efflux and assimilation, with separate transporters for the influxes and effluxes [38–40]. The increase in external nitrate concentration from 0.5 to up to 15 mg NO_3-N/L implied enhanced NO_3^- inflow to the cells [38]. Although some efflux of NO_3^- was produced, the net import would be positive and would go into the vacuoles, or to the reduction pathway into protein production [38], explaining the increase in %N in the biomass. However, when the external nitrate concentrations were higher than 15 mg NO_3-N/L, the NO_3^- inflow continued, the cells having an excess of cytoplasmic NO_3^- (the N assimilation could be limited by the low P concentrations in the water) and, then, the efflux of this anion would increase considerably. Thus, the internal concentration also depends on the efflux of nitrate from the cells [39], and this might be the reason why the %N in charophyte biomass did not increase proportionally with the availability of external N when it was in very high concentrations. However, focussing in the lower nitrate concentrations (up to 3 mg NO_3-N/L) the N content in the charophyte biomass increased significantly and linearly with increasing external nitrate only in *C. hispida* and *C. vulgaris* from Somolinos. These specimens, which come from a low-nitrate environment, reacted by absorbing more nitrate when there was more nitrate in the water in the range of lower nitrate concentrations and when nitrate was the sole N source. This fact was repeated

in both species, although *C. vulgaris* increased the %N two-fold quicker than *C. hispida* in the range up to 3 mg NO_3-N/L. Thus, the environment where the charophytes grow imposes a selection of mechanisms to acquire nitrate, as was observed in other macroalgae [20,21]. However, the patterns of increase of %N in the biomass, depending on the origin of the specimens, are not clearly reflected in the growth features. For example, *C. vulgaris* from the low-nitrate environment showed a completely opposite pattern of %N enhancement in biomass and growth rate in the range 0.5–7.5 mg NO_3-N/L. This would suggest that nitrate accumulates in the cells when they are exposed to enhanced concentrations of nitrate in the surrounding water, and that they expend energy to regulate nitrate uptake under such conditions, negatively affecting growth. Details of the mechanisms for nitrate transport and assimilation need further study in charophytes.

When the charophytes grew planted in sediment, a more realistic situation where other substances, such as ammonium, were present and interfere with nitrate in water and rhizoids play a relevant role in nutrient absorption, the results of %N in the charophyte biomass were quite different. We did not observe the clear linear increase in %N when increasing external nitrate in both *C. hispida* populations. It has been described how, in spite of the apparent intimate link that is likely to exist between aquatic plants and the surrounding water/sediment environment, a poor correlation often exists between aquatic plant tissue N concentrations and the ambient nutrient supply [41]. The presence of ammonium, an energetically more advantageous source of N, in our sediments may have regulated the net uptake of nitrate through stimulation of the NO_3^- efflux [39]. Cedergreen and Madsen [42] also reported how submerged macrophytes considerably take up NH_4 through their roots from the sediment. Box [43] found that rhizoids of *C. hispida* took up a fraction of the charophyte's N that was disproportionate to their surface area and mass. We have proof that ammonium is consumed from the sediment, since the concentration at the end of the experiments was lower than at the start (a reduction of approximately 0.2

mg N/kg sed. day). Moreover, Vermeer *et al.* [33] reported preferential uptake of ammonium over nitrate by the rhizoids, and translocation of N predominantly from below- to above-ground parts, even when plants were exposed to high concentrations of nitrate or ammonium in the water column. Since ammonium concentration in our sediment was the same, independently of the species and their origin, this would explain the smaller differences in %N with the different nitrate treatments in every population, in comparison to the larger difference encountered in %N due to the different external nitrate when the charophytes grew unrooted. In this case, the different response patterns in %N with increasing nitrate supply in the water between *C. hispida* and *C. vulgaris* biomass, regardless of site origin, indicate that the response depends more on phylogenetic reasons than on local adaptation to origin environmental conditions. Species-specific differences have been reported for the complex interaction between nitrate and ammonium uptake, which is related to both preference for one of these N sources and inhibition of ammonium on nitrate uptake [44].

4.3. Nitrate-reductase activity and respiration

Deane-Drummond [38] reported how some induction is necessary to produce nitrate-reductase in the cells, and this author reported nitrate concentrations in the water of 2.8 mg NO_3-N/L resulting in high induction of the enzyme. The peak of NR activity was at 3 mg NO_3-N/L in both *C. hispida* and *C. vulgaris* specimens from the low-nitrate system, but this particular pattern was not found in the specimens from the high-nitrate waterbody. The nitrite produced in the first step in nitrate reduction has to be exported or neutralised to prevent an increase in cytoplasmic pH [38]. In our experiment with unplanted charophytes some nitrite was exported to the water, as indicated by the nitrite concentrations measured at the end of the experiment. This nitrite must be originated by the charophyte activity since the reduction of nitrate to nitrite by chemical and/or microbiological transformations was not expected due to the aerobic conditions of the cultures (9 mg/L of dissolved oxygen in the water). When

charophytes grew planted, the nitrate-reductase activity was also low, particularly in *C. hispida* from the high-nitrate origin. There were no statistical differences among treatments in *C. hispida* from the low-nitrate origin. Zhao *et al.* [45] also reported that nitrate-reductase activity in macrophyte leaves and roots did not change significantly with the N loading (1, 3 and 5 mg/L). Cedergreen and Madsen [42], in a survey of 12 species of aquatic macrophytes, reported that all plants exhibited low-nitrate reductase activity (<2 µmol NO_2/g DW h) in both roots and shoots, except for the amphibious species. Overall, our results were also lower than this level (0.06–1.08 µmol NO_2/g DW h when nitrate was the sole N source, and 0.09–0.24 µmol NO_2/g DW h when there was ammonium and nitrate in the sediment) and only both species from the low-nitrate habitat slightly surpassed this value when growing at 3 mg NO_3-N/L as the sole N source (2.4 µmol NO_2/g DW h). It has been described how, NH_4^+ or the products of NH_4^+ assimilation, can inhibit the induction of nitrate-reductase or even inactivate it [46]. NH_4^+ and its assimilation products were probably transported to the shoots after uptake by the roots from the sediment, and this fact would explain the low NR activity measured when the charophytes grew planted.

Increases in respiration rates (RR) have been described by ammonium transport costs that consume more energy by decreasing protein and sugar content, consequently plants increase their respiration in order to maintain a normal metabolism [47]. If, in our situation, charophytes are growing mainly using the ammonium from the sediment, the lack of difference in RR with increasing nitrate concentrations in water, as happened in the populations from the low-nitrate lake, would be expected, since the cost of ammonium transport would be the same for all nitrate treatments. The other two populations from the high-nitrate system showed a slightly different pattern. The RR was statistically different within the nitrate treatments, and the pattern was coincident with the tendency in the growth rate (higher respiration rates at higher growth rates). Once again, there is a difference in one of the physiological biomarkers [48] depending on the origin of the populations.

The possibility that other aspects are affecting the respiration and growth rate as well as the %N in the charophytes, and not necessarily by a linked pathway, has to be considered. This could include other micronutrients, physical responses either to the substrate or to orientation, and biotic interactions with a microbiome.

4.4. Concluding remarks

Our study has contributed to the debate about ecological *versus* physiological factors as causes of reduced growth of charophytes with increased N loading: we have proved that nitrate in the water *per se* is not detrimental to two particular charophytes species, no direct nitrate toxicity existed until 30 mg NO_3-N/L. However, despite *C. hispida* and *C. vulgaris* specimens being vital and growing under these very high-nitrate concentrations in short-term laboratory experiments, such a situation in the environment may eventually not be sustainable, since ecological factors are acting in the field (competition and higher shading produced by filamentous algae, phytoplankton and periphyton with N loading, as described for the angiosperm macrophytes [49]).

The response of *C. hispida* and *C. vulgaris* when faced with a nitrate increase was different depending both on their origin and between them, therefore our second and third hypotheses are verified. This supports other studies on the specificity of macroalgae responses to nutrient increases [21,50]. Under the most realistic situation (planted) the growth of both *C. vulgaris* populations was higher than that of *C. hispida*. This is in accordance with the pioneer features of *C. vulgaris* [51]. Moreover, the *C. vulgaris* specimens from the nitrate-rich environment adapted best to the highest nitrate concentrations when grew floating. Therefore, facing the future scenario of increased nitrate in shallow waters [11] it would be interesting to carry out a screening study to discover the maximum nitrate thresholds for each charophyte species, and to consider the evolution of distinct mechanisms to deal with high-nitrate concentrations. High-nitrate concentrations in aquatic ecosystems would cause a

biodiversity loss [52], because the environment would be selective for the more nitrate-tolerant species (*e.g. C. vulgaris*, *C. hispida* in detriment of *C. globularis* [13]). While decreasing eutrophication in the first place is the most useful, other factors (mainly ecological interactions) should be given close scrutiny in studies aimed at ameliorating diversity loss.

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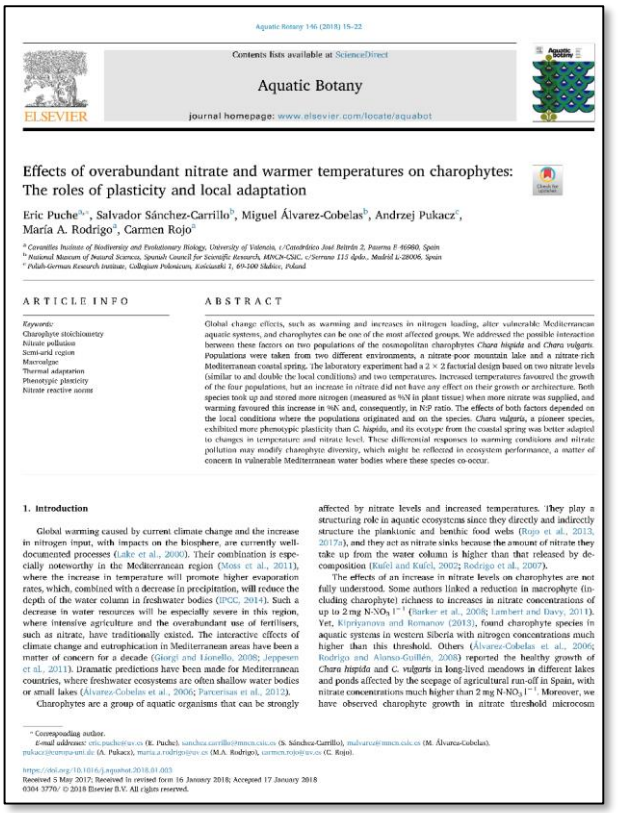
|CHAPTER 2|

Effects of overabundant nitrate and warmer temperatures on charophytes: the roles of plasticity and local adaptation



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Effects of overabundant nitrate and warmer temperatures on charophytes: The roles of plasticity and local adaptation

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ABSTRACT

Global change effects, such as warming and increases in nitrogen loading, alter vulnerable Mediterranean aquatic systems, and charophytes can be one of the most affected groups. We addressed the possible interaction between these factors on two populations of the cosmopolitan charophytes *Chara hispida* and *Chara vulgaris*. Populations were taken from two different environments, a nitrate-poor mountain lake and a nitrate-rich Mediterranean coastal spring. The laboratory experiment had a 2 × 2 factorial design based on two nitrate levels (similar to and double the local conditions) and two temperatures. Increased temperatures favoured the growth of the four populations, but an increase in nitrate did not have any effect on their growth or architecture. Both species took up and stored more nitrogen (measured as %N in plant tissue) when more nitrate was supplied, and warming favoured this increase in %N and, consequently, in N:P ratio. The effects of both factors depended on the local conditions where the populations originated and on the species. *Chara vulgaris*, a pioneer species, exhibited more phenotypic plasticity than *C. hispida*, and its ecotype from the coastal spring was better adapted to changes in temperature and nitrate level. These differential responses to warming conditions and nitrate pollution may modify charophyte diversity, which might be reflected in ecosystem performance, a matter of concern in vulnerable Mediterranean water bodies where these species co-occur.

1. Introduction

Global warming caused by current climate change and the increase in nitrogen input, with impacts on the biosphere, are currently well-documented processes (Lake et al., 2000). Their combination is especially noteworthy in the Mediterranean region (Moss et al., 2011), where the increase in temperature will promote higher evaporation rates, which, combined with a decrease in precipitation, will reduce the depth of the water column in freshwater bodies (IPCC, 2014). Such a decrease in water resources will be especially severe in this region, where intensive agriculture and the overabundant use of fertilisers, such as nitrate, have traditionally existed. The interactive effects of climate change and eutrophication in Mediterranean areas have been a matter of concern for a decade (Giorgi and Lionello, 2008; Jeppesen et al., 2011). Dramatic predictions have been made for Mediterranean countries, where freshwater ecosystems are often shallow water bodies or small lakes (Álvarez-Cobelas et al., 2006; Parcerisas et al., 2012).

Charophytes are a group of aquatic organisms that can be strongly affected by nitrate levels and increased temperatures. They play a structuring role in aquatic ecosystems since they directly and indirectly structure the planktonic and benthic food webs (Rojo et al., 2013, 2017a), and they act as nitrate sinks because the amount of nitrate they take up from the water column is higher than that released by decomposition (Rafiel and Rafiel, 2002; Rodrigo et al., 2007).

The effects of an increase in nitrate levels on charophytes are not fully understood. Some authors linked a reduction in macrophyte (including charophyte) richness to increases in nitrate concentrations of up to 2 mg $N\text{-}NO_3\ l^{-1}$ (Barker et al., 2008; Lambert and Davy, 2011). Yet, Kirpichnikova and Romanov (2013), found charophyte species in aquatic systems in western Siberia with nitrogen concentrations much higher than this threshold. Others (Álvarez-Cobelas et al., 2006; Rodrigo and Alonso-Guillén, 2008) reported the healthy growth of *Chara hispida* and *C. vulgaris* in long-lived meadows in different lakes and ponds affected by the seepage of agricultural run-off in Spain, with nitrate concentrations much higher than 2 mg $N\text{-}NO_3\ l^{-1}$. Moreover, we have observed charophyte growth in nitrate threshold microcosm

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Abstract

Global change effects, such as warming and increases in nitrogen loading, alter vulnerable Mediterranean aquatic systems, and charophytes can be one of the most affected groups. We addressed the possible interaction between these factors on two populations of the cosmopolitan charophytes *Chara hispida* and *Chara vulgaris*. Populations were taken from two different environments, a nitrate-poor mountain lake and a nitrate-rich Mediterranean coastal spring. The laboratory experiment had a 2 × 2 factorial design based on two nitrate levels (similar to and double the local conditions) and two temperatures. Increased temperatures favoured the growth of the four populations, but an increase in nitrate did not have any effect on their growth or architecture. Both species took up and stored more nitrogen (measured as %N in plant tissue) when more nitrate was supplied, and warming favoured this increase in %N and, consequently, in N:P ratio. The effects of both factors depended on the local conditions where the populations originated and on the species. *Chara vulgaris*, a pioneer species, exhibited more phenotypic plasticity than *C. hispida*, and its ecotype from the coastal spring was better adapted to changes in temperature and nitrate level. These differential responses to warming conditions and nitrate pollution may modify charophyte diversity, which might be reflected in ecosystem performance, a matter of concern in vulnerable Mediterranean waterbodies where these species co-occur.

Keywords: charophyte stoichiometry; nitrate pollution; semi-arid region; macroalgae; thermal adaptation; phenotypic plasticity; nitrate reactive norms

Resum

Els efectes del canvi global, com l'escalfament i l'increment en la càrrega de nitrogen, altera els ecosistemes aquàtics vulnerables de la regió mediterrània, i els caròfits poden ser un dels grups més afectats. Nosaltres hem abordat la possible interacció entre aquests factors en dues poblacions de les espècies cosmopolites de caròfits Chara hispida *i* Chara vulgaris*. Les poblacions foren recol·lectades de dos ambients diferents, un llac de muntanya pobre en nitrogen i una surgència costanera rica en nitrogen. L'experiment de laboratori va tindre un disseny factorial 2x2 basat en dos nivells de nitrat (similar a i el doble de les condicions locals de les poblacions) i dues temperatures. L'increment de la temperatura va afavorir el creixement de les quatre poblacions, però l'increment de nitrat no va tindre cap efecte en el seu creixement i l'arquitectura. Ambdues espècies assimilaren i emmagatzemaren més nitrogen (mesurat com a %N al teixit vegetal) quan més nitrat estava disponible, i l'escalfament va afavorir aquest increment en %N i, conseqüentment, en la proporció N:P. Els efectes d'ambdós factors depengueren de les condicions locals dels llocs d'origen de les poblacions, així com de l'espècie.* C. vulgaris*, una espècie pionera, va exhibir una major plasticitat fenotípica que* C. hispida*, i el seu ecotip de la surgència costanera estava millor adaptat als canvis en la temperatura i la concentració de nitrat. Aquestes respostes diferencials a condicions d'escalfament i contaminació per nitrat podrien modificar la diversitat de caròfits, la qual cosa es pot reflectir en la resposta de l'ecosistema, un tema de preocupació en els vulnerables ecosistemes aquàtics mediterranis, on aquestes espècies conviuen.*

***Paraules clau**: estequiometria dels caròfits; contaminació per nitrat; regió semiàrida; macroalgues; adaptació tèrmica; plasticitat fenotípica; normes de reacció front al nitrat*

1. Introduction

Global warming caused by current climate change and the increase in nitrogen input, with impacts on the biosphere, are currently well-documented processes (Lake *et al.*, 2000). Their combination is especially noteworthy in the Mediterranean region (Moss *et al.*, 2011), where the increase in temperature will promote higher evaporation rates, which, combined with a decrease in precipitation, will reduce the depth of the water column in freshwaterbodies (IPCC, 2014). Such a decrease in water resources will be especially severe in this region, where intensive agriculture and the overabundant use of fertilisers, such as nitrate, have traditionally existed. The interactive effects of climate change and eutrophication in Mediterranean areas have been a matter of concern for a decade (Giorgi and Lionello, 2008; Jeppesen *et al.*, 2011). Dramatic predictions have been made for Mediterranean countries, where freshwater ecosystems are often shallow waterbodies or small lakes (Álvarez-Cobelas *et al.*, 2006; Parcerisas *et al.*, 2012).

Charophytes are a group of aquatic organisms that can be strongly affected by nitrate levels and increased temperatures. They play a structuring role in aquatic ecosystems since they directly and indirectly structure the planktonic and benthic food webs (Rojo *et al.*, 2013, 2017a), and they act as nitrate sinks because the amount of nitrate they take up from the water column is higher than that released by decomposition (Kufel and Kufel, 2002; Rodrigo *et al.*, 2007).

The effects of an increase in nitrate levels on charophytes are not fully understood. Some authors linked a reduction in macrophyte (including charophyte) richness to increases in nitrate concentrations of up to 2 mg $N\text{-}NO_3$ l^{-1} (Barker *et al.*, 2008; Lambert and Davy, 2011). Yet, Kipriyanova and Romanov (2013), found charophyte species in aquatic systems in western Siberia with nitrogen concentrations much higher than this threshold. Others (Álvarez-Cobelas *et al.*, 2006; Rodrigo and Alonso-Guillén, 2008) reported the healthy growth of *Chara hispida* and *C. vulgaris* in long-lived meadows in different lakes and ponds affected by the seepage of agricultural run-off in Spain, with

nitrate concentrations much higher than 2 mg $N\text{-}NO_3$ l^{-1}. Moreover, we have observed charophyte growth in nitrate threshold microcosm experiments (without microalgae competition) at concentrations ranging from 0.5 to 50 mg $N\text{-}NO_3$ l^{-1} (Rodrigo *et al.*, 2017).

A few studies tested both the direct relationship between the nitrogen concentration in the medium and its uptake and storage by *Chara* spp. and the differences between aboveground and belowground uptake (Vermeer *et al.*, 2003; Rodrigo *et al.*, 2017). Different populations of *Chara vulgaris* responded to temperature changes according to the altitude of their habitat, implying different genetic capacities for adaptation and different reaction norms depending on the local conditions (Rojo *et al.*, 2015). Recently, the interactive and antagonistic effect of warmer temperatures and increases in salinity has been shown for two *Chara* species (Rojo *et al.*, 2017b).

Warmer temperatures led to an increase in the growth and metabolic rates of charophytes, and these increases modified charophyte stoichiometry (Rojo *et al.*, 2015, 2017b). However, it is currently unclear what occurs when more nitrate is available. The novelty of the current study is the analysis of the response of two cosmopolitan charophyte species (*Chara hispida* and *Chara vulgaris*) to sudden and concomitant, but realistic, changes in nitrate concentration and temperature. *Chara hispida* and *C. vulgaris* co-occur in many ecosystems of southern Europe (*e.g.*, Spain; Cirujano *et al.*, 2008). Although both have been described as 'generalist' species (Rey-Boissezon and Auderset Joye, 2015), they are not redundant species, as their autecology is somewhat different. *Chara vulgaris* is clearly a pioneer species, as it is the first to germinate from seedbanks. It has great expansion ability, with high fertility and high growth rates (Moore, 1986; Rodrigo *et al.*, 2017), while *C. hispida* has lower growth rates, although it can form dense and monospecific meadows in a wide range of habitats (Barinova *et al.*, 2014; Rojo *et al.*, 2017b). Populations of both species co-occurring in the same ecosystem differ in their response to salinization and increased

temperatures, and *C. vulgaris* was shown to have faster growth rates in all the conditions tested (Rojo *et al.*, 2017b). The response of charophytes to changes in environmental conditions depends on the phenotypic plasticity of populations and the existence of ecotypes (Rojo *et al.*, 2015, 2017b). Such differential responses to local environmental variation would result in changes in the diversity of charophyte communities. There are important relationships between charophytes and the abiotic or biotic environment which are species-specific, such as the nutrients incorporation or the allelopathy and its effects over plankton and epipyhtic community (Kufel and Kufel 2002; Rodrigo *et al.*, 2017; Rojo *et al.*, 2013, 2017a). Therefore, the loss of biodiversity, finally, may alter ecosystem functioning (*e.g.* clear water phase, biogeochemical cycles, carbon sink), with shallow ecosystems being particularly vulnerable to the aforementioned global changes (Auderset Joye and Rey-Boissezon, 2015; Rodrigo *et al.*, 2013; Rojo *et al.*, 2017b). Thus, it is necessary to consider populations originating from different environmental conditions when studying the interactive effect of two factors, such as increases in nitrate concentrations and temperature (Hyldgaard and Brix, 2012; Cross *et al.*, 2015). For this reason, we chose *C. hispida* and *C. vulgaris* populations from two Spanish sites that clearly differ in their nitrate loading, Somolinos mountain Lake and Quartons coastal Spring. In a laboratory experiment, we subjected the four populations to increases in nitrate concentration and temperature that are foreseeable based on current global change predictions: a two-fold increase in nitrate concentration with respect to their habitats of origin and a 4°C increase in temperature. Our first hypothesis is that the charophyte species will show an increase in growth and/or morphological or physiological changes in response to an increase in nitrate concentration. The second hypothesis is that higher growth rates mediated by warmer temperatures will favour nitrate uptake and that the synergistic effect of temperature and water nitrate concentration can affect charophyte stoichiometry. We expect that the population responses will depend on the phenotypic plasticity of the charophyte species, and might depend on the local conditions of origin.

2. Materials and methods

2.1. Population origin and culture

The specimens of *C. hispida* and *C. vulgaris* used in the experiment were collected from two different sites: Somolinos Lake (Sierra de Ayllón Protected Area, 1270 m a.s.l., 41°15'04"N, 3°03'54"W), an oligotrophic, deep (7 m maximum depth), mountain lake located in a cold climate, and Quartons Spring (Almenara, Castellón, 0 m a.s.l., 39°45'16"N, 0°11'27"W), a eutrophic, shallow (0.6 m maximum depth) waterbody located in a warmer climate (Fig. 1, Table 1). In Somolinos Lake, *C. hispida* (CHS) grows in a dense meadow in the littoral zone, while *C. vulgaris* (CVS) is located much deeper, close to the lake bottom, forming scattered patches. In Quartons Spring, *C. vulgaris* (CVQ) is the dominant charophyte throughout its extension and forms a dense meadow that almost reaches the water surface. Scattered among this species, *C. hispida* (CHQ) also forms dense patches.

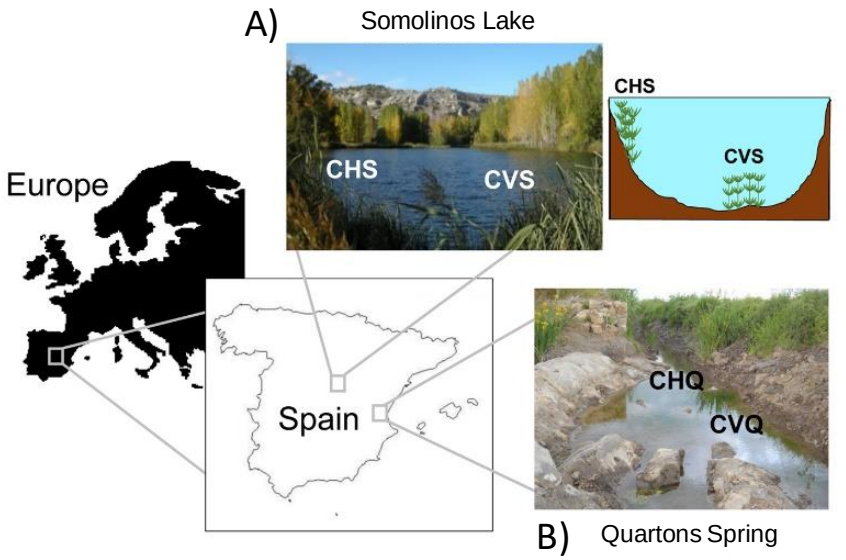

Fig. 1. Location of studied charophyte populations, showing sampling sites: A) the Somolinos mountain Lake and B) the Quartons coastal Spring. There were meadows of *Chara hispida* and *Chara vulgaris* in both sites: CHS and CVS (*C. hispida* and *C. vulgaris* from the Somolinos Lake), CHQ and CVQ (*C. hispida* and *C. vulgaris* from the Quartons Spring). Source: Miguel Álvarez-Cobelas photographed the mountain lake and Acció Ecologista-Agró took the photograph of the coastal spring, both pictures taken in 2016.

The harvested charophytes were transported to the laboratory at the University of València. The plants were gently washed, and the apical parts with a few nodes were cut and planted in small pots containing a mixture of sand and sediment (2:1 ratio); the sediment used was a 50% mixture of sediment from each place of origin (Table 1). The pots were placed in containers filled with dechlorinated tap water until the charophytes began to grow (Rojo *et al.*, 2015). These stock cultures were maintained in an indoor culture room at a constant temperature (20°C) under artificial illumination provided by Sylvania Gro-Lux F58W tubes (100 µmol photons m^{-2} s^{-1}; light:dark schedule 13:11 h). These conditions have been found to be non-limiting to the growth of these charophytes (Rodrigo *et al.*, 2013; Rojo *et al.*, 2015, 2017b; Rubio *et al.*, 2015).

Table 1. Variables measured in the two sampling sites of the studied charophyte populations. Annual mean (monthly sampled) and standard deviation (Mean±SD) from 2013 to 2015 and ranges of values reached considering only three vegetative periods (March-August) are shown. Values for sediment stoichiometry correspond to October 2015.

	Site			
	Somolinos Lake		Quartons Spring	
Variable	Mean±SD	Range	Mean±SD	Range
Temperature (°C)	11.4±3.2	9.0-15.0	21.4±4.2	19.3-28.2
Conductivity (µS cm^{-1})	444±10	433-469	2479±1089	1366-2570
pH	7.9±0.2	7.5-8.1	7.9±0.4	7.4-8.3
Nitrate (mg N-NO_3 l^{-1})	1.6±0.1	1.5-1.7	7.4±2.9	4.5-11.1
TN (mg N l^{-1})	1.8±0.2	1.4-1.9	8.1±1.5	4.6-11.9
TP (mg N l^{-1})	0.010±0.005	0.003-0.020	0.045±0.005	0.046-0.053
Sediment %C	14.2		9.4	
Sediment %N	0.3		0.1	
Sediment %P	0.02		0.02	

2.2. Experimental design

The experimental design consisted of growing individuals from the four populations (2 species × 2 origins) at two temperature and nitrate concentration levels. The temperature levels were 20°C, which was referred to as the low temperature treatment (LT), and 24°C, the high temperature treatment (HT). This increase is in accordance with the expected increase in temperature for the Mediterranean region

by the end of this century (IPCC, 2014), and has been used previously in other experiments addressing the effects of warming on charophytes (Rojo *et al.*, 2015, 2017b). The nitrate treatment consisted of two levels: the lower concentrations of each site of origin during the vegetative period of the populations (Table 1), which were 1.5 and 5 mg $N\text{-}NO_3$ l^{-1} for Somolinos Lake and Quartons Spring, respectively, referred to as the low nitrate treatment (LN) and a two-fold increase in these concentrations (3 and 10 mg $N\text{-}NO_3$ l^{-1}), referred to as the high nitrate treatment (HN). The combination of the temperature and nitrate concentration treatments resulted in four conditions: LTLN, HTLN, LTHN and HTHN.

Pre-experimental acclimatisation consisted of growing several individuals from each population under the four different conditions for two weeks, which is sufficient time for the charophytes to grow and acclimatise to the new environment (Rojo *et al.*, 2015; Rubio *et al.*, 2015). When the acclimatisation period had ended and before the experiment started, the dry weight (DW; 24 h at 70°C) and morphological variables (explained below) of 3 randomly selected shoot tips of each population from each of the four conditions were measured to obtain the initial biomass for each treatment at time zero.

We used six replicates for each population and condition (Rojo *et al.*, 2015). Shoot tips from each population and condition were randomly selected from the acclimatisation cultures.

96 shoot tips were individually planted in small pots, avoiding pseudo-replication, and using the same substrate used for the stock cultures. After planting, the initial length above the sediment for each individual was measured. Each pot was placed in a tall plastic beaker filled with 1 L of one of the four nitrate solutions (two for LN and two for HN; one from each origin). These four solutions were prepared by adding the necessary amount of sodium nitrate ($NaNO_3$) to dechlorinated tap water. The beakers were then placed in plastic containers (buckets) filled with ~40 L of tap water. The water in the buckets and their beakers reached the LT temperature under the

temperature and illumination conditions of the culture room. The HT temperature was achieved using aquarium heaters (Eheim Jäger 125W/150W for 100 L) in the corresponding buckets. The experiment lasted 26 days, which is sufficient time to observe changes related to warming (Rojo *et al.*, 2015, 2017b) and nutrients (Vermeer *et al.*, 2003; Rodrigo *et al.*, 2007).

The positions of the beakers were carefully changed every second day in order to avoid site effects. The lack of a 'bucket effect' or 'position effect' was tested as in previous experiments (Rojo *et al.*, 2015). The volume of 1 L was maintained in each beaker during the experimental period by adding the corresponding nitrate solutions and/or dechlorinated tap water every three days to compensate for evaporation. The physical and chemical variables were measured periodically to detect and subsequently rectify deviations from the experimental conditions. For example, the nitrate concentrations two days after the experiment began were the desired values, and so no nitrate addition was performed. Eleven days after start, these concentrations were 60-86% of the initial concentrations in the beakers. Therefore, a few millilitres of a concentrated solution of sodium nitrate were added to obtain the initial concentrations. Mann-Whitney tests showed that the temperatures were significantly different ($p < 0.05$) between the two levels of the temperature treatments and Kruskal-Wallis test showed that the nitrate concentrations measured at each of the four levels (low and high for charophytes from both the Somolinos and Quartons sites) were consistently different ($p < 0.05$).

2.3. Growth rate and morphological architecture

At the end of the experiment, each shoot was carefully removed from its pot and immediately placed on a tray with a gridded background and water. The individuals were extended as much as possible, and then a picture was taken in order to obtain the morphological variables by means of the image analysis software ImageJ (Schneider *et al.*, 2012). The plants were then dried at 70°C for 24 h and weighed to obtain the final DW of each individual.

The initial DW determined from control shoots was subtracted from the total final DW and normalised using the initial DW to obtain the normalised dry weight (NDW), which provides a measure of the production (growth rate) based on the unit weight of each specimen. The relative growth rate (RGR, d^{-1}) was determined using the equation (ln final DW–ln initial DW)/time (days) (Van der Berg *et al.*, 2002). The morphological variables measured were the length of the main axis (LMA, in cm), the number of lateral ramifications (B, branches hereafter) and the number of nodes (N). The calculated variables were the final minus the initial LMA (LMAV, in cm), which can be used as a measure of the absolute elongation. Moreover, to get an idea of changes in the shape or architectural complexity (Rojo *et al.*, 2015; Schneider *et al.*, 2015a), we calculated the weight distribution as the final DW/LMA ratio (in mg cm^{-1}), the internodal distance (LMA/N, in cm) and the number of branches per node (B/N).

2.4. Photosynthetic pigments and metabolic activity

At the end of the experiment, chlorophylls (a and b) and carotenoids were extracted from the apical parts of three replicates (upper 0.5-1 cm) using acetone (80%). Fresh apices were weighed after gently blotting dry with tissue paper. Then they were extracted using acetone solvent according to the detailed method in Rubio *et al*. (2015). Moreover, their concentrations (µg mg^{-1} org DW) were calculated based on the dry weight of the macroalgae without the calcium carbonate from incrustations (organic DW).

Immediately after the experiment ended, the *in vivo* respiration rates were assessed using an adaptation of the Winkler method (Golterman *et al.*, 1978) based on changes in the water dissolved oxygen (DO) concentration due to the respiratory activity of charophytes in short-term incubations (Rojo *et al.*, 2015). Three whole specimens (without rhizoidal systems) from each population and treatment were removed from the pots, rinsed (to remove possible epiphytes and the remaining sediment) and introduced into dark Winkler flasks (120 ml) containing the respective nitrate concentration and temperature of each treatment. The incubation started at

noon, four hours after the period of illumination in the culture room began. An optical O_2 probe (Hach USA IntelliCAL™, LDO101) with a special adaptor on the flask mouth (which prevented oxygen exchange with the air) was used to measure the dissolved oxygen concentrations (mg l^{-1}) and the incubation time was 45 min. The dissolved oxygen measurements were normalised using the dry weight of each shoot.

2.5. Calcium carbonate content and stoichiometric composition (C:N:P)

The calcium carbonate incrustation (% $CaCO_3$) of samples was determined from shoots dried at 105°C for three hours. These dry samples were analysed using the two-step weight loss on ignition method by Pukacz *et al.* (2014).

To analyse the organic stoichiometric composition of the specimens at the beginning and at the end of the experiment, the calcium carbonate from incrustations was removed. Several individuals from each population and treatment were dried (24 h at 70°C) and then washed with HCl (0.5 M) (Rojo *et al.*, 2015). Once the carbonate was removed, the samples were crushed by means of an automatic tissue grinder (TissueLyser II Qiagen) in two series of 15 s at 4500 rpm and kept desiccated in plastic tubes until the stoichiometric analyses were conducted. Total C and N were determined using a Perkin-Elmer CHN/O-2400 elemental autoanalyser. The P contents were measured using standard ICP methods following the thorough digestion of the samples using a mixture of nitric and perchloric acids (Rubio *et al.*, 2015). All stoichiometric ratios were calculated on a molar basis.

2.6. Statistical analysis

During the experiment, we compared the average temperature and nitrate concentrations measured in each beaker using Mann-Whitney or Kruskal-Wallis tests to verify that the charophytes were growing under the conditions stipulated in the experimental design.

The normality and the homoscedasticity of data were tested using the Kolmogorov-Smirnov and Levene tests, respectively. When both conditions were met, two-way

ANOVAs were carried out to determine the sensitivity of charophytes to temperature and nitrate concentration. We analysed the data from the four populations separately by taking into account the site of origin. When the assumptions for ANOVA were not met, we used the non-parametric Mann–Whitney *U* and Kruskal-Wallis (χ^2) tests for comparisons between two or more than two groups, respectively.

Statistically significant differences were considered to be present at $p < 0.05$. All analyses were conducted using the SPSS Statistics v.22 software (IBM Corp, Armonk, NY).

3. Results

In Somolinos Lake populations, doubling the nitrate concentration in the water only has significant effects on morphological variables of CVS (Tables S1 and S2 Supplementary material Chapter 2). In this population, morphological changes can be observed in B and N, with increases of 50 and 21% respectively under HN treatment (Fig. 2C, Tables 2, S1 and S2). The $CaCO_3$ content significantly increased in CVS due to nitrate supply from 21 to 26% (Table S1 and S2). However, temperature increase affected the growth and the morphology of both CHS and CVS (Tables S1 and S2). The RGR of CHS increased from 0.11 d-1 to 0.13 d^{-1} with warming regardless of the nitrate concentration, and that of CVS increased from 0.16 d^{-1} to 0.20 d^{-1}. This trend was followed by other variables related to growth and architectural morphology such as LMAV, NDW and LMA/N (in CHS) and DW/LMA and B (in CVS, Tables S1 and S2). Furthermore, we observed a neutralising effect of warming on B in CVS, as the increase in this variable between LN and HN was reduced from 163% (under LT) to 6% (under HT), showing an antagonistic effect of temperature and nitrate concentration (Fig. 2C, Tables 2, S1 and S2).

The nitrate reaction norms were similar for the populations from the coastal Mediterranean spring, with no changes observed for any growth- or morphology-related variable, although the CVQ values were always higher than those for CHQ (Fig.

2, Tables 2, S1 and S2). Warming caused the most significant change in the RGR of CVQ, which increased from 0.18 to 0.21 d^{-1} (Tables S1 and S2). Comparable to the Somolinos Lake populations, the other morphological and growth-related variables were significantly increased under HT treatment (Tables S1 and S2). Moreover, the increase in temperature also produced changes in physiological variables such as the increase in pigment concentration or the respiratory rate (Tables S1 and S2). No significant interaction effects have been observed between the two factors in neither of the two populations (Table S2).

With respect of the stoichiometric variables of the four populations, individuals of CHS and CVS had N contents that were not very different between the two nitrate treatments (Fig. 2D, Table 2). In fact, the increase in this variable between nitrate treatments was only significant in CVS (6% of increase, Table 2). Individuals of CHQ and CVQ showed higher and significant changes in %N (Fig. 2D, Table 2). The relative N content in the charophytes increased by 20% in CHQ and by 30% in CVQ when the nitrate concentration was doubled and consequently the C:N ratio decreased in both populations. Temperature and %N covaried only in the two populations from Somolinos Lake (CHS and CVS, Table 2). Moreover, warming favoured an increase in %N as a response to the nitrate supply in CVS, CHQ and CVQ (Table 2). As a consequence, the N:P ratio of CHQ and CVQ were significantly higher under the HTHN condition than under LTLN, following the same trend as %N (Table S1 and S2).

After comparing the reaction norms and phenotypic plasticity of charophytes that co-occur in the same environment, we analysed the differences in the responses of populations of the same species when facing a changing environment. CHS showed similar growth, architecture and %N when growing under the extreme assayed conditions, LTLN and HTHN (Fig. 2, Tables 2 and S2) while CHQ significantly increased its %N from 1.9 to 2.4% between the two extreme conditions. Differences in CVS growing under the LTLN and HTHN conditions were noticeable in terms of RGR (from 0.16 d^{-1} to 0.20 d^{-1}), the number of branches (which increased from 2.4 to 6.3) and %N

(which varied from 2.1 to 2.4, Tables 2, S1 and S2). CVQ, whose features were similar to those of CVS (Fig. 2, Table S1), experienced greater changes in growth and N content than CVS when cultivated under the two extreme conditions (Fig. 2).

4. Discussion

4.1. Population responses to increased nitrate

We had chosed two study sites for this work with very different nutrient loading. Somolinos Lake is considered an oligotrophic system with a TP concentration that limits microalgal growth (lower than 0.01 mg P l^{-1}) while Quartons coastal Spring has moderate phosphorus concentrations (lower than 0.06 mg P l^{-1}). *Chara hispida* and *C. vulgaris* populations both form meadows both in the lake with nitrate concentrations of almost 2 mg $N\text{-}NO_3$ l^{-1} (maximum tolerance limit proposed by Lambert and Davy, 2011) and in the coastal spring, which is located in an agricultural catchment area with an over-abundance of nitrate (more than 5 mg $N\text{-}NO_3$ l^{-1}). We demonstrated experimentally that, under low phosphorus concentrations to limit microalgal development, which might shade the charophytes, nitrate at double the concentration of the sites of origin was not harmful for these species, even if the populations came from oligotrophic sites. Others have observed negative effects on macrophytes caused by an increase in nutrients, which resulted in an increase in seston (González-Sagrario *et al.*, 2015; Olsen *et al.*, 2015). Yet, our results are more in accordance with those of Yu *et al.* (2015) who did not find any relationship between the nitrogen content in the water and the development of macrophytes.

In our experiments, there were no or only weak relationships between charophyte growth, morphology or physiology variables to such as photosynthetic pigment concentration or respiration and the nitrate content in the culture water. Similar results have been found for submerged angiosperms due to the higher nitrogen uptake

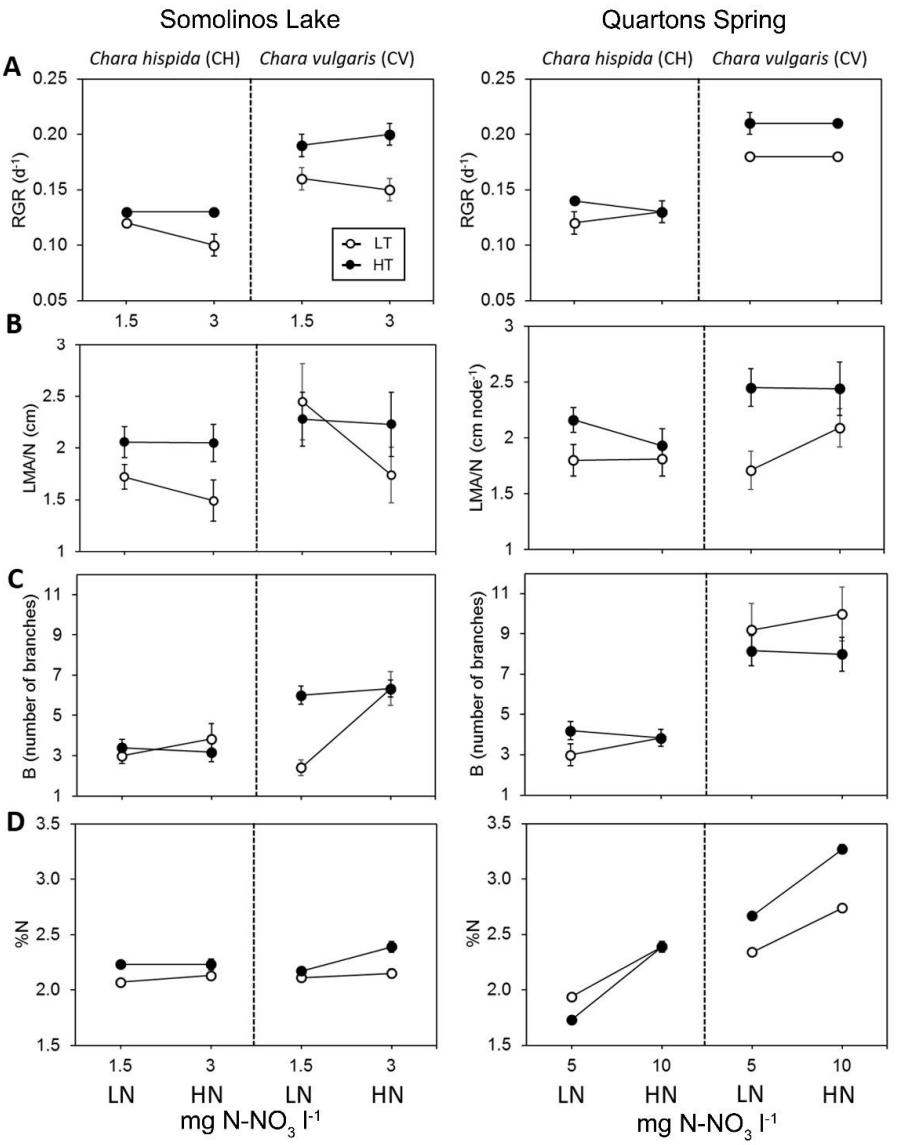

Fig. 2. Variables measured, at the end of the experiment, in the two populations of *Chara hispida* and *Chara vulgaris*, from the Somolinos mountain Lake and the Quartons coastal Spring, cultivated under four experimental conditions of temperature and nitrate concentrations. Low temperature (20°C, LT) and high temperature (24°C, HT) and low or high nitrate concentration (LN, HN, respectively). RGR is relative growth rate, LMA/N means internodal distance and %N is the percentage of nitrogen in the charophytes. Bars show standard errors.

Table 2. Comparison of mean values of internodal distance in cm (LMA/N), number of branches (B), relative growth rate in d^{-1} (RGR) and percentage of nitrogen (%N) between the four populations of charophytes. The four populations of the experiment: *Chara hispida* from the Somolinos mountain Lake (CHS) and the Quartons coastal Spring (CHQ) and *C. vulgaris* from the same sites (CVS and CVQ). Measures taken at the end of the experiment. F or *U* values of both two-way parametric ANOVA or non-parametric Mann–Whitney tests to analyse the effect of factor temperature (T, two levels), nitrate (N, two levels) and their novel interaction; 1 degree of freedom. F or χ^2 values of both one-way parametric ANOVA or non-parametric Kruskal-Wallis tests to analyse the effect of the four culture conditions, combination of two temperatures and two nitrate concentrations; 3 degrees of freedom. p < 0.05:*, p < 0.01:**, p < 0.001:***. Results of these tests on all analysed variables are in Table S2 (Supplementary material Chapter 2).

	Somolinos Lake															
	CHS								CVS							
	T		N		TxN		4 conditions		T		N		TxN		4 conditions	
Variable	F/*U*		F/*U*		F/*U*		F/χ^2		F/*U*		F/U		F/*U*		F/χ^2	
LMA/N	22.0	*	49.0				2.7		0.3		1.5		1.2		1.0	
B	33.5		56.5				3.5		9.4	**	13.2	**	9.4	**	10.1	***
RGR	14.0	**	59.0				9.5		28.1	***	0.0		0.3		9.7	***
%N	17.8	**	0.9		0.9		6.5		17.3	**	12.1	**	5.7	*	11.7	**
	Quartons Spring															
	CHQ								CVQ							
	T		N		TxN		4 conditions		T		N		TxN		4 conditions	
Variable	F/*U*		F/*U*		F/*U*		F/χ^2		F/*U*		F/*U*		F/*U*		F/χ^2	
LMA/N	2.7		0.6		0.7		1.2		8.9	**	1.0		1.1		3.5	
B	34.5		55.5				4.8		36.0		52.0				0.8	
RGR	44.0		55.0				0.9		11.0	***	50.0				8.5	**
%N	16.2	**	452.6	**	15.2	**	161.3	***	260.3		351.3	***	13.2	**	207.4	***

by roots than by aboveground parts and the preference of angiosperms for sedimentary ammonia rather than nitrate in the water column (Touchette and Burkholder, 2000; Cedergreen and Madsen, 2003). Vermeer *et al.* (2003) also confirmed these preferential sediment-based uptake mechanisms in *Chara* spp., as they observed a more important nitrogen flux from belowground to aboveground parts, highlighting that the translocation of ^{15}N in this direction occurred even when charophytes were exposed to high concentrations of nitrate in the water column.

Also by only increasing water nitrate levels, we observed increases in the nitrogen content of the charophytes. The possibility of nitrate uptake from the water column and retention by marine macroalgae and freshwater charophytes is well known (Vermeer *et al.*, 2003; Rodrigo *et al.*, 2007; Rodrigo and Alonso-Guillén, 2008). Both target charophyte species showed an increased percentage of N in their cells with higher nitrate availability, with a %N range of 2.3 - 2.6% for both species. Such elevated nitrogen percentages have been described for *C. hispida* in oligotrophic lakes in central Spain (2.9±0.3%) when high nitrate concentrations in the water column were measured (8 mg $N\text{-}NO_3$ l^{-1}; Álvarez-Cobelas *et al.*, 2007; Rodrigo *et al.*, 2007). In addition, the inter-annual variability of %N in *C. hispida* was also directly related to nitrate contamination events in the abovementioned lakes (Álvarez-Cobelas *et al.*, 2007).

Moreover, *C. hispida* and *C. vulgaris* populations from the Somolinos mountain Lake, the lower nitrate site, showed a lower accumulation of nitrogen when nitrate is supplied to the medium than their counterparts from the nitrogen-rich site (1 to 6% in the Somolinos Lake populations *versus* 20 to 30% in the Quartons Spring populations). The accumulation of nitrogen in charophytes when nitrate is abundant in the medium could be explained by the capacity for the storage of nitrate taken up from the water column, as was demonstrated for other macroalgal groups (Touchette and Burkholder, 2000; Naldi and Viaroli, 2002; Bracken *et al.*, 2015). This storage capacity is strongly dependent on the origin of populations, suggesting that both ecotypes inhabiting the

coastal ecosystem, with a wide range of nitrate concentrations throughout the year, have higher phenotypic plasticity in response to nitrate concentration variability. This observation is in accordance with the known relationships between ranges of environmental factors and the adaptation of local populations to them (Peipoch *et al.*, 2014) which we demonstrated in charophytes for thermal phenotypic plasticity (Rojo *et al.*, 2015).

4.2. Effects of increased temperature on populations

Our results show that the different populations of the two species increased in growth as a response to warming but in different ways depending on their origin. The populations of the two species living in the same place do not necessarily share their thermal reaction norms, and this discrepancy between cohabiting species has been demonstrated also in other organisms (Nilsson-Örtman *et al.*, 2013). We found that *C. vulgaris* from the colder environment showed the steepest slope of the thermal reaction norm (Fig. S1 Supplementary material Chapter 2) and in the warmer coastal environment, *C. vulgaris* grew better in response to warming temperatures. Yet, no temperature-induced changes in the relative growth rate of *C. hispida* were observed (Fig. S1). These results are coherent with the pattern of response to thermal changes that we have been establishing for some years for these macroalgae (Rojo *et al.*, 2015, 2017b). We previously observed that the relative growth rate of *C. vulgaris* from coastal Mediterranean ponds kept increasing up to at least 27°C; however, the relative growth rate of *C. hispida* from the same sites was invariant with warming (Rojo *et al.*, 2017b). The described trend is in accordance with the thermal reaction norms of *C. vulgaris* populations, which showed wide phenotypic plasticity (Rojo *et al.*, 2015) and could explain the wide geographical distribution of this species on the Iberian Peninsula (Cirujano *et al.*, 2008). Populations from the colder environment were able to respond to warming, but the *C. hispida* reaction norms were always (Rojo *et al.*, 2015) flatter than those of the pioneer *C. vulgaris* (Rey-Boissezon and Auderset Joye, 2015; Rodrigo *et al.*, 2017). Hence, according to our previous and current results, if a

temperature increase occurs during spring (IPCC, 2014), it is likely that *C. vulgaris* will produce more biomass in less time than co-occurring *C. hispida*, both in warm coastal ponds and cold mountain lakes.

4.3. Synergistic interaction of warming and nitrate levels on charophyte stoichiometry

Regarding temperature as a stressor related to global change, the issue that deserves the most attention is its interaction with other drivers of change, such as salinization or the over-abundance of nutrients (Moss *et al.*, 2011; Jeppesen *et al.*, 2011). The species-specific response of charophytes to concomitant changes in water temperature and salinity has recently been demonstrated (Rojo *et al.*, 2017b). They showed that temperature-enhanced growth compensated for the damaging effect of increasing salinity. In contrast, the test of the interactive effect of warming and increased nitrate supply showed that the greatest percentage of nitrogen in plants occurred in *C. vulgaris* from the most nitrate-polluted site at higher temperature. These results are in accordance with Coppens *et al.* (2016) who showed that higher temperatures enhanced the growth and therefore the N and P uptake of macrophytes and algae, and lowering the nutrient concentrations in the water. However, our results highlight that N accumulation in charophytes was not related to enhanced growth (see Fig. 2A and 2D for Quartons Spring). The higher nitrogen content is not (directly) translated into faster growth. We consider that the capacity for N storage or the accumulation of N increased (Touchette and Burkholder, 2000; Naldi and Viaroli, 2002; Bracken *et al.*, 2015). The two populations from the oligotrophic nitrate-poor environment were not able to accumulate nitrogen. We demonstrate this relationship for two taxonomically very different species (Schneider *et al.*, 2015b); therefore, this capacity seems to be more dependent on the development of local population abilities in response to the environment rather than differences among species. In other words, we suggest that the capacity to store overabundant nitrate depends on the environment inhabited by the population rather than on the species itself. This

hypothesis, which requires more testing, is in accordance with the suggestions of Peipoch *et al.* (2014) indicating that the nitrogen incorporated from the water is strongly influenced by the environmental conditions in the location inhabited by the population of algae (intrinsic capacity) and the over-abundance of N in the water (extrinsic factors). In accordance with these suggestions, the findings regarding the molar N:P ratios were related to the interaction between temperature and nitrate concentration and were not only dependent on the species but also, and more importantly, on the local conditions of the sites of origin of the populations. These stoichiometric changes under the extreme conditions assayed might represent a competitive advantage. According to Jeppesen *et al.* (2011), the higher molar N:P ratio of macroalgae in comparison to microalgae can enhance their competitiveness in a world with more nitrate over time. In this sense, better adapted charophyte populations that increase their N:P ratio through nitrogen incorporation or storage when nitrogen levels rise would be able to cope with such pollution. However, although our stoichiometric values are in accordance with the little data available for charophytes (Duarte, 1992; Kufel and Kufel, 2002; Puche and Rodrigo, 2015), understanding nutrient limitation and thus the competitive fitness of these macroalgae deserves more attention and more specific experimentation (Townsend *et al.*, 2008).

5. Conclusion

Our results indicate that both *C. hispida* and *C. vulgaris* have ecotypes with clear differences in phenotypic plasticity. Those ecotypes adapted to higher nitrate and temperature levels (*e.g.* individuals from a coastal lagoon), possess the ability to react in response to increases in this parameters in the medium. Such ecotypes would benefit at the expense of those that are not able to adapt to such changes. Our results imply that the population responses to foreseeable changes in nitrogen and temperature depended on their adaptations to previous conditions. With respect to global change, this might result in changes in the charophyte community structure that

could, in turn, affect ecosystem functioning (Rodrigo *et al.*, 2013; Peipoch *et al.*, 2014; Rojo *et al.*, 2015; Rojo *et al.*, 2017a). The observed different response patterns are particularly important since both *Chara* species are widely distributed and co-occur in lakes, ponds, lagoons and springs with very different local nutritional and thermal conditions (Álvarez-Cobelas *et al.*, 2007; Cirujano *et al.*, 2008; Calero *et al.*, 2016). We hope that this knowledge will help charophyte conservation and restoration in vulnerable Mediterranean freshwater systems. A deeper understanding of specific charophyte responses under global change will allow us to predict the implications for freshwater systems.

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|CHAPTER 3|

The antagonistic effect of UV radiation on warming or nitrate enrichment depends on ecotypes of freshwater macroalgae (charophytes)

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THE ANTAGONISTIC EFFECT OF UV RADIATION ON WARMING OR NITRATE ENRICHMENT DEPENDS ON ECOTYPES OF FRESHWATER MACROALGAE (CHAROPHYTES)[1]

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Increases in ultraviolet radiation (UVR), a negative global change factor, affect aquatic primary producers. This effect is expected to be modulated by other global change factors, and to be different for populations adapted to different environments. A common garden experimental approach using freshwater green macroalgae, the cosmopolitan charophyte species *Chara hispida* and *C. vulgaris*, allowed us to test whether the beneficial increases in water temperature (T) and nitrate concentration (N) mitigate negative UVR effects. Also, whether these interactions would be not only species-specific but also according to the origin of the population; therefore, two populations of each species were used: one from a coastal wetland and the other from a mountain lake. Two factorial-design experiments were performed: (i) the presence and absence of UVR × lower and higher T × four populations, and (ii) the presence and absence of UVR × lower and higher N × four populations. Response variables were: growth, morphometry, UVR-protective compounds, photosynthetic pigments, and stoichiometric composition. There were consistent response patterns in the key variables that represent different organization levels. Our main results showed that both warming and, to a lesser extent, the increase in nutrients ameliorated the negative effects of UVR on the molecular processes involved in acclimation to UVR, and that such a mitigating effect depended on the different phenotypic plasticity of each species and each ecotype. The coastal populations, being from a more variable environment, were more resilient than the mountain populations, mainly because of changes in growth and morphology.

Key index words: **Charophyceae; common garden; global change; local adaptation; Mediterranean region; photoprotection; plasticity**

***Abbreviations*: CHQ, *Chara hispida* from Quartons Spring; CHS, *Chara hispida* from Somolinos Lake; C, total carbon content; CVQ, *Chara vulgaris* from Quartons Spring; CVS, *Chara vulgaris* from Somolinos Lake; DW/LMA, dry weight per unit of length of the main axis; HN, high nitrate concentration; HT, high temperature; LMA/Nod, length of the main axis per node; LMA, length of the main axis; LMAV, variation of the length of the main axis; LN, low nitrate concentration; LT, low temperature; Nod, number of nodes; PAB, photosynthetically active radiation + ultraviolet A radiation + ultraviolet B radiation; RGR, relative growth rate; SUVACs, methanol-soluble ultraviolet radiation absorbing compounds; T, temperature; UVACs, total ultraviolet radiation absorbing compounds; UVAR, ultraviolet A radiation; UVBR, ultraviolet B radiation; UVR, ultraviolet radiation; WUVACs, methanol-insoluble ultraviolet radiation absorbing compounds**

Charophytes (green macroalgae from the Family Characeae, Order Charales, Class Charophyceae, Division Chlorophyta) are benthic primary producers of key relevance in aquatic habitats all over the world (Blindow et al. 2014), and have proven to be highly vulnerable to changes in their environment (i.e., Auderset Joye and Rey-Boissezon 2015, Rojo et al. 2015, Puche et al. 2018). For this reason, they are a key group to predict the effects of global change on the function and structure of freshwater ecosystems (Rodrigo et al. 2010, Pelechaty et al. 2015).

Environmental factors, considered drivers of global change, such as eutrophication, drought, increased ultraviolet radiation (UVR), or global warming (IPCC 2014, Williamson et al. 2014, EEA 2015), are receiving increasing attention because they interactively affect the biodiversity and the functioning of aquatic ecosystems (Sala et al. 2000, Jackson et al. 2016). A well-described example of these related factors is the concomitant effect of warm temperatures and low precipitation in the Mediterranean region where freshwater ecosystems are especially vulnerable as they are often shallow water bodies or small lakes (Álvarez-Cobelas et al. 2005, Parcerisas et al. 2012). In this climatic region, it is expected that the average temperature will increase by 4–5°C, due to sudden warm days (Christensen et al. 2007, Giorgi and Lionello 2008) accompanied by a decrease in precipitation by the end of the century (IPCC 2014). Moreover, detailed analyses

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Abstract

Increases in ultraviolet radiation (UVR), a negative global change factor, affect aquatic primary producers. This effect is expected to be modulated by other global change factors, and be different for populations adapted to different environments. A common garden experimental approach using freshwater green macroalgae, the cosmopolitan charophyte species *Chara hispida* and *C. vulgaris*, allowed us to test whether the beneficial increases in water temperature (T) and nitrate concentration (N), mitigate the negative UVR effects. Also, whether these interactions would be not only species-specific but also according to the origin of the population; therefore, two populations of each species were used: one from a coastal wetland and the other from a mountain lake. Two factorial-design experiments were performed: (i) the presence and absence of UVR x lower and higher T x four populations, and (ii) the presence and absence of UVR x lower and higher N x four populations. Response variables were: growth, morphometry, UVR-protective compounds, photosynthetic pigments and stoichiometric composition. There were consistent response patterns in the key variables that represent different organization levels. Our main results showed that both warming and, to a lesser extent, the increase in nutrients ameliorated the negative effects of UVR on the molecular processes involved in acclimation to UVR, and that such a mitigating effect depended on the different phenotypic plasticity of each species and each ecotype. The coastal populations, being from a more variable environment, were more resilient than the mountain populations, mainly because of changes in growth and morphology.

Keywords: Charophyceae; common garden; global change; local adaptation; Mediterranean region; photoprotection; plasticity

Resum

Els increments de la radiació ultraviolada (RUV), un factor negatiu del canvi global, afecta als productors primaris. S'espera aquest efecte estiga modulat per altres factors del canvi global i que siga diferent per a poblacions adaptades a diferents ambients. Una aproximació experimental de jardí comú usant algues verdes d'aigua dolça, les espècies de caròfit cosmopolites Chara hispida *i* C. vulgaris*, ens va permetre comprovar si els efectes beneficiosos de l'increment de la temperatura de l'aigua (T) i de la concentració de nitrat (N) mitiguen els efectes negatius de la RUV. També, si aquestes interaccions podrien ser no sols específiques d'espècie sinó també respecte a l'origen de les poblacions; per tant, dues poblacions de cada espècie foren utilitzades: una d'un aiguamoll costaner i altra d'un llac de muntanya. Es van realitzar dos experiments amb disseny factorial: (i) presència i absència de RUV x baixa i elevada T x quatre poblacions, i (ii) presència i absència de RUV x baixa i elevada N x quatre poblacions. Les variables resposta foren: creixement, morfometria, compostos protectors de la RUV, pigments fotosintètics i composició estequiomètrica. Hi va haver patrons de resposta coherents en les variables clau que representen nivells d'organització diferents. Els nostres resultats principals mostraren que tant l'escalfament com, en menor mesura, l'increment de nutrients, van minorar els efectes negatius de la RUV en els processos moleculars involucrats en l'aclimatació a la RUV, i que aquesta mitigació depengué de la diferent plasticitat fenotípica de cada espècie i cada ecotip. Les poblacions costaneres, provinents d'un ambient més variable, foren més resilients que les de muntanya, principalment degut als canvis en el creixement i la morfologia.*

***Paraules clau**: Charophyceae; jardí comú; canvi global; adaptació local; regió mediterrània; fotoprotecció; plasticitat*

Abbreviations: CHQ, *Chara hispida* from Quartons Spring; CHS, *Chara hispida* from Somolinos Lake; C, total carbon content; CVQ, *Chara vulgaris* from Quartons Spring; CVS, *Chara vulgaris* from Somolinos Lake; DW/LMA, dry weight per unit of length of the main axis; HN, high nitrate concentration; HT, high temperature; LMA/Nod, length of the main axis per node; LMA, length of the main axis; LMAV, variation of the length of the main axis; LN, low nitrate concentration; LT, low temperature; Nod, number of nodes; PAB, photosynthetically active radiation + ultraviolet A radiation + ultraviolet B radiation; RGR, relative growth rate; SUVACs, methanol-soluble ultraviolet radiation absorbing compounds; T, temperature; UVACs, total ultraviolet radiation absorbing compounds; UVAR, ultraviolet A radiation; UVBR, ultraviolet B radiation; UVR, ultraviolet radiation; WUVACs, methanol-insoluble ultraviolet radiation absorbing compounds

1. Introduction

Charophytes (green macroalgae from the Family Characeae, Order Charales, Class Charophyceae, Division Chlorophyta) are benthic primary producers of key relevance in aquatic habitats all over the world (Blindow *et al.* 2014), and have proven to be highly vulnerable to changes in their environment (Auderset Joye and Rey-Boissezon 2015, Rojo *et al.* 2015, Puche *et al.* 2018). For this reason, they are a key group to predict the effects of global change on the function and structure of freshwater ecosystems (Rodrigo *et al.* 2010, Pełechata *et al.* 2015).

Environmental factors, considered drivers of global change, such as eutrophication, drought, increased ultraviolet radiation (UVR), or global warming (IPCC 2014, Williamson *et al.* 2014, EEA 2015), are receiving increasing attention because they interactively affect the biodiversity and the functioning of aquatic ecosystems (Sala *et al.* 2000, Jackson *et al.* 2016). A well-described change of these related factors is the concomitant effect of warm temperatures and low precipitation in the Mediterranean region where freshwater ecosystems are especially vulnerable as they are often shallow waterbodies or small lakes (Álvarez-Cobelas *et al.* 2005, Parcerisas *et al.* 2012). In this climatic region, it is expected that the average temperature will increase by 4-5°C, due to sudden warm days (Christensen *et al.* 2007, Giorgi and Lionello 2008) accompanied by a decrease in precipitation by the end of the century (IPCC 2014). Moreover, detailed analyses of the decadal variations and trends of global solar

radiation over areas of the Mediterranean region have shown a widespread increase related to the air quality associated with anthropogenic alterations (Sánchez-Lorenzo *et al.* 2013a). For example, a significant positive trend of +3.9 $W \cdot m^{-2}$ per decade during the period 1985–2010 has been reported throughout Spain (Sánchez-Lorenzo *et al.* 2013b). The combination of the above-mentioned factors results in a severe decline in the water column thickness due to higher evaporation (Mariotti *et al.* 2008, Lelieveld *et al.* 2012). This loss of water causes both a concentration of nutrients, such as overabundant agricultural nitrate and salts (Giorgi and Lionello 2008, Jeppesen *et al.* 2011), and enables greater amounts of UVR to penetrate into the water, sometimes reaching the bottom of these systems (Rubio *et al.* 2015). On the other hand, the increase in global change factor variability affecting ecosystems raises topics that have received less attention (EEA 2015, Jickells and Moore 2015, Mateos *et al.* 2016).

Studies on the effects of UVR carried out directly in nature include a complex set of interacting factors that make them difficult to repeat observationally, and in these studies it is difficult to isolate the variance that the UVR intensity can explain from the population features (Pessoa 2012). Experimentation on this cause-effect relationship can help to achieve this goal and minimizes unwanted interactions (Álvarez-Gómez *et al.* 2017). This approach is also supported by the importance of developing predictions concerning population ecological responses to multiple and simultaneous drivers of global change (Kreyling and Beierkuhnlein 2007, Jackson *et al.* 2016, Carrillo *et al.* 2017). There is also a need to prove the differences in the response of distinct populations, due to adaptations that can be tested with a common garden experimental approach, as has been done on marine macroalgae (Figueroa *et al.* 2014, Celis-Plá *et al.* 2015).

In freshwater macroalgae, Rubio *et al.* (2015) demonstrated, in a short-term experiment, how increasing UVR had a negative impact on charophytes, and how this effect varied among species. Increased UVR damaged DNA, slowed growth rate, and resulted in morphologies which favoured more horizontal than apical growth, and

produced a higher bulk of UV-absorbing compounds (UVACs; Rubio *et al.* 2015). Schneider *et al.* (2006, 2015) experimentally established that *Chara intermedia* and *Chara contraria* change their morphology (orientation of branches or elongation) as defensive strategies against damaging changes in radiation (*i.e.* an increase in the intensity of photosynthetically active radiation, PAR). In field studies comparing charophytes living in shallow and deeper zones, the light climate is considered to be the main force that promotes morphological changes in shoots (Asaeda *et al.* 2007, Wang *et al.* 2015). Nevertheless, until now, UVR experiments have been carried out using different lighting conditions and species, meaning that the results are difficult to compare. Some experiments check the effect of ultraviolet B radiation (UVBR) plus PAR on several charophyte species (*e.g. Chara baltica*, *Chara hispida*, *Chara vulgaris* and *Nitella hyalina*) and an angiosperm species (*Myriophyllum spicatum*; Rubio *et al.* 2015). Others, such as this study and that of Álvarez-Gómez *et al.* (2017), use PAR plus UVBR and ultraviolet A radiation (UVAR), hereafter PAB, with *Gracilariopsis longissima* (marine rodophyte). Therefore, until now, the information that has been obtained demonstrates the different negative aspects that UVR causes on different species of macroalgae, but it could not establish an unquestionable comparison of the response capacity of the different target species or populations.

Regarding temperature increases, this has a positive effect on the growth of several primary producer groups (Barko and Smart 1981, O'Neal and Lembi 1995, Graham *et al.* 1996, Berry and Lembi 2000) including charophytes (Puche *et al.* 2018). In the latter, it has been found that the response to warming is species-specific and even varies with the origin of the populations (population-specific), the low altitude-populations being the most reactive (Rojo *et al.* 2015). Nitrate concentration (N) increases also generate a positive response in terms of growth in macroalgae (Luo *et al.* 2012, Rodrigo *et al.* 2017), up to a threshold (Touchette and Burkholder 2000). Within intensively cultivated lands, such as those in the Mediterranean region, this threshold should be very high. Rodrigo *et al.* (2017) reported that *Chara hispida* and *Chara vulgaris* from

Mediterranean ecosystems were able to grow under N of up to 50 mg $N\text{-}NO_3 \cdot L^{-1}$ and Puche *et al.* (2018) tested how both mentioned species had a higher percentage of nitrogen in the biomass when more nitrate was supplied in the medium.

In order to get more realistic interpretations, the effects of global change factors should be studied by taking their interactions into account (Jackson *et al.* 2016, Villar-Argaiz *et al.* 2018). More specifically, their possible antagonistic effects, for example, the mitigating effect of nutrients or temperature increases on the damaging UVR observed in benthic marine algae (Marcoval *et al.* 2008, Zheng and Gao 2009, Heinrich *et al.* 2015, Álvarez-Gómez *et al.* 2017), microalgae (Carrillo *et al.* 2017) and cyanobacteria (Gao *et al.* 2008). Nevertheless, to our knowledge, few studies regarding these interactions (UVR and T) have been carried out on freshwater macroalgae (Berry and Lembi 2000, Aigner *et al.* 2017), and none on charophytes. In fact, there are few studies concerning the effects of temperature on these macroalgae (Anderson and Lommasson 1956, Rojo *et al.* 2015, 2017) and few focus on the interaction between UVR and other stressors (Cabello-Pasini *et al.* 2011, Heinrich *et al.* 2015).

In addition, the interactive effect of these mentioned factors (UVR, T, or nutrient availability) may be relevant from an evolutionary point of view. The aim is to unravel whether responses to the abiotic interaction are due to local adaptation to specific sets of environmental conditions or to a more generalist increase of phenotypic plasticity (Avia *et al.* 2017, Pierangelini *et al.* 2017). Therefore, to develop an experiment on interactive effects, we should consider not only the different response of species (*e.g.* Roleda *et al.* 2009) but also the origin of populations as confirmed by the meta-analysis by Jin *et al.* (2017) on the photosynthetic organism's response to UVR. Related to this, studies were carried out on the intraspecific differences in phenotypic plasticity of the macrophyte *Ceratophyllum demersum* (Hyldgaard and Brix 2012), or how the concomitant positive effect of an increase in T and N can occur depending on the charophyte population origin (Puche *et al.* 2018).

In this study, our main goal is an understanding of the interactive effect on charophytes of UVR with warming and nutrient (such as nitrate) increases; this represents a predictable scenario for the Mediterranean region. Specifically, we aim to prove: i) that an increase in T and N mitigates the harmful effect of UVR on charophytes, and ii) that this mitigation will depend not only on the charophyte species but also on local adaptations of the populations, being more resilient those inhabiting the more variable environment.

2. Materials and methods

2.1. Charophyte cultivation

Original specimens from both charophyte species (*Chara hispida* and *Chara vulgaris*) were collected from two different sites. One of these sites was the Somolinos Lake (Sierra de Ayllón Protected Area, 1270 m a.s.l. 41°15'04"N 3°03'54"W), which is an oligotrophic, moderately deep (7 m maximum depth) mountain lake in a cold climate. The other site was the Quartons Spring (Almenara, Castelló, 0 m a.s.l. 39°45'16"N 0°11'27"W), which is a meso-eutrophic shallow (1 m maximum depth) waterbody fed by ground water located in a warmer climate (Puche *et al.* 2018). With these specimens, stock cultures were established planting them individually in small pots containing a mixture of sand and sediment (2:1 ratio). This sediment was, in turn, a mixture (50%) of sediments from the two study sites. The pots were placed in containers filled with sufficient dechlorinated tap water (Rojo *et al.* 2015). The stock cultures were maintained for several months in an indoor room in the laboratory at the University of València before the beginning of the experiments. They were maintained at 20°C under artificial illumination provided by Sylvania Gro-Lux F58W fluorescent tubes (22 W · m^{-2} or 5.1 mol photons · m^{-2} · d^{-1} or 1108 KJ · m^{-2} · d^{-1} of incident PAR) in a light:dark cycle of 14:10 h. In previous studies (Rodrigo *et al.* 2013, Rojo *et al.* 2015, 2017, Rubio *et al.* 2015) it has been demonstrated that these conditions are non-limiting to the growth of charophytes. Therefore, we had four stock

"population" cultures (2 species x 2 sites: *C. hispida* and *C. vulgaris* from Somolinos Lake (CHS and CVS, respectively), and the same species from Quartons Spring (CHQ and CVQ, respectively).

2.2. UVR x T experimental design

The UVR x T experimental design consisted of growing individuals from the four population cultures at two levels of radiation and T. Radiation levels were PAR and PAB. In the PAR treatment, the individuals only received this type of radiation, while in the PAB treatment, the individuals received the same PAR plus UVAR and UVBR doses. Radiation was provided by Philips TL40W/12 RS SLV tubes for UVBR, Philips Cleo 40W tubes for UVAR and Agro-Lite SHP GRO&FLO 600W-T sodium high-pressure lamps for PAR. In the PAB treatment, the UVAR and UVBR tubes were covered by an Ultraphan 295 filter (Digefra GmbH, Munich, Germany) to completely remove the ultraviolet C radiation. Furthermore, three Sylvania Gro-Lux F58W fluorescent tubes located at the back of the set up were turned on in the two radiation treatments. The underwater radiation was measured at different depths of the experimental container (detailed below: 5, 10, 15 and 20 cm) by means of a modular spectroradiometer (JAZ, Ocean Optics, Inc., Dunedin, FL, USA) supplied with a submersible optical fibre with a cosine corrected sensor. Values from 280 to 320 nm, from 320 to 400 nm and from 400 to 700 nm were integrated for UVBR, UVAR and PAR dose calculations, respectively (Table 1). The light:dark period was 14:10 h. In order to avoid light stress, and to try to emulate the natural solar cycle, the back lights were turned on first (dawn conditions), later the sodium high-pressure lamps which provided most of the PAR, and finally UVAR and UVBR beginning with 2 and 3 h, respectively, after the onset of the light period (*i.e.* macroalgae were exposed to UVAR and UVBR for 12 h and 10 h, respectively).

With respect to T, the two levels were: 23°C, referred to as the low T treatment (LT hereafter), and 4°C warmer (27°C), or high T treatment (HT hereafter). This increase is in accordance with the expected increase in T for the Mediterranean region by the end

of this century (Christensen *et al.* 2007, Bussotti *et al.* 2014), and was used before in other experiments on the effects of warming on charophytes (Rojo *et al.* 2015, 2017). Therefore, the combination of radiation and T treatments resulted in four conditions: PAR-LT, PAR-HT, PAB-LT and PAB-HT.

Table 1. Average underwater doses of photosynthetic active radiation (PAR) and ultraviolet A and B radiation (UVAR and UVBR) in UVR x T and UVR x N experiments. The average doses were calculated from measurements made at depths of 5, 10, 15, and 20 cm in the culture containers. The PAR:UVR and UVBR:UVAR ratios are provided for each experiment.

	PAR	UVAR	UVBR
UVR x T experiment	400-700 nm	320-400 nm	280-320 nm
$W \cdot m^{-2}$	86	1.5	0.1
$KJ \cdot m^{-2} \cdot d^{-1}$	4334	67	4.8
$KJ \cdot m^{-2} \cdot d^{-1}$ (effective dose)	-	1.3	3.8
mol photons $\cdot m^{-2} \cdot d^{-1}$	19.9	-	-
PAR:UVR	52		
UVBR:UVAR	0.08		
UVR x N experiment			
$W \cdot m^{-2}$	55	1.2	0.1
$KJ \cdot m^{-2} \cdot d^{-1}$	2772	53	3.7
$KJ \cdot m^{-2} \cdot d^{-1}$ (effective dose)	-	1.0	2.7
mol photons $\cdot m^{-2} \cdot d^{-1}$	12.8	-	-
PAR:UVR	41		
UVBR:UVAR	0.08		

The shoot tips of the organisms of the four charophyte populations required for the pre-experimental acclimatization period came from the stock cultures described above and were randomly selected to be used in the experiment (Fig. 1a). These specimens were planted individually in small pots using the same substrate as in the stock cultures (Fig. 1a). To ensure equivalent initial conditions for all experimental treatments, charophyte shoot tips were cut just below the third node and then planted upright (introducing the third node into the substrate). The planted pots were then introduced into cylindrical methacrylate beakers (30 cm high; 5 cm diameter; Fig. 1a) filled with tap water; the combination of tap water and sediment resulted in a N of 0.5 mg $N\text{-}NO_3 \cdot L^{-1}$. The beakers were used in order to avoid the individuals becoming pseudo-

replicates due to a "bucket effect" (Hurlbert 1984). Then, these beakers were placed in plastic buckets (4 L) filled with tap water (Fig. 1b). Both the beakers and the buckets were UVR-transparent. In the containers corresponding to the HT treatment, the T was raised by means of aquarium heaters (Eheim Jäger 25 W for 20 L). The positions of the buckets, and of the beakers within the buckets, were changed periodically in order to avoid a site effect (Niu *et al.* 2012). The pre-experimental period lasted 15 d, which is time enough for the charophytes to grow and acclimatize to the new environment (Rojo *et al.* 2015, Rubio *et al.* 2015). After this, the shoots were removed from the pots, the apical parts cut and planted again in order to equalize the characteristics of the individuals at the start of the experimental stage. The dry weight (DW) –24 h at 70°C– and morphological variables (explained below) of three randomly selected individuals of each population from each of the four conditions were measured to obtain the initial biomass of the macroalgae for each treatment group.

Physical and chemical variables were measured periodically in each beaker to detect deviations to the experimental conditions and to rectify them. The experiment ended after 15 d, which is sufficient time to observe changes related to radiation (Rubio *et al.* 2015, Álvarez-Gómez *et al.* 2017) and T (Rojo *et al.* 2015, 2017, Puche *et al.* 2018).

2.3. UVR x N experimental design

Both the pre-experimental acclimatization and the experimental design of the UVR x N experiment followed the same methodology as in the UVR x T experiment (explained above). However, the setup was slightly different: the cylindrical beakers (with the planted pots) filled with the corresponding nitrate solution (explained below) were put in a perforated structure where they were adjusted vertically (Fig. 1b). To ensure that all the individuals were receiving the same radiation, this structure was on a rotatory platform (Fig. 1b) and fans were used to avoid an increase in T and keep it at the room levels. There was one perforated structure for each radiation treatment (PAR and

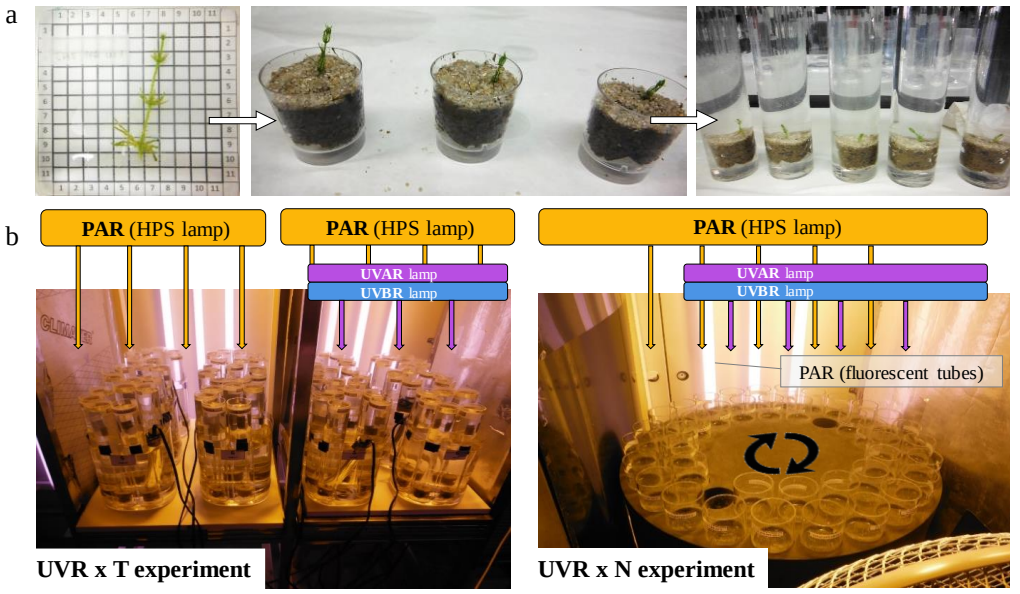

Fig. 1. Images of the experimental set-up: a) the selected shoot was measured on a gridded tray, then planted in small pots and put into the cylinders filled with tap water or the corresponding nitrate solution (depending on the experiment), b) on the left the UVR x T experimental setup with the cylindrical beakers in buckets to allow the different T supplied, and on the right the UVR x N experimental setup with the beakers in a rotatory platform with a fan to avoid any unwanted increase in T. In both experiments a set of lamps and fluorescent tubes (PAR, UVBR, and UVAR radiation) were placed to achieve the corresponding radiation doses for each treatment.

PAB). Owing to this setup, radiation doses were slightly different from the UVR x T experiment, although the PAR:UVR and UVBR:UVAR ratios were maintained in both experiments (Table 1). The lamps and periods of the different types of radiation were the same as in the UVR x T experiment.

The nitrate treatment also consisted of two levels: 1.5 and 15.0 mg $N\text{-}NO_3 \cdot L^{-1}$ (referred to as low nitrate –LN– and high nitrate treatment –HN–, hereafter). The nitrate solutions were prepared by adding the necessary amount of sodium nitrate ($NaNO_3$) to dechlorinated tap water. The N in the beakers were measured weekly in order to detect and correct deviations from experimental conditions. Therefore, the combination of radiation and N treatments resulted in four conditions: PAR-LN, PAR-HN, PAB-LN and PAB-HN. The experimental period lasted 15 d. As mentioned above,

this is enough time to observe changes related to radiation as well as nutrients (Vermeer *et al.* 2003, Rodrigo *et al.* 2017).

2.4. Growth rate and morphological architecture

Immediately after the completion of the experiments, each shoot was carefully removed from its pot and placed on a tray with a gridded background and water to leave the individual as extended as possible. Then a picture was taken in order to obtain the morphological variables using the image analysis software ImageJ (Schneider *et al.* 2012). After this, the apical part was separated from the rest of the shoot (for photosynthetic pigments and UVACs analyses, explained below) and the DW of the individuals without the apical part was measured, drying them for 24 h at 70°C. The normalized dry weight (NDW) was calculated as (final DW – initial DW)/initial DW, and the relative growth rate (RGR) was also determined as (ln final LMA - ln initial LMA)/t (days); LMA being the length of the main axis, in cm (van den Berg *et al.* 2002).

The morphological variables measured with ImageJ were: LMA and the number of nodes (Nod). Furthermore, other variables were calculated: final minus initial LMA or variation in LMA (LMAV, in cm), as a measurement of the absolute elongation; the ratio DW/LMA (in mg · cm^{-1}) and the internodal distance (LMA/Nod, in cm), in order to get an idea of changes in the shape or architectural complexity (Schneider *et al.* 2006, 2015).

2.5. Photosynthetic pigments and UV-absorbing compounds (UVACs)

At the end of the UVR x T experiment, chlorophylls a and b (chl-a and chl-b, respectively) and carotenoids were extracted from the apical parts of the macrophytes (upper 0.5–1.0 cm) using acetone (80%). Apices were weighed (FW, fresh weight after gently pressing the plants with drying paper) and introduced into test tubes. The samples were then deep-frozen by means of liquid nitrogen and immediately ground with an automatic tissue grinder (Precellys® 24, Bertin Technologies, France) in two series of 15 s at 1470 *g* to disrupt cell walls. The crushed samples were transferred to

centrifuge tubes with 4 mL of extractant and placed in a freezer (-20˚C) in darkness. After 24 h, the tubes were centrifuged, and the spectral absorption of the supernatant was measured by means of a Genesys 10S UV-VIS spectrophotometer at 470, 630, 645 and 665 nm. Pigment concentrations (µg · mg FW^{-1}) were calculated using the Lichtenthaler (1987) formulas.

Furthermore, at the end of both experiments, the levels of UVACs, both methanol-soluble and methanol-insoluble (SUVACs and WUVACs, respectively), were measured in the charophytes following Fabón *et al.* (2010). These compounds are located in different cell fractions, SUVACs being within vacuoles and WUVACs within cell walls (Clarke and Robinson 2008). The analyzed samples consisting of the whole apical part (in UVR x N experiment) and half of the apical part (in UVR x T experiment, because the other half was used for the analysis of the photosynthetic pigments, explained above) were ground with the automatic tissue grinder. The SUVACs were extracted by adding acidified methanol to the comminuted tissues in test tubes (methanol:water:12 M HCl, 79:20:1, v:v:v). The tubes were stored overnight at 4°C and then centrifuged, and the supernatant (containing the SUVACs) was preserved. The pellet remaining after SUVACs extraction was then subjected to WUVACs extraction by digesting the cell wall with 2 mL of 1 M NaOH in a water bath at 80°C for 3 h. After acidification to a pH of 1.0 using HCl, the absorbing compounds were extracted three times in acetyl acetate and, eventually, using a rotatory evaporator they were resuspended in methanol and preserved at -20°C. The contents of both the SUVACs and the WUVACs (and consequently total UVACs as the sum of both fractions), were measured by means of the spectrophotometer, in order to determine the amount of these compounds in the samples. The results are given in arbitrary units, as the area under the curve (AUC) normalised per unit of DW, described by the absorbance spectrum between 280 and 400 nm (Rubio *et al.* 2015).

2.6. Carbon and nitrogen content and C:N ratio

As mentioned earlier, at the end of both experiments the shoots of each population and condition (except for the apical part that had been used for the analysis of photosynthetic pigments and UVACs) were dried (24 h at 70°C). After drying, the samples were crushed by means of the automatic tissue grinder in two series of 15 s at 1470 *g*, and kept desiccated in plastic tubes until stoichiometric analyses were carried out. Total carbon (C) and nitrogen content were determined using a Perkin-Elmer CHN/O-2400 Elemental Autoanalyser. Their stoichiometric ratio (C:N) was expressed on a molar basis.

2.7. Statistical analyses

For each common garden experiment, a three-way analysis of variance (ANOVA) was used to analyze the effect of the three factors (explanatory variables), UVR, T or N, and Population, as well as their interactive effects on all dependent variables. The explanatory variables were treated as fixed categorical variables. A QQ plot, residual plot, Shapiro-Wilk test, and Levene test were used to assure normality and homoskedasticity of data. When these assumptions were not met, variables were transformed.

Once the interactive effect of UVR x T (or N) x Population had been tested, and to assess the possible effect of the origin site, we used another three-way ANOVA considering only the two populations of the same species (CHS *vs* CHQ or CVS *vs* CVQ), thus UVR x T (or N) x Origin.

Finally, the individualized response of each population was assessed to highlight their differences. This was tested by means of two-way ANOVAs, whose factors were UVR and T (or N), for each population separately, and each variable. For all significant findings, standardized effect sizes (partial η^2 values, range 0-1) are presented to help understand the biological importance of the results (Piggott *et al.* 2015). The partial η^2 values were calculated dividing the sum of squares for the interaction effect (UVR x T

or N) by the sum of squares of that effect plus the sum of squares for the error associated with that effect (Cohen 1988).

The effects of the single factors were classified as positive or negative when compared with the baseline condition (PAR-LT or PAR-LN, depending on the experiment). In those variables where UVR x T (or N) interaction was significant, and following Piggott *et al.* (2015), this effect was classified as: i) additive (AD) when the result of the interaction represents the sum of the individual effects of the factors, ii) positive antagonistic (+A) when the result is less positive than predicted additively, iii) negative antagonistic (-A) when the result is less negative than predicted additively, and iv) positive synergistic (+S) when the result is more positive than predicted additively. The level of significance was set for all statistical analyses to a $P < 0.05$. All analyses were performed with the SPSS Statistics-22 software (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Mitigation of UVR effect by warming or increased nitrate concentration

An interactive effect of T and UVR on growth, plant morphology, UVACs, photosynthetic pigments, and stoichiometry related to nitrogen content was found (Table 2). The supply of UVR caused a reduction in growth in both elongation and weight of 36-66% when the temperature was lower (Fig. 2a). But this harmful effect of UVR was mitigated when T rose, to the extent that no significant differences in these variables were found between PAR and PAB treatments (Fig 2a). This fact highlighted the antagonism in the interaction between these two factors (A in Table 2). This pattern was also shown by morphology-related variables. UVR produced a reduction of 32% in the internodal distance and an increase of 13% in DW/LMA under LT, but these differences became not significant under HT (Fig. 2b).

Ultraviolet radiation induced the production of total UVACs under LT (increases of ~170%), but this was significantly slowed down with warming (increases of only 30%;

Fig. 2c); a similar pattern was observed in both vacuoles (SUVACs) and cell wall fractions (WUVACs; Fig. S1 Supplementary material Chapter 3). There were no remarkable changes in photosynthetic pigment concentration between radiation levels under LT (Fig. S1); this pattern was reversed under HT, with UVR and T acting synergistically (S+ in Table 2).

The UVR supply under LT produced an increase of 39% in %N and a subsequent 34% reduction in the C:N ratio (Fig. 2d). Again, these stoichiometric changes between radiation treatments were weaker when the temperature was higher (the %N increased only 13% and the C:N ratio decreased 15%; Fig. 2d). Therefore, the antagonism of the increase in temperature over the effect of UVR was stronger on metabolic variables than on growth and morphology (see the standardized effect sizes in Table 2).

As with temperature, N and UVR had an antagonistic effect on growth and morphological variables (Table 2). The decrease in growth produced by the UVR supply (40-60% decrease in RGR, LMAV and NDW) was reduced under HN (25-35% decrease; Fig. 2e). The shortening of the internodal distance and the increase in DW/LMA due to UVR (a reduction of 54% and an increase of 73%, respectively) under LN were counteracted because of the nitrate enrichment (Fig. 2f). The same pattern was shown by the C:N ratio, which was reduced by 15% due to UVR supply under LN, but this was not significantly different between radiation treatments under HN. Neither the UVACs concentration nor %N were significantly modified by the UVR x N interaction (Fig. 2g and h).

Furthermore, the interactive relationship between UVR and the mitigating factors was different between populations (Table 2), showing clear results in growth, morphology and stoichiometric features in both experiments.

3.2. Mitigation of UVR effect by warming or increased nitrate concentration: role of the populations' origin

The relationship between mitigation and populations in growth, morphometry and stoichiometry was explained by the origin of the populations (CHS *vs* CHQ or CVS *vs* CVQ; Table 3a). This interactive effect (UVR mitigation) was always more pronounced in the coastal populations, with regard to the number of features significantly affected (Table 3b).

In the UVR x T experiment, both coastal populations (CHQ and CVQ) showed the strongest negative effect of UVR on growth variables (see the steeper slopes in Fig. 3a). With the supply of UVR when the T was lower, the RGR of CHQ and CVQ decreased by 47% and 58%, respectively, and NDW by 75% and 84%. In addition, and only in these populations, the UVR negative effect was significantly counteracted under HT (Fig. 3a, Table 3b). The effect of N on growth was also more evident in the coastal populations (Table 3b); for example, LMAV was reduced by 66% and 72% in CHQ and CVQ, respectively, under UVR and LN, but under HN, these reductions did not exceed 50% (Fig. 3b). However, these changes were not reflected in RGR (Fig. S2 Supplementary material Chapter 3).

The morphology of both coastal populations experienced the greatest amount of modification due to UVR under LT (Fig. 3c, Fig. S2, Table 3b); for example, DW/LMA increased by 29% and 65% in CHQ and CVQ, respectively; however, in lake populations no significant change due to UVR was observed. The higher T reversed the effect of UVR for the coastal populations (Fig. 3c, Table 3b). A similar interactive effect with N was also observed on the morphological variables (Fig. 3d and Fig. S2, Table 3b).

The concentration of UVACs under the UVR x T interaction did not differ depending on the origin, in either of the two species (Fig. 3e, Table 3a). Under LT and UVR, all populations significantly increased their UVACs concentration almost 3-fold (Fig. 3e). Under HT and UVR, the increase in UVACs compounds was only double in coastal

populations, and no significant variation was found in the compounds in the populations from the lake (Fig. 3e). This pattern was also shown by the fraction of these compounds located within vacuoles and those within the cell wall (SUVACs, WUVACs; Fig. S2). In the UVR x N experiment, the nitrate enrichment of the medium did not exert any mitigation on the positive effect of UVR on UVACs concentrations in any of the populations (Fig. 3f, Table 3b). An acute increase in pigment concentration under UVR and HT was observed in all populations except for CVS (Fig. 4a and b, Table 3b).

The supply of UVR caused a significant increase in %N in all populations, no matter the T or N treatment (Fig. 4c and d); the only exception was the almost zero response to UVR under HT in CHS. In all T or N treatments, the increase of %N caused by UVR supply was higher in the coastal populations (Fig. 4c and d). The C:N ratio showed a specular pattern compared to that of %N in all populations, and in both experiments (Fig. 4e and f). For example, in the UVR x N experiment, the C:N ratio decreased up to 30% in the coastal populations under UVR and LN , but only up to 13% under HN (Fig. 4f, Table 3b).

4. Discussion

The first hypothesis concerning the mitigation of the harmful effects of UVR on freshwater green macroalgae by increasing temperature and nutrient supply has been shown here on a wide diversity of response variables. The resilience was different in the morphological variables in comparison to the molecular composition ones. The mitigation of UVR varied depending on the beneficial factors considered (warming or nitrate supply), on charophyte species and on local adaptation of the populations. Faced with a similar variation in UVR, an increase in temperature of a few degrees Celsius was more successful than a 10-fold increase in N in the culture medium. The life history of the charophyte species was a key factor to understand the magnitude of the antagonistic interactions between the two pairs of stressors; thus, the different

Table 2. Three-way ANOVA results with the factors radiation (UVR), temperature (T) or nitrate (N), and Population (species and their origin) and their interactions on dependent variables of growth, morphology, UV-absorbing compounds, photosynthetic pigments and stoichiometry.

	UVR			T			Population		UVR x T			UVR x Population		T x Population		UVR x T x Population	
Dependent variable	P (F)	η^2_p		P (F)	η^2_p		P (F)	η^2_p	P (F)	η^2_p		P (F)	η^2_p	P (F)	η^2_p	P (F)	η^2_p
Growth and morphology																	
RGR	**0.000 (28.3)**	0.31	-	**0.007 (7.76)**	0.11	+	**0.000 (30.2)**	0.59	**0.000 (20.9)**	0.25	-A	**0.000 (7.5)**	0.26	0.910 (0.2)		**0.000 (6.8)**	0.25
sqrt (LMAV)	0.281 (1.2)			0.275 (1.2)			**0.000 (32.2)**	0.61	**0.000 (31.1)**	0.33	-A	**0.000 (11.3)**	0.35	**0.020 (3.5)**	0.15	**0.001 (6.7)**	0.25
log (NDW)	**0.000 (31.6)**	0.34	-	**0.000 (16.2)**	0.21	+	**0.000 (34.7)**	0.63	**0.000 (23.9)**	0.28	-A	**0.003 (5.1)**	0.20	0.630 (0.58)		**0.002 (5.6)**	0.22
log (LMA/Nod+1)	0.371 (0.8)			0.254 (1.3)			**0.000 (21.8)**	0.51	**0.000 (55.0)**	0.47	-A	0.096 (2.2)		**0.000 (7.3)**	0.26	**0.002 (5.7)**	0.21
DW/LMA	0.846 (0.0)			**0.005 (8.5)**	0.12	-	**0.000 (34.3)**	0.62	**0.006 (8.1)**	0.11	-A	0.480 (0.84)		0.220 (1.5)		**0.012 (4.0)**	0.16
UV-absorbing compounds																	
UVACs	**0.000 (127.1)**	0.67	+	**0.000 (36.0)**	0.36	-	0.142 (1.9)		**0.000 (46.4)**	0.42	+A	**0.019 (3.6)**	0.15	0.561 (0.7)		0.114 (2.1)	
log (SUVACs+1)	**0.000 (137.6)**	0.69	+	**0.000 (50.6)**	0.45	-	0.276 (1.3)		**0.000 (55.6)**	0.47	+A	**0.007 (4.5)**	0.18	0.534 (0.7)		**0.043 (2.9)**	0.12
WUVACs	**0.000 (141.2)**	0.70	+	**0.000 (30.7)**	0.34	-	0.136 (1.9)		**0.000 (72.4)**	0.54	+A	**0.003 (5.3)**	0.21	**0.037 (3.0)**	0.13	**0.017 (3.7)**	0.15
Photosynthetic pigments																	
chl-a	**0.000 (29.6)**	0.34	+	0.308 (1.1)			**0.000 (9.3)**	0.33	**0.009 (7.)**	0.12	+S	**0.112 (2.1)**	0.10	**0.271 (1.3)**	0.07	0.081 (2.4)	
chl-b	**0.005 (8.6)**	0.15	+	0.105 (2.7)			0.062 (2.6)		0.069 (3.5)		AD	**0.968 (0.1)**	0.01	**0.075 (2.4)**	0.13	**0.001 (6.8)**	0.29
Carotenoids	**0.007 (7.9)**	0.14	+	0.246 (1.4)			0.005 (4.9)		**0.000 (22.1)**	0.32	+S	0.123 (2.0)		0.237 (1.5)		0.068 (2.5)	
Stoichiometry																	
%N	**0.000 (1640.5)**	0.98	+	**0.000 (58.5)**	0.65	+	**0.000 (850.7)**	0.99	**0.000 (308.1)**	0.91	+A	**0.000 (41.9)**	0.80	**0.000 (504.0)**	0.98	**0.000 (141.1)**	0.93
C:N	**0.000 (1543.5)**	0.98	-	**0.000 (151.0)**	0.83	-	**0.000 (641.1)**	0.98	**0.000 (172.2)**	0.84	-A	**0.000 (54.9)**	0.84	**0.000 (501.1)**	0.98	**0.000 (213.6)**	0.95

Table 2. Continued.

Dependent variable	UVR P (F)	η^2_p		N P (F)	η^2_p		Population P (F)	η^2_p	UVR x N P (F)	η^2_p		UVR x Population P (F)	η^2_p	N x Population P (F)	η^2_p	UVR x N x Population P (F)	η^2_p
Growth and morphology																	
RGR	**0.000 (15.2)**	0.63	-	0.425 (0.6)			**0.000 (34.3)**	0.64	**0.018 (5.9)**	0.09	-A	0.185 (1.7)		0.174 (1.7)		0.913 (0.2)	
LMAV	**0.000 (134.3)**	0.70	-	0.06 (3.7)			**0.000 (36.1)**	0.65	**0.000 (14.7)**	0.20	-A	**0.000 (6.9)**	0.26	**0.028 (3.3)**	0.14	0.246 (1.4)	
NDW	**0.000 (36.7)**	0.63	-	**0.009 (7.4)**	0.12	-	**0.000 (33.0)**	0.64	**0.018 (5.9)**	0.10	-A	**0.023 (3.4)**	0.16	**0.000 (8.5)**	0.31	0.796 (0.3)	
LMA/Nod	**0.000 (32.3)**	0.61	-	**0.016 (6.0)**	0.09	-	**0.000 (11.3)**	0.36	**0.000 (14.9)**	0.20	-A	**0.017 (3.6)**	0.15	0.404 (1.0)		0.358 (1.1)	
log (DW/LMA)	**0.001 (12.4)**	0.18	+	0.747 (0.1)			**0.000 (98.3)**	0.84	**0.010 (7.0)**	0.11	+A	0.288 (1.3)		**0.012 (4.0)**	0.18	**0.000 (12.8)**	0.41
UV-absorbing compounds																	
UVACs	**0.000 (28.5)**	0.32	+	0.328 (0.9)			**0.000 (21.0)**	0.51	0.709 (0.1)		AD	**0.001 (5.8)**	0.23	0.398 (1.0)		0.120 (2.0)	
SUVACs	**0.000 (43.8)**	0.42	+	0.253 (1.3)			**0.000 (45.8)**	0.70	0.285 (1.2)		AD	**0.001 (6.2)**	0.24	0.241 (1.4)		0.061 (2.6)	
WUVACs	**0.003 (9.7)**	0.14	+	0.605 (3.7)			**0.013 (3.9)**	0.16	0.816 (0.1)		AD	**0.010 (4.1)**	0.17	0.567 (0.7)		0.300 (1.2)	
Stoichiometry																	
%N	**0.000 (151.4)**	0.81	+	**0.000 (70.9)**	0.67	+	**0.000 (31.1)**	0.73	0.486 (0.5)		AD	**0.000 (47.1)**	0.80	**0.000 (33.0)**	0.74	**0.008 (4.6)**	0.28
C:N	**0.000 (196.1)**	0.85	-	**0.000 (62.5)**	0.64	-	**0.000 (52.3)**	0.82	**0.000 (20.9)**	0.37	-A	**0.000 (99.9)**	0.90	**0.000 (55.9)**	0.83	**0.000 (16.9)**	0.59

Significance of the analysis (P) and F statistic (in parentheses) are reported. The main effects of the factors are classified directionally as positive (+) or negative (-) compared to a baseline condition (PAR-LT or PAR-LN, depending on the experiment). UVR x T (or N) interaction effects are classified directionally (+ or -) as antagonistic (A), synergistic (S), or additive (AD; no interaction). Effect sizes (partial η^2 squared values; range 0-1) are shown when $P < 0.05$. Abbreviations are: relative growth rate (RGR), length of the main axis variation (LMAV), normalized dry weight (NDW), internodal distance (LMA/Nod), dry weight per unit of LMA (DW/LMA); total, methanol-soluble and methanol-insoluble UV-absorbing compounds (UVACs, SUVACs and WUVACs, respectively), percentage of nitrogen (%N) and carbon *vs* nitrogen molar ratio (C:N). Square root, logarithmic and logarithmic plus one transformations are indicated (sqrt, log and log+1).

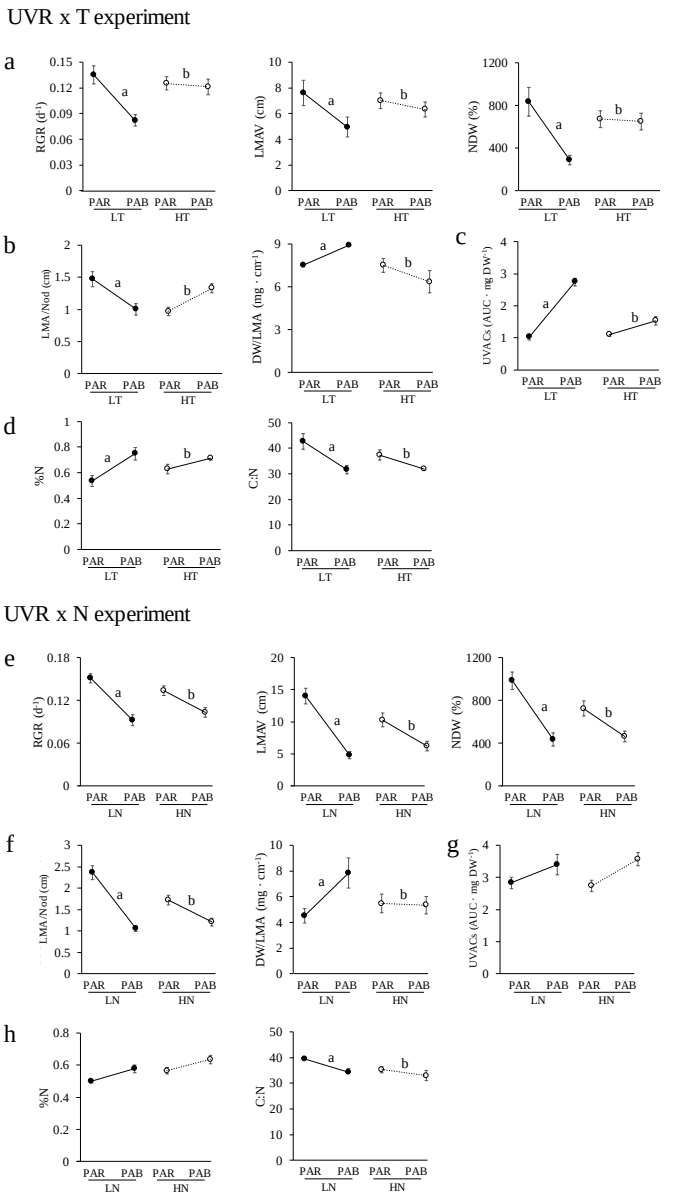

Fig. 2. Growth variables (a), morphological variables (b), UV-absorbing compounds concentration, UVACs (c), and stoichiometric variables (d) in charophytes (all the populations together) of the UVR x T experiment cultivated under four experimental conditions: photosynthetic active radiation (PAR) and PAR plus UVBR and UVAR radiation (PAB), and low temperature (LT, black dots) and high temperature (HT, white dots). Growth variables (e), morphological variables (f), UV-absorbing compounds concentration, UVACs (g), and stoichiometric variables (h) in charophytes (all the populations together) of the UVR x N experiment cultivated under four experimental conditions: the same radiation treatments as in the UVR x T experiment and low (black dots) and high (white dots) nitrate concentration (LN and HN, respectively). Abbreviations as in Table 2. Variation of data between two radiation levels were lineally fitted; a continuous line indicates significant differences ($P < 0.05$) whereas a dotted one shows that the adjustment is not significant. When letters above the lines appear, an interactive effect between the two factors (radiation and T or nitrate) is significant. Bars show standard error.

Table 3. a) Three-way ANOVA results of the interaction between the factors ultraviolet radiation (UVR), temperature (T) or nitrate (N) and origin (both lake (S) and spring (Q) populations of the same species, CHS-CHQ for *Chara hispida* and CVS-CVQ for *Chara vulgaris*). b) Two-way ANOVA results of the interaction between UVR and T (or N) in each population separately. Significance of the analysis (P) and F statistic (in parentheses) are reported. Effect sizes (partial eta squared values; range 0-1) are shown when P < 0.05. Abbreviations as in Table 2.

	a) UVR x T x Origin		UVR x T x Origin		b) UVR x T											
	CHS-CHQ		CVS-CVQ		CHS			CHQ			CVS			CVQ		
Dependent variable	P (F)	η^2_p	P (F)	η^2_p	P (F)	η^2_p		P (F)	η^2_p		P (F)	η^2_p		P (F)	η^2_p	
Growth and morphology																
RGR	**0.000 (19.8)**	0.38	0.052 (4.1)		0.741 (0.1)			**0.000 (25.1)**	0.61	+A	0.688 (0.2)		AD	**0.005 (10.5)**	0.40	-A
sqrt (LMAV)	0.079 (3.3)		**0.001 (13.7)**	0.31	**0.012 (8.2)**	0.35	-A	**0.001 (18.6)**	0.54	-A	0.431 (0.7)		AD	**0.000 (33.0)**	0.67	-A
log (NDW)	**0.007 (8.4)**	0.21	**0.007 (8.3)**	0.22	0.362 (0.9)			**0.001 (25.4)**	0.50	-A	0.956 (0.0)		AD	**0.000 (30.3)**	0.67	-A
log (LMA/Nod+1)	**0.031 (5.1)**	0.14	**0.002 (11.8)**	0.28	**0.017 (7.1)**	0.31	-A	**0.000 (23.8)**	0.60	-A	0.456 (0.6)		AD	**0.000 (150.8)**	0.91	-A
DW/LMA	**0.017 (6.4)**	0.17	0.251 (1.4)		0.210 (1.7)			**0.040 (5.0)**	0.24	+A	**0.020 (6.6)**	0.29	+A	**0.012 (8.3)**	0.36	+A
UV-absorbing compounds																
UVACs	0.258 (1.3)		0.056 (3.9)		**0.000 (33.8)**	0.64	+A	**0.004 (11.1)**	0.41	+A	**0.001 (15.5)**	0.51	+A	0.125 (2.6)		AD
log (SUVACs+1)	0.078 (3.3)		**0.041 (4.6)**	0.13	**0.001 (23.8)**	0.53	+A	**0.008 (9.3)**	0.37	+A	**0.000 (34.0)**	0.71	+A	**0.017 (7.1)**	0.31	-A
WUVACs	0.271 (1.3)		**0.003 (10.6)**	0.27	**0.000 (29.6)**	0.65	+A	**0.005 (10.6)**	0.40	+A	**0.000 (63.6)**	0.82	+A	0.099 (3.1)		AD
Photosynthetic pigments																
chl-a	0.512 (0.4)		**0.017 (6.4)**	0.18	0.841 (0.0)			0.184 (1.9)		AD	0.665 (0.2)		AD	**0.001 (20.7)**	0.52	+S
chl-b	**0.026 (5.6)**	0.19	0.869 (0.0)		**0.000 (38.8)**	0.81	+S	0.576 (0.3)		AD	0.341 (0.5)		AD	0.244 (1.5)		AD
Carotenoids	0.443 (0.6)		**0.015 (6.8)**	0.21	**0.013 (8.7)**	0.44	+S	**0.033 (5.7)**	0.31	+S	0.872 (0.0)		AD	**0.007 (12.7)**	0.44	+S
Stoichiometry																
%N	**0.000 (263.8)**	0.94	**0.000 (152.0)**	0.91	**0.000 (501.4)**	0.98	+A	0.097 (3.5)		AD	0.488 (0.5)		AD	**0.000 (253.8)**	0.06	+A
C:N	**0.000 (366.0)**	0.96	**0.000 (43.9)**	0.73	**0.000 (562.3)**	0.99	-A	**0.005 (15.1)**	0.65	+A	0.075 (4.2)		AD	**0.000 (78.3)**	0.34	+A

Table 3. Continued.

	a) UVR x N x Origin				b) UVR x N											
	CHS-CHQ		CVS-CVQ		CHS		CHQ			CVS			CVQ			
Dependent variable	P (F)	η^2_p	P (F)	η^2_p	P (F)	η^2_p	P (F)	η^2_p		P (F)	η^2_p		P (F)	η^2_p		
Growth and morphology																
RGR	0.911 (0.0)		0.411 (0.7)		0.358 (0.9)		0.249 (1.4)		AD	0.357 (0.9)		AD	0.070 (3.8)			AD
sqrt (LMAV)	0.069 (3.6)		0.326 (1.0)		0.402 (0.7)		**0.007 (9.7)**	0.39	-A	0.301 (1.2)		AD	**0.024 (6.2)**	0.28		-A
log (NDW)	0.669 (0.2)		0.396 (0.7)		0.074 (3.7)		0.208 (1.7)		AD	0.175 (2.0)		AD	0.550 (0.4)			AD
log (LMA/Nod+1)	0.166 (2.0)		0.367 (0.8)		0.393 (0.8)		**0.042 (4.9)**	0.23	-A	0.077 (3.6)		AD	**0.008 (9.4)**	0.39		-A
DW/LMA	**0.002 (11.2)**	0.29	0.291 (1.2)		0.823 (0.1)		**0.002 (15.3)**	0.52	-A	**0.010 (8.8)**	0.37	-A	0.849 (0.0)			AD
UV-absorbing compounds																
UVACs	0.177 (1.9)		0.061 (3.8)		0.327 (1.0)		0.368 (0.9)		AD	0.119 (2.8)		AD	0.322 (1.0)			AD
log (SUVACs+1)	0.448 (0.6)		**0.018 (6.2)**	0.17	0.678 (0.2)		0.510 (0.5)		AD	**0.043 (4.9)**	0.26	+A	0.312 (1.1)			AD
WUVACs	0.128 (2.5)		0.266 (1.3)		0.168 (2.0)		0.425 (0.7)		AD	0.444 (0.6)		AD	0.426 (0.7)			AD
Stoichiometry																
%N	**0.005 (10.1)**	0.34	0.154 (2.3)		0.142 (2.5)		**0.003 (16.3)**	0.64	+A	0.483 (0.3)		AD	0.200 (2.0)			AD
C:N	**0.000 (27.5)**	0.58	**0.000 (23.5)**	0.61	0.354 (0.9)		**0.000 (51.2)**	0.85	-A	0.161 (2.5)		AD	**0.001 (27.3)**	0.77		-A

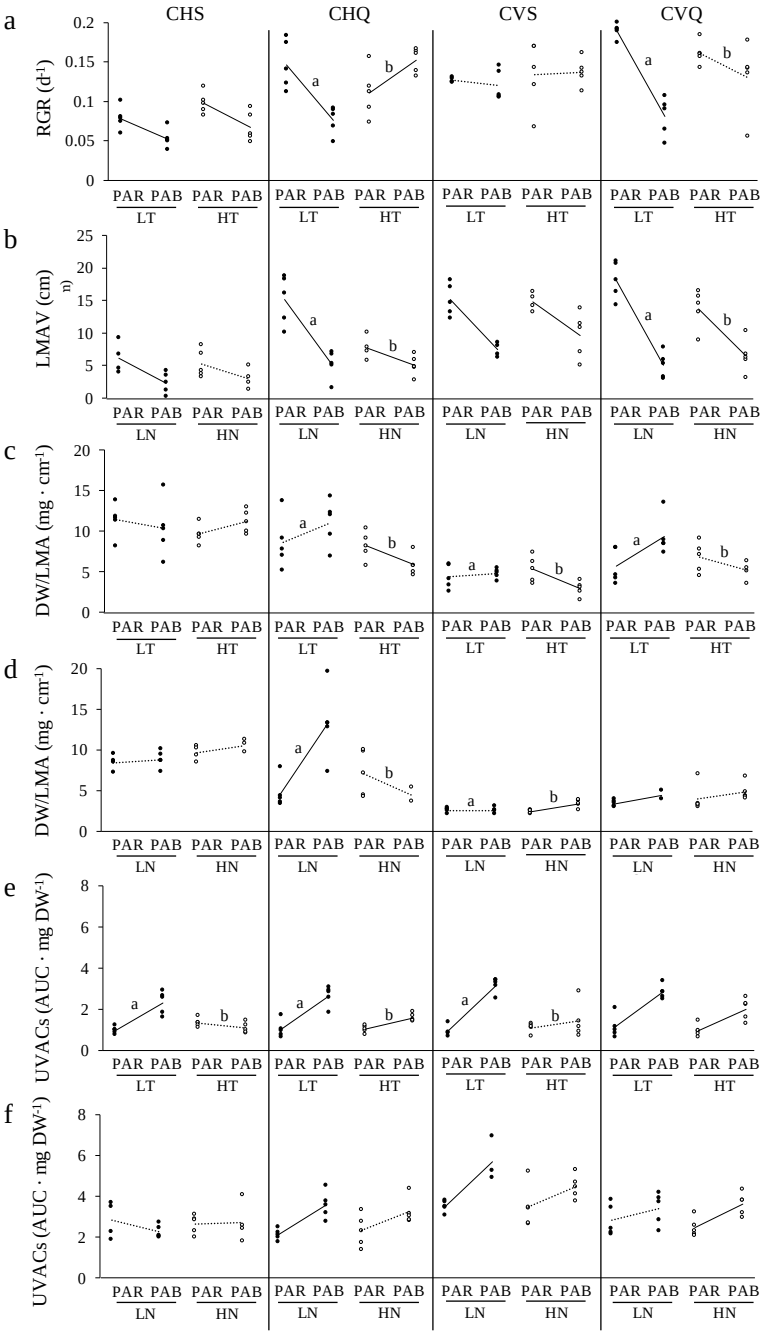

Fig. 3. Growth (a, b), morphological (c, d), and UV-absorbing compounds (UVACs) concentration (e, f) in charophytes in the UVR x T and UVR x N experiments, in the two populations of *Chara hispida* and *C. vulgaris* from the Somolinos Lake (CHS and CVS) and the Quartons Spring (CHQ and CVQ), cultivated under four experimental conditions. Details of experimental conditions in Fig. 2. Abbreviations as in Table 2. Variation of data between two radiation levels were lineally fitted; a continuous line indicates significant differences ($P < 0.05$) whereas a dotted one shows that the adjustment is not significant. When letters above the lines appear, an interactive effect between the two factors (radiation and T or N) is significant. Each dot represents a replicate.

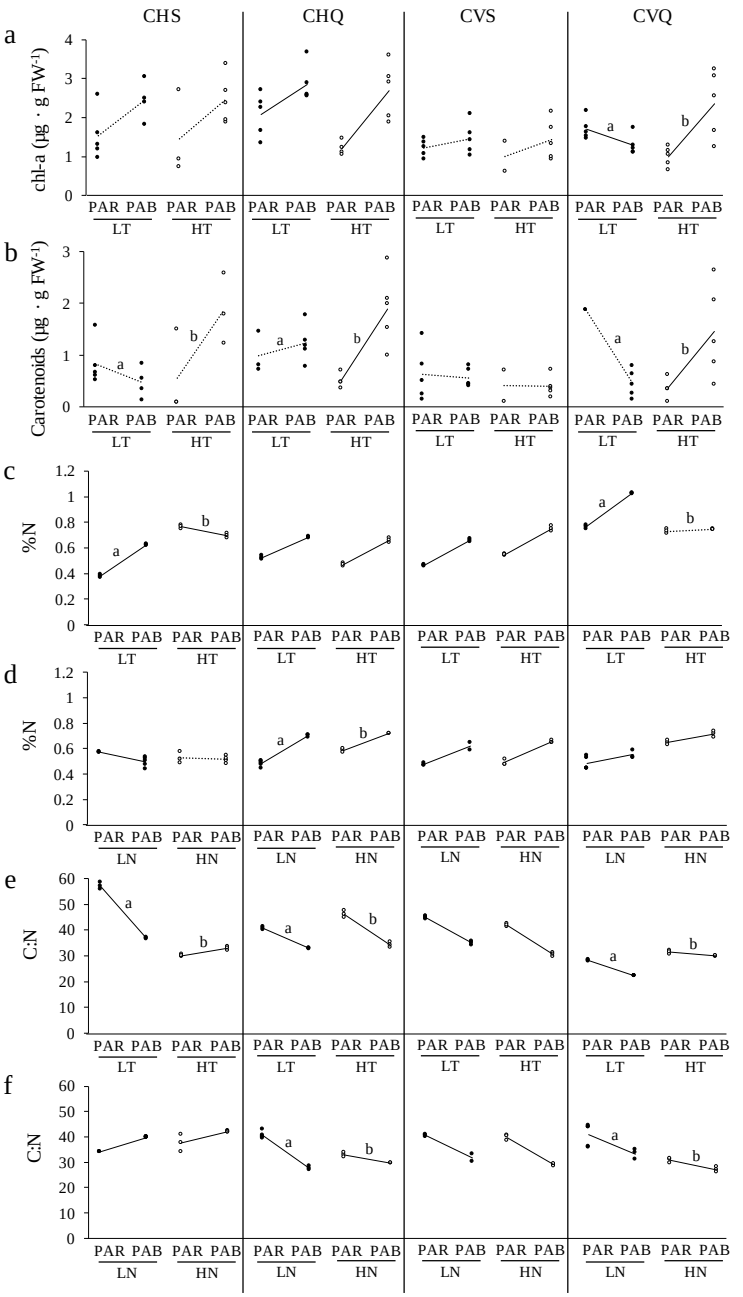

Fig. 4. Photosynthetic pigments (a, b), percentage of nitrogen in cells (c, d), and C:N ratio (e, f) in charophytes in the UVR x T and UVR x N experiments, in the two populations of *Chara hispida* and *C. vulgaris* from the Somolinos Lake (CHS and CVS) and the Quartons Spring (CHQ and CVQ), cultivated under four experimental conditions. Details of experimental conditions in Fig. 2. Abbreviations as in Table 2. Variation of data between two radiation levels were lineally fitted; a continuous line indicates significant differences ($P < 0.05$) whereas a dotted one shows that the adjustment is not significant. When letters above the lines appear, an interactive effect between the two factors (radiation and T or N) is significant. Each dot represents a replicate.

phenotypic plasticity of populations to acclimatize and survive under rapid factor variations depended on the origin of populations, verifying our second hypothesis.

Individuals of both studied species responded to UVR by increasing the number of nodes and reducing their internodal distance; they became more flattened structures as a defensive strategy under harmful radiation. These morphological changes have been attributed, both in charophytes and mosses, as defensive strategies against damaging changes in radiation (Schneider *et al.* 2006, 2015, Asaeda *et al.* 2007, Rubio *et al.* 2015, Wang *et al.* 2015, Hyyryläinen *et al.* 2018). These morphological or plant-architecture changes implied a reduced relative growth rate of charophytes due to the effect of UVR. This mechanism is an evolutionary stress response in an increasing UVR environment that, in aquatic photosynthetic organisms with higher levels of organization than unicellular organisms, complements the more ancient cellular stress response (Pierce *et al.* 2005, Hyyryläinen *et al.* 2018).

In both of our experiments, charophytes, like other aquatic organisms, developed protective and repairing strategies against UVR, such as the synthesis of UVACs and DNA repair, as expected (Roy 2000, Rubio *et al.* 2015). *Chara hispida* and *Chara vulgaris* faced with the implementation of UVR increased their concentration of UVACs, mainly in the cell wall-bound where protective compounds are transported quickly and are more efficient (Rubio *et al.* 2015). Moreover, this production was facilitated in our experiments by the UVAR supplied in addition to UVBR that stimulates photosynthesis (Gao *et al.* 2007, Carrillo *et al.* 2017). The production of UVACs, molecularly considered N-compounds with photoprotection and antioxidant capacities (Adamczyk *et al.* 2017), implied a higher relevance of nitrogen accumulation in tissues in the studied charophytes. These mechanisms of maintaining the integrity of DNA in response to genotoxic stress, such as increasing UVR, are largely considered a conservative, ancient, and general adaptation of cellular stress (Pierce *et al.* 2005, Vágnerová *et al.* 2017); and they have also been described in marine macroalgae (the rhodophytes

Hypnea musciformis [Schmidt *et al.* 2010] and *Gracilariopsis longissima* [Álvarez-Gómez *et al.* 2017]).

The present study bears out, in both *Chara hispida* and *Chara vulgaris*, that a moderate increase in temperature is positive for charophytes as it mitigates the harmful effect due to UVR. Some mechanisms whereby warming mitigates UVR stress in macroalgae have been proposed. In charophytes, the increase in temperature accelerates the photosynthetic metabolism causing sudden changes in morphology and increasing growth (Rojo *et al.* 2015), and these changes are able to modulate the effects of radiation variability (Schneider *et al.* 2006).

However, this acceleration of the metabolism due to a temperature increase could imply a potential metabolic cost (Rojo *et al.* 2017) that prevented charophytes from producing other needed molecules, such as UVACs. Therefore, some trade-off should be taken into account in the molecular response to UVR mediated by temperature; the photorepair mechanisms have been described as temperature-dependent while the photochemical damage processes are independent of temperature (Li *et al.* 2002), for example in macroalgae (Pakker *et al.* 2000). In general, when confronted with stress, and particularly stress due to UVR, photorepair mechanisms seem to imply less energetic cost than production and storage of photoprotective compounds (Pierce *et al.* 2005, Vágnerová *et al.* 2017). Therefore, an increase in temperature triggers DNA repair by photoreactivation and production of, for example, vitamins and enzymes thanks to the activation of proper genes (Pierce *et al.* 2005, Heinrich *et al.* 2015). We have observed that these mechanisms improve algae growth without having to increase the concentration of photoprotective compounds. In charophytes, another interactive effect tested here has been the lower production of UVACs in the presence of UVR when the temperature was higher, mitigating the loss of growth; in fact, the increase in %N in the biomass, which has been related to greater defence against UVR, is reduced under warming conditions, supporting this idea.

With respect to the amount of nitrate in water as a UVR-mitigating factor, its effectiveness did not seem conclusive in charophytes. This interactive effect, recently dealt with in marine macroalgae, offered contradictory results. The production of photorepairing and photoprotective molecules, such as the N-compound polyphenols or mycosporines, is promoted by the N increase in marine phaeophytes *Ascophyllum nodosum* and *Fucus vesiculosus* and chlorophyte *Ulva rigida* (Pavia and Toth 2000, Cabello-Pasini *et al.* 2011). However, this interactive effect was not evident in an experiment with rhodophyte *Gracilariopsis longissima*, where Álvarez-Gomez *et al.* (2017) verified that the higher the UVR, the more nitrogen was incorporated, but this incorporation was at maximum levels under LN. The results of our experiments do not demonstrate, in a reliable way, that the increase in nitrogen in the biomass due to UVR is also favoured by a higher N in the medium.

Thus, we can confirm that warming conditions counteracted the charophytes stress due to the foreseeable consequences of climatic change in the Mediterranean region (*i.e.* the loss of water level), with the consequent increase in UVR and the concentration of solutes stressing the benthic macroalgae (Rojo *et al.* 2017).

We highlight that molecular changes due to UVR x T interaction were common in all studied populations, but the morphological changes were not. These latter traits were different between the populations from the same species, and this difference was mainly due to the reactiveness of coastal populations. These results were in accordance with the evolution of plant strategies (Pierce *et al.* 2005). As we have mentioned before, intracellular changes in molecular composition (cellular stress) occurred earlier, and were more general and conservative than morphological changes developed by multicellular organisms. Populations living in conditions of variability, which have achieved sufficient phenotypic plasticity to respond to short-term changes, are the ones most able to react to change factors, for example, the plasticity of the photosynthetic apparatus (Necchi 2005) as described for freshwater red algae (Necchi and Vis 2005). Moreover, a study on brown algal species has suggested that coastal

populations of macroalgal species, as they are sessile organisms subjected to higher environmental variability and need to be adapted to a wide range of conditions, reflected how natural selection acts on different sets of genes implied in stress response (Teng *et al.* 2017). In this way, the growth of the charophyte populations from the spring was more negatively affected by UVR than that of the populations from the lake under LT, but it was in those organisms where this effect is totally offset by warming and the implementation of UVR increased the concentration of UVACs (Rubio *et al.* 2015). Thus, this fact suggests a greater protective-restorative response in the populations from the coastal spring, compared to the lake populations. The local adaptation to a shallow environment with higher incidence and variability of UVR allows responses that would agree with a greater phenotypic plasticity of populations from the spring. Moreover, despite the fact that UVR-protective compounds and their stoichiometric trace were related to an increase in UVR in all the studied populations, only the coastal populations from both species had enough plasticity to substantially modify their morphology and growth due to factor interactions.

While being aware of the limitations of extrapolating an experimental study to natural conditions, this kind of research on the interactive effects of existing global change factors might allow us to predict possible changes in the distribution of these important macroalgae in continental aquatic systems (Jeppesen *et al.* 1997, Rodrigo *et al.* 2013, 2015). We encourage studies that genetically test the relative impacts of local adaptation to specific environments, and an increase in phenotypic plasticity in charophytes governed by stressor interactions as is occurring in marine macroalgae (Avia *et al.* 2017, Pierangelini *et a.* 2017, Vágnerová *et al.* 2017).

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|BLOCK 2| ECOLOGY OF INTERACTIONS: THE NETWORK

(MESOCOSM SCALE)

|CHAPTER 4|

Structure and vulnerability of the multi-interaction network in macrophyte-dominated lakes

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Research

Structure and vulnerability of the multi-interaction network in macrophyte-dominated lakes

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The network approach is crucial to understand how ecosystems are structured and how they will respond to the disturbances (e.g. the current global change). We have recreated the multi-interaction network of a shallow freshwater lake dominated by submerged macrophytes (Charophytes), a known system very vulnerable to environmental changes, considering both trophic and non-trophic relationships among its elements. To minimize the environmental variability, we established it in an experimental mesocosm, including three habitats: the pelagic, the habitat around the meadow and the periphytic community living on macrophytes. We aimed to study the structure of this network and the roles of its elements, as well as the response of this system to a foreseeable decrease in charophytes due to the global change. Thus, we tested whether there are species in the system that, due to the connections they establish, have central or connecting roles and if the reduction of charophytes affects more the elements that live intimately associated with them. Our results confirm that charophytes are the most central node in the network and that the high-mobility large planktonic herbivores living within the meadow are acting as bridges between the conformant compartments. This suggests a structurally crucial tandem macrophytes-herbivores with the former playing a foundation role (i.e. basal and abundant species centralizing non-trophic interactions) and the latter being connectors in this network. Interestingly, we found that the periphytic elements where those with the highest capacity to affect the other elements of the network when being disturbed. Furthermore, an eventual decrease in the abundance of charophytes will cause a major direct damage to the meadow and periphyton, compartments to which they provide refuge and life support, respectively. Our study highlights the need of approaches encompassing the complex structure of the ecological networks to identify crucial species (such as foundation or connecting species) for their topology and vulnerability geared towards conservation biology.

Keywords: aquatic network, charophyte meadows, foundation species, non-trophic interactions, periphyton, plankton, topology

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Abstract

The network approach is crucial to understand how ecosystems are structured and how they will respond to the disturbances (*e.g.* the current global change). We have recreated the multi-interaction network of a shallow freshwater lake dominated by submerged macrophytes (charophytes), a known system very vulnerable to environmental changes, considering both trophic and non-trophic relationships among its elements. To minimize the environmental variability, we established it in an experimental mesocosm, including three habitats: the pelagic, the habitat around the meadow and the periphytic community living on macrophytes. We aimed to study the structure of this network and the roles of its elements, as well as the response of this system to a foreseeable decrease in charophytes due to the global change. Thus, we tested whether there are species in the system that, due to the connections they establish, have central or connecting roles and if the reduction of charophytes affects more the elements living intimately associated with them. Our results confirm that charophytes are the most central node in the network and that the high-mobility large planktonic herbivores living within the meadow are acting as bridges between the conformant compartments. This suggests a structurally crucial tandem macrophytes-herbivores with the former playing a foundation role (*i.e.* basal and abundant species centralizing non-trophic interactions) and the latter being connectors in this network. Interestingly, we found that the periphytic elements where those with the highest capacity to affect the other elements of the network when being disturbed. Furthermore, an eventual decrease in the abundance of charophytes will cause major direct damage to the meadow and periphyton, compartments to which they provide refuge and life support, respectively. Our study highlights the need of approaches encompassing the complex structure of the ecological networks to identify crucial species (such as foundation or connecting species) for their topology and vulnerability geared towards conservation biology.

Keywords: aquatic network; charophyte meadows; foundation species; non-trophic interactions; periphyton; plankton; topology

Resum

*L'aproximació de xarxa és crucial per a entendre com estan estructurats i com respondran a les pertorbacions (*e.g. *l'actual canvi global) els ecosistemes. Nosaltres hem recreat la xarxa multi-interacció d'un ecosistema aquàtic somer d'aigua dolça dominat per macròfits submergits (caròfits), un tipus d'ecosistema molt vulnerable a canvis ambientals, considerant tant interaccions tròfiques com no-tròfiques entre els seus elements. Per tal de minimitzar la variabilitat ambiental, nosaltres vam establir aquest sistema en un mesocosm experimental, que incloïa tres hàbitats: el pelàgic, l'hàbitat al voltant de la pradera de caròfits i la comunitat perifítica que vivia sobre els caròfits. Ens vam proposar estudiar l'estructura d'aquesta xarxa i els rols dels seus elements, així com la resposta d'aquest sistema a una previsible disminució dels caròfits deguda al canvi global. Per tant, testàrem si hi havia espècies al sistema que, degut a les connexions que estableixen, tenen un paper central o connector i si la reducció dels caròfits afecta més a aquells elements que viuen íntimament associats a ells. Els nostres resultats confirmen que els caròfits són el node més central de la xarxa i que els herbívors planctònics amb elevada mobilitat que viuen entre la pradera actuen com a ponts entre els diferents compartiments de la xarxa. Açò suggereix un tàndem macròfits-herbívors estructuralment crucial amb els macròfits jugant un paper fundacional (*i.e. *espècies basals i abundants que centralitzen les interaccions no-tròfiques) i els herbívors sent connectors en la xarxa. És interessant a més, que els elements perifítics són els que tenen una major capacitat d'afectar a altres en la xarxa quan són pertorbats. A més, la disminució en l'abundància de caròfits causarà un major impacte en els elements de pradera i perifítics, als quals els macròfits els proveeixen de refugi i suport vital, respectivament. El nostre estudi remarca la necessitat d'aproximacions que engloben l'estructura complexa de les xarxes ecològiques per a identificar espècies crucials (com les espècies fundacionals o connectores) per a la seua topologia i vulnerabilitat, orientades a l'àmbit de la conservació.*

Paraules clau: *xarxa aquàtica; praderes de caròfits; espècies fonamentals; interaccions no-tròfiques; perifiton; plàncton; topologia*

1. Introduction

Aquatic ecosystems comprise numerous habitats or compartments (Tokeshi and Arakaki 2012). These compartments can be defined from pelagic (in the free-water) to benthic environments (over the sediment), including the macrophyte meadows and their planktonic and periphytic associated communities. The connections established intra- and inter-compartments by means of matter and energy flows, contribute to the structural and functional complexity characterizing these systems (Lodge *et al.* 1988). The role and influence of each compartment in the functioning of aquatic ecosystems is related to their size and shape, *e.g.* macrophyte meadows are a relatively large part of the habitat in shallow ecosystems and thus an important component (Jeppesen *et al.* 1998). Moreover, in these ecosystems, where there are two possible alternative states (one dominated by macrophytes and the other dominated by plankton; Scheffer and Jeppesen 2007), the importance of the different compartments, and the shift of one state towards the other, is determinant for the maintenance of the biodiversity and the functioning of the ecosystem (Scheffer and Jeppesen 2007).

The freshwater planktonic (pelagic) food web structure, and its response to disturbances, has been largely studied (Carpenter *et al.* 1987, Christoffersen *et al.* 2008). However, the network associated with the macrophyte meadows is less well-known. Charophytes are one of the most widespread macrophyte groups in shallow freshwater ecosystems, which perform a critical ecosystem role (Jeppesen *et al.* 1997, Hilt and Gross 2008, Rodrigo *et al.* 2013). By establishing dense meadows, these organisms are capable of modifying not only the abiotic environment (van Donk and van de Bund 2002, Rodrigo *et al.* 2007), but also the whole community through establishing non-trophic interactions such as competition (direct or indirect) with other primary producers (van Donk and van de Bund 2002, Rojo *et al.* 2013a, b), providing physical refuge to zooplankton (Blindow *et al.* 2002), or being inhabited by very specific periphytic assemblages (Rojo *et al.* 2017).

Regarding non-trophic interactions, in the last few years emphasis has been placed on these types of relationships as an important component of ecosystems (Bascompte *et al.* 2003, Ings *et al.* 2009, Kéfi *et al.* 2012). However, merging non-trophic interactions with the commonly studied trophic ones is not an easy issue to solve and efforts must be done in this direction (Vasas and Jordán 2006, Kéfi *et al.* 2015). In addition, the role of foundation species is receiving increasing attention (Borst *et al.* 2018, Ellison 2019). These species are considered crucially important for the ecosystems they inhabit and are distinguished by three features: 1) they are abundant in the system in terms of biomass, 2) they are normally basal species (*e.g.* primary producers) and 3) they stablish mainly non-trophic interactions with the other elements of the system (*e.g.* providing support or refuge for other species or altering ecosystem properties to damage other species; Ellison 2019). Based on these criteria, the submerged macrophytes are a strongly good candidate to exert such a role in freshwaters. Therefore, a complex aquatic network that includes pelagic, meadow and periphytic habitats emerges with a myriad of imbricated relationships of different nature, both trophic and non-trophic. The construction and analysis of this network is one of the main objectives of this study.

Furthermore, these shallow macrophyte-dominated freshwater systems are particularly vulnerable to global change, and they will see their biodiversity decreased and their biogeochemical cycles altered (Álvarez-Cobelas *et al.* 2005, Parcerisas *et al.* 2012). All the habitats in these freshwater systems are expected to be affected, in a direct or indirect way, by environmental changes. In this vein, through experimental approaches at a mesocosm scale (Stewart *et al.* 2013), the sensitivity of the pelagic communities in these systems has been studied (Carrillo *et al.* 2017, Deininger *et al.* 2017, Rojo *et al.* 2017) as well as the response of macrophytes (Short and Neckles 1999, Barker *et al.* 2008, Zhang *et al.* 2019) and benthic communities (Lepori and Robin 2014, Piggott *et al.* 2015, Cao *et al.* 2019). Among macrophytes, charophytes have been proved to be very sensitive to changes in environmental factors related to global

change such as warming, eutrophication, salinization and ultraviolet radiation (Calero *et al.* 2017, Rodrigo *et al.* 2017, Puche *et al.* 2018, Rojo *et al.* 2019). These changes are expected to be more acute in shallow ecosystems in Mediterranean semi-arid regions (Jeppesen *et al.* 2014). However, most of these studies have focused on populations, rather than on higher levels of organization (Woodward *et al.* 2010). This gap limits our ability to disentangle what elements of these complex networks are more relevant to the system's stability, when faced with the foreseeable changes (IPCC 2014). It is in this context that tackling these systems with a network approach provide a useful tool for recognizing structurally important species, and lead for stablishing the extent of their influence on the response of the whole system to disturbances such as those related with the current global change, thus, allowing a better understanding of the community structure and the ecosystem functioning (Ings *et al.* 2009, Kéfi *et al.* 2015, Poisot *et al.* 2016, Delmas *et al.* 2017, García-Callejas *et al.* 2017, Ellison 2019).

Our aims in this study are: 1) to recreate the multi-interaction network organized around the charophyte meadows in a freshwater shallow ecosystem; 2) to characterize the global structure of this network and the topological importance of its elements and, 3) to project the effects that a reduction in the abundance of the charophyte meadows would lead to for the constituent species of the network, and the structure of the network as a whole. We hypothesize that: 1) charophytes will exhibit a central role in the network, mainly due to the set of non-trophic interactions in which they participate; 2) among the three considered compartments, the meadow compartment, and specifically the organisms with greater mobility will play an important connecting role in the system and, 3) faced with a reduction in the abundance of charophytes, the periphyton compartment and elements of the meadow that benefit from the shelter and support provided by these macrophytes will be adversely affected. We developed an experimental shallow ecosystem whose elements and interactions we know well (Fig. 1). The experimental control of the abiotic environment in the mesocosm avoid the great variability that this type of

shallow ecosystems can exhibit in nature (Stewart *et al.* 2013), allowing us to address our goals and to test the hypotheses focused in its multi-interaction network.

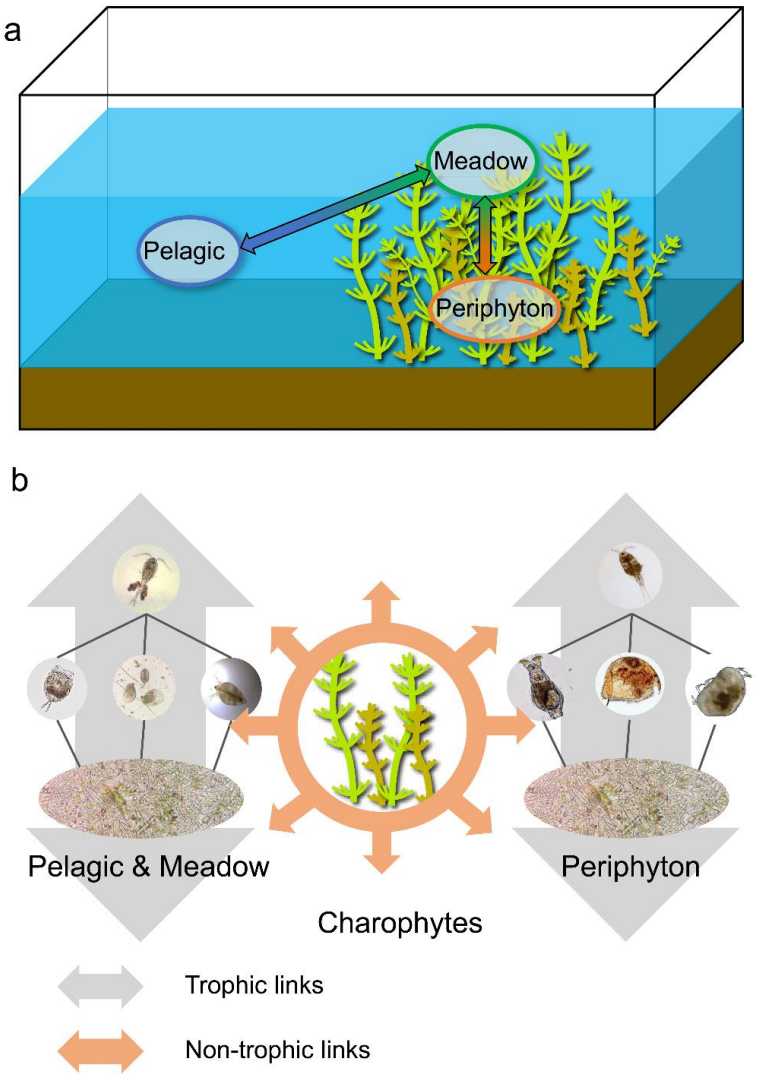

Fig. 1. a) Scheme of the mesocosm where the experimental community was set up with the three compartments represented, b) the experimental model community with the compartments (pelagic, meadow and periphyton), representing the "vertical" trophic links and the non-trophic links in all directions. The components in each compartment are organized in rows as autotrophs and heterotrophs (herbivores and carnivores). Charophytes (submerged macrophytes) are presented in the center (although they belong to the periphyton compartment) to highlight their key role in non-trophic interactions in this system.

2. Material and methods

2.1. The ecological community and its multi-interaction network construction

A freshwater ecosystem was recreated in an experimental mesocosm. In order to build its multi-interaction network and assess its structure and vulnerability, it was crucial to have tight control over the conditions to which the system was submitted and to better delimit the compartments considered. These needs are covered by the use of mesocosms, a useful tool that offers greater tractability than whole-ecosystem manipulations (Stewart *et al.* 2013). The mesocosm consisted of a 0.5 m^2 enclosure (length 0.8 m x width 0.6 m x height 0.4 m) containing 165 L of tap water plus an inoculum of 5 L of water from a coastal lagoon. The bottom of the mesocosm was covered with a substrate layer, the width being 10 cm. The substrate was a mixture of organic compost and gravel in the proportion 2:1. On this base, a layer of sediment from a coastal lagoon (sediment inoculum) was scattered. A charophyte meadow was planted in one of the halves of the mesocosm. The meadow was monospecific, formed by the species *Chara hispida*, a green cosmopolitan macroalgae with erect thallus and regular nodes and internodes. Individuals of this species were planted as groupings (packets) in three rows of three packets each one (a total of nine packets). For the plantation, part of the main axis of the individuals was buried in the sediment. This buried part served to form the rhizoidal system that allowed the fixation of the individuals to the sediment. This plantation method has been described in other studies with these macroalgae (Rojo *et al.* 2015, Rodrigo *et al.* 2017, Puche *et al.* 2018, Rojo *et al.* 2019). There were no charophytes on the remaining half of the mesocosm surface, allowing a more pelagic environment (Fig. 1a). From the water and sediment inoculum, as well as from the planted charophytes, a planktonic and periphytic community emerged. Several aquatic gastropods arose from the sediment in the mesocosm, which were also sampled and considered at the time of building the network. In this recreation of a shallow freshwater ecosystem, as happens naturally in most of them due to their temporary nature, predators such as fish were not present.

The mesocosm was maintained at 21˚C in a light:dark cycle of 14:10 h. In previous studies (Rodrigo *et al.* 2013, Rojo *et al.* 2015, 2017, Rubio *et al.* 2015) it was demonstrated that these conditions are non-limiting to the growth of charophytes. The physical and chemical variables were measured periodically to detect and subsequently rectify possible deviations from the experimental conditions (Table S1 Supplementary material Chapter 4). The community in the mesocosm was allowed to grow for two months before the sampling process. This period of time was determined based on previous studies claiming that charophytes are well fixed to the sediments and grow properly about two weeks after being planted (Rojo *et al.* 2015, Rodrigo *et al.* 2017, Puche *et al.* 2018). In addition, it is known that plankton, in an undisturbed system, can reach a state of equilibrium before two months (Naselli-Flores *et al.* 2003). Moreover, we did some previous tests in the mesocosm to ensure the feasibility of this recreation.

In this experimental system, three connected compartments were distinguished: 1) periphyton, a compartment formed by charophytes and all the organisms living on them; 2) meadow, the plankton inhabiting free-water within the meadow, and 3) pelagic, the planktonic compartment in the pelagic habitat, furthest from the charophytes (Fig. 1a). Each of these compartments was sampled for autotrophs (phytoplankton/phytobenthos and cyanobacteria) and heterotrophs (bacteria, zooplankton/zoobenthos and gastropods). All the taxa were sampled following the methods described in previous studies (Rodrigo *et al.* 2003, Villaescusa *et al.* 2010, Rojo *et al.* 2012, 2017), and they were identified at the highest possible resolution (Table S2 Supplementary material Chapter 4).

To construct the multi-interaction network of this experimental system, we grouped the identified taxonomic species according to functional criteria (such as mobility, edibility or toxicity) to define the nodes (Table 1). In the network, (inorganic) nutrients were considered as a node. In this way, exploitation competition between autotrophic organisms is defined by trophic links going from the nodes that represent

the autotrophic organisms to the node that represents the nutrients, as suggested by Kéfi *et al.* (2012). In addition, charophytes, represented also as a node, performed a function that goes beyond the autotrophic role, as they are also the physical support for the entire periphyton compartment considered in the network (Rojo *et al.* 2017).

The establishment of the links between the nodes of the network was based on the literature and on expert knowledge. These links encompass both trophic and non-trophic relationships (Table 2; Fig.1b).

2.2. The structure of the network at a global-scale

The arrangement of nodes and links of the network was reflected in a SxS matrix A (where S is the number of nodes in the network). The entries of matrix A, a_{ij}, represent ecological interactions among species (Cohen 1978). Specifically, a_{ij}, represents the effect (1 positive, -1 negative and zero otherwise) of node *j* (in the column) on node *i* (in the row). For instance, if charophytes (*j*) provide refuge for zooplankton (*i*), then the effect of charophytes over the zooplankton will be 1. For trophic links, the effect of the predator over the prey was coded as -1, and the effect of the prey over the predator as 1. For example, it is well known that cyclopoid adult copepods are mainly carnivores. They can prey on, for example, rotifers of the *Lecane* genus. So that, the effect of the copepods over the rotifers will be -1 and the effect of the rotifers over the copepods will be 1. All node dynamics were assumed to be self-damped so the diagonal elements a_{ii} were assigned a negative value for the construction of the net effect matrix N (see below). Non-trophic effects were either positive or negative. For network visualization we used the software Gephi©.

The topological features of the network were assessed by means of global descriptors. We first recorded the number of nodes (S) and links (L). From these basic variables, we calculated the directed connectance (C; Table 3). This is the proportion of realized interactions relative to the potential number of possible interactions in the

network (Martínez 1992). Furthermore, the modularity coefficient (Table 3) was calculated using the algorithm developed by Guimerà and Amaral (2005). This

Table 1. List of the criteria used to define the nodes in the network and the experimental compartment to which they belong. From these compartments, a nutritional classification of the nodes into "Nutrients", "Autotrophic" and "Heterotrophic" is carried out to clarify the different groups of organisms considered. The first column separates the elements that appear in the three compartments from those that are unique to the periphyton compartment.

Compartment	Nutritional criteria	Taxonomic classification	Functional criteria	Nodes in the network
Pelagic, Meadow and Periphyton	Nutrients	Nutrients	nutrients	nutrients
	Autotrophic	Class Chlorophyceae	unicellular, edible	unicellular chlorophytes
			colonial, edible	colonial chlorophytes
			filamentous, non-edible	filamentous chlorophytes
		Class Bacillariophyceae	small (<20µm), edible	small diatoms
			large (>20µm) edible	big diatoms
		Class Cyanophyceae	colonial, edible	colonial cyanobacteria
			filamentous, non-edible	filamentous cyanobacteria
	Heterotrophic	Domain Bacteria	bacteria	bacteria
		Phylum Ciliophora and Nauplii	protists, bacterivore	ciliates
		Class Eurotatoria	small herbivore	rotifers
		Class Branchiopoda	large herbivore	cladocerans
		Class Hexanauplia	large herbivore	copepodites
			carnivore	copepods
Periphyton	Autotrophic	Class Charophyceae	macrophyte	charophytes
	Heterotrophic	Class Gastropoda	large, benthic herbivore	benthic gastropod

Table 2. List of the non-trophic interactions considered to build the multi-interaction network. For each interaction, the source and the target of the interaction as well as a short description and a reference are shown.

ID	Source	Target	Interaction	Desciption of interaction	Reference
1	Cyanobacteria (0)	Bacteria (+)	Stimulation	Cyanobacteria release a variety of organic molecules that could stimulate heterotrophic bacteria's growth	Lange 1967 Baines and Pace 1991 Kirkwood *et al.* 2006
2	Cyanobacteria (0)	Microalgae (-)	Allelopathy	Some groups of cyanobacteria has an antialgal allelopathic activity	Flores and Wolk 1986 Schlegel *et al.* 1999 Smith and Doan 1999
3	Meadow microalgae	Charophyte	Shading	Phytoplankton development causes a shading effect on macrophytes reducing the amount of light reaching the bottom of the systems	Sand-Jensen and Søndergaard 1981 Ozimek *et al.* 1991
4	Meadow herbivore zooplankton (+)	Charophyte (+)	Relaxing competition	Grazing by herbivore zooplankton slows microalgal growth benefiting the macrophytes	Zuo *et al.* 2014
5	Benthic microalgae (+)	Charophyte (-)		Microalgae living on macrophytes colonizing them and limiting the amount of light that they receive	Sand-Jensen and Søndergaard 1981
6	Benthic cyanobacteria (0)	Charophyte (-)	Allelopathy	The same effect as in interaction 2.	
7	Zoobenthos (+)	Charophyte (+)	Cleaning	Zoobenthos "clean" macrophytes from epiphytes and provide them with CO_2 for photosynthesis	Cheng *et al.* 2017
8	Charophyte (0)	Meadow microalgae and cyanobacteria (-)	Allelopathy	Macrophytes release allelopathic compounds that inhibit or slow the growth of several groups of microalgae	Gross *et al.* 2007 Rojo *et al.* 2013a
9	Charophyte (0)	Meadow zooplankton (+)	Refuge	Charophytes meadows serve as a refuge for zooplankton, protecting them from their predators	van Donk and van de Bund 2002 Rodrigo *et al.* 2015
10	Charophyte (0)	Benthic organisms (+)	Vital support	Charophytes meadows provide benthic organisms a substrate for living	Rojo *et al.* 2017

Table 3. Global-scale and node-scale structural network attributes measured. The formulae used to calculate their values with a description and the references are provided.

Network global-scale variables	Equation		Reference
Directed connectance, C	$C = \frac{L}{S(S-1)}$	Where L is the number of links and S is the number of nodes	Martínez 1992
Modularity, M	$M = \sum_{S=1}^{N_M} \left[\frac{L_m}{L} - \left(\frac{D_m}{2L} \right)^2 \right]$	Where N_M is the number of modules, L is the number of links in the network, L_m is the number of links between nodes in module m and D_m is the sum of the degrees of the nodes in module m.	Guimerà and Amaral 2005
Nestedness, NODF	$NODF = \frac{\sum D_{paired}}{\left[\frac{c(c-1)}{2}\right] + \left[\frac{r(r-1)}{2}\right]}$	Where D_{paired} is the averaged paired degrees of nestedness of columns and rows, c is the number of columns and r is the number of rows in the matrix.	Almeida-Neto *et al*. 2008
Network node-scale variables			
Degree centrality, CD	$CD_i = L_i$	Where L_i is the number of links of node i.	Freeman 1977
Closeness centrality, CC	$CC_i = \frac{S-1}{\sum_{j=1}^{S} d_{ij}}$	Where S is the number of nodes and d_{ij} is the shortest path length between nodes i and j.	Freeman 1978 Freeman 1979
Betweenness centrality, BC	$CB_i = 2 \times \sum_{j<k;i \neq j} \frac{g_{jk}(i) / g_{jk}}{(S-1)(S-2)}$	Where g_{jk} is the number of paths between j and k, while $g_{jk}(i)$ is the number of these paths that include node i and S is the number of nodes.	Freeman 1977
Within module z-score, z	$z_i = \frac{L_i - \bar{L}_{m_i}}{\sigma_{L_{m_i}}}$	Where L_i is the total number of links of node i to other nodes in its module m, L_{mi} is the average of links over all nodes in m_i and σL_{mi} is the standard deviation of L_i in m.	Guimerà and Amaral 2005
Participation coefficient, P	$P_i = 1 - \sum_{S=1}^{N_M} \left(\frac{L_{im}}{L_i} \right)^2$	Where N_M is the number of modules, L_{im} is the number of links of node i to nodes in module m and L_i is the total number of links of node i.	Guimerà and Amaral 2005
Effectiveness, E	$E_i = \frac{\Sigma_{i \neq j} \lvert a_{ij} \rvert}{S-1}$	Where aij is the effect of a perturbation in node j over the node i (taken from the net effects matrix), and S is the number of nodes in the network.	This study
Sensitivity, s	$s = \frac{\Sigma_{j \neq i} \lvert a_{ij} \rvert}{S-1}$	Where aij is the effect of a perturbation in node j over the node i (taken from the net effects matrix), and S is the number of nodes in the network.	This study

algorithm finds a particular partition of the network that maximizes a function called modularity, bunching closely connected nodes into modules (*i.e.* subsystems of non-overlapping strongly interacting species). In our network, four modules emerged by means of this algorithm: module 1, including the charophytes and the entire periphytic community (with primary producers, herbivores and carnivores), modules 2 and 3 consisted of pelagic and meadow-related primary producers, respectively, and module 4 which was mainly formed by the planktonic herbivores and carnivores (both pelagic and meadow-related). We also checked the presence of nestedness in the network (Table 3). This metric was defined by Almeida-Neto *et al.* (2008) and it is based on two features of the matrices: the overlap and the decreasing fill. In a completely nested matrix, overlap means that there is a full overlap of 1 s from right to left columns and from down to up rows; while decreasing fill means that there is a decreasing marginal totals (sum of 1 s) between all pairs of columns and all pairs of rows (Almeida-Neto *et al.* 2008). The significance of this metric was evaluated after 1000 randomizations of the network using the software ANHIDADO (version Bangu 3.0; Guimarães and Guimarães 2006).

2.3. The structure of the network at a node-scale

At a node-scale, we determined the importance of each node in the directed matrices of the network by means of 1) different centrality measures and 2) the alteration of global descriptors that the removal of each node caused in the network.

The centrality measures were: degree centrality (C_D, the number of interactions established by a node; Freeman 1977, Table 3); closeness centrality (C_C) which is a measure of the proximity of a node to all other nodes in the network, and it is based on the shortest path length between pairs of nodes (Freeman 1978; Freeman *et al.* 1979, Table 3) and betweenness centrality (C_B) which gives information about how central a node is, in the sense of being incident to many shortest paths in the network (Freeman 1977, Table 3).

The other approach to the importance of the nodes was the assessment of the response of the global descriptors of the network to the elimination of each node (Solé and Montoya 2001). We performed removals with replacement (one different node each time). After each elimination, we calculated the global descriptors of the network (connectance, modularity and nestedness). In this way, we calculated the alteration in these global parameters by eliminating each node as the difference between their value in the network without the node, and their value in the complete network, normalized by the latter. It should be highlighted that the node "charophytes" was not eliminated since it is the vital support for all the periphytic community considered and, therefore, its elimination would automatically lead to the elimination of all those nodes in the network. By the same way, the elimination of the node "nutrients" was not considered for this analysis, since it does not make ecological sense to remove the nutrients from a biological community.

Moreover, based on the modules defined by the modularity algorithm, we assessed the universal roles played by the nodes in the network by means of the within-module degree (z) and the participation coefficient (*P*) of each node to determine how important a node is for its module and for connecting modules, respectively (Guimerà and Amaral 2005, Olesen *et al.* 2007, Table 3).

All the calculations for these descriptors (except for nestedness) were performed in MATLAB© using the Brain Connectivity Toolbox.

2.4. Net effects matrix: dynamic importance of the nodes and effects of reducing charophytes

As explained above, the community matrix A shows the direct relationships between the elements that comprise it. These relations can have values 1, -1 or 0. From this matrix A, we have calculated the net effect matrix N to assess both direct and indirect influences (*i.e.* chains of connections) among the elements. To do that, and under the assumption that the system is at an equilibrium state, we simulated 5000 random

matrices from matrix A by multiplying each off-diagonal element by a random value sampled from a uniform distribution within the interval (1/2, 2). To the elements within the diagonal (a_{ii}, self-regulation elements) a value of -3 was assigned. From each random community matrix A, the net effect matrix N was calculated as $N = -A^{-1}$ (Novak *et al.* 2016), thus obtaining 5000 net effects matrices, from which an average net effect matrix was obtained. Its elements n_{ij} represent the expected long-term change in the equilibrium value of node *i* due to a constant pressure exerted on node *j* (Nakajima, 1992). With this net-effects matrix, we calculated two metrics of dynamic importance related to the incidence and susceptibility of the nodes in the network. These metrics were effectiveness (*i.e.* the average capacity of a node to affect the others when being disturbed; Table 3) and sensitivity (*i.e.* the average susceptibility of a node to be affected by the others when these are disturbed; Table 3). Mathematically, the effectiveness of an element *i* is calculated as a summation of the net effects of this element over the rest of the elements of the network (sum of rows) and the sensitivity of the element *i* is the summation of the net effect of the other elements over this element (summations of columns; Table 3). Note that other kinds of "net effects" have been used in the literature. For example, Ulanowicz and Puccia (1990) presents their MTI (mixed tophic impact) analysis based on the paths between source and target species in the network. Conversely, our calculations summarize the asymptotic responses of species abundances after parameter disturbances in any species. While Ulanovicz's analysis only considers the paths involved in connecting source and target species, our analysis (based on Levins 1974) also considers the set of species and their interconnections not included in those paths, wich Levins (1974) call "Complementary subsystem" (see also Dambacher *et al.* 2003). This is a key difference that determines not only differences in the values of net effects but also in their signs, as compared with Ulanovicz's MTI.

Furthermore, a principal component analysis (PCA) was carried out considering these metrics as a multivariate descriptor of the compartments, each node being a

variable. In this way, we intended to assess if the compartments considered in the network differ in terms of the values of the nodes for these metrics and which nodes contribute the most to this differentiation.

3. Results and discussion

3.1. Characterization of the multi-interaction network in a macrophyte-dominated shallow lake

The recreated multi-interaction network of charophyte meadows consisted of a total of 42 nodes (Table 4), distributed into three trophic levels and a nutrients node at a separate level (at the bottom of the network; Fig. 2a). Of these nodes, 52% were primary producers (microalgae, cyanobacteria and charophytes), 31% were herbivores (ciliates, rotifers, cladocerans, cyclopoid copepodites and gastropods) and 7% were carnivores (adult cyclopoid copepods). In addition, the bacteria in each compartment were considered (7% of the nodes), and represented at the row of primary producers, since they are consumers of inorganic nutrients, despite not being photosynthetic organisms. These nodes were interconnected by a total of 240 links. These links represented trophic connections (66%) and non-trophic connections, the latter being positive (21%) and negative (13%). The periphyton and meadow compartments contained the majority of non-trophic interactions (Fig. 2a). In addition, among these, the negative non-trophic relationships occurred mainly among the primary producers (*e.g.* allellopathy; Gross *et al.* 2007), while in the positive non-trophic ones the herbivorous organisms were also involved (*e.g.* the refuge provided by charophytes to zooplankton, or the cleaning of the periphytic microalgae on charophytes carried out by zooplanktonic and zoobenthic herbivores such as the abundant organisms of the genus *Lecane* or the bigger organisms of the genera *Simocephalus* and *Pleuroxus*; Fig. 2a; van Donk and van de Bund 2002, Cheng *et al.* 2017). Each node was involved in 11±7 links (mean±standard deviation), the connectance of the network resulted in 0.14 and the modularity coefficient was 0.26 (Table 5). Furthermore, the network showed a significant nested structure (with a NODF of 9.1 and $p < 0.001$; Table 5).

Table 4. List of nodes in the network with their ID and the compartment to which they belong.

ID	Compartment	Node	Main genus/order
1		Nutrients	
2	*Pelagic*	Bacteria	
3	*Pelagic*	Unicellular chlorophytes	*Tetraedron*
4	*Pelagic*	Colonial chlorophytes	*Scenedesmus*
5	*Pelagic*	Filamentous chlorophytes	*Oedogonium*
6	*Pelagic*	Small diatoms	*Cyclotella*
7	*Pelagic*	Big diatoms	*Rhopalodia*
8	*Pelagic*	Colonial cyanobacteria	*Chroococcus*
9	*Pelagic*	Filamentous cyanobacteria	*Oscillatoria*
10	*Pelagic*	Ciliates	
11	*Pelagic*	Rotifers	*Lecane*
12	*Pelagic*	Cladocerans	*Simocephalus*
13	*Pelagic*	Copepodites	*Cyclopoida*
14	*Pelagic*	Copepods	
15	*Meadow*	Bacteria	
16	*Meadow*	Unicellular chlorophytes	*Chlorella*
17	*Meadow*	Colonial chlorophytes	*Scenedesmus*
18	*Meadow*	Filamentous chlorophytes	*Oedogonium*
19	*Meadow*	Small diatoms	*Cyclotella*
20	*Meadow*	Big diatoms	*Diploneis*
21	*Meadow*	Colonial cyanobacteria	*Gomphosphaeria*
22	*Meadow*	Filamentous cyanobacteria	*Oscillatoria*
23	*Meadow*	Ciliates	
24	*Meadow*	Rotifers	*Lecane*
25	*Meadow*	Cladocerans	*Simocephalus*
26	*Meadow*	Copepodites	*Cyclopoida*
27	*Meadow*	Copepods	
28	*Periphyton*	Bacteria	
29	*Periphyton*	Unicellular chlorophytes	*Chlorella*
30	*Periphyton*	Colonial chlorophytes	*Coelastrum*
31	*Periphyton*	Filamentous chlorophytes	*Oedogonium*
32	*Periphyton*	Small diatoms	*Cyclotella*
33	*Periphyton*	Big diatoms	*Ulnaria*
34	*Periphyton*	Colonial cyanobacteria	*Chroococcus*
35	*Periphyton*	Filamentous cyanobacteria	*Ulothrix*
36	*Periphyton*	Ciliates	
37	*Periphyton*	Rotifers	*Lecane*
38	*Periphyton*	Cladocerans	*Simocephalus*
39	*Periphyton*	Copepodites	*Cyclopoida*
40	*Periphyton*	Copepods	*Cyclopoida*
41	*Periphyton*	Charophyceae	*Chara*
42	*Periphyton*	Gastropoda	*Physella*

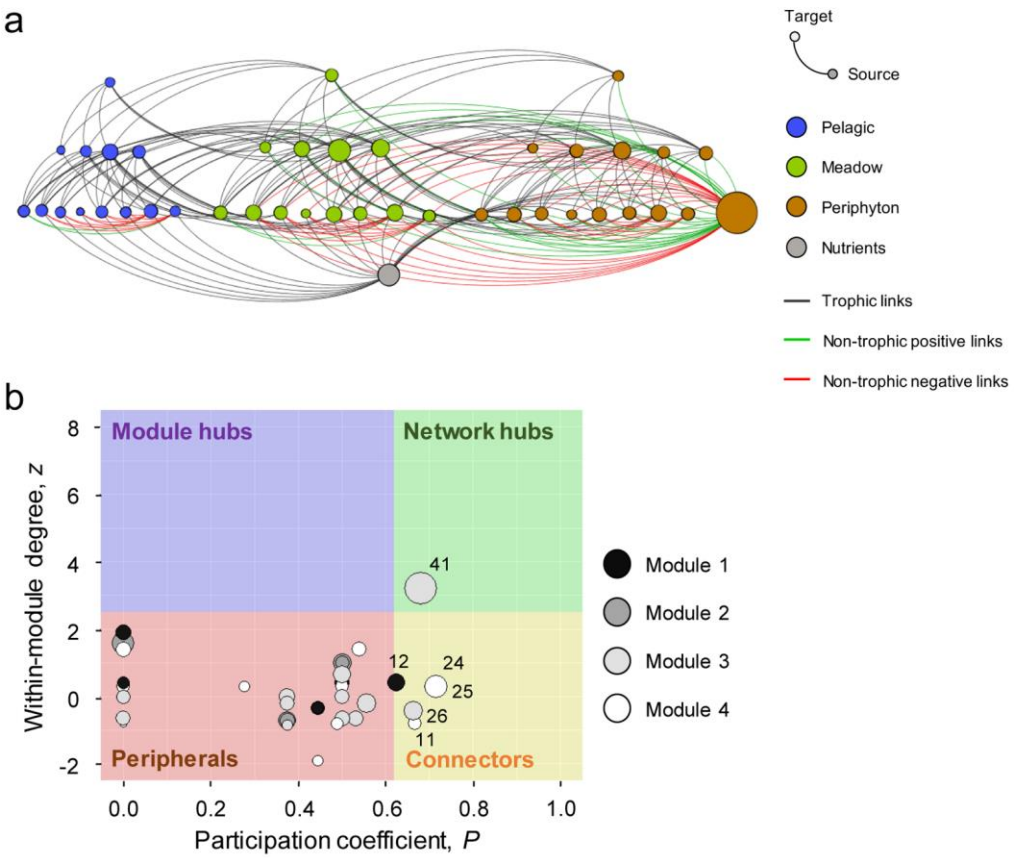

Fig. 2. a) Graphical representation of the multi-interaction functional network. The size of the nodes is proportional to their degree (number of links in which they are involved), and the color represents the experimental compartment to which they belong. Nodes are horizontally distributed in groups according to which compartment they belong to. The vertical distribution corresponds to the trophic position of the nodes, with nutrients at the bottom. The line colors represent the different types of interactions: trophic (black), non-trophic negative (red) and non-trophic positive (green). The curvature of lines connecting the nodes represents the directionality of the interaction, with lines arcing clockwise from the source to the target species. b) Roles of the nodes of each defined module according to their within-module, z (y-axis) and their participation coefficient, *P* (x-axis). Each circle is a node of the network, their size represents their degree and their color represents the module they belong to. The numbers are the ID of the nodes next to them (see Table 4). The parameter regions considered follow those proposed by Olesen *et al.* (2007).

Table 5. Global structural descriptors of the network. S is the number of nodes, L is the number of links, C is the directed connectance, M is the modularity coefficient and NODF is the descriptor measuring the nestedness of the network with the p-value associated.

S	42
L	240
Mean degree (mean ± SD)	11±7
C	0.1394
M	0.2578
Number of modules	4
NODF (p)	9.1 (0.0)

3.2. Roles of the nodes in the multi-interaction network

We found a significant correlation between the centrality measures C_D, C_C and C_B ($p < 0.001$; Fig. S1 Supplementary material Chapter 4). That is, a node involved in many links (degree), is both very accessible (closeness) and acts as an intermediary for other nodes in the network (betweenness). Charophytes were the element of the network with the highest values of these metrics (Table S3 Supplementary material Chapter 4), followed by the large herbivores (such as cladocerans of the genera *Simocephalus*, *Pleuroxus* and *Chydorus* and cyclopoid copepodites) living within the meadow. As confirmed by Jordán (2006), these measures of centrality are complementary and end by giving a realistic idea of the importance of the nodes in the network. With this information, decisions related to conservation can be focused on these key nodes.

Analysing the effect of removing each node on the global metrics of the network (connectance, modularity and nestedness) it can be observed that, in absolute value, the nodes of the planktonic compartments (both pelagic and meadow) are those with a greater influence on the global structure of the network (Fig. 3). Going into nodes in more detail, it is remarkable that by eliminating large herbivores in the meadow there is a loss of connectance and nestedness, while the network increases its modularity (Fig. 3). This is because these elements, as mentioned above, have high values of centrality (specifically of degree centrality), that is, they are involved in many interactions and when they are eliminated, the network becomes less connected. The interactions in which large herbivores participate occur in the three considered

compartments (pelagic, meadow and periphyton) since they are organisms with high mobility. These organisms living around the macrophytes use them as a refuge, going in and out of the meadow (Blindow *et al.* 2002, Meyer *et al.* 2019), they have a broad-spectrum diet (*e.g.* those of the genus *Simocephalus*) and can feed on virtually all the planktonic primary producers (both in the pelagic, and in the meadow and periphyton compartments; Sterner 1989, Stewart *et al.* 2017). Therefore, after removing them, the network becomes more modular (the different modules become more isolated by losing those “bridge” connections between them) and this triggers the loss of the nested structure characterized by the presence of more specialist nodes whose links are "nested" within the links of more generalist species. On the contrary, the nodes of the periphyton do not seem to have a noticeable influence on the overall structure of the network when they are eliminated (Fig. 3). This reflects that the latter are highly specialist nodes in their relationships (*e.g.* the periphytic microalgae require the charophytes’ branches as a substrate; Rojo *et al.* 2017). Changes in the global structure of the network when removing a node have been related to the effects on the stability of the system. In this way, Solé and Montoya (2001) stated that the elimination of central species causes the decrease of the robustness of the network (measured as secondary extinctions generated from the elimination of a node).

Taking into account the modules defined by the algorithm (explained in Material and methods section) and considering the parameter regions proposed by Olesen *et al.* (2007), it can be observed that, consistently with the importance measures, the charophytes and the large planktonic herbivores living within the meadow play important roles in the network. The charophytes’ node was classified as a network hub (Olesen *et al.* 2007), being very important for their own module and with high participation in the rest of the modules (Fig. 2b). The nodes representing the large meadow-related herbivores (*e.g.* cladocerans and copepodites) were classified as connector nodes (Olesen *et al.* 2007), which play an important role connecting the different modules in the system (Fig. 2b). This habitat-coupler role has been similarly

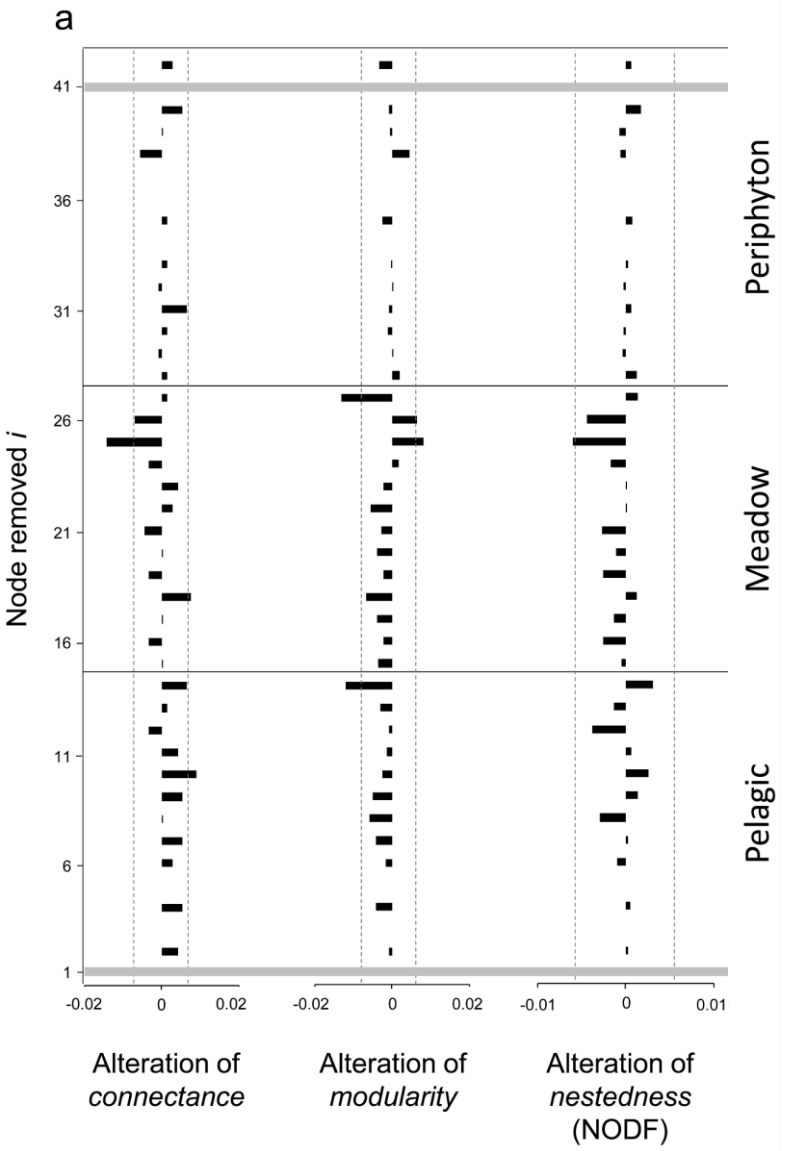

Fig. 3. a) Alteration of connectance, modularity and nestedness of the network after the removal of node *i* (calculated as the difference between the values of these descriptors in the network without node *i*, and in the network with all the nodes). Dashed lines represent ±95th percentile of the absolute value of deviations from the whole network. Gray bands indicate the nodes not considered for these analyses. The correspondence between the number and the name of the nodes is shown in Table 4. b) Net effects of reducing charophytes on the rest of the nodes in the network.

described for fish in several freshwater systems (Schindler and Scheuerell 2002). The rest of the nodes played peripheral roles, being nodes immersed in their modules with

few connections to the other modules (Fig. 2b). These results are consistent with what was previously mentioned regarding the importance of the nodes in the network, and highlights the crucial role as an influencer that the charophyte-large herbivores tandem plays in the whole system. This role is close to that of the topological keystone species suggested by Jordán *et al.* (2006). Thus it is highlighted that knowing the "biological content" of the modules defined in an ecological network is necessary to understand the functioning of these complex systems (Olesen *et al.* 2007, Jordán *et al.* 2018).

3.3. Dynamic importance of the nodes in the multi-interaction network

From the net effect matrix N, both the direct and non-direct influences of a node over the others are considered (Nakajima 1992). In this way, the average of the effectiveness of the nodes was greater in the periphyton than in the meadow, and lowest in the pelagic compartment ($F = 3.8$, $p < 0.05$; Fig. 4a). This means that, on average, a sustained and constant disturbance on the nodes of the periphytic community (among which are the charophytes) has the greatest effect on the whole system (Fig. 4b). The non-trophic interactions are key in this effect, since, as we said previously, it is in this compartment where the majority of these types of interactions occurs.

Considering the effectiveness as a multivariate descriptor of the compartments (each node being a variable), these can be ordered in a first axis that explains 88% of the total variance (PCA; Fig. S2 Supplementary material Chapter 4). The nodes that, due to their effectiveness, classify to a greater extent the compartments on this axis are the charophytes and the filamentous chlorophytes (Fig. S2). The charophytes were those with the greatest effectiveness (Fig. 4b), that is, they have the greatest capacity to affect the nodes of the system and do so basically through non-trophic interactions. This feature logically segregates the periphyton compartment (Fig. S2). In addition, the effectiveness of the filamentous chlorophytes (filaments commonly attached to the

thallus of the charophytes; Rojo *et al.* 2017) characterizes the meadow compartment compared to the pelagic compartment (Fig. S2).

Regarding sensitivity, charophytes again demonstrated the highest value, followed by benthic carnivore copepods of the genus *Cyclopoida* (Fig. 4b). Thus, despite the charophytes having the greatest capacity to affect the different elements that make up the system, they are also the most susceptible to being affected by changes in the other members of the community. However, there were no significant differences between the average sensitivity of the nodes depending on the compartment they belong to.

3.4. Projecting the net effect of a charophyte reduction in the network

Charophytes are very vulnerable to global ghange factors (Rojo *et al.* 2015, Calero *et al.* 2017, Rodrigo *et al.* 2017, Puche *et al.* 2018, Rojo *et al.* 2019) and, here, we project the potential chain effects of their depletion. Our analyses revealed that the reduction of the equilibrium abundance of this group of macrophytes negatively affects 69% and 47% of the nodes of the meadow and periphyton compartments, respectively (Fig. 5a). In the pelagic compartment there is a lower percentage of nodes harmed by the decrease in charophytes (31%), while in this compartment a higher percentage of nodes are favored (54%; Fig. 5a). A detailed analysis of the nodes in each compartment shows that in the meadow compartment the main beneficiaries were the colonial and filamentous cyanobacteria, since they are competing with the charophytes establishing negative non-trophic interactions, such as allelopathy (Rojo *et al.* 2013a,b; Fig. 5b), and they are, indirectly, strong competitors of the periphytic microalgae that inhabit on the charophytes (Rojo *et al.* 2017). On the other hand, large herbivores in this compartment, such as cyclopoid copepodites, and carnivores, such as cyclopoid adult copepods, are harmed (Fig. 5b). Again the non-trophic interactions that the charophytes establish with these zooplanktonic organisms play an important role in this effect; by reducing the density in the equilibrium of charophytes, the refuge

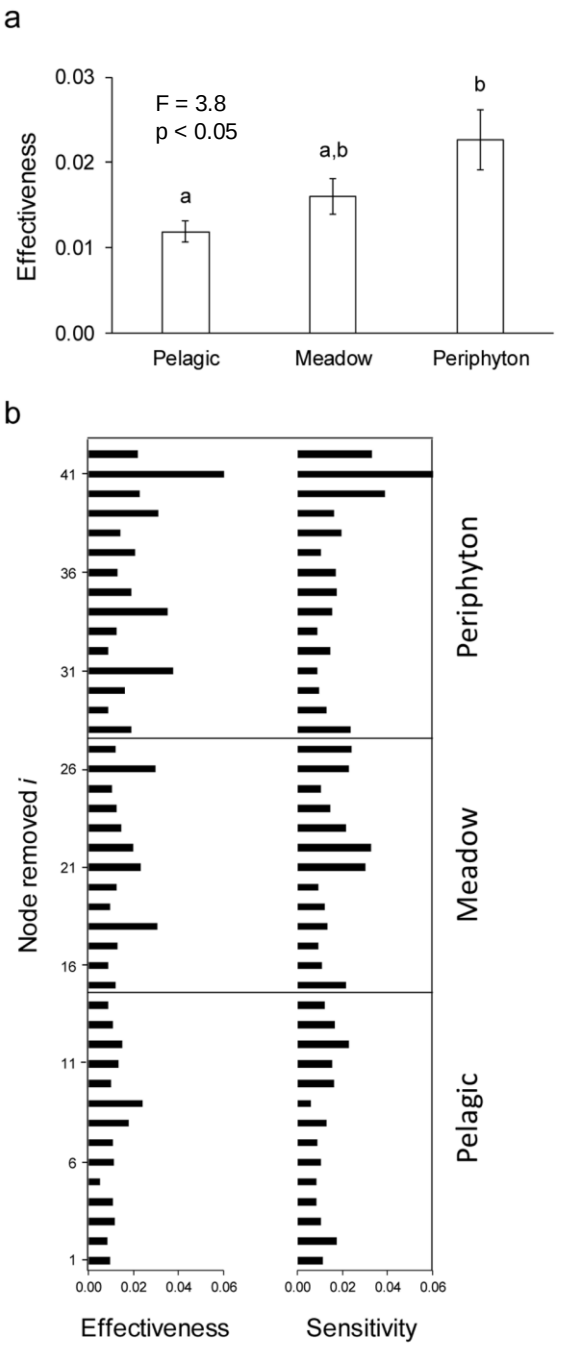

Fig. 4. a) Average values of the node effectiveness in the three compartments. We conducted an ANOVA test to assess the significant differences. Lower-case letters indicate significant differences ($p < 0.05$) within conditions after the Tukey *post hoc* test. Bars show standard error. b) Values of effectiveness and sensitivity of each node in the network. The compartments are indicated to the right.

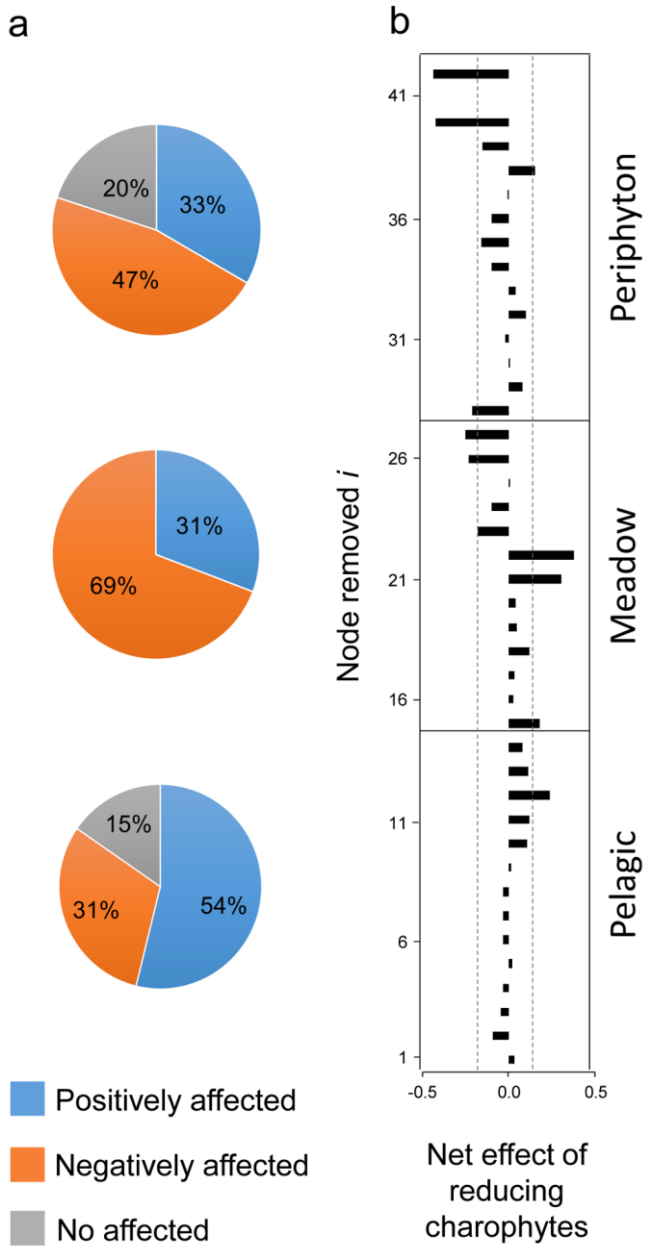

Fig. 5. a) Percentage of positively, negatively and unaffected nodes in each compartment of the network after the reducing the abundance of charophytes, and b) detail of the net effects of reducing the equilibrium abundance of charophytes on equilibrium levels of each node of the network. Dashed lines represent ±95th percentile of the absolute value of deviations from the whole network. The correspondence between the number and the name of the nodes is shown in Table 4.

that these macrophytes provide is lost, and the edible microalgae disappear in favor of non-edible cyanobacteria (both filamentous and colonies; van Donk and van de Bund 2002, Hilt and Gross 2008). The negative effect of the reduction in charophytes on the nodes of the periphyton compartment is mainly due to the fact that these macroalgae are the life support for the elements of this community (Rojo *et al.* 2017). Among these elements, the gastropods are seriously damaged (Fig. 5b), since in addition to benefiting from their support they feed on the charophytes (Brönmark and Vermaat 1998, Semenchenko *et al.* 2008). Copepods are also negatively affected, for reasons similar to the effect on their homologues in the meadow compartment (Fig. 5b).

Conclusions

Through the study of the structure and sensitivity of the network of a complex aquatic community in a shallow environment dominated by macrophyte meadows recreated in a mesocosm, we were able to identify which elements play critical roles for the integrity of the whole system. Our results highlight the importance of submerged macrophytes (such as charophytes) as a key highly-influential element on the rest of the elements in this system. These macrophytes are playing a foundation role, structuring the whole system. Furthermore, the determining function of the littoral habitats in these waterbodies and, particularly, the key role played by large herbivores (such as cladocerans or copepodites) living within the submerged meadows, introduces the idea of a macrophyte-large herbivores tandem structurally crucial. The functioning of the lake with alternative states (macrophyte-plankton dominance) has been described for years, we now quantify both the relevance of their main agents and the shifts on their network due to the foreseeable global change. Our numerical characterization of the multi-interaction network in this system, contributes to better identification of species extremely relevant in conservation biology and open the gate to more complex views that encompass dynamics, environmental factors and relevant tandems between species with different roles in ecological networks.

Speculations

Macrophyte-dominated shallow lakes exposed to changing climate will likely suffer from a negative impact on their constituent species, including charophytes. The loss of macrophytes would harm the efficiency of the macrophyte-herbivore tandem since much of the non-trophic relationships, along with the connections between the different habitats generated by these elements, would be lost. Consequently, the system will increase its modularity and, thus, become more vulnerable, favoring the shift towards a phytoplankton-dominated system. Therefore, the deterioration of ecosystem services provided by these ecosystems, such as the necessary maintenance of good water quality, as much as other cultural services associated with it, would occur.

In this context in which the network elements and the relationships they establish can be altered differentially by environmental changes, it is essential to accurate the measure of strengths of both trophic and non-trophic relationships. Moreover, the macrophyte-dominated multi-interaction network includes elements of very different body size, from bacteria to plants, the latter being also, as we have described here, the foundation species. Thus, we expect to obtain substantial differences in link strength depending on whether they are measured: on a population basis or a per-individual o per-unit biomass basis. Establishing which of these metrics will be more sensitive to environmental disturbances suffered by the network and introducing tools such as the size spectrum of the community in its calculation seems to us exciting challenges.

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|CHAPTER 5|

Multi-interaction network performance under global change: a shallow ecosystem experimental simulation

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PRIMARY RESEARCH PAPER

Multi-interaction network performance under global change: a shallow ecosystem experimental simulation

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Abstract Shallow freshwater ecosystems are structurally complex with different, highly-coupled habitats: the pelagic, the within-macrophyte-meadow, and the benthic. Submerged macrophyte meadows support benthic microorganisms and provide the trophic network with non-trophic relationships. Multi-interaction network analysis disentangles how these systems respond to changes in global-change-related factors. We examined whether (i) populations' responses to such disturbances are habitat-dependent, and (ii) if whole-community configurations are different. We performed an indoor-mesocosm experiment ("control" plus two disturbed scenarios: enhanced ultraviolet radiation (UVR) or temperature), recreating shallow freshwater ecosystems. We assessed the population-nodes' carbon biomass, their resistance and resilience to the disturbances, and global- and node-scale structural parameters of the multi-interaction network. Under the UVR-scenario, the phytoplankton C-biomass (from pelagic and within-meadow habitats) was significantly the highest, with mixotrophs dominating. Warming favoured macrophyte growth and significantly increased the network's size and nestedness, with zooplanktonic herbivores playing a connector role. The within-meadow and benthic habitats' nodes were highly influential for the network, whatever the scenario. The benthic nodes were the most resistant to the disturbances. Therefore, a phytoplankton- and a macrophyte-dominated configuration was attained under UVR and warming scenarios, respectively. The macrophyte meadows, and the community linked to them, were pivotal in the achievement of these contrasting configurations.

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Keywords Food web · Non-trophic interactions · Charophytes · Plankton · Benthos

Introduction

Current global change (GC hereafter) alters the structure of ecosystems around the world by differentially affecting their elements (Steffen et al., 2004)

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Abstract

Shallow freshwater ecosystems are structurally complex with different, highly-coupled habitats: the pelagic, the within-macrophyte-meadow, and the benthic. Submerged macrophyte meadows support benthic microorganisms and provide the trophic network with non-trophic relationships. Multi-interaction network analysis disentangles how these systems respond to changes in global change-related factors. We examined whether (i) populations' responses to such disturbances are habitat-dependent, and (ii) if whole-community configurations are different. We performed an indoor-mesocosm experiment ("control" plus two disturbed scenarios: enhanced ultraviolet radiation (UVR) or temperature), recreating shallow freshwater ecosystems. We assessed the population-nodes' carbon biomass, their resistance and resilience to the disturbances, and global- and node-scale structural parameters of the multi-interaction network. Under the UVR-scenario, the phytoplankton C-biomass (from pelagic and within-meadow habitats) was significantly the highest, with mixotrophs dominating. Warming favoured macrophyte growth and significantly increased the network's size and nestedness, with zooplanktonic herbivores playing a connector role. The within-meadow and benthic habitats' nodes were highly influential for the network, whatever the scenario. The benthic nodes were the most resistant to the disturbances. Therefore, a phytoplankton- and a macrophyte-dominated configuration was attained under UVR and warming scenarios, respectively. The macrophyte meadows, and the community linked to them, were pivotal in the achievement of these contrasting configurations.

Keywords: food web; non-trophic interactions; charophytes; plankton; benthos

Resum

*Els ecosistemes aquàtics continentals somers són estructuralment complexos amb hàbitats diferents i altament acoblats: l'hàbitat pelàgic, l'hàbitat entre-pradera i l'hàbitat bentònic. Les praderes de macròfits submergits donen suport a organismes bentònics i proveeixen a la xarxa tròfica amb relacions no-tròfiques. L'anàlisi de la xarxa multi-interacció desentranya com responen aquests sistemes a canvis en factors relacionats amb el canvi global. Nosaltres examinàrem si (i) les respostes poblacionals a aquestes pertorbacions són dependents de l'hàbitat i (ii) si la configuració de la comunitat sencera és diferent. Vam realitzar un experiment a escala de mesocosmos (amb un escenari "control" i dos escenaris pertorbats: un increment de radiació ultraviolada (RUV) o de temperatura), simulant ecosistemes aquàtics continentals somers. Vam avaluar la biomassa en carboni de les poblacions-nodes, la seua resistència i resiliència davant les pertorbacions, i paràmetres estructurals de la xarxa multi-interacció a escala global i de node. Sota l'escenari RUV, la biomassa en carboni del fitoplàncton (dels hàbitats pelàgic i entre-pradera) fou la més elevada significativament, amb dominància dels mixòtrofs. L'escalfament va afavorir el creixement dels macròfits i va augmentar significativament la grandària i l'aniuament (*nestedness*) de la xarxa, amb els herbívors zooplanctònics exercint un rol connector. Els hàbitats entre-pradera i bentònics foren altament influents per a la xarxa, independentment de l'escenari. Els nodes bentònics foren els més resistents a les pertorbacions. Per tant, es va aconseguir una configuració dominada pel fitoplàncton i pels macròfits sota l'escenari RUV i l'escenari d'escalfament, respectivament. Les praderes de macròfits, així com la comunitat associada a ells, foren essencials per a l'assoliment d'aquestes configuracions contrastants.*

Paraules clau: *xarxa tròfica; interaccions no-tròfiques; caròfits; plàncton; bentos*

1. Introduction

Current global change (GC hereafter) alters the structure of ecosystems around the world by differentially affecting their elements (Steffen *et al.*, 2004) and, consequently, their functioning and the services they provide (Hautier *et al.*, 2015). Freshwater shallow ecosystems, which house a high biodiversity (Williams *et al.*, 2004) and provide crucial ecosystem services on a global scale (Zedler & Kercher, 2005), constitute the majority of waterbodies in the especially vulnerable to GC semi-arid Mediterranean regions (Parcerisas *et al.*, 2012; IPCC, 2014).

The GC-related factors differentially affect the populations of these ecosystems (*e.g.* Gerten & Adrian, 2002; Langer *et al.*, 2006) through different mechanisms, and these define their resistance and resilience to environmental disturbances (Cabrerizo *et al.*, 2019). On the one hand, a temperature (T) increase (up to a threshold) can reduce the phytoplankton biomass by altering competition among microalgae and promoting higher predation rates of herbivores (Velthuis *et al.*, 2017), or by favouring the growth of submerged macrophytes (Puche *et al.*, 2018). On the other hand, higher doses of ultraviolet radiation (UVR) suppose an oxidative stress for many planktonic elements (Carrillo *et al.*, 2017; Wolf & Heuschele, 2018) favouring mixotrophs, which can cope with UVR increases (Rojo *et al.*, 2012). Furthermore, macrophytes reduce their growth to produce UVR-protecting compounds (Rubio *et al.*, 2015; Rojo *et al.*, 2019). For their part, the periphytic populations have shown weak responses when facing environmental changes such as UVR increases (Hill *et al.*, 1997; Mcnamara & Hill, 2000) or warming (Alsterberg *et al.*, 2012; Brose *et al.*, 2012), and protecting morphologic and physiologic mechanisms have been advocated.

These population-specific responses occur in ecosystems that, despite their reduced dimensions and shallowness, have a high structural complexity (Tokeshi & Arakaki, 2012). In shallow freshwater ecosystems, three highly coupled habitats (Wetzel, 2001) can be defined based on the presence of submerged macrophyte meadows: (i) the pelagic, consisting of organisms living in the free-water column where

there are not macrophyte meadows at the bottom, (ii) the within-meadow, which is made up of organisms inhabiting the free water within the macrophyte meadows and, (iii) the benthic, which encompasses organisms that are highly linked to the bottom of the system (*e.g.* submerged macrophytes and all the organisms attached to their surface).

Thus, in this mosaic of interconnected habitats composing the ecological network of these ecosystems, meadows of submerged macrophytes play a key role (Carpenter & Lodge, 1986). They can occupy part, or all of the bottom of these shallow systems, influencing the entire water column (Sand-Jensen & Borum, 1991; Rodrigo *et al.*, 2015) by incorporating a set of non-trophic interactions to the trophic connections among the planktonic-benthic community. Some of these interactions are: allelopathy against primary producers (van Donk & van de Bund, 2002; Rojo *et al.*, 2013a, b); refuge for zooplankton and macroinvertebrates (Hampton *et al.*, 2000; Rodrigo *et al.*, 2015), or vital support for periphyton (Vadeboncoeur & Steinman, 2002; Rojo *et al.*, 2017). Thus, to better understand the effect of current GC in shallow freshwater ecosystems, we must unravel if this effect is due to the habitat-dependent response of populations to the changing environmental factors, and if the network structure is involved in this effect.

The network approach allows these systems to be addressed through a community perspective, *i.e.* taking into account not only the elements (*i.e.* populations-nodes) and the habitats within a system, but also the interactions or feedbacks established among them (Berlow *et al.*, 2004). Networks considering only direct trophic interactions (*i.e.* food webs) have been widely studied (Williams & Martínez, 2000). However, non-trophic interactions could be as important as trophic ones (Bertness & Callaway, 1994), and have recently been considered in ecological models (Vasas & Jordán, 2006; Kéfi *et al.*, 2012). Merging them with trophic interactions (*i.e.* a multi-interaction network; Ings *et al.*, 2009; Puche *et al.*, 2020) supposes a challenge that must be tackled to

better understand the performance of a complex ecosystem facing environmental disturbances.

Some studies have attempted to define node roles (*e.g.* peripherals, connectors or hubs) in the ecological networks, since the node-scale structure could drive the global structure of the network (Bascompte *et al.*, 2003; Capocefalo *et al.*, 2018). Moreover, studies by Borst *et al.* (2018) and Ellison (2019) established the foundation role played by nodes centralizing the non-trophic relationships, which are abundant (in terms of biomass) and are usually at the base of the network (*e.g.* primary producers). In this vein, Puche *et al.* (2020) have recently suggested a structurally crucial tandem between foundational (charophytes, green macroalgae) and connector elements (zooplanktonic herbivores) in an experimental multi-interaction network as a model for shallow freshwater ecosystems.

Now, we put forward the need to combine the network approach with experimentation on GC-related factors at a mesocosm scale (Benton *et al.*, 2007; Spivak *et al.*, 2010). Mesocosm experiments, although being a simplification of the natural environment and therefore, providing conclusions that should be taken with caution, allow the study of systems at a high level of complexity, while maintaining tight control over the conditions to which they are subjected, and making it possible to apply models at different organisational levels, from individuals to interaction networks and even to entire ecosystems (Stewart *et al.*, 2013). This combination will allow us to disentangle the relative importance and the influence of the different habitats in shallow freshwater ecosystems, potentially applicable to better understand their structure and functioning when facing current and foreseeable GC.

Our main goal is to assess the performance of a reproduced macrophyte-dominated freshwater shallow system under GC-related scenarios, with UVR and T as stressors, tested separately to avoid the overlapping of their effects. We hypothesize that: (1) the differential response of populations-nodes to these stressors will depend on their habitat; those from the pelagic and within-meadow being more vulnerable to

change than those from the benthic. Therefore, we also hypothesize that: (2) the habitats in the network will be differentially affected, changing their relative importance in the system, with the habitats most related with macrophytes (within-meadow and benthic) being the most influential for all the multi-interaction network differences among scenarios. And finally, (3) these effects will result in contrasting configurations under the tested environmental disturbances: phytoplankton-dominance under a UVR increase, and macrophyte-dominance under warming conditions.

2. Material and methods

2.1. Experimental setup

The mesocosm experiment was conducted in tanks that allowed a water volume of 170 l (length 0.75 m × width 0.48 m × height of water column 0.47 m). Twelve independent tanks were setup in order to simulate replicates of a shallow freshwater system dominated by charophytes (submerged macrophytes; Fig. 1a).

The bottom of each mesocosm was covered with a substrate layer (thickness 10 cm), then charophytes collected from a shallow coastal lagoon were planted and the mesocosm was carefully filled with tap water (165 l), plus an inoculum of water from the same shallow coastal lagoon (5 l). The substrate layer was obtained by mixing organic compost and gravel in the proportion 2:1. Over this substrate, a layer of natural sediment from the same shallow coastal lagoon was scattered to include a natural sediment inoculum. The cosmopolitan species *Chara hispida* Linnaeus was planted in three rows of three bundles each, as evenly as possible, to form a monospecific charophyte meadow, covering half the tank (Fig. 1b). The meadow grew in the half of the mesocosm where it was planted, and at the end of the experiment the average surface occupied by the meadows (12 mesocosms) was 1766 ± 109 cm^2 (mean ± standard error), approximately 50% of the total surface of the mesocosm (3600 cm^2). For the methods of planting charophytes see Rodrigo *et al.* (2018), Rojo *et al.* (2019)

and Puche *et al.* (2020). This design allowed us to define three connected habitats in the mesocosms: i) the pelagic, with organisms living in free water in the half of the mesocosm with no meadow; ii) the within-meadow, where organisms inhabit the free water within the charophyte meadow, and iii) the benthic, the charophytes and all the organisms living attached to them (Fig. 1b).

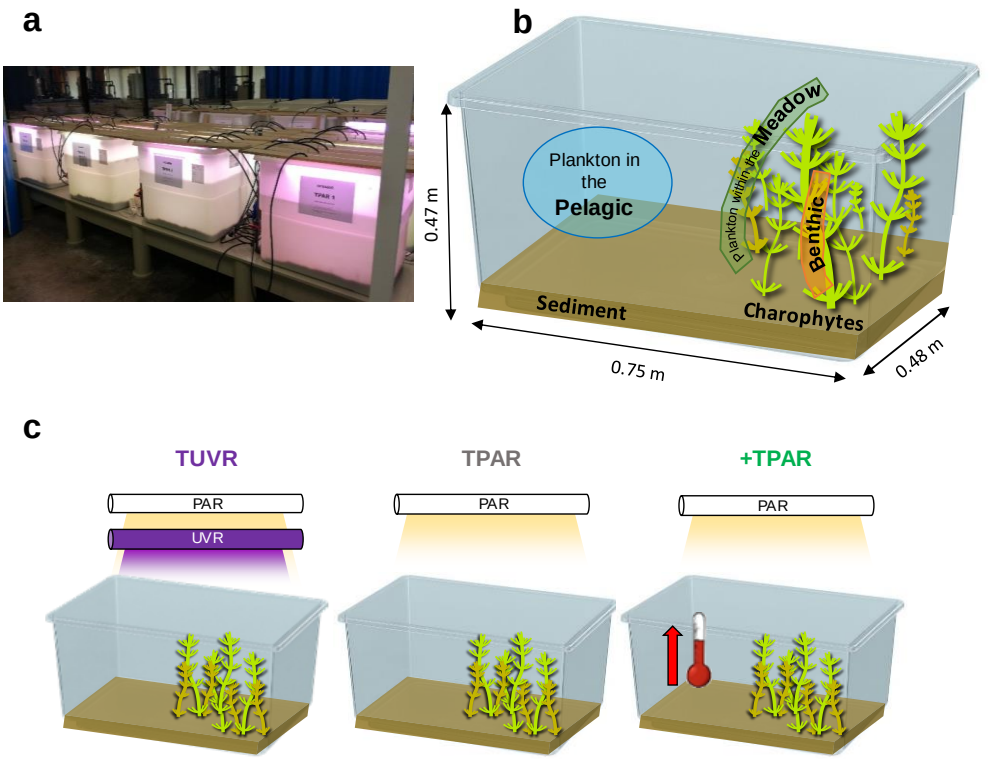


Fig. 1. Arrangement of experimental mesocosms, including a) the location of the mesocosms in the room. The distance between each row of tanks was 30 cm. Within each row, the tanks were separated from each other by 15 cm. The radiation setup on top of each mesocosm is shown. b) The three considered habitats (pelagic, within-meadow and benthic). And c) the experimental design with the three scenarios: TUVR (temperature 22°C and a supply of UV radiation to the photosynthetically active radiation, PAR), TPAR (control scenario, temperature 22°C and only PAR supplied) and +TPAR (temperature 26°C and only PAR supplied) with four replicates each.

We established a control scenario (TPAR), from which UVR-enhanced (TUVR) and T-increased (+TPAR) scenarios were defined in quadruplicate (Fig. 1c). The TPAR scenario consisted of a temperature of 22°C and photosynthetically active radiation

(PAR) only; the same temperature was used in the TUVR scenario, but a high dose of UVR to PAR was supplied (representing a stressful increase in the ratio of UVR per dose of PAR which is typically found in very shallow Mediterranean freshwater ecosystems; Rojo *et al.*, 2012). The +TPAR scenario consisted of supplying only PAR, but increasing the temperature by 4°C (to 26°C) in accordance with the expected increase in temperature for the Mediterranean region by the end of this century (IPCC, 2014). Average temperature in low T scenarios (TPAR and TUVR) and high temperature scenario (+TPAR) were significantly different over the study period (Table S1 Supplementary material Chapter 5). With regard to radiation, PAR (400–700 nm) was provided by Sylvania Gro-Lux F58W fluorescent tubes. In the TUVR scenario, the supply of UVR was provided by Philips TL40W/12 RS SLV tubes (for UVBR, 280–320 nm) and Philips Cleo 40W tubes (for UVAR, 320–400 nm). These UVR tubes were covered by an Ultraphan 295 filter (Digefra GmbH, Munich, Germany) to completely remove the UVCR. The doses of PAR and UVR, and their ratios, are detailed in Table 1. All the tubes were placed at the top of each mesocosm (Fig. 1a). The desired temperature in +TPAR was achieved by means of aquarium heaters (Eheim Jäger 50 W for 1000 l). The temperature in the other scenarios was the result of the room temperature, plus the heat given off by the radiation tubes. The mesocosms were maintained under the corresponding environmental conditions in a light:dark cycle of 14:10 h.

Table 1. Average underwater doses of photosynthetically active radiation (PAR), ultraviolet radiation A (UVAR) and B (UVBR) in the mesocosms. TPAR (with temperature 22°C) and +TPAR (with temperature 26°C) scenarios were only supplied with PAR radiation. The TUVR scenario was supplied with PAR and UVR. The measurements were taken at depths of 0.5, 15 and 25 cm in the mesocosms. PAR:UVR and UVBR:UVAR ratios are provided.

	PAR (400-700 nm)	UVAR (320-400 nm)	UVBR (280-320 nm)
W m^{-2}	12.0	1.3	0.4
KJ m^{-2} d^{-1}	605	66	20
mol photons m^{-2} d^{-1}	3		
PAR:UVR	7.0		
UVBR:UVAR	0.3		

Mesocosms were placed completely independent of each other. The four replicates of each scenario were allocated occupying a total area of 7 m^2 (Fig. 1a); this experimental area was isolated and located indoors in the 500-m^2 aquarium plant facilities of the University of València; thus, a site effect on mesocosms was not expected. The logistics of the UVR installation oblige (*e.g.* for safety), to place the four mesocosms with UVR radiation in a row. We corroborated that these UVR conditions do not affect the mesocosms of the other two scenarios, in which no-detectable doses of UVR were measured.

The experiment lasted two months. After the disturbance caused by filling the mesocosms, the environmental conditions were undisturbed and constant for each scenario. In such undisturbed conditions it is reasonable to consider that during the first month the result of competition and predation between microorganisms (*e.g.* plankton) would allow them to achieve equilibrium, or a post-disturbance steady state (Sommer *et al.*, 1993; Naselli-Flores *et al.*, 2003; Ortega-Mayagoitia *et al.*, 2003; Rojo & Álvarez-Cobelas, 2003; Rodrigo *et al.*, 2009). Moreover, during the first weeks the charophytes would be well fixed to the sediment by the rizhoids and be able to attain their highest growth rate during the first month (Rojo *et al.*, 2015; Rodrigo *et al.*, 2018; Puche *et al.*, 2018). Then, by extending the experiment to two months, we would be able to compare the state of the community indicators over time. For instance, resistance and resilience between scenarios can be analysed by taking into account the data from the end of the first and the second months (Cabrerizo *et al.*, 2019).

The weekly maintenance of the mesocosms consisted of measurements of physical and chemical variables, and refilling the fraction of evaporated water. These periodic measurements allowed us to rectify possible deviations from the experimental conditions, and to maintain the same values of the variables not directly involved in the definition of the scenarios in all the mesocosms.

2.2. Biological community sampling

In the mesocosms, a planktonic-benthic community was established in the considered habitats from the water and sediment inoculums, as well as from the biological content attached to the charophytes themselves. We are aware that there may be a high degree of connection between the defined habitats due to the small space in which they were found, something that could occur naturally in shallow ecosystems. Aquatic gastropods were attached to the planted charophytes and in the sediment inoculum, thus, they were taken into account for the definition of the biological community in the mesocosms. It has to be noted that in these experimental mesocosms fish were not present. This situation is quite frequent in natural shallow freshwater systems, since many of them are temporary.

In the middle of the experiment (day 33, or the end of the first month; considering that the community has achieved an equilibrium state) and at the end of the experiment (day 60, or the end of the second month), each habitat was sampled for autotrophs (phytoplankton/phytobenthos, cyanobacteria and charophytes) and heterotrophs (heterotrophic bacteria, zooplankton/zoobenthos and gastropods). To this end, for phytoplankton, 250 ml were collected from each mesocosm in the middle of the water column from the pelagic and within-meadow habitats and fixed with Lugol's solution. For zooplankton, 4 l from the same locations as for phytoplankton were filtered through 37 µm Nytal mesh, and the samples were fixed with formaline (Rodrigo *et al.*, 2015). For benthic organisms, several charophyte individuals were sampled and washed carefully with tap water. The material obtained from this first wash was kept in small tubes and fixed with formaline in order to count and identify zoobenthos. After this, the charophyte shoots were gently scrubbed with a toothbrush, and the resulting sample was fixed with Lugol's solution for phytobenthos. The dry weight (DW) of charophytes (after drying them for 24 h at 70°C) was calculated to refer the benthic organisms to this weight (Rojo *et al.*, 2017). The different fractions of organisms were identified at the finest possible taxonomic resolution, and then

counted. Thus, the density of each taxon was calculated as individuals per litre (in the case of planktonic organisms), or as individuals per gram of DW of charophytes (in the case of periphytic organisms living attached to the macrophytes). Afterwards, in order to compare the densities of the organisms from the different habitats, we express their densities as organisms per surface unit (ind m^{-2}). The abundance of plankton inhabiting free water can be expressed by surface unit (LeCren & Lowe-McConnell, 1980) if its density by water volume (ind L^{-1} or ind dm^{-3}) is multiplied by the depth of the water column (m). To express the density of benthic organisms per surface unit, we took a photograph from the top of each mesocosm to assess the area (m^2) occupied by the meadow at the end of the experiment. Then, the total biomass (DW) of the meadow was measured at the end of the experiment and divided by the area occupied. Multiplying the ind g^{-1} DW of charophytes by the g DW of charophytes m^{-2} we obtained the ind m^{-2} for benthic organisms. On day 33 (first month), we also took a photograph of each mesocosm to assess the meadow area occupied at this time. Then with the correlation biomass-area of charophytes at the end of the experiment (second month), we extrapolated the biomass of the meadows at the end of the first month.

From the density of the different taxa in the considered habitats, the carbon biomass per surface unit was calculated. For autotrophs (phytoplankton/ phytobenthos), the equations proposed by Menden-Deuer & Lessard (2000) were applied depending on the taxonomic group. For the heterotrophs (zooplankton/ zoobenthos), five individuals from each taxon were measured and the equations proposed by Dumont *et al.* (1975), Rutner-Kolisko (1977), Malley *et al.* (1989) and Anderson & Hessen (1991) were applied. For ciliates, specifically, the equations of Sherr *et al.* (1986), Putt & Stoecker (1989) and Bojanić *et al.* (2006) were used. Bacteria were assumed as spheres of 1 µm in diameter, and following Nagata (1986), a carbon content of 106 fgC μm^{-3} was considered. In order to assess the carbon content of charophytes, several individuals from each mesocosm (after brushing away the periphytic organisms on their surface) were dried (24 h at 70°C), crushed by means of

an automatic tissue grinder (Precellys® 24, Bertin Technologies, France) in two series of 15 s at 4500 rpm, and stored in plastic tubes until carbon analyses were conducted. For gastropods, the same methodology of dry-crushing was followed, taking into account only the soft parts of these organisms. The total carbon content was determined using a Perkin-Elmer CHN/O-2400 Elemental Autoanalyser.

2.3. The multi-interaction network and global scale parameters

The definition of the nodes in the network followed a mix between taxonomic and functional (*e.g.* size, mobility, edibility and toxicity) criteria. Thus, the identified taxa were grouped into a total of 48 nodes (Table 2). The trophic and non-trophic links among the nodes were established based on our expertise and on the literature. For a detailed explanation of the establishment of links in the network see Puche *et al.* (2020). To highlight the differences between scenarios, and based on the results of carbon biomass, we eliminated from the networks of a particular scenario those nodes whose mean biomass had a lower value than the minimum value of the distribution in the scenario with greater biomass for these nodes. Thus, we eliminated the nodes meeting this criterion in the networks of TPAR (Cr_p, DS_p, DB_p, Cil_p, Cr_m, DS_m, DB_m, O_m, B_b), TUVR (DS_p, DB_p, C_p, Co_p, DS_m, DB_m, O_m) and +TPAR (Cr_p, Cil_p, Co_p, Cr_m, B_b; the meaning of these abbreviations is in Table 2).

The set of nodes and links were embodied in an SxS matrix of interactions A, where S is the number of nodes and each element a_{ij} represents the ecological interaction between two nodes (Cohen, 1978). The value of these matrix entries can be 1 (positive interaction), -1 (negative interaction) or 0 (no interaction). Trophic relationships were coded bidirectionally (*i.e.* − 1 for the effect of the predator on the prey, and 1 for the effect of the prey on the predator). Non-trophic relationships were coded unidirectionally, as the effect of the agent on the target. Gephi© software was used for the network visualization.

Table 2. List of the 48 nodes defined in the networks.

Abbreviation	Node	Autotroph (A) / Heterotroph (H) / Mixotroph (M)
N	Nutrients	
$ClU_{p,m,b}$	Unicellular chlorophytes	A
$ClC_{p,m,b}$	Colonial chlorophytes	A
$ClF_{p,m,b}$	Filamentous chlorophytes	A
$Cr_{p,m,b}$	Cryptophytes	M
$DS_{p,m,b}$	Small diatoms	A
$DB_{p,m.b}$	Big diatoms	A
$CiC_{p,m,b}$	Colonial cyanobacteria	A
$CiF_{p,m,b}$	Filamentous cyanobacteria	A
$B_{p,m,b}$	Bacteria	H
$Cil_{p,m,b}$	Ciliates	M
$R_{p,m,b}$	Rotifers	H
$C_{p,m,b}$	Cladocerans	H
$Cop_{p,m,b}$	Copepodites	H
$O_{p,m,b}$	Ostracods	H
$Co_{p,m,b}$	Copepods	H
G_b	Gastropods	H
$Char_b$	Charophytes	A

The correspondence between the abbreviation in the network and the identity of the node, as well as their classification as autotrophs, heterotrophs or mixotrophs, are provided. In the abbreviations, the subscript indicates the compartment the node belongs to: p for pelagic, m for within-meadow and b for benthic.

The global structure of the networks was assessed by means of five descriptors: number of nodes (S), number of links (L), directed connectance (C), modularity coefficient (M) and nestedness (N). Connectance (C) is the proportion of realized interactions relative to the potential number of possible interactions in the network (Martínez, 1992). The modularity coefficient (M) arises from a particular partition of the network that maximizes its division into modules (non-overlapping strongly interacting set of nodes; Guimerà & Amaral, 2005). Nestedness (N) looks for a structure in the network in which nodes with few interactions are a subset of nodes with a higher number of interactions (Almeida-Neto *et al.*, 2008). The calculations of these parameters were performed in MATLAB using the Brain Connectivity Toolbox

and, in the case of nestedness, using the software ANHIDADO (ver. Bangu 3.0; Guimarães & Guimarães, 2006).

2.4. Structural and dynamic importance of nodes

To analyse the role played by the nodes in the structure of the network, first we applied the above-mentioned modularity algorithm proposed by Guimerà & Amaral (2005). Based on the modules defined by the algorithm, we assessed the roles that nodes played in the network by calculating the within-module degree (*z*) and the participation coefficient (P). The former indicates the importance of the node within its own module, and the latter assess the importance of the node for connecting different modules (Olesen *et al.*, 2007). For details of the equations of these parameters, see Olesen *et al.* (2007). Then, the nodes were represented in a z–P parameter space. Initially, Guimerà & Amaral (2005) proposed seven node roles according to these parameters, but later Olesen *et al.* (2007) simplified this classification into four groups that cover all the combinations between the importance within their own module (*z*) and the importance connecting modules (P): peripherals (low *z* and P), connectors (low *z* and high P), module hubs (high *z* and low P) and network hubs (high *z* and P). The calculations of these parameters were performed in MATLAB using the same package as for global parameters.

Moreover, we assessed the importance of the nodes facing disturbances in other nodes and in the environment. We called this the dynamic importance of nodes (Puche *et al.*, 2020), as we are summarizing the asymptotic responses of species abundances after parameter disturbances in any species of the network. We first calculated the net effects matrix N from the interaction matrix A. Matrix N encompasses both direct and indirect effects among the nodes. A direct effect between two nodes occurs when there is a link connecting them. While an indirect effect means that there are one or more intermediaries between these two nodes. We followed the Novak *et al.* (2016) procedure: under the assumption that the system (matrix) is in an equilibrium state, we randomized matrix A 5000 times by multiplying each off-diagonal element by a

random value sampled from a uniform distribution within (1/2 and 2). The diagonal elements were set to a value of − 3. In each randomization, matrix N was calculated as $N = -A^{-1}$. Then, an average N matrix was obtained. In this net effects matrix, each element n_{ij} represents the expected long-term pressure in the equilibrium value of node *i*, when node *j* is constantly pressured (Nakajima, 1992). With this matrix, and following Puche *et al.* (2020), we calculated two node-scale parameters: sensitivity, which represents the susceptibility of a node to be affected when other nodes are disturbed; and effectiveness, which indicates the capacity of a node to affect other nodes when being disturbed. The sensitivity of node *i* is simply the sum of the values of the i^{th} row in N divided by (S − 1), while the effectiveness of node *i* is the sum of values of the i^{th} column in N divided by (S − 1).

Furthermore, with the carbon biomass of the nodes obtained in the middle and at the end of the experiment, we calculated the resistance (Rt) and resilience indices (Rl) of each node to an increase in UVR or T following the methodology of Orwin & Wardle (2004), applied by Cabrerizo *et al.* (2019) in a mesocosm experiment:

$$\text{Resistance index (Rt)} = 1 - (2\,|D_0| \,/\, (C_0 + |D_0|)$$

where C_0 is the carbon biomass of the node in the control scenario (TPAR) in the middle of the experiment (day 33, or the end of the first month); and $|D_0|$ is the absolute difference between the biomass of this node in the control scenario and in the perturbed scenarios (TUVR or +TPAR), also in the middle of the experiment (day 33).

$$\text{Resilience index (Rl)} = (2\,|D0|) \,/\, (|D0| + |Dx|) - 1$$

where |Dx| is the absolute difference between the carbon biomass of the node in the control scenario and in the perturbed scenarios at the end of the experiment (day 60).

We calculated average resistance and resilience indices for each node by pairwise comparisons of all the possible combinations between the replicates of the control and disturbed scenarios.

The values of these indices range between 1 and -1. A value of 1 means that the node is totally resistant (not affected by the disturbance) or totally resilient (fully recovered after the disturbance). Values below 1 mean less resistance or resilience.

2.5. Statistical analyses

Several one-way ANOVA tests were performed (after corroborating that the assumptions of normality and homoscedasticity were fulfilled) to assess significant differences among the environmental scenarios regarding the set of variables considered in this study: overall carbon biomass of phytoplankton/benthos and zooplankton/benthos, carbon biomass of each node in the network separately, and global-structure parameters of the networks. Other one-way ANOVA tests were carried out to assess differences among the habitats regarding the resistance and resilience indices, facing T or UVR increases. Furthermore, a two-way ANOVA test was performed to analyse the effect of the scenario and habitat, as well as their interaction, on the sensitivity and effectiveness of the nodes.

All the statistical analyses were conducted using SPSS Statistics v.22 software (IBM Corp, Armonk, NY), considering statistically significant differences at $P < 0.05$.

3. Results

3.1. Plankton and periphyton carbon biomass

At the end of the experiment, focusing on planktonic organisms (both from the pelagic and within-meadow habitats), phytoplankton carbon biomass in the TUVR scenario (12 mgC m^{-2}) was, on average, more than six times higher than in the TPAR and +TPAR scenarios (Fig. 2a) and also showed the highest variability. Phytoplankton in the TUVR scenario was dominated by the flagellate mixotrophic cryptophyte of the species *Cryptomonas marsonii* Skuja (75 % carbon biomass; Fig. 3a). The biomass of the nodes corresponding to planktonic cryptophytes (termed Cr_p and Cr_m in the network) was significantly higher in the TUVR networks, as occurred with benthic bacteria (B_b) and pelagic ciliates (Cil_p; Fig. 4). Total zooplankton carbon biomass did not show

remarkable differences between the scenarios (Fig. 2b), and was dominated by cladocerans of the genus *Simocephalus* (Fig. 3b). However, the carbon biomass of pelagic cladocerans (C_p) and pelagic cyclopoid copepods (Co_p) was significantly lower in the TUVR scenario (Fig. 4). Finally, the carbon biomass of planktonic bacteria (B_p and B_m) was also significantly lower in the communities in the TUVR scenario (Fig. 4).

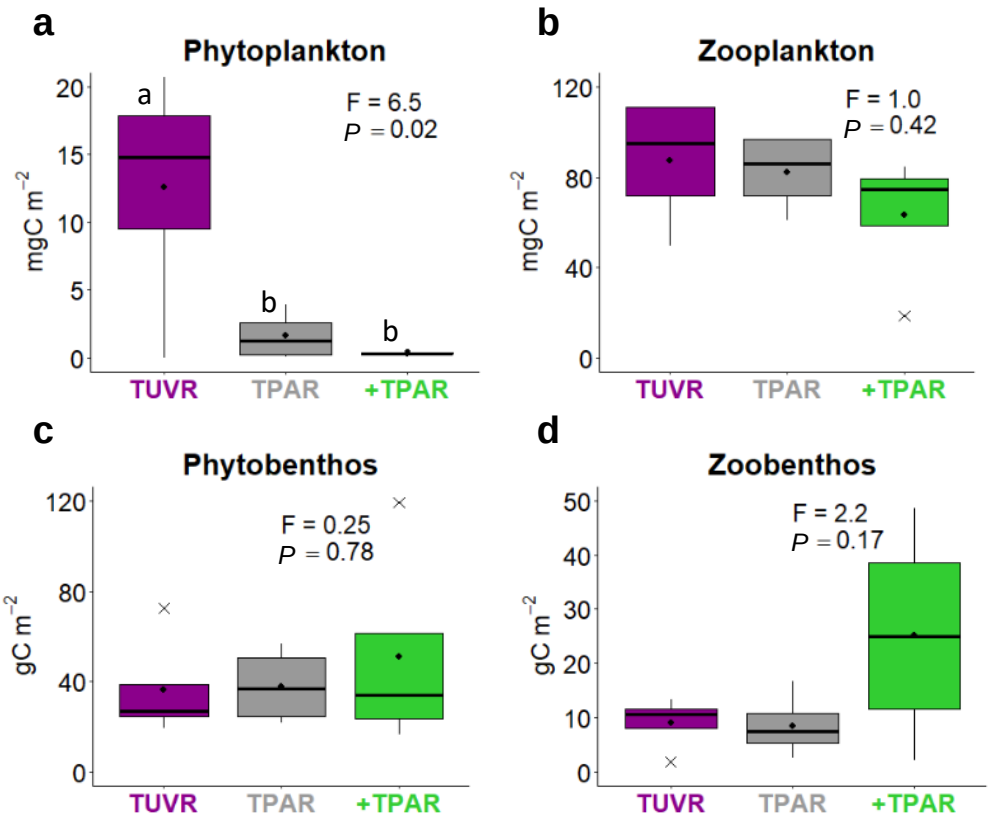


Fig. 2. Box-plot of the carbon biomass (mg C m^{-2} or g C m^{-2}) of a) phytoplankton, b) zooplankton, c) phytobenthos and d) zoobenthos under the tested scenarios (TUVR, TPAR and +TPAR). Lower and upper box boundaries represent 25th and 75th percentiles, respectively. The line inside box is the median, and the dot inside the box is the mean. Lower and upper error lines indicate 10th and 90th percentiles, respectively. Crosses represent values falling outside 10th and 90th percentiles. ANOVA F statistic and *P*-value are shown in each graph. Lower-case letters represent significant differences ($P < 0.05$) between scenarios after the Tukey *post hoc* test

For the TPAR and +TPAR scenarios, the phytoplankton carbon biomass of the communities was similar and did not exceed 2 mgC m^{-2} (Fig. 2a), but there were differences in the community taxonomic composition between these scenarios. In TPAR, cryptophytes were dominant (60% of the carbon biomass; Fig. 3a), with two species sharing this dominance (*Cryptomonas marsonii* and *C. rostratiformis* Skuja; Fig. 3a). This fact is reflected in the significantly higher biomass of the corresponding node in these networks compared to those of +TPAR (Fig. 4). In addition, chlorophytes (*Scenedesmus aculeolatus* Reinsch and *Tetraedron minimum* A.Braun), diatoms (the big centric *Diploneis parma* Cleve and the small centric *Cyclotella meneghniana* Kützing) and colonial cyanobacteria (*Chroococcus* sp.) accounted for 40% of the phytoplankton carbon biomass in TPAR (Fig. 3a). However, in +TPAR, the dominance shifted towards diatoms (70% of the carbon biomass; Fig. 3a). The carbon biomass of the diatom nodes (DB_p and DB_m, and DS_p and DS_m) was significantly higher in the +TPAR scenario than in the others (Fig. 4).

Regarding the benthic organisms, the phytobenthos carbon biomass did not vary among scenarios (Fig. 2c), and was always dominated by filamentous chlorophytes of the genus *Oedogonium* (Fig. 3c). Charophytes (Char), despite belonging to the benthic habitat of the network, were not considered in this calculation since being macroalgae their biomass was disproportionately superior to that of the other benthic elements of the community. The carbon biomass of the charophytes was significantly lower in the TUVR scenario (Fig. 4).

For the zoobenthos, there were no differences in the carbon biomass in the three scenarios (Fig. 2d). Compositionally, all the communities were dominated by cladocerans. However, differences at a genus level occurred: under the TUVR scenario the genus *Simocephalus* dominated (79% of the cladoceran carbon biomass; Fig. 3d);

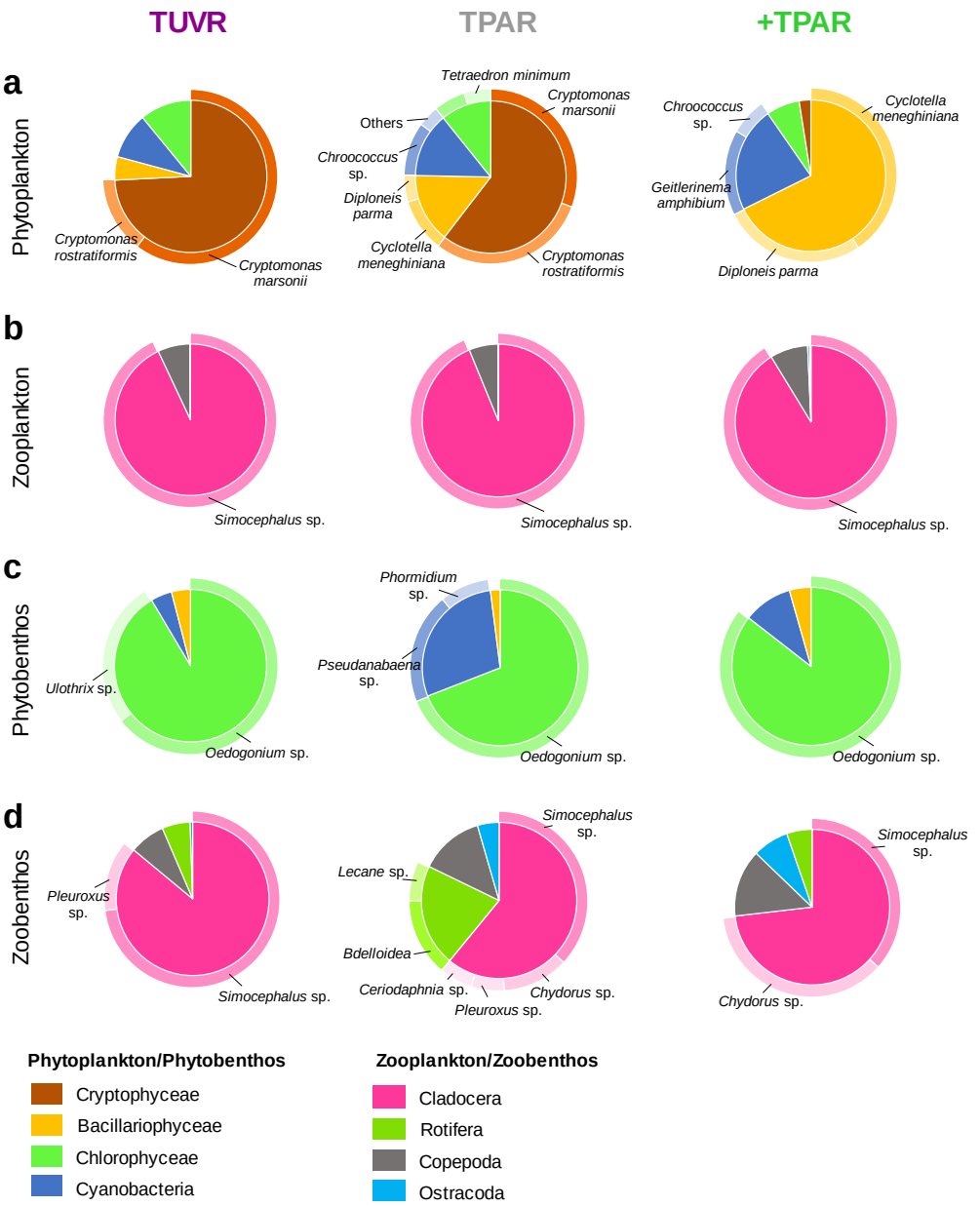
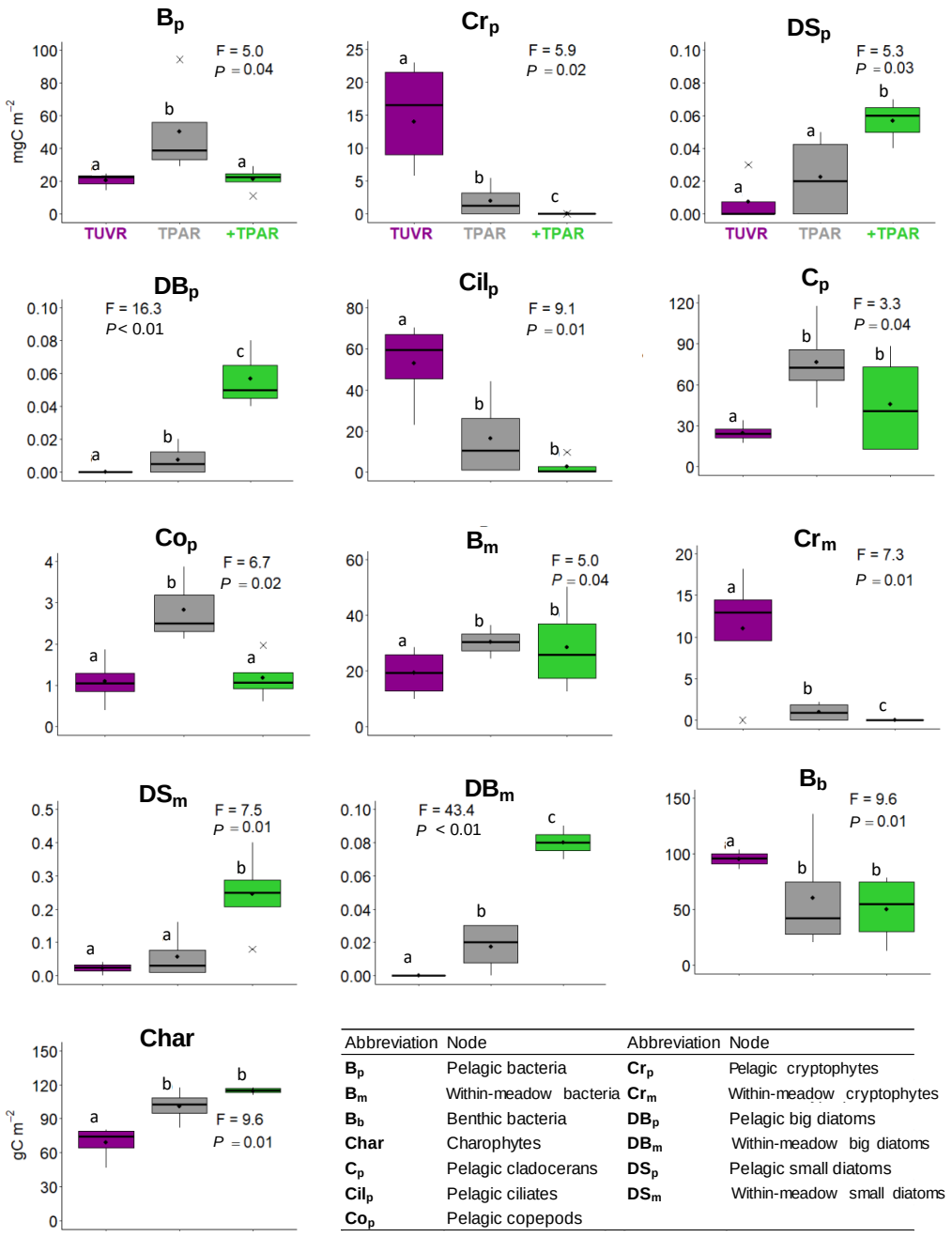


Fig. 3. Pie charts of the percentage of carbon biomass in the different taxonomic groups at the end of the experiment (day 60) within a) phytoplankton, b) zooplankton, c) phytobenthos and d) zoobenthos under the tested scenarios (TUVR, TPAR and +TPAR). Outer sectors in pie charts show the main genera/species in the most abundant taxonomic groups. Gastropods are not considered in the graphs of zoobenthos as they are macroorganisms and would mask the results of the other zoobenthic elements



Abbreviation	Node	Abbreviation	Node
B_p	Pelagic bacteria	**Cr_p**	Pelagic cryptophytes
B_m	Within-meadow bacteria	**Cr_m**	Within-meadow cryptophytes
B_b	Benthic bacteria	**DB_p**	Pelagic big diatoms
Char	Charophytes	**DB_m**	Within-meadow big diatoms
C_p	Pelagic cladocerans	**DS_p**	Pelagic small diatoms
Cil_p	Pelagic ciliates	**DS_m**	Within-meadow small diatoms
Co_p	Pelagic copepods		

Fig. 4. Box-plot of carbon biomass (mgC m^{-2} or gC m^{-2}) of the network nodes that showed significant differences within the tested scenarios (TUVR, TPAR and +TPAR). Details of box-plot graphs as in Fig. 2. ANOVA F statistic and *P*-value are shown in each graph. Lower-case letters represent significant differences ($P < 0.05$) between scenarios after the Tukey *post hoc* test.

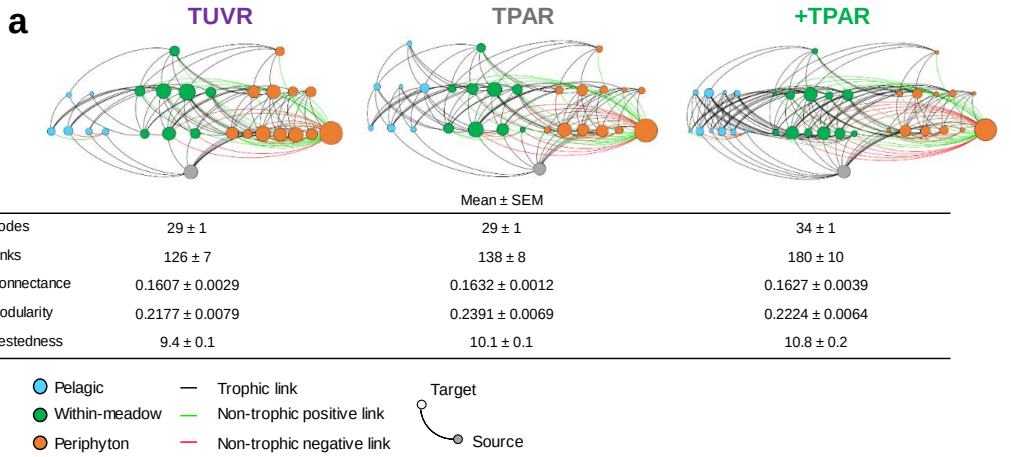
in TPAR, although *Simocephalus* was the dominant genus (40%), the rest of the cladoceran carbon biomass was shared by the genera *Chydorus*, *Pleuroxus* and *Ceriodaphnia* (Fig. 3d); and in the +TPAR scenarios, the cladoceran carbon biomass was split mainly between the genera *Simocephalus* and *Chydorus* (41% and 48 %, respectively; Fig. 3d). The benthic bacteria (B_b) carbon biomass was two times higher in the TUVR scenario compared to the TPAR and +TPAR scenarios (Fig. 4). As for charophytes, gastropods are not included in these calculations since they are macroorganisms compared to the rest of considered elements. Their average carbon biomass was not significantly different between scenarios (85 ± 10 mgC m^{-2}; mean ± standard error).

These described compositions of populations and taxonomical groups, in relative abundance (Fig. 3), were reached at the end of the first month of the experiment (Fig. S1 Supplementary material Chapter 5).

3.2. Global structure of the networks and the roles of the nodes

According to the global-structure parameters of the networks, there were also differences among scenarios. Networks under the +TPAR scenario had a significantly higher number of nodes (S) and links (L; Fig. 5). The connectance (C; related to S and L) remained the same among scenarios (Fig. 5). Regarding modularity (M), there were no statistically significant differences among scenarios (Fig. 5). For nestedness (N; related to a network configuration with generalists and specialists' nodes), there were significant differences among TUVR (lowest values), TPAR (intermediate values) and +TPAR (highest values; Fig. 5).

Analysing the structural roles played by nodes in the networks (Fig. 6), it can be observed that differences occurred in the networks among scenarios regarding the "connector" nodes. While in networks under the TUVR and TPAR scenarios none of the nodes was a connector, in +TPAR networks the connector role was played by zooplanktonic herbivores in the pelagic and within-meadow habitats (C_p and C_m, R_p and R_m, and O_p; Fig. 6).



	Mean ± SEM		
Nodes	29 ± 1	29 ± 1	34 ± 1
Links	126 ± 7	138 ± 8	180 ± 10
Connectance	0.1607 ± 0.0029	0.1632 ± 0.0012	0.1627 ± 0.0039
Modularity	0.2177 ± 0.0079	0.2391 ± 0.0069	0.2224 ± 0.0064
Nestedness	9.4 ± 0.1	10.1 ± 0.1	10.8 ± 0.2

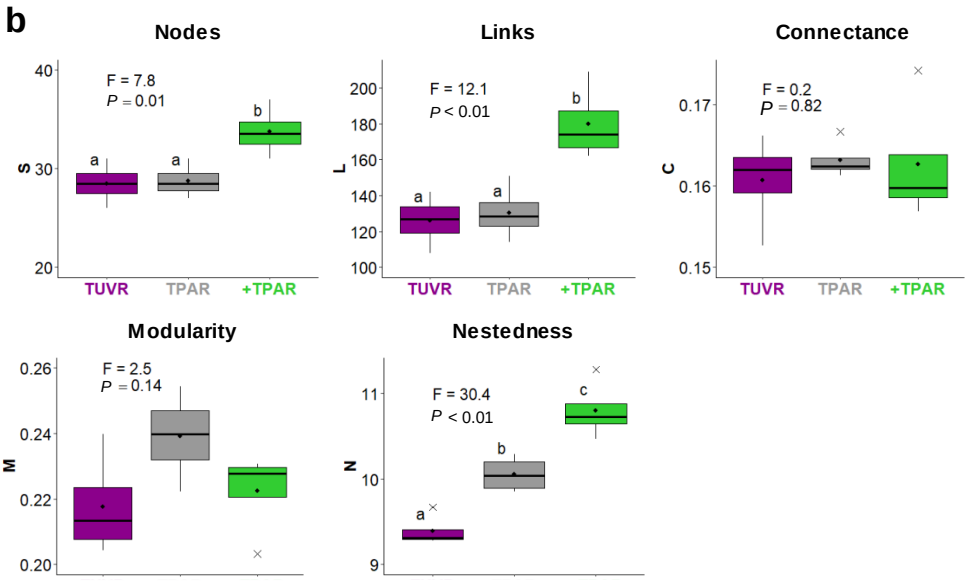


Fig. 5. Results of the networks' global structure analysis. a) Representative multi-interaction network of each tested scenario (TUVR, TPAR and +TPAR) and values (mean ± SE) of the global-scale descriptors of the network distributed in groups according to the habitat they belong to (pelagic, within-meadow or benthic), and vertically corresponding to the trophic position, with nutrients at the bottom. Node colour represents the habitat the node belongs to (Nutrients node is represented in grey), line colour represents the type of interaction, curvature of links represents the directionality of the interaction clockwise from the source to the target. b) Box-plot of global-scale network parameters within the tested scenarios (TUVR, TPAR and +TPAR). Details of box-plot graphs as in Fig. 2. ANOVA F statistic and *P*-value are shown in each graph. Lower-case letters represent significant differences ($P < 0.05$) between scenarios after the Tukey *post hoc* test.

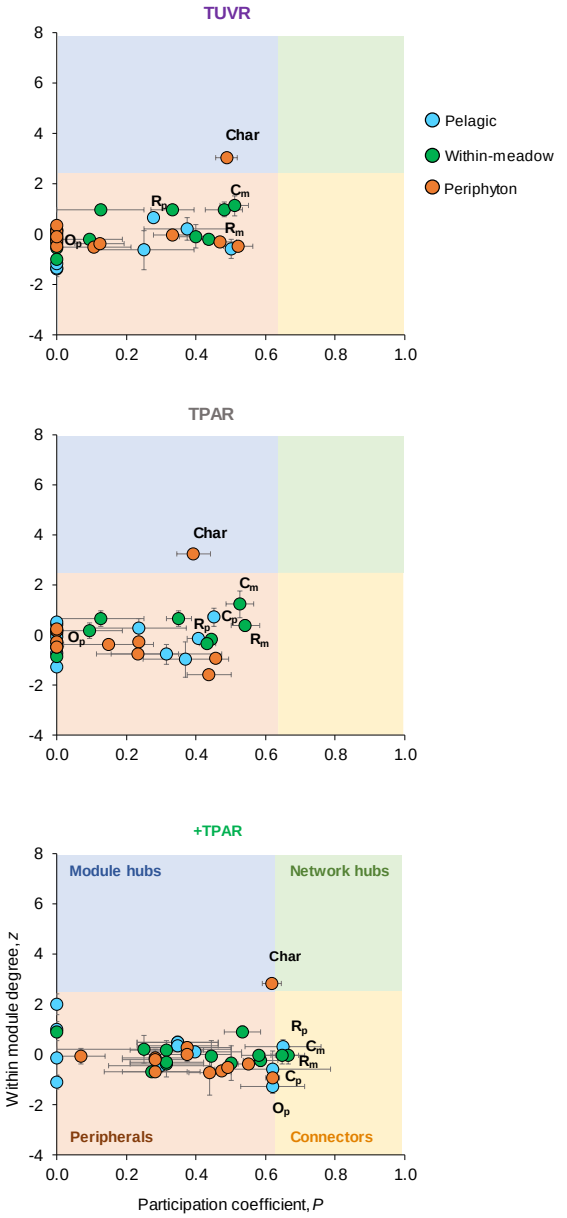


Fig. 6. Classification of nodes after partitioning the networks into modules (by modularity algorithm; Guimerà & Amaral, 2005). The nodes' roles were defined according to the within-module *z* score (importance within its module, y-axis) and the participation coefficient P (importance between modules, x-axis) in each of the tested scenarios (TUVR, TPAR and +TPAR). Each circle is a node in the multi-interaction networks, and the colour represents the defined habitat they belong to (pelagic, within-meadow or benthic). The classification regions (nodes' roles) in the graphs follow those proposed by Olesen *et al.* (2007). Vertical and horizontal error bars are the standard errors among the four replicates per scenario of within-module *z* score and P, respectively. Nodes with connector role in +TPAR are highlighted (abbreviations of the nodes as in Table 2).

3.3. Dynamic importance of nodes

The effect of habitat was significant regarding sensitivity (F = 5.1, *P* = 0.01, df = 2) and effectiveness (F = 7.8, *P* < 0.01, df = 2). The mean values of sensitivity and effectiveness in nodes from the within-meadow and benthic habitats were higher compared to those from the pelagic habitat (Fig. 7). Neither the scenario (F = 2.3, *P* = 0.12, df = 2 for sensitivity and F = 1.9, *P* = 0.17, df = 2 for effectiveness) nor the habitat x scenario interaction (F = 0.4, *P* = 0.81, df = 4 for sensitivity and F = 0.3, *P* = 0.92, df = 4 for effectiveness) had a significant effect on these node parameters.

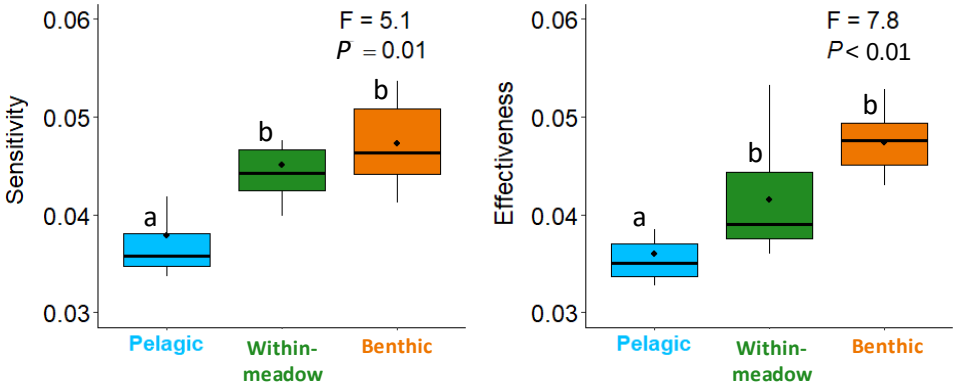


Fig. 7. Box-plot of sensitivity and effectiveness of nodes in the networks of the three tested scenarios together according to the habitat they belong to (pelagic, within-meadow and benthic). Details of box-plot graphs as in Fig. 2. ANOVA F statistic and *P*-value are shown in each graph. Lower-case letters represent significant differences (*P* < 0.05) between habitats after the Tukey *post hoc* test.

Regarding the resistance and resilience indices (Rt and Rl, respectively), the response to the tested GC-related factors (UVR and T) was similar. The benthic habitat (averaging its nodes) was significantly more resistant and less resilient than the pelagic and within-meadow habitats for the tested disturbances (Fig. 8). Although belonging to the benthic habitat, charophytes were not considered in the calculations for this habitat. Their attributed features within the network, and the fact that they are macroorganisms, meant that they had disproportionately different values of these parameters and indices compared to the other benthic elements, and this would have

masked their response. Their sensitivity and effectiveness values were 0.13 ± 0.00, mean ± SE, for both parameters, considering all the networks, regardless of the environmental factor. Their Rt to UVR and to T was 0.5, 0.7, respectively, and their Rl to UVR and T was − 1.

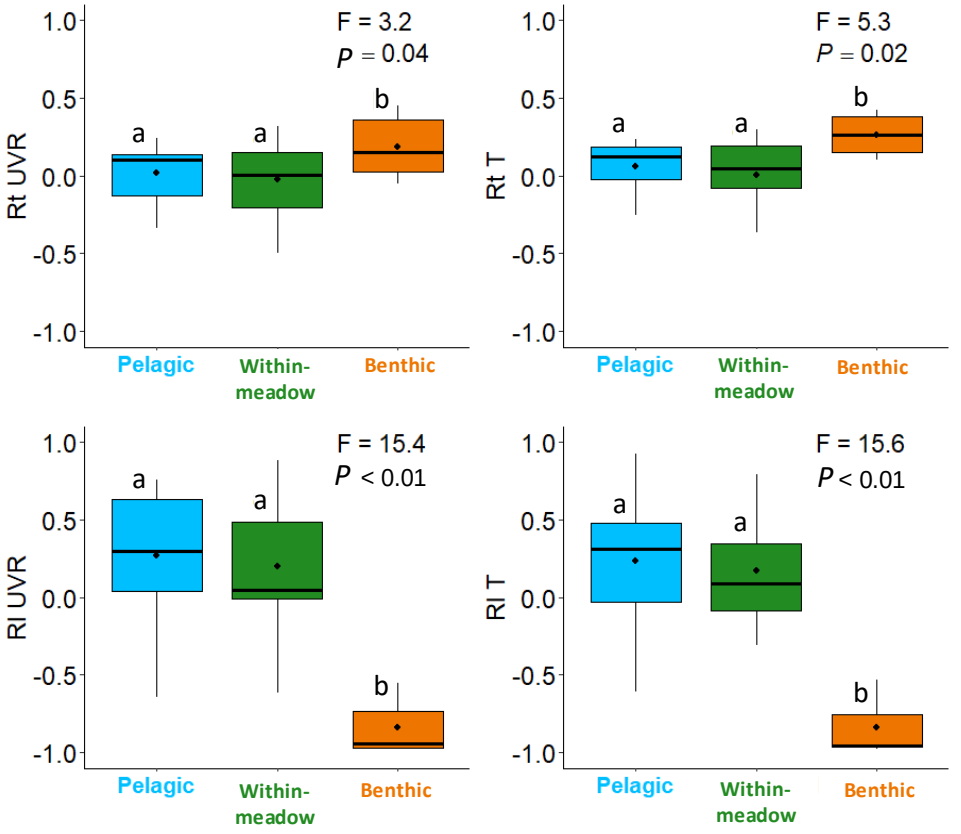


Fig. 8. Box-plot of resistance index (Rt) and resilience index (Rl) to a UVR increase (left column) and to a T increase (right column) of nodes in the networks according to the habitat they belong to (pelagic, within-meadow and benthic). Details of box-plot graphs as in Fig. 2. ANOVA F statistic and *P*-value are shown in each graph. Lower-case letters represent significant differences ($P < 0.05$) between habitats after the Tukey *post hoc* test.

4. Discussion

This study highlights the importance of addressing the performance of a shallow freshwater ecosystem facing GC-related scenarios considering different levels of complexity. The organisms (population level) respond differentially to environmental

changes. These responses are habitat-dependent (pelagic and within-meadow *vs* benthic) and lead to changes in the relative importance of the habitats within the system (habitat level), thus, culminating in a particular configuration of the whole ecosystem (ecosystem level).

4.1. Populations' responses to experimental scenarios

In our study, the response of populations to the tested environmental factors provided a contrasting set of "winners" and "losers" under the two disturbed scenarios (TUVR and +TPAR). The network approach is a useful tool that can shed light on these configurations. Under TUVR, the favoured organisms were the pelagic and within-meadow mixotrophs (cryptophytes and ciliates) and the benthic bacteria, while the harmed ones were, mainly, the charophytes and the pelagic large herbivores (cladocerans) and carnivores (copepods). However, in the warming scenario (+TPAR), the charophytes achieved the highest growth, and the zooplanktonic herbivores and the planktonic diatoms were also favoured. The damage to organisms at higher trophic levels (*e.g.* cladocerans and copepods) due to an oxidative stress by high UVR doses (Huebner *et al.*, 2006; Wolf & Heuschele, 2018) such as those under TUVR scenario, produced a lack of top-down effects favouring the proliferation of organisms at lower levels such as cryptophytes and ciliates, which are mixotrophs and well-adapted to increases in UVR (Rojo *et al.*, 2012; Domingues *et al.*, 2017; González-Olalla *et al.*, 2019). However, as large herbivores and copepods are favoured under the warming scenario (+TPAR), they exerted a trophic control over the basal species (Jeppesen *et al.*, 1997).

Furthermore, our results make evident the influence of non-trophic interactions over trophic relationships, with charophytes being pivotal (Rodrigo *et al.*, 2015; Puche *et al.*, 2020). Under the TUVR scenario, damage to planktonic cladocerans and copepods occurred only in the pelagic habitat, which indicates that those who inhabit the meadow habitat were "protected" against the UVR increase. It is known that submerged macrophytes provide refuge to zooplankton against predators (Jeppesen

et al., 1998; Hampton *et al.*, 2000; Rodrigo *et al.*, 2015). However, we suggest an extension of the refuge effect offered by charophytes not only against predators, but also against adverse environmental conditions (*e.g.* high doses of UVR). Furthermore, under a UVR increase, these macroalgae are capable of synthesizing UVR-protecting compounds (rich in nitrogen and phosphorus), but this compromises their growth and morphology (Rubio *et al.*, 2015; Rojo *et al.*, 2019), even accelerating their decomposition (Måns *et al.*, 1998; Bastidas-Navarro *et al.*, 2009). It is likely that, due to their decomposition, these compounds, as well as other organic substances, are released and used as resources by benthic bacteria (Murray *et al.*, 1986; Belova, 1993) as their greater carbon biomass under the UVR-scenario suggests. In the case of warming (+TPAR), the higher growth of charophytes reinforced their allelopathic capacity against other primary producers (van Donk & van de Bund, 2002; Rojo *et al.*, 2013a,b) favouring phytoplankton dominated by small centric diatoms related to clear waters and higher temperatures in wetlands, as Izaguirre *et al.* (2004) reported.

4.2. Implications for multi-interaction network structure

The reciprocal influence between the populations differentially responding to changes in GC-related factors, and the interactions established among these populations, imply alterations in the network structure and in the relative importance of the habitats. Networks under the TUVR scenario significantly lost nodes and links compared to the other scenarios (*i.e.* the network became smaller) although the connectance remained unaltered. Connectance is theoretically related to the complexity and persistence of species in a community (Dunne *et al.*, 2002), and it has been considered to be sensitive to a small network size (Russo *et al.*, 2013) in trophic networks. In our case, the lack of effect on the connectance, despite losing nodes and links under TUVR, could be attributed to the non-trophic interactions centralized by charophytes that would be buffering the loss of nodes involved in trophic interactions, as suggested by Kéfi *et al.* (2015).

Other structural parameters such as modularity and nestedness have recently been related to the complexity and stability (in terms of proportion of persisting species under equilibrium) of networks, although with different results depending on the type of network (Bascompte & Stouffer, 2009; Thébault & Fontaine, 2010; Fortuna *et al.*, 2010). The increase in nestedness and/or decrease in modularity enhances the stability of mutualistic networks, while the opposite promotes stability in trophic networks (Thébault & Fontaine, 2010). Furthermore, Kéfi *et al.* (2015) showed variations in nestedness and modularity of a natural network when considering different types of interactions. Our multi-interaction networks include both trophic and non-trophic interactions, being half-way between the trophic and mutualistic networks; thus, a different pattern would be expected. In fact, in our study modularity did not change among scenarios, but the nestedness of networks under the warming scenario (+TPAR) was the highest. Furthermore, in this scenario the greatest biomass of the nodes of generalist herbivores (*e.g.* cladocerans) are achieved, while this node was lost in the UVR-scenario. These results agree with the idea that nestedness in ecological networks is typically acquired by the presence of generalists and specialists, the interactions of the latter being a subset of those of the former, reducing effective interspecific competition and enhancing the number of coexisting species (Nielsen & Bascompte, 2007; Bastolla *et al.*, 2009). Moreover, in this warming scenario the connector role of the meadow-related herbivores emerged in the structural analysis of the network at a node-scale. The emergence of this role was stated by Puche *et al.* (2020), and was considered as highly important for the structure of these networks, as it represents a coupling among the habitats defined in these systems. Here we are able to add that this structurally important role is environment-dependent and favoured by climate warming.

4.3. Implications for community responses (nodes' influence, resistance and resilience)

Therefore, the community performances, under the tested environmental scenarios transferred to a network perspective, demonstrate changes in the relative importance of the different habitats in these systems. Due to the morphometric features of shallow freshwater ecosystems, the free-water habitats (pelagic and, mainly, within-meadow) and the benthic habitat are highly coupled (Verspagen *et al.*, 2005; Rautio & Vincent, 2006). This coupling is more pronounced with the presence of dense macrophyte meadows which act as a bridge between these habitats (Carpenter & Lodge, 1986; Celewicz-Gołdyn & Kuczyńska-Kippen, 2017; Rojo *et al.*, 2017). With the network approach (*i.e.* considering the connections among the nodes), we found that the within-meadow and benthic nodes turned out to be those with the highest capacity to affect, and be affected, by disturbances in other nodes of the network (*i.e.* they have, on average, the highest sensitivity and effectiveness), thus placing themselves in a central position in the multi-interaction network. Furthermore, when considering their resistance and resilience indices (in terms of biomass changes) when faced with the tested environmental disturbances, the benthic nodes appeared to be the most capable of coping with the disturbances (highest resistance) and had the lowest resilience. This could be related to the difference in the scale of the ecological processes occurring in this habitat compared to those in the free-water habitats (Raffaelli *et al.*, 2003). Therefore, combining the high influence of within-meadow and benthic nodes on the network with their different level of resistance against changes in environmental factors, we highlight the decisive importance of the macrophyte meadows and the elements tightly coupled with them (*i.e.* within-meadow and benthic habitats; Carpenter & Lodge, 1986; Vadeboncoeur & Steinman, 2002; Rodrigo *et al.*, 2015; Puche *et al.*, 2020) when facing changes in stressors related to GC.

4.4. Whole-community configurations under environmental scenarios

Gathering these results, and considering the whole system (*i.e.* wrapping-up the habitat-dependent populations' responses under the umbrella of the multi-interaction network), two markedly differentiated configurations were observed between the disturbed scenarios: a phytoplankton-dominance configuration under TUVR, and a macrophyte-dominance configuration under +TPAR. From a control scenario, the disturbances imposed by changes in GC-related factors (UVR and T) led to the achievement of one or another configuration that pivoted on the macrophyte meadows and the community associated with them. These pivoting configurations bring to mind the alternative states of shallow freshwater ecosystems (Scheffer *et al.*, 1993), and support the central position assigned to macrophytes in these shifts (Su *et al.*, 2019).

Conclusions

The performance of ecosystems facing GC is based on the differential capacity of the populations to respond to changes in the environment, these responses being contingent on their planktonic or benthic nature. Therefore, the inter-habitat connections are affected, modifying their relative importance within the ecosystem. These forces led the community of a reproduced freshwater shallow ecosystem towards contrasting configurations, depending on whether it faced enhanced UVR or a temperature increase in the environment. The macrophyte meadows, and their associated community, are pivotal in the achievement of one or another configuration.

We attempt to strengthen the importance of the complex set of interactions (trophic and non-trophic) and the relationship between different habitats, which occur in shallow freshwater ecosystems. Furthermore, we encourage their study through a multi-interaction network perspective, linked to mesocosm experimentation. This design, as a methodological combination, improves the understanding of the structure-function relationships of these valuable and threatened ecosystems, and

offers potentially transferable results to the real world. We also strongly advocate the combination of single- or few-species experiments, combined with this whole-community approach to delve deeply into the mechanisms by which environmental disturbances spread through the community. Furthermore, our results open the door for future research to tackle the interactive effect of GC-related factors on the response of shallow freshwater communities.

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|CHAPTER 6|

Non-trophic key players in aquatic ecosystems: a mesocosm experiment

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Research

Non-trophic key players in aquatic ecosystems: a mesocosm experiment

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The trophic network (TN) has been well stablished, and recently knowledge concerning non-trophic relationships (NTRs) is receiving increasing attention. Although NTRs can influence trophic ones, network models, including both types of interactions (multi-interaction network, IN) and changes in the role of nodes when NTRs are added to TN, are scarce. To evaluate the role of NTRs in freshwater shallow ecosystems, where these interactions are relevant mainly in the benthic habitat, we constructed, from the same communities, the two mentioned networks and compared them focusing on the nodes' topological roles. Our approach is based on empirical data from a mesocosm experiment where aquatic communities inhabited coupled habitats (pelagic, within-meadow and benthic) under three environmental scenarios: warming, increased ultraviolet radiation, plus control conditions. The experiment allowed us to assess: the topological roles of the nodes from different habitats when NTRs were added to the TN, and the relative impact of adding NTRs according to environmental scenarios. We calculated a set of node indices by considering both direct and indirect connections up to an ecologically meaningful number of steps. Our results highlight significant differences in the nodes' roles between both network versions. When NTRs were added: i) pelagic nodes lost relevance in the network; ii) the number of within-meadow relevant nodes increased and iii) the large benthic consumers in TN were substituted by charophytes, plus a chain of small within-meadow predators/preys, as the most relevant to the IN. Furthermore, the scenarios modulated changes in the nodes' roles when including NTRs. The warming scenario promotes the central position of some nodes (e.g. charophytes) and harms others (e.g. benthic cladocerans), and UVR modulates changes in benthic filamentous primary producers' roles. Therefore, the inclusion of NTRs in ecological models seems crucial to better understand the functioning of complex communities and their response to environmental disturbances.

Keywords: centrality, food web, global change, mesoscale indices, multi-interaction network, non-trophic effects

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Abstract

The trophic network (TN) has been well stablished, and recently knowledge concerning non-trophic relationships (NTRs) is receiving increasing attention. Although NTRs can influence trophic ones, network models, including both types of interactions (multi-interaction network, IN) and changes in the role of nodes when NTRs are added to TN, are scarce. To evaluate the role of NTRs in freshwater shallow ecosystems, where these interactions are relevant mainly in the benthic habitat, we constructed, from the same communities, the two mentioned networks and compared them focusing on the nodes' topological roles. Our approach is based on empirical data from a mesocosm experiment where aquatic communities inhabited coupled habitats (pelagic, within-meadow and benthic) under three environmental scenarios: warming, increased ultraviolet radiation, plus control conditions. The experiment allowed us to assess: the topological roles of the nodes from different habitats when NTRs were added to the TN, and the relative impact of adding NTRs according to environmental scenarios. We calculated a set of node indices by considering both direct and indirect connections up to an ecologically meaningful number of steps. Our results highlight significant differences in the nodes' roles between both network versions. When NTRs were added: i) pelagic nodes lost relevance in the network; ii) the number of within-meadow relevant nodes increased and iii) the large benthic consumers in TN were substituted by charophytes, plus a chain of small within-meadow predators/preys, as the most relevant to the IN. Furthermore, the scenarios modulated changes in the nodes' roles when including NTRs. The warming scenario promotes the central position of some nodes (*e.g.* charophytes) and harms others (*e.g.* benthic cladocerans), and UVR modulates changes in benthic filamentous primary producers' roles. Therefore, the inclusion of NTRs in ecological models seems crucial to better understand the functioning of complex communities and their response to environmental disturbances.

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Resum

*La xarxa tròfica (XT) ha estat ben establida i, recentment, el coneixement respecte a les relacions no-tròfiques (RNT) està rebent cada vegada més atenció. Encara que les RNT poden influir les relacions tròfiques, els models de xarxa, incloent ambdós tipus d'interaccions (xarxa multi-interacció, XI) així com els canvis en els rols dels nodes quan les RNT són incorporades a la XT, són escassos. Per tal d'avaluar el rol de les RNT en ecosistemes aquàtics continentals somers, on aquestes interaccions són rellevants principalment en l'hàbitat bentònic, nosaltres vam construir, per a les mateixes comunitats, el dos tipus de xarxa mencionats i els vam comparar basant-nos en els rols topològics dels nodes. La nostra aproximació es basa en dades empíriques a partir d'un experiment a escala de mesocosmos on comunitats aquàtiques ocupaven hàbitats acoblats (pelàgic, entre-pradera i bentònic) sota tres escenaris ambientals: escalfament, increment de la radiació ultraviolada (RUV) i un escenari control. L'experiment ens va permetre avaluar: els rols topològics dels nodes en els diferents hàbitats quan les RNT foren afegides a la XT, i l'impacte relatiu d'afegir RNT depenent de l'escenari ambiental. Vam calcular un conjunt d'índexs de nodes que consideren les connexions directes i indirectes fins a un nombre de passos amb un sentit ecològic. Els nostres resultats remarquen diferències significatives en els rols dels nodes entre les dues versions de la xarxa. Quan les RNT foren afegides: i) els nodes pelàgics perderen la rellevància en la xarxa; ii) els consumidors bentònics grans en la XT foren substituïts pels caròfits juntament amb la cadena de depredadors-preses menuts de l'hàbitat entre-pradera, com a nodes més rellevants en la XI. A més, els escenaris modularen els canvis en els rols dels nodes quan s'inclogueren les RNT. L'escenari d'escalfament promou la posició central d'alguns nodes (*e.g. caròfits*) i perjudica a altres (*e.g. cladòcers bentònics*), i l'escenari RUV modula els canvis en els rols dels productors primaris filamentosos bentònics. Per tant, la inclusió de les RNT en els models ecològics sembla crucial per a entendre millor el funcionament de comunitats complexes així com la seua resposta a les pertorbacions ambientals.*

Paraules clau: *centralitat; xarxa tròfica; canvi global; índexs mesoescala; xarxa multi-interacció; efectes no-tròfics*

1. Introduction

Food webs, representing networks of trophic interactions, have been traditionally used as a powerful tool to depict the complexity of ecosystems by means of predator-prey interactions among the coexisting species in a community (Paine 1980, Pimm *et al.* 1991, Berlow *et al.* 2004). Furthermore, over the past decade there has been a growing interest in ecological networks of non-trophic relationships (NTRs), such as host-parasitoid or plant-pollinator networks (Jordán *et al.* 2003, Ramos-Jiliberto *et al.* 2020). In fact, species are immersed in an intricate array of direct and indirect interactions of both trophic and non-trophic nature (Bascompte *et al.* 2003, Ings *et al.* 2009, Melián *et al.* 2009, Pocock *et al.* 2012). Some species can promote or prevent the presence of others through diverse non-trophic mechanisms, such as mutualism (Fortuna and Bascompte 2006, Fath 2007), facilitation (Borst *et al.* 2018) and allelopathy (Rojo *et al.* 2013a, b). Although these interactions play roles as crucial as the trophic ones, they have been largely ignored, or under-emphasized, in a wide variety of ecosystems (Pocock *et al.* 2012). This bias could be attributed to the difficulty of direct observation of the NTRs and the lack of a common currency between them and the trophic ones; their incorporation into trophic models is a challenge that researchers must address (Vasas and Jordán 2006, Majdi *et al.* 2013, Zhao *et al.* 2016).

In aquatic ecosystems, attempts to include different types of interactions in a network are still rare. Among the few attempts, Kéfi *et al.* (2015) assessed how NTRs are mapped onto the trophic network (TN) of an intertidal ecosystem. Also, Puche *et al.* (2020a) established the multi-interaction network (IN) model of an experimental shallow freshwater ecosystem with submerged macrophytes, testing their effect, mainly due to their NTRs, on the structure and vulnerability of the whole network.

In fact, shallow freshwater ecosystems have a high structural complexity (Tokeshi and Arakaki 2012) with both planktonic and benthic habitats being highly coupled, due to the presence of dense meadows of submerged macrophytes (Søndergaard *et al.* 2005). Planktonic-benthic connections, both trophic and non-trophic (Vadeboncoeur

and Steinman 2002, Vadeboncoeur *et al.* 2002), are able to modulate the top-down and bottom-up effects (Vasconcelos *et al.* 2018). Furthermore, it is in this context of a heterogeneous system (*i.e.* with different coupled habitats), comprising of different types of relationships among its elements, where the IN approach seems to be decisive (Puche *et al.* 2020a).

The distribution of NTRs is neither random nor uniform, but typically centralized around certain species (Kéfi *et al.* 2015, Puche *et al.* 2020a) which, in addition, usually have few trophic interactions (Jordán *et al.* 2006, Kéfi *et al.* 2012). The NTRs may connect species both horizontally, at the same trophic level (*e.g.* allelopathy among primary producers), and vertically, species at different trophic levels (*e.g.* refuge provided by macrophytes to zooplanktonic herbivores). The inclusion of NTRs will increase the presence of nodes with this centralized character of multidirectional interactions in the network. These sets of interactions in all directions, and the topologically central nodes, seem to be the most influential in the network (Kéfi *et al.* 2015), making it more redundant and strongly determining its dynamics and stability in response to environmental changes (Vasas and Jordán 2006, Jordán and Osváth 2009, Martín-González *et al.* 2010, Kéfi *et al.* 2016).

The responses to environmental stresses, such as those driven by global change, are species-specific and must be dealt with in a network context to understand their effects on the whole community (Sala *et al.* 2000, Steffen *et al.* 2004). These differential effects could be related to the degree of the trophic or non-trophic role of a node in the network (Kéfi *et al.* 2015), and will have implications concerning how disturbances propagate through the community (Krause *et al.* 2003, Memmot *et al.* 2004, Fortuna and Bascompte 2006). In a previous study (Puche *et al.* 2020b), we experimentally assessed how different disturbed scenarios (warming and increased ultraviolet radiation, UVR) modified the IN of shallow macrophyte-dominated freshwater communities in a mesocosm experiment. That experiment allowed us to state that this response to disturbances depended on nodes (functional groups from

bacteria to macroinvertebrates) and habitats (pelagic, within-meadow and benthic). The results highlighted that, for example, warming increased the size of the networks, their nestedness and favoured the connector role of large zooplanktonic herbivores between pelagic and within-meadow habitats. The nodes from the within-meadow and benthic habitats were highly influential for the whole network, regardless of the scenario, and the benthic nodes were the most resistant to both disturbances. The macrophyte meadows and the community linked to them were pivotal in the achievement of contrasting configurations (phytoplankton-dominance *versus* macrophyte-dominance) under the disturbed scenarios.

Related to this, the question that now arises is the particular role of NTRs in the responses to the stressors. Here, we want to answer this question and, based on the same mesocosm experiment, we compare the different topological roles of nodes between TN and IN in different environmental conditions. To assess the relevance of the topological function of each node in the network, we calculated a set of node-topological-importance indices which give information about the nodes' connections with others in the network, their sensitivity to changes in other nodes and their capacity to affect others. Some of these indices provide a mesoscale perspective, by considering not only the direct connections of a node, but also the indirect effects up to an ecologically meaningful path length (Yodzis *et al.* 2000, Williams *et al.* 2002, Jordán *et al.* 2006, 2019).

Therefore, in this study we specifically aim to assess that: 1) there are changes in the relative topological importance of the nodes in a reproduced shallow freshwater system dominated by macrophytes, when NTRs are taken into account and added to the TN, and that 2) the environmentally disturbed conditions can modulate the non-trophic effects. In addition, as corollaries, we would expect that the incorporation of NTRs would reduce the importance in the network of the nodes that were only considered as predators or prey; for instance, a lower effect of herbivory, the main basis of the relationships in TN, in the IN. At the same time, habitats related to

macrophyte meadows (*i.e.* within-meadow and benthic habitats) will host the topologically central nodes of the community. The inclusion of multidirectional relationships will make the IN more connected and accessible than the TN was. Furthermore, we expect that differential positive (warming) and negative (increasing UVR) factors will mainly affect primary producers which are the main contributors to NTRs, modifying these relationships, and hence their effect on TN.

2. Material and methods

2.1 Experimental design

The experiment consisted of twelve mesocosms (capacity 170 l) which we set up in the aquarium facilities of the Central Service for Experimental Research belonging to the University of València (Spain), to reproduce shallow freshwater ecosystems dominated by charophyte (submerged macrophytes) meadows (Fig. 1a). We planted bundles of charophytes, sourced from a coastal lagoon, in one half of the mesocosm over a sediment layer (a mixture of artificial substrate and natural sediment) and filled the mesocosms with tap water, plus an inoculum of water from the same lagoon (Puche *et al.* 2020a). Thus, planktonic and periphytic communities (with organisms living in the free-water and attached to charophytes, respectively) were established (Fig. 1a). Three habitats were defined: the pelagic, consisting of organisms in the free-water, in the half without charophytes; the within-meadow, which is made up of planktonic organisms highly associated or living within the charophyte meadows; and the benthic, composed of the charophytes themselves, and all the living periphytic organisms attached to their surface (Fig. 1a).

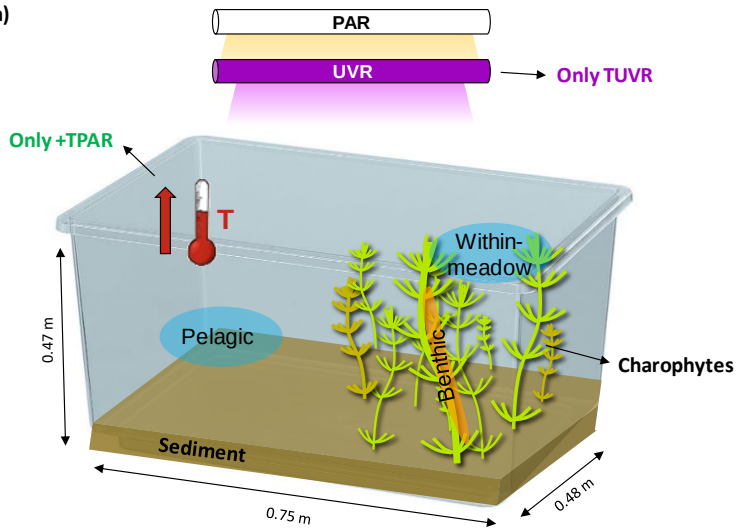
Four mesocosms (replicates) were set up for three experimental scenarios, with temperature (T) and ultraviolet radiation (UVR) as the tested factors (Fig. 1b). The scenario called TPAR was considered as the control and consisted of a water temperature of 22 ˚C and only photosynthetically active radiation provided (PAR; Fig. 1b). The scenario called TUVR used the same temperature and PAR, but a high dose of

UVR was added (Fig. 1b). This was a stressful increase in the ratio of UVR per dose of PAR, found typically in very shallow Mediterranean freshwater ecosystems (Rojo *et al.* 2012). The scenario called +TPAR consisted of supplying only PAR, but increasing the water temperature by 4˚C (26˚C) in accordance with the expected increase in temperature for the Mediterranean region by the end of this century (IPCC 2014; Fig. 1b). For radiation, Sylvania Gro-Lux F58W fluorescent tubes provided the PAR doses. In the TUVR scenario, the supply of UVR was provided by Philips Cleo 40W tubes (for UVAR) and Philips TL40W/12 RS SLV tubes (for UVBR). These UVR tubes were covered by an Ultraphan 295 filter (Digefra GmbH, Munich, Germany) to completely remove the UVCR. All the tubes were placed at the top of each mesocosm. To achieve the desired temperature in +TPAR scenario, we placed aquarium heaters in the mesocosms (Eheim Jäger 50W for 1000 l). The temperature in the other scenarios (22˚C) was the result of the room temperature plus the heat provided by the radiation tubes. The mesocosms were maintained under the corresponding environmental conditions in a light:dark cycle of 14:10 h. The experiment lasted two months, and we carried out periodic measurements of the experimental conditions to control possible deviations. We also tested the independence of conditions between scenarios (Puche *et al.* 2020b).

2.2. Biological sampling and network construction

At the end of the experiment, we performed a sampling for planktonic and benthic autotrophs (phytoplankton/phytobenthos, cyanobacteria and charophytes) and heterotrophs (heterotrophic bacteria, zooplankton/zoobenthos and gastropods). All these organisms were identified at the highest possible taxonomic resolution to better include the populations in the different nodes. More information about the composition of the experimental communities is available in Puche *et al.* (2020b).

(a)



(b)

		TUVR	TPAR	+TPAR
Temperature	°C	22	22	26
Radiation*				
PAR	W m^{-2}	12	12	12
UVAR	W m^{-2}	1.3	-	-
UVBR	W m^{-2}	0.4	-	-
UVR (UVAR+UVBR)	W m^{-2}	1.7	-	-

* Average underwater dose from measurements at depths of 0.5, 15 and 25 cm

Fig. 1. (a) Scheme of an experimental mesocosm with the three considered habitats (pelagic, within-meadow and benthic) and the conditions imposed by the environmental scenarios, showing the increase in temperature (T) in +TPAR and the supply of ultraviolet radiation (UVR) in TUVR. Dimensions of the mesocosm are provided. b) Summary of temperature and radiation (photosynthetically active radiation, PAR and ultraviolet radiation both A, UVAR and B, UVBR) conditions in the three experimental scenarios (TUVR, TPAR and +TPAR).

We aggregated the sampled organisms in a total of 41 nodes to construct the networks (Table 1, Table S1 Supplementary material Chapter 6) by means of taxonomic and functional criteria (see Puche *et al.* 2020a). Then, we constructed two versions of the network in each mesocosm (Fig. 2): 1) the trophic network (TN), only considering trophic links among the nodes, and 2) the multi-interaction network (IN), merging trophic and non-trophic links in the same network. The latter version of the network comprised of several types of NTRs: allelopathy among primary producers, organic

exudates as a resource from cyanobacteria to heterotrophic bacteria, the shading effects of phytoplankton, refuge and vital support provided by macrophytes, and the cleaning effect from zoobenthos over macrophytes (Puche *et al.* 2020a).

The set of nodes and links in each version of the network were embodied in a binary SxS matrix of interactions A, where S is the number of nodes and each element a_{ij} represents the ecological interaction between two nodes (Cohen 1978). In order to facilitate the calculations of the topological indices used (explained below), the matrices were symmetrized (*i.e.* considered undirected). Thus, in TN, 1 means a trophic link between two nodes (one node preys on or is prey to the other), and in IN, 1 means that two nodes are connected by trophic link, or by either a positive non-trophic link (*e.g.* refuge) or a negative non-trophic link (*e.g.* allelopathy). The absence of interactions between two nodes was coded as 0.

2.3. Topological importance (TI) and topological overlap (TO) indices

The topological importance index (TI) was based on that of Müller *et al.* (1999) for two-step-long apparent competition in host-parasitoid communities, and later generalized for indirect effects of *n* steps by Jordán *et al.* (2003). Consider that *i* and *j* are connected, so the direct effect of *i* on *j* (a_{ij}) is:

$$a_{ij} = 1/D_j$$

where D_j is the degree of *j* (the number of direct neighbours). So, if *i* is the only neighbour of *j*, its effect will be the maximum value, but if *j* has more neighbours the effect of *i* will be only a proportion of this maximum value. We can put this direct effect between all pairs of nodes in a matrix A, and generalize it to an *n*-steps effect just by calculating A^n. As different paths of different lengths between two nodes may exist, we can calculate the effects of node *i* on *j*, up to a defined number of steps, and then average them over the maximum number of steps considered (*i.e. n*):

$$AE_{n,ij} = \frac{1}{n}\left(A_{ij} + A_{ij}^2 + A_{ij}^3 + \cdots A_{ij}^n\right)$$

Table 1. List of the nodes defined in the networks. The name of the nodes, as well as their abbreviation and the compartment in the network they belong to, are provided.

Abbrev.	Node
Pelagic habitat	
B_p	Bacteria
ClU_p	Unicellular chlorophytes
ClC_p	Colonial chlorophytes
DS_p	Small diatoms
DB_p	Big diatoms
Cr_p	Cryptophytes
CiC_p	Colonial cyanobacteria
CiF_p	Filamentous cyanobacteria
Cil_p	Ciliates
R_p	Rotifers
C_p	Cladocerans
O_p	Ostracods
Cop_p	Copepodites
Co_p	Copepods
Within-meadow habitat	
B_m	Bacteria
ClU_m	Unicellular chlorophytes
ClC_m	Colonial chlorophytes
DS_m	Small diatoms
DB_m	Big diatoms
Cr_m	Cryptophytes
CiC_m	Colonial cyanobacteria
CiF_m	Filamentous cyanobacteria
Cil_m	Ciliates
R_m	Rotifers
C_m	Cladocerans
O_m	Ostracods
Cop_m	Copepodites
Co_m	Copepods
Benthic habitat	
B_b	Bacteria
ClF_b	Filamentous chlorophytes
DS_b	Small diatoms
DB_b	Big diatoms
CiC_b	Colonial cyanobacteria
CiF_b	Filamentous cyanobacteria
R_b	Rotifers
C_b	Cladocerans
O_b	Ostracods
Cop_b	Copepodites
Co_b	Copepods
$Char_b$	Charophytes
G_b	Gastropods

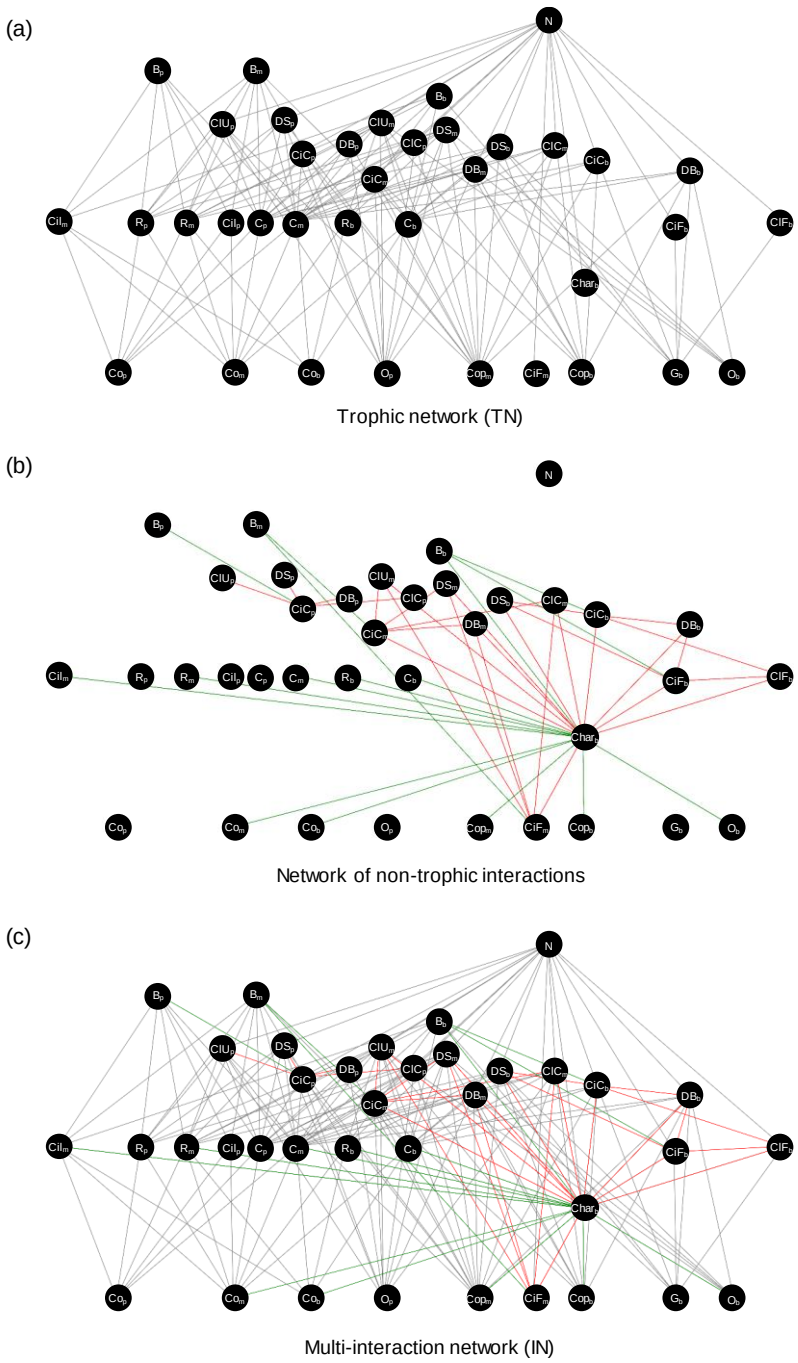


Fig. 2. Models of: a) trophic network (TN), b) non-trophic interactions and c) multi-interaction network (with trophic and non-trophic interactions together; IN). Black lines represent trophic links, red and green lines represent non-trophic negative and positive links, respectively. Each node is labelled with its abbreviation (for correspondence between abbreviations and the name of the node, see Table 1). Note that the nutrients node (N) is represented, although it was not considered for the calculations.

With this average effect of all pairs in the network, we can construct an interaction matrix IM_n, where the ij^{th} element is the $AE_{n,ij}$. Then the sum of the values in row i^{th} is the topological importance of *i*, as it is the sum of effects up to *n*-steps on the other nodes of the network.

With the IM_n matrix, we can assess the overlapping in the neighbours of two nodes, quantifying the uniqueness or redundancy of nodes (Jordán *et al.* 2009, Lai *et al.* 2012, 2015). We have to define the value of a threshold (t) and construct the AM_t matrix as follows: if $AE_{n,ij}>t$, then AM_{tij} is labelled as St (meaning "strong" interactor) and if $AE_{n,ij}<t$, then AM_{tij} is labelled as W (meaning "weak" interactor). Then, we focus on the i^{th} and the j^{th} rows and compare the number of St matches which indicate the overlap between *i* and *j* (TO_{tij}). We generalized this for all the pairs in the network, and constructed the TO_t matrix (we used a threshold t=0.02). Then, the sum of the i^{th} row in this matrix is the total overlap between node *i* and all other species in the network. The TI and TO values for each node were normalized by dividing the value by the sum of the values of the index of all the nodes in all the replicates.

The importance of an organism in the network is given by its condition of being central (they are connected to many others), or unique (they cannot be replaced by any other one; Jordán *et al.* 2003). High TI values clearly indicate central species. A high TO can be associated with a high TI (important for being central), and a low TO can really indicate unique positions (important for being non-replaceable). We calculated the TI and TO by using CoSBiLab Graph (Valentini and Jordán 2010).

2.4. Closeness and betweenness centrality (CC, BC)

The closeness centrality index (CC) measures the proximity of a node *i* to all other nodes in the network, quantifying how short the minimal path is between pairs of nodes (Freeman 1978). A node with a large CC_i is able to more rapidly affect others in the network (Vasas and Jordán 2006). The normalized CC_i is:

$$CC_i = \frac{S - 1}{\sum_{j=1}^{S} d_{ij}}$$

where S is the number of nodes and d_{ij} is the shortest path length between nodes *i* and *j*.

The betweenness centrality index (BC) is widely used in social network analysis (Wassermann and Faust 1994). It shows how central node *i* is in terms of being incident to many shortest paths in the network (*i.e.* this index is measuring the number of shortest paths between two nodes that pass through node *i*). If node *i* has a large BC_i, it means that this node is highly mediating the rapid spread of effects in the network (*i.e.* it has a high intermediation capacity; Vasas and Jordán 2006). The normalized BC_i is:

$$BC_i = 2\,x \sum_{j<k;i\neq j} \frac{g_{jk}(i) \,/\, g_{jk}}{(S-1)(S-2)}$$

where g_{jk} is the number of paths between nodes *j* and *k*, and $g_{jk}(i)$ is the number of these paths that include node *i*.

CC and BC were calculated by using UCINET (Borgatti *et al.* 2002).

2.5. Statistical analysis

We calculated the topological indices described above for each node in each of the constructed networks (2 types of network x 3 scenarios x 4 replicates). The nodes were ranked according to the values of the indices. In total, there were 24 ranks for each index. We performed Kendall rank correlations between TN and IN ranks for each index to detect significant relationships (Jordán *et al.* 2006, Table S2 Supplementary material Chapter 6). Furthermore, Pearson correlations were carried out among the values of all the pairs of indices to find possible covariance. After corroborating the fulfilment of normality and homoscedasticity, we carried out two-way ANOVAs to find significant

effects of the type of network, the scenario and the interaction between them on the indices' values of the nodes. We conducted all the statistical analyses using SPSS Statistics v.22 software (IBM Corp, Armonk, NY), considering statistically significant differences at $P < 0.05$.

3. Results

The rankings of nodes by scenario (averaging the four replicates) in trophic networks (TN) and in multi-interaction networks (IN) were not significantly correlated for any of the considered indices in any of the scenarios (Table 2). In other words, the role of the same node in TN and IN was significantly different whatever the topological index considered. These differences can be summarized by taking into account the top four nodes of each index (Table 3): when NTRs were added (IN), half of the top nodes changed, whatever the index. Large herbivores (generalist consumers such as benthic cladocerans of the genus *Simocephalus* and gastropods of genus *Physella*), which were top in TN ranks, were mainly replaced by charophytes and smaller organisms, both primary producers and herbivores, from the within-meadow habitat (Table 3). Charophytes, which were in a low position in the TN ranks, as they were only eaten by benthic gastropods (Fig. 2), became a top-ranking node in all the indices in IN, due to the allelopathic and refuge interactions they provide the network with. Small diatoms (*e.g. Cyclotella meneghiniana*) and colonial cyanobacteria (*e.g. Chroococcus* sp. that can allelopathically compete with other primary producers), as well as small herbivores such as rotifers (*e.g. Lecane* sp. and Bdelloidea) inhabiting the within-meadow habitat, which were underestimated in TN, emerged as top nodes in IN (Table 3, Fig. 2). Large herbivores (*e.g.* cladocerans) and the most edible microalgae (*e.g.* unicellular chlorophytes such as *Tetraedron minimum*) from the within-meadow habitat, continued to play an important role when including NTRs (Table 3). All these substitutions of nodes in the rank of role relevance implied that when NTRs were added: i) pelagic nodes lost relevance in the network; ii) the number of within-meadow top nodes in the ranking increased, and iii) the large benthic consumers in TN were

replaced by the charophytes, plus a chain of small predators and preys associated with its meadow, as the most relevant to the IN.

Table 2. Kendall rank correlation coefficients between the ranks of the indices of trophic and multi-interaction networks in the three experimental scenarios (TUVR, TPAR and +TPAR). Abbreviations of the indices: TI (topological importance index), TO (topological overlap index), CC (closeness centrality index) and BC (betweenness centrality index). None of the correlation coefficients were significant.

	TUVR	TPAR	+TPAR
TI	-0.15	0.11	-0.11
TO	-0.07	0.04	0.09
CC	0.08	0.10	-0.06
BC	0.00	0.05	0.04

Additionally, when comparing the nodes found in the control and disturbed scenarios, some differences were apparent. For instance, the exclusive presence of mixotrophs (*e.g.* cryptophytes and ciliates) or the disappearance of pelagic cladocerans and copepods in TUVR (Table S1). Furthermore, when comparing +TPAR with the control scenario, the main difference was the exclusive presence of planktonic diatoms under the warming scenario (Table S1). Thus, for some nodes, the changes in the ranking when NTRs were added, were different between scenarios. For example, regarding the overlapping (TO; Table 3), within-meadow rotifers were in the top four of the ranking under the UVR scenario, while within-meadow colonial cyanobacteria and small diatoms ranked at the top under both PAR scenarios when NTRs were added to the network. The CC ranking resulted more homogenous in IN whatever the scenario, due to a convergence in the substitution. With respect to this index, charophytes plus within-meadow small primary producers replaced the set of small diatoms and cladocerans from the benthic habitat (in low T scenarios, Table 3) as well as the set of small benthic diatoms (*e.g. Navicymbula pusilla* and *Navicula* sp.) and the within-meadow copepodites (in the high T scenario; Table 3).

Table 3. Top four nodes (highest values of each index) of the average ranking of trophic and multi-interaction networks in the three experimental scenarios (averaging the four replicates per scenario). Each node is shaded according to the habitat it belongs to. Abbreviations of nodes are provided. Abbreviations of indices and scenarios as in Table 1 and 2.

Trophic networks

Position ranking	TI			TO			CC			BC		
	TUVR	TPAR	+TPAR	TUVR	TPAR	+TPAR	TUVR	TPAR	+TPAR	TUVR	TPAR	+TPAR
1	G_b	G_b	C_m	C_m	C_b	C_m	C_m	C_m	C_m	G_b	C_m	G_b
2	C_m	C_m	G_b	C_b	C_m	C_b	DS_b	DS_b	DS_b	C_m	G_b	C_m
3	R_m	ClU_m	Cop_m	DS_b	ClU_m	Cop_m	R_m	ClU_m	Cop_m	DS_b	DS_b	DS_b
4	C_b	C_p	C_p	ClU_m	C_p	ClU_m	C_b	C_b	ClU_m	ClU_m	ClU_m	Cop_m

Multi-interaction networks

Position ranking	TI			TO			CC			BC		
	TUVR	TPAR	+TPAR	TUVR	TPAR	+TPAR	TUVR	TPAR	+TPAR	TUVR	TPAR	+TPAR
1	$Char_b$	$Char_b$	$Char_b$	C_m	C_m	C_m	$Char_b$	$Char_b$	$Char_b$	$Char_b$	$Char_b$	$Char_b$
2	C_m	C_m	C_m	$Char_b$	$Char_b$	$Char_b$	C_m	C_m	C_m	C_m	C_m	C_m
3	R_m	ClU_m	ClU_m	R_m	ClU_m	ClU_m	R_m	ClU_m	ClU_m	ClU_m	ClU_m	Cop_m
4	ClU_m	DS_b	DS_m	ClU_m	CiC_m	DS_m	ClU_m	CiC_m	DS_m	R_m	R_m	ClU_m

Nodes' abbreviations

Pelagic habitat	
C_p	Cladocerans

Within-meadow habitat	
C_m	Cladocerans
CiC_m	Colonial cyanobacteria
ClU_m	Unicellular chlorophytes
Cop_m	Copepodites
DS_m	Small diatoms
R_m	Rotifers

Benthic habitat	
C_b	Cladocerans
$Char_b$	Charophytes
DS_b	Small diatoms
G_b	Gastropods

These observed changes between types of network were modulated by the scenario not only for the top ranking ones (Table 4). With regard to nodes from different habitats, for the pelagic habitat nodes this interactive effect was mainly observed regarding the TO of primary producers (Table 4, Fig. S1 Supplementary material Chapter 6). The increase in the TO of these nodes (*e.g.* pelagic filamentous cyanobacteria) when NTRs were added (*e.g.* potential allelopathy of this group) was favoured by the warming scenario. In the within-meadow habitat, we did not observe any interactive effect for any index or, when this occurred, it was weak (Table 4, Fig. S1). For benthic nodes, the interaction scenario x type of network was more conspicuous for TI and TO, affecting filamentous primary producers such as chlorophytes (*Oedogonium* sp.) and cyanobacteria (*Pseudanabaena* sp.); for the latter, the BC was also modified. The value of these indices for these almost inedible nodes (there was only a trophic link with gastropods in TN) increased when NTRs were added (*e.g.* the previously mentioned allelopathic effects from cyanobacteria to other benthic primary producers such as filamentous chlorophytes or the organic compounds they release for benthic bacteria). This increase was sharper under TUVR (Table 4, Fig. S1). Another change enhanced by UVR, and also in the benthic habitat, was the increase in the charophytes' intermediary capacity (BC), when NTRs were added (compared to TN; Table 4, Fig. S1).

The warming scenario interacted on a greater number of changes (Fig. S1). For the benthic habitat, both, the TO increase in the charophyte node and the TO decrease of the cladoceran node, were enhanced (Table 4, Fig. S1). Regarding the small benthic diatoms and small colonial cyanobacteria, they lost BC when NTRs were added. They are prey for both within-meadow and benthic consumers; this implies a connector role between these two habitats of the network through trophic mechanisms. This intermediary capacity decreased when NTRs were added and several non-trophic ways connected these two habitats. This loss was sharper under the warming scenario (Table 4, Fig. S1).

Averaging the replicates of all the scenarios, and considering the nodes with a significant type of network effect, we observed a significant increase in the values of all indices when the NTRs were added (factor network type; Table 4). CC and TO were the indices that, on average, increased the most between TN and IN (25% and 29%, respectively) while the BC and TI had a smaller increase (7% and 1%, respectively; Fig. S1). Intentionally, these percentages exclude the results concerning charophytes as this node, which incorporates the majority of NTRs, increased the values of all the indices disproportionately compared to the other nodes in the network.

4. Discussion

In our study, we highlight, with the different topological indices applied and considering both direct and indirect interactions among nodes, the relevance of NTRs on the network structure. We corroborate the importance of taking into account both trophic relationships and NTRs to better understand the roles of the nodes from aquatic communities facing current global change.

Our results confirm that the incorporation of NTRs into a trophic network completely changes the topological importance of the nodes (our first hypothesis). The inclusion of NTRs is known to generate a heterogeneous distribution of node connections, with highly-connected and poorly-connected nodes (Kéfi *et al.* 2012) and we have corroborated this in our study. But also, the IN (*i.e.* the most realistic network) shows, in general, higher values of topological and centrality indices, becoming more connected and accessible (Vasas and Jordán 2006, Kéfi *et al.* 2016). These new enhanced properties would suggest aquatic communities with a greater stability (Jordán and Osváth 2009, Martín-González *et al.* 2010, Kéfi *et al.* 2016) in the face of the foreseeable environmental disturbances related to global change.

Table 4. Summary of the two-way ANOVA results. For each node of the networks, the significant effect of the Scenario with three levels (TUVR, TPAR and +TPAR), the Type of network with two levels (TN and IN) and the interaction "Scenario x Type of network" for each index is marked with a cross. Nodes in which there is a significant effect of interaction on any of the indices are shaded grey. Abbreviations as in Tables 1 and 2.

		TI			TO			CC			BC		
		Scenario	Type of network	Scenario x Type of network	Scenario	Type of network	Scenario x Type of network	Scenario	Type of network	Scenario x Type of network	Scenario	Type of network	Scenario x Type of network
Pelagic habitat	B_p	X			X		X		X		X		
	ClU_p				X	X		X	X				
	$ClCp$				X		X		X			X	
	DS_p^1					X			X				
	DB_p^1								X			X	
	CiC_p^2		X	X		X			X		X	X	
	CiF_p		X		X	X	X		X	X		X	
	Cr_p^3								X				
	Cil_p^3		X			X			X				
	R_p	X	X			X			X		X		
	C_p^2		X		X	X		X	X			X	
	O_p	X	X					X	X		X		
	Cop_p	X	X		X	X		X	X		X		
	Co_p^4		X			X			X			X	
Within-meadow habitat	B_m	X	X		X				X		X		
	ClU_m				X			X	X		X		
	ClC_m	X			X			X	X		X		
	DS_m^1								X				
	DB_m^1								X				
	CiC_m	X	X	X		X		X	X		X	X	
	CiF_m		X		X	X			X			X	
	Cr_m^3								X				
	Cil_m	X			X			X	X	X	X	X	
	R_m	X	X		X			X	X		X	X	
	C_m		X		X	X			X		X	X	
	O_m^1		X						X				
	Cop_m	X	X		X			X	X		X	X	
	Co_m	X	X		X	X			X		X		

Table 4. continuation.

		TI			TO			CC			BC		
		Scenario	Type of network	Scenario x Type of network	Scenario	Type of network	Scenario x Type of network	Scenario	Type of network	Scenario x Type of network	Scenario	Type of network	Scenario x Type of network
Benthic habitat	B_b[3]		X						X			X	
	ClF_b	X	X	X	X	X	X	X	X			X	
	DS_b		X		X	X			X		X	X	X
	DB_b				X				X		X	X	X
	CiC_b	X	X		X	X		X	X		X	X	X
	CiF_b	X	X	X	X	X	X	X	X		X	X	X
	R_b	X	X		X			X	X		X	X	
	C_b	X	X			X	X		X		X	X	
	O_b		X						X			X	
	Cop_b	X	X			X			X		X	X	
	Co_b	X	X		X	X		X	X			X	
	$Char_b$		X		X	X	X	X	X			X	X
	G_b	X	X		X			X	X			X	

[1] This node is only in +TPAR networks
[2] This node is only in TPAR and +TPAR networks
[3] This node is only in TUVR networks
[4] This node is only in TPAR networks

In the aquatic TN, the zooplanktonic and zoobenthic top herbivores (such as cladocerans, copepodites and gastropods) stood out as the most influential players, with the greatest capacity of spreading their effects through the community, by means of direct and indirect connections with the other elements, supporting the relevance of top-down control (Sommer and Stibor 2002, Sommer and Sommer 2006). However, when NTRs were incorporated into the models, other players such as charophytes emerged as highly-connected nodes (*sensu* Kéfi *et al.* 2012), scaling up to the top positions of importance ranks. If there are "non-trophic ways" connecting the nodes in the network, the intermediary capacity of some of them, linking elements by trophic mechanisms, can be diluted, hence losing their alleged capacity to transmit impacts through the network (Vasas and Jordán 2006). This alteration of the overestimated top-down control by means of NTRs has been recently addressed, for example, in some terrestrial ecosystems (Miyashita and Niwa 2006, Kalinkat *et al.* 2013), in aquatic detritus-based food web ecosystems (Majdi *et al.* 2013), and in the recovery of sea otters (Moxley *et al.* 2019). Therefore, we concur the demand for more complex and realistic models that has been going on for a decade (Fontaine *et al.* 2011, Kéfi *et al.* 2012, Gsell *et al.* 2016).

Charophytes become a central element regarding their connections with other elements in the community thanks, for example, to their allelopathic capacity, and the provision of refuge against predators (van Donk and van de Bund 2002, Rojo *et al.* 2013a, Rodrigo *et al.* 2015). This fact is of great importance to the system because it explains the intermediary role of this node within the community that was observed in the IN, and its key role between different attained configurations of the community under disturbed environments (Puche *et al.* 2020a, b). In addition, other underestimated nodes emerged as relevant to the network, such as the members of the within-meadow autotrophic chain of small organisms, rather than the chain related to large herbivores mainly from the pelagic habitat. Other pelagic nodes of TN became poorly-connected (Kéfi *et al.* 2012) when NTRs were added. In fact, none of

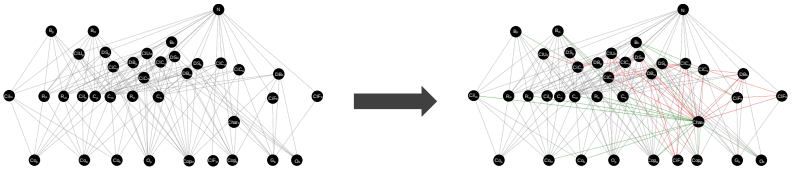
the pelagic nodes reached top positions in the topological indices' rankings. These changes occurring between the TN and the IN clearly suggest the overestimation of the pelagic habitat with respect to the rest of the ecosystem (within-meadow and benthic habitats; Vadeboncoeur *et al.* 2002).

Thus, considering the IN of shallow freshwater ecosystems with macrophyte meadows, the great relevance of these meadows and the habitats linked to them (within-meadow and benthic habitats) is revealed. Disentangling the relevance of these habitats within the whole network helps to understand the pivotal function of the macrophyte meadows that couples the pelagic and benthic habitats, enhancing the matter and energy flows from sediment to the water column (Schindler and Scheuerell 2002, Søndergaard *et al.* 2005). Moreover, the inclusion of NTRs has allowed the unravelling of the importance of the benthic habitat which, until recently, had been largely ignored. Over the past 20 years, there have been several studies which have praised its role in the functioning of aquatic ecosystems (Vadeboncoeur and Steinman 2002, Vadeboncoeur *et al.* 2002; Puche *et al.* 2020b). Under a network perspective, we demonstrated that benthic organisms are highly influential within the network, due to the connections they establish with other nodes, and their capacity to cope with environmental disturbances (Puche *et al.* 2020b). In this study, we have delved deeply into this relevance, comparing the roles of these nodes in both a trophic and a multi-interaction context, facing global change-related disturbances. These results are in accordance with the idea stated by Vadeboncoeur *et al.* (2002) of considering plankton-benthos coupling in aquatic ecosystems, to achieve a less skewed perception of the structure and functioning of these systems. We support this idea, and go further by calling for the incorporation of NTRs into the models, as they are a conspicuous fraction of the interactions occurring in aquatic systems which are being affected by changes in the environment, thus implying changes in their structure and functioning (Vasas and Jordán 2006, Zhao *et al.* 2016, Kéfi *et al.* 2012, Puche *et al.* 2020a, b).

Furthermore, the environmental disturbances to which the community is subjected (*i.e.* the environmental scenarios) modulated these changes in the topological roles of nodes between the TN and the IN (our second hypothesis). This fact was evident for some benthic primary producers, such as diatoms and colonial cyanobacteria, whose intermediary capacity (BC) in the IN (compared to the trophic network) decreased more under the warming scenario (Fig. 3). This could be explained, as mentioned before, by the presence of a myriad of NTRs between the benthic and planktonic habitats which reduced their relative BC value. They are prey for both within-meadow and benthic consumers, implying a connector role between these two habitats of the network through trophic mechanisms, but this was diluted when NTRs were added and several non-trophic ways connected these two habitats. Moreover, under the warming environment, charophytes increased their TO to a greater extent, and benthic small primary producers decreased it. However, these last populations (*i.e.* small primary producers such as colonial chlorophytes) in the pelagic habitat, where charophytes exert less influence, increased their TO, favoured by warming, at the same time that large herbivores decreased it. The warming scenario had a greater influence on the growth of primary producers than on the large consumers; this well-known fact was not only observed in the nodes' biomass (Puche *et al.* 2020b), but also in the connectivity of the network, since the favoured planktonic nodes (such as diatoms) are particularly edible by herbivores occupying a central position and highly influencing the IN.

Contrarily, the increase in importance of other benthic primary producers, such the inedible filamentous organisms (*e.g.* chlorophytes and cyanobacteria) when NTRs were considered, was favoured by the UVR scenario (Fig. 3). The value of topological indices for these almost inedible nodes (filamentous organisms only had a trophic link with gastropods in TN) increased when NTRs were added. These nodes are the main contributors of NTRs to the network, by means of different mechanisms such as allelopathy (Rojo *et al.* 2013a, b) which links cyanobacteria to other benthic primary

producers (*e.g.* filamentous chlorophytes). Moreover, cyanobacteria can release organic compounds for benthic bacteria, and this is another non-strictly trophic link (Kirkwood *et al.* 2006). This inevitably puts these benthic elements in a central position in the network. These changes in the network structure would be reflect the selective effect of UVR, with pelagic herbivores (*e.g.* cladocerans) being harmed (Huebner *et al.* 2006; Wolf and Heuschele 2018) and larger primary producers and mixotrophs (*e.g.* cryptophytes) being able to cope with the UVR (Rojo *et al.* 2012, Carrillo *et al.* 2017).



	TUVR	TPAR	+TPAR
Phytoplankton	=	=	+
Zooplankton	=	=	=
Phytobenthos	+	+	++
Zoobenthos	=	=	=
Benthic filamentous cyanobacteria	++	+	+
Macrophytes	++	++	++

Fig. 3. Summary of the changes which occurred in the topological importance of nodes (compiling the results of the considered indices) between the trophic network (TN) and the multi-interaction network (IN) in the three environmental scenarios (TUVR, TPAR and +TPAR). Nodes have been gathered into five large groups typically used in aquatic ecology: phytoplankton and zooplankton (primary producers and consumers in the free-water), phytobenthos and zoobenthos (primary producers and consumers attached to macrophytes surface), benthic filamentous cyanobacteria and macrophytes. The signs of the cells represent the relative amount of change between the network versions: ++ (large change), + (small change), = (no-change).

We thus highlight that with our approach, comparing TN and IN from the same complex communities, we can define sets of keystone species, based on different criteria which go beyond the charismatic, unique or trophic nature, namely, the need

to consider their capacity to generate habitats, to influence, in a non-trophic way, the other elements and their trophic relationships. Furthermore, we have experimentally confirmed that, facing environmental disturbances, the topological roles of nodes, and the connections of different habitats in shallow freshwater ecosystems, are differentially affected. Assessing the degree of trophic and non-trophic interactions in which the elements are involved has turned out to be decisive.

Speculations

The presence of submerged macrophyte meadows in shallow freshwater ecosystems forces us to conduct studies concerning the functioning of these systems, making use of a multi-interaction network approach (*i.e.* considering different types of interactions such as trophic and non-trophic ones). A lot of work has been done to explain the lack of evidence of top-down control (a mechanism related to the trophic chain), based on the amount of resource-nutrients in the system. Would it not be better to explain or unravel processes by adopting a multi-interaction network perspective? If we use this approach in the set of studied shallow lakes, we will be able to model not only a more realistic network, including both trophic and non-trophic agents and relationships, but it could also explain the modification or the real position of elements which were underestimated (such as those from the benthic habitat). We strongly believe that choosing this approach could allow us to understand the connection between the structure and function of these systems in a better way, rather than developing evidence of top-down/bottom-up control.

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|CHAPTER 7|

Habitat coupling mediated by the multi-interaction network linked to macrophyte meadows: ponds *versus* lakes

Puche, E., Rodrigo, M.A., Segura, M., Rojo, C. Habitat coupling mediated by the multi-interaction network linked to macrophyte meadows: ponds versus lakes. Submitted to *Aquatic Sciences*.

Abstract

Morphometric differences between ponds and lakes have implications in habitat-dimensioning and -coupling. The prevalence of pelagic over benthic habitats in lakes differs from ponds, where macrophytes dominate, offering both within-meadow free water and support for benthic organisms. We assessed four Mediterranean waterbodies (two ponds and two lakes) combining a model based on taxonomic composition with a functional perspective of habitat-coupling (*i.e.* multi-interaction network). Compositionally, the two habitats (benthic and within-meadow) emerged as coupled in both ponds, while in the lakes the highest similarity occurred between planktonic habitats (pelagic and within-meadow), with benthic habitats having exclusive populations. However, the network approach disentangled three functional modules in the ponds coupled by macrophytes, herbivores and mixotrophs: a microbial loop, an autotrophic food chain, and macrophytes hosting benthic microalgae. In the lakes, two disconnected modules emerged: the pelagic plankton plus the within-meadow herbivores, and the benthos plus the within-meadow primary producers. Topologically, within-meadow herbivores and small phytoplankton nodes were central in pond and lake networks. Furthermore, benthic nodes showed high functional redundancy and were highly influential for spreading the disturbances' effects. All these results point to two contrasting patterns of habitat-coupling between ponds and lakes, and highlight: i) the functional disaggregation in ponds despite the shared composition; ii) the importance of within-meadow organisms as connectors; iii) the relevance of benthos which has the greatest diversity, redundancy and also the most influential elements within a network, and iv) that the functional modules' coupling may be essential for the ecosystem's function and responsiveness to disturbances.

Keywords: benthos; charophytes; modularity; food web; plankton; topology

Resum

*Les diferències morfomètriques entre tolles i llacs tenen implicacions en les dimensions i l'acoblament entre hábitats. La prevalencia de l'hàbitat pelàgic sobre el bentònic en llacs difereix de la de les tolles, on els macròfits submergits dominen, oferint aigua lliure entre les praderes així com suport per als organismos bentònics. Nosaltres hem avaluat quatre sistemes aquàtics mediterranis (dos tolles i dos llacs) combinant un model basat en la composició taxonómica amb una perspectiva funcional d'acoblament entre hábitats (*i.e. *xarxa multi-interacció). Composicionalment, els dos hábitats (bentònic i entre pradera) van emergir com a acoblats en les dues tolles, mentre que en els llacs la major similaritat va ocórrer entre els hábitats planctònics (pelàgic i entre pradera), quedant l'hàbitat bentònic amb espècies exclusives. No obstant, l'aproximació de xarxa va desentranyar tres mòduls funcionals acoblats pels macròfits, els herbívors i els mixòtrofs en les tolles: un bucle microbià, una cadena autotròfica, i els macròfits junt a les microalgues bentòniques. En els llacs, van emergir dos mòduls desconnectats: el plàncton pelàgic junt als herbívors de l'hàbitat entre pradera, i els organismes bentònics junt als productors primaris de l'hàbitat entre pradera. Topològicament, els nodes dels herbívors d'entre pradera i el fitoplàncton menut eren centrals tant en les xarxes de les tolles com en les dels llacs. A més, els nodes bentònics mostraren una elevada redundància funcional i foren molt influents per a difondre els efectes de les pertorbacions. Tots aquests resultats apunten a dos patrons contrastats d'acoblament entre hàbitats en tolles i llacs, i remarquen: i) la disgregació funcional en tolles malgrat la similaritat en quant a composició; ii) la importància dels organismes de l'hàbitat entre pradera com a connectors; iii) la rellevància del bentos el qual té la major diversitat, redundància i els organismes més influents de la xarxa, i iv) que l'acoblament entre els mòduls funcionals deu ser essencial per al funcionament dels ecosistemes i la seua capacitat de resposta front a pertorbacions.*

Paraules clau: *bentos; caròfits; modularitat; xarxa tròfica; plàncton; topologia*

1. Introduction

Lakes and ponds are conspicuously distinguished by their morphometry (*e.g.* area and depth). Differences in morphometry drive changes in the relative importance of their habitats as well as in their degree of connection, such as benthic-pelagic coupling (Schindler and Scheuerell 2002, Søndergaard *et al.* 2005, Dolson *et al.* 2009). However, comparative studies of benthic-pelagic coupling in different types of aquatic ecosystems are still scarce. In this regard, the review by Schindler and Scheuerell (2002) highlighted that benthic-pelagic coupling depends on the perimeter:area (or depth) ratios, small lakes or ponds being those with greater coupling between these habitats.

The pelagic habitat, the most prevalent in lakes, is the free-water far from the shores and the bottom where macrophyte meadows thrive establishing other communities and abiotic features. However, in ponds the pelagic habitat could be negligible while the presence of meadows (and their associated community) becomes the most relevant (Lyche-Solheim *et al.* 2013). Macrophyte meadows comprise two different habitats (Rojo *et al.* 2017): the free water within the meadow (within-meadow habitat) and the benthic habitat, represented by the macrophytes themselves and the organisms attached to their surface (periphyton).

Therefore, these morphometry-based structural differences between lakes and ponds will have implications in the inhabiting biological communities and their response to environmental changes. Some of the environmental characteristics linked to the system's morphometry differentially affecting the described habitats include, among others: the quality and variability of light for primary producers (Vadeboncoeur *et al.* 2014, Rojo *et al.* 2019); wave disturbances in surface water and changes in water level (Bucak *et al.* 2012); the presence of stabilizing and/or protecting macrophyte meadows (Palma-Silva *et al.* 2002, Gebrehiwot *et al.* 2017); nutrient availability (Søndergaard *et al.* 2017), and the influence of allelopathic metabolites (van Donk and van de Bund 2002, Rojo *et al.* 2013a, b). Hence, two main ideas emerge: i)

environmental changes (*e.g.* those produced by global change) will differently affect not only the distinct types of aquatic ecosystems (Kosten *et al.* 2011, Jeppesen *et al.* 2014) but also the habitats included in them as well as their coupling, which is essential for the system's functioning, and ii) the role of each functional group, for example, the magnitude of the herbivory effect or the relevance of macrophytes as a refuge will depend on the ecological network in which they are immersed (Shurin *et al.* 2002, Puche *et al.* 2020a, b, c).

In the semi-arid Mediterranean region, these issues become even more important since lakes are medium sized and the majority of waterbodies are small, shallow and/or temporary (ponds and coastal lagoons), and are highly vulnerable to current global change (Álvarez-Cobelas *et al.* 2006, Naselli-Flores and Barone 2012, Parcerisas *et al.* 2012). It seems crucial to undertake studies focusing on the degree of connection between habitats within a waterbody and the possible differences in this connection depending on the type of ecosystem (*i.e.* pond, lake). The approach of these studies must rely on the shared species between habitats and the degree of functional habitat-coupling through their multi-interaction network models. This complementary information would allow a better understanding of the different mechanisms related to the function and stability of lake and pond communities.

Consequently, considering the biological elements which compose the communities of the different habitats in ecosystems not as isolated entities but interconnected by a myriad of trophic and non-trophic relationships, assessing the ecosystem-dependent benthic-pelagic coupling is decisive (Ings *et al.* 2009). The analysis of the multi-interaction networks allows a more functional perspective of the community, providing complementary information to that obtained by the taxonomic description. It is a priority to elucidate the ecological roles played by the different elements in the community to depict the functioning of ecosystems facing environmental changes (Jones and Lawton 1995, Berlow *et al.* 2004, Olesen *et al.* 2007, Puche *et al.* 2020b). In this vein, networks can be divided into functional modules (*i.e.*

subsystems of tightly connected nodes; Guimerà and Amaral 2005) which can go beyond the pre-defined habitats. The connector nodes establish many interactions between the different modules, and their extinction would fragment the network into isolated modules with implications for network stability (Olesen *et al.* 2007, Allesina and Pascual 2008). Recently, a structurally important macrophytes-zooplanktonic herbivores tandem has been experimentally suggested for shallow freshwater ecosystems (Puche *et al.* 2020a). Therefore, we expect this tandem to be more relevant in ponds than in lakes, corroborating the high influence of macrophyte meadows in small waterbodies. Furthermore, the assessment of the topological roles of nodes by means of commonly used centrality indices such as: closeness and betweenness (Freeman 1978, Martín-González *et al.* 2010); the more sophisticated topological importance index (Jordán *et al.* 2003), and the sensitivity and effectiveness of the nodes (Puche *et al.* 2020a, b, c), provides information about how important a node is for spreading the effects of a disturbance through the community, or how sensitive it is to any change in the network due to its topological position.

In this study, we provide a detailed description, and analysis of the composition of the communities from the different habitats (pelagic, within-meadow and benthic) in two contrasting types of aquatic ecosystems (lake *versus* pond) in the Mediterranean region. Furthermore, we add the multi-interaction network approach considering the trophic and non-trophic interactions among the biological elements from the different habitats (Puche *et al.* 2020a, b, c) to this snapshot, and assess their topological role by means of global and node-scale indices. We hope that applying the network approach to the compositional description of lakes and ponds will pathe the way for discerning key players in the functioning of these systems and their connected modules, helping us to predict the response of these contrasting ecosystems to environmental changes.

2. Materials and methods

2.1. Origin and sampling of aquatic communities: ponds and lakes

In this study we selected four Mediterranean ecosystems (Fig. 1). Two of them were Mediterranean shallow interdunal ponds within the Albufera de València Natural Park: Pond Llacuna del Dossel (PD, hereafter; 3 m a.s.l. 39°12'30"N; 0°14'5"W; Ballester *et al.* 2006) and Pond Llacuna Nova del Canyar (PNC, hereafter; 3 m a.s.l., 39°19'41"N; 0°18'16"W; Calero *et al.* 2017). The other two ecosystems were lakes in the centre of the Iberian Peninsula: Lake Somolinos (LS, hereafter; Sierra de Ayllón Protected Area, 1270 m a.s.l., 41°15'04"N; 3°03'54"W; Sánchez-Carrillo and Álvarez-Cobelas 2019), and Lake Tinaja (LT, hereafter; Ruidera lakes Protected Area, 842 m a.s.l., 38°58'32"N; 2°53'3"W; Álvarez-Cobelas *et al.* 2006).

The criteria for their selection were that i) they had dense charophyte (submerged macrophytes) meadows; ii) they were situated in contrasting geographical locations, and iii) their ecology related to the benthic aquatic community had been studied (Cirujano and Medina 2002, Álvarez-Cobelas *et al.* 2006, Cirujano 2013, Calero *et al.* 2017, Rojo *et al.* 2017, Puche *et al.* 2018). Following the European Water Framework Directive (W.F.D. 2000) and according to the Spanish Lakes Typology (B.O.E. 2015), the two ponds are considered as type 29 (coastal lakes developed on dunes, permanent) and the two lakes as type 12 (calcareous karst, permanent, travertine closure).

The sampling at the study sites was carried out in spring, when the submerged vegetation was at its growth peak (Calero *et al.* 2017, Rojo *et al.* 2017). Some physical and chemical features of the subsurface water (the epilimnion layer in the lakes) were measured in each site *in situ* with portable field equipment: a WTW Meter (WTW GmbH, Weilheim, Germany) for temperature, pH, conductivity and salinity. Water samples were collected and transported to the laboratory to analyse total nitrogen (TN), total phosphorus (TP) and sestonic chlorophyll-a (Chla) concentrations.

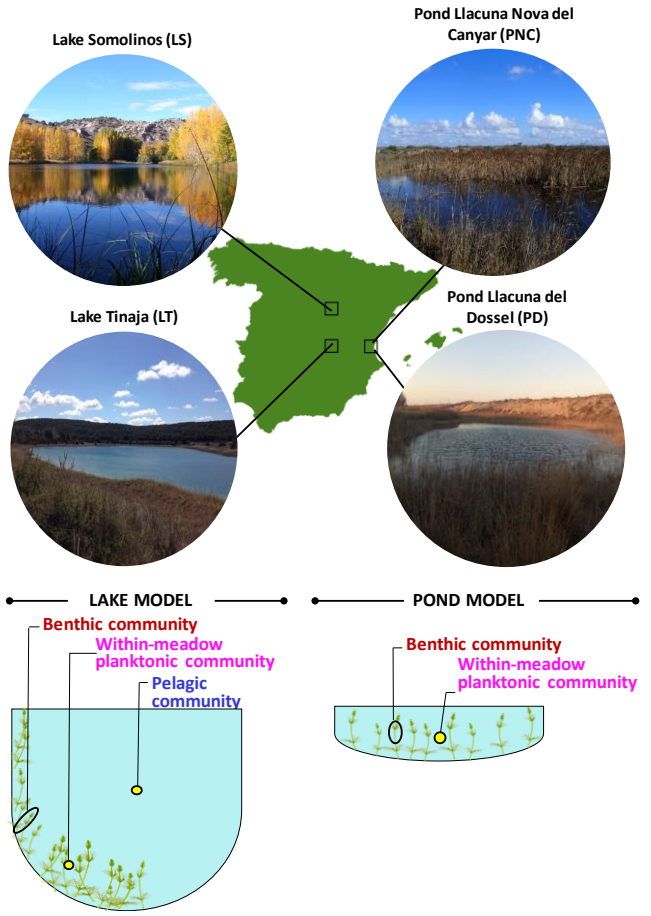

Fig. 1. Location in Spain of the four Mediterranean study sites, two ponds and two lakes (abbreviations as in Table 1). The diagram represents the different habitats from which the communities that are compared in this study are obtained.

The ultraviolet radiation data of these sites were collected from the nearby meteorological stations (a station in València for the ponds and in Navacerrada for the lakes). In total, data from 15 variables were obtained to describe the abiotic conditions in the four studied sites related to their geographical position, morphometry, light conditions, physical and chemical water features and biotic variables, such as Chla concentration or meadow position (Table 1).

We analysed three connected habitats (Søndergaard *et al.* 2005, Rojo *et al.* 2017) in the selected waterbodies: i) the pelagic (only in the two lakes), with organisms living

in the free-water away from the meadow; ii) the within-meadow, where organisms inhabit the free-water within the charophyte meadows, and iii) the benthic, encompassing the charophytes themselves and all the organisms living attached to them (Fig. 1). These latter two habitats were found both in the lakes and ponds.

Table 1. Limnological variables in spring (sampling time) of the four studied aquatic ecosystems (PD: Pond llacuna del Dossel, PNC: Pond llacuna Nova del Canyar, LS: Lake Somolinos and LT: Lake Tinaja). Abbreviations for variables are shown. MxM is the maximum depth of the waterbody where there are macrophyte meadows.

Abbr.	Variable	Units	PD	PNC	LS	LT
	Geomorphology					
Altit	Altitude	m a.s.l.	1	3	1239	842
Area	Area	m^2	680	5900	28000	80400
MxD	Max. Depth	cm	100	150	800	1700
	Physical conditions					
UVR	UVR in spring (average of monthly total)	J m^{-2}	130739		147023	
MxT	Max. Temperature	°C	31	34	17	23
SprT	Spring Temperature	°C	19	20	12	17
Trans	Transparency	cm	100	150	550	850
pH	pH		8.0	7.4	7.8	8.0
Cond	Conductivity	µS cm^{-1}	1648	3435	445	620
	Chemical conditions					
Sal	Salinity	g L^{-1}	0.6	1.8	0.4	0.4
TN	Total nitrogen	mg N^{-1}	0.752	0.950	1.750	9.500
TP	Total phosphorus	mg P^{-1}	0.026	0.030	0.010	0.042
	Biotic conditions					
Chla	Sestonic chlorophyll-a	µg L^{-1}	1.5	2.0	0.9	0.2
MxM	Max. depth with meadow	cm	100	150	800	1700
DistM	Meadow distance from the shore	cm	100	100	100	200

For the planktonic assemblages, water samples from the middle depth of epilimnion were taken in the centre of the waterbody (assemblage from pelagic habitat in lakes) and/or within the charophyte meadows (assemblage from within-meadow habitat). When the meadows were located at great depths, a limnological bottle (Niskin) was used to collect the samples (Rojo *et al.* 2017). For phytoplankton, these samples consisted of 250 mL fixed with Lugol's solution. For zooplankton, 4 L were filtered through 37 µm Nytal mesh and the samples were fixed with formaline (Rodrigo *et al.* 2015). For benthic organisms associated with the meadows of the four-studied waterbodies (assemblage from the benthic habitat), ten shoots of charophytes, always including pieces from the apical to basal parts, were collected by hand in the ponds or by means of a Van Veen grab in the lakes, and then stored in plastic bags. In the laboratory, these shoots were gently washed with tap water and the obtained material was kept in small tubes and fixed with formaline to identify and count zoobenthos. Then, the shoots were scrubbed with a toothbrush to analyse the benthic microalgae and cyanobacteria. The dry weight (DW) of charophytes (after drying them for 24 h at 70˚C) was calculated to refer the benthic organisms to this weight (Rojo *et al.* 2017). All the organisms in the different fractions were identified at the finest possible taxonomic resolution, and then counted by means of Utermöhl chambers with an inverted microscope (Olympus CK2) from 100x to 1000x magnifications. In the case of samples of benthos associated with macrophytes, individuals of each species found in each microscopic field were recorded, which enabled us to obtain an area-species plot that would later be used as saturating criteria. Populations of a genus that could not be determined as a species were named as sp1, sp2, etc., to represent the maximum richness per sample. The abundance of bacteria and charophytes was not quantified, but their corresponding nodes were considered for the construction of multi-interaction networks in the studied ecosystems (explained below).

2.2. Description of the communities and their possible control factors

The set of taxa found in the sample of a determined habitat was considered to be from this habitat. Thus, for example, large benthic diatoms found in free-water samples within the meadow were considered to be from the within-meadow habitat. Under this criterion, we want to highlight the connection among habitats naturally occurring in these ecosystems (Søndergaard *et al.* 2005, Rojo *et al.* 2017).

Each habitat required specific sampling protocols and analyses, thus, the planktonic populations (*i.e.* from the pelagic and within-meadow habitats) were expressed as ind L^{-1}, and those from the benthic habitat as ind g^{-1} DW of charophytes. To ensure consistency among the measurements, and to make them comparable between ecosystems, for the six possible groups (3 habitats x primary producers or consumers) we expressed the percentage represented by each population with respect to the total number of individuals in each group (Table S1 Supplementary material Chapter 7). This percentage was the variable used in all the assemblage analyses.

In order to transfer the obtained taxonomical information to the node-based multi-interaction network (the functional view of the community), we first grouped the taxa into nodes following the criteria established by Puche *et al.* (2020a). Briefly, these criteria discriminate, by taxonomic group, functional features and habitat (Table S2 Supplementary material Chapter 7). Then, the percentage of each node in the network was the sum of the percentages of the populations that it was made up of.

We also calculated the diversity (based on both taxa and nodes) of each assemblage as the richness, the dominance (Dominance = 1-Simpson index) which ranges from 0 (all element are equally present) to 1 (one element dominates the community completely), and the Shannon-Wiener index (using natural logarithms), which is sensitive to less frequent elements (Shannon and Weaver 1949). Exclusiveness, complementarity and shared taxa were calculated between pairs of habitats from the

same ecosystem (Colwell and Coddington 1994, Rojo *et al.* 2012). The diversity indices were calculated using PAST 3.14 software (Hammer *et al.* 2001).

To reflect biodiversity and ecosystem function relationships (BEF), we assessed the different populations included in each node, that is, how many populations-species supposedly have the same function in the ecosystem's multi-interaction network (Wellnitz and Poff 2001).

2.3. Multi-interaction network analysis

The defined nodes in each system were connected through trophic and non-trophic links to construct the multi-interaction networks (Puche *et al.* 2020a). Non-trophic links comprised effects such as allelopathy among primary producers, shading of phytoplankton over macrophytes or the refuge or vital support provided by macrophytes to planktonic and benthic organisms.

The set of nodes and links was arranged in a SxS matrix A for each ecosystem (where S is the number of nodes in the network). The entries of matrix A (a_{ij}) represent ecological interactions among nodes (Cohen 1978) as the effect of node *j* (in the column) on node *i* (in the row). The values in this matrix for trophic interactions can be 1 (positive; the effect of prey on the predator) or -1 (negative; the effect of predator on prey), while positive and negative non-trophic interactions were coded separately as 1. When there was no interaction between nodes, this was coded as 0.

Then the structure of the whole network in the four ecosystems was assessed by means of global descriptors. The number of nodes (S) and links (L) allowed us to calculate the directed connectance (C; Martínez 1992). We also checked the nestedness of the networks (N; Almeida-Neto *et al.* 2008). The significance of this metric was calculated after 1000 randomizations of the matrices using the software ANHIDADO (ver. Bangu 3.0; Guimarães and Guimarães 2006). Moreover, the modularity coefficient (M) was calculated following the algorithm by Guimerà and Amaral (2005). This algorithm finds the best partition of the network in groups of non-

overlapping tightly connected nodes (*i.e.* modules). Based on the emergent modules, we assessed the roles of nodes considering their within-module z-score and the between-modules connections (participation coefficient, P) following the roles proposed by Olesen *et al.* (2007).

We also calculated node-scale indices to quantify the relative topological importance of each node. The topological importance index (TI) provides a mesoscale perspective, considering the direct and indirect effects of a node up to n steps (*i.e.* it allows you to assess how effects from this node can spread through the network to reach nodes within a pre-defined step length; Jordán *et al.* 2003). In our case, the considered number of steps was three. Closeness centrality (CC) is a measure of the proximity of a node to other nodes in the network, based on the shortest paths between pairs of nodes (Freeman 1978). Betweenness centrality (BC) represents how incident or intermediary a node is in the shortest paths between other nodes in the network (Freeman 1977).

Furthermore, from matrix A we calculated the net effect matrix N (as $N=-A^{-1}$; Novak *et al.* 2016) and randomized it 5000 times to obtain an average net effect matrix that encompasses all the direct and indirect effects among nodes in the network (*i.e.* global effects of nodes in the network; Puche *et al.* 2020a). Briefly, the entries of this matrix represent the expected long-term change in the equilibrium value of node *i* due to constant pressure exerted on node *j* (Nakajima 1992). This matrix allowed us to calculate two node indices: effectiveness (E, the capacity of a node to affect others when being disturbed) and sensitivity (Sens., the susceptibility of a node of being affected when others are disturbed; Puche *et al.* 2020a).

The global network's descriptors (except for nestedness) were calculated in MATLAB using the Brain Connectivity Toolbox. TI values were calculated by using CoSBiLab Graph (Valentini and Jordán 2010), while CC and BC indices were calculated with UCINET (Borgatti *et al.* 2002). Net effect matrices and Sens. and E indices were calculated in MATLAB.

2.4. Statistical analysis

A canonical correspondence analysis (CCA) was performed to discriminate the four studied ecosystems based on the 15 measured environmental variables. Multivariate analyses (Euclidean distance; paired groups) performed a cluster of the sites' origin of assemblages based on the relative abundance of both taxa and nodes. Principal component analysis (PCA based on variance-covariance coefficients) arranged taxa (or nodes) and ecosystems, highlighting the most discriminant populations. Correspondence analysis, including the 15 environmental variables of the four ecosystems, confirmed they belong to two different types, and indicated which variables were more significantly implied in their differences. We also carried out a non-parametric MANOVA to assess the differences between the multi-interaction networks of the four ecosystems considering the calculated indices (TI, CC, BC, Sens. and E) as independent variables. All multivariate analyses were calculated using PAST 3.14 software (Hammer *et al.* 2001).

3. Results

3.1. Assemblages and their environment in systems with macrophyte meadows

The studied aquatic communities came from four ecosystems, clearly distinguished by their geomorphology (Table 1; Fig. S1 Supplementary material Chapter 7): two coastal shallow ponds and two deeper and larger lakes located at a higher altitude. The maximum annual temperature of the ponds was 11-14°C higher than that of the lakes; conductivity values and salinity were 4-fold greater in the ponds compared to the lakes, and their sestonic chlorophyll-a concentration was also higher (Table 1; Fig. S1). The values of TN (range 0.8-9.5 mg N L^{-1}) or TP (0.01-0.04 mg P L^{-1}) were higher in lakes than in ponds. Submerged meadows of the charophyte *Chara hispida* L. occupied the shores and spread to the maximum depth of the ponds and were mainly concentrated at the bottom of the lakes. The transparency values and the presence of charophytes

suggest that photosynthetic active radiation reached the bottom of the systems (Table 1).

The total identified taxa varied from 79 to 102 in the studied ecosystems (Table 2 and Table S1). Considering all the ecosystems, the complementarity range between within-meadow and benthic habitats was 50-83%, and the range of common taxa between these habitats was 17-50%. In the ponds there were few differences between the diversity of within-meadow and benthic habitats. In the within-meadow of PNC, richness was the greatest mainly due to a higher biodiversity of filamentous cyanobacteria (Table S1). Diversity (Shannon-Wiener index) in the ponds was around 2.7-3.0 nats, and 1.0-1.8 nats for primary producers and consumers, respectively. The percentage of common taxa between the two habitats in the ponds varied between 38-50%. In the lakes, the benthic habitat showed values of richness and diversity similar to those obtained from the ponds (Table 2). In both lakes, the benthic habitat had the greatest degree of richness, followed by the within-meadow habitat, and finally the pelagic habitat. The loss of richness coincided with an increase in dominance. Therefore, Shannon-Wiener index values also decreased from the benthic to the pelagic habitats (Table 2). The percentage of common taxa between pelagic and within-meadow habitats in the lakes was similar to that shared between benthic and within-meadow habitats in the ponds. The habitats which shared less taxa in the lakes (17-18%) were both linked to the meadow (*i.e.* within-meadow and benthic habitats).

The four studied ecosystems were discriminated according to their most relevant taxa (Fig. 2a). The two first components explained 69% of variance. The first component singled out the ponds, due to their high percentage of Bdelloidea species (benthic rotifers), the second separated one lake from the other due to the composition of their dominant taxa in pelagic and within-meadow habitats. In LT, the dominant taxa were the rotifers of the genus *Polyarthra* and the small centric diatom *Cyclotella distinguenda*, while in LS, copepod *Cyclops* cf. *abyssorum* and small cryptophytes *Plagioselmis nannoplanctica* stood out (Fig. 2a). A third component

explains 31% of the variance separating the ponds based on their main small herbivores: bacterivores (nauplii of the cyclopoid copepod) in PD and herbivore rotifers of the *Collotheca* genus in PNC. A dendrogram of assemblages from the different habitats allowed the clustering of the benthic ones (both from the ponds and lakes) as well as allowing them to be linked to the within-meadow assemblages of the ponds (Fig. 2b). In addition, pelagic plus within-meadow from each of the two lakes were joined in two more clusters (Fig. 2b).

These dominant species, and those discriminating the ecosystems, were reflected in the community structure described from their functional groups (nodes; Table S2, Fig. S2 Supplementary material Chapter 7). Diversity of consumers was lower in the benthic than in the within-meadow habitat in the ponds, because more than 90% corresponded to benthic herbivore rotifers (RH_b; Table S2; Fig. S2). The ponds differed in the within-meadow habitat, which was dominated by nauplii and copepodites (Nau_m+ Cop_m; 88%) in PD, and by herbivore rotifers and copepodites (RH_m+ Cop_m; 98%) in PNC. With respect to primary producers, LS had lower ecological diversity than the other ecosystems due to the dominance of small mixotrophic algae and small diatoms (Mxs_p, 80%, Mxs_m, 83%, DS_b, 83%). Moreover, in LS pelagic carnivore copepods (CoC_p) accounted for 89%, while in LT RH_p+Nau_p represented 97%. These differences were enough to order the ecosystems in a similar way to that achieved with species (Fig. 2A; Fig. S2), but the explained variance was higher in the PCA based on nodes (80% of the variance was explained by the two first components).

An estimation of redundancy, as the number of populations included in each node (*i.e.* functional group), showed differences between both types of ecosystem. On average, the redundancy of primary producer nodes from both habitats in the ponds were similar (Table 3), while in the lakes a clear increase of redundancy was observed, in the following order, pelagic, within-meadow and benthic habitats; with almost four

Table 2. Measurements of taxa diversity (richness, dominance and Shannon-Wiener index) for primary producers and consumers inhabiting the different habitats in the four aquatic studied ecosystems during spring. The same is shown for the nodes (structural elements). Exclusive and common taxa between habitats and their complementarity in percentage are also indicated (in brackets when exclusivity is calculated between within-meadow and benthic habitats in lakes). Abbreviations for ponds and lakes as in Table 1; Shannon is Shannon-Wiener index expressed in nats.

		PD		PNC		LS			LT		
Taxa		Within-meadow	Benthic	Within-meadow	Benthic	Pelagic	Within-meadow	Benthic	Pelagic	Within-meadow	Benthic
Total taxa richness		79		102		91			101		
Primary producers	Richness	44	44	72	45	10	17	46	22	28	60
	Dominance	0.08	0.12	0.09	0.08	0.61	0.68	0.11	0.29	0.21	0.09
	Shannon	2.99	2.67	3.02	2.91	0.88	0.76	2.63	1.76	2.04	2.97
Consumers	Richness	10	11	7	11	6	15	21	3	5	18
	Dominance	0.59	0.23	0.29	0.46	0.40	0.12	0.20	0.57	0.64	0.13
	Shannon	0.90	1.81	1.48	1.09	1.10	2.38	1.88	0.70	0.74	2.37
Exclusive taxa (%)		44	45	43	20	25	63 (25)	(82)	24	42 (48)	(78)
Common taxa (%)		38		50		33	17		49	18	
Complementarity (%)		62		50		67	83		51	82	
Nodes											
Total nodes richness		15		17		17			14		
Primary producers	Richness	8	6	8	6	5	5	6	8	7	6
	Dominance	0.42	0.30	0.22	0.31	0.66	0.71	0.65	0.40	0.40	0.36
	Shannon	1.34	1.32	1.74	1.40	0.69	0.60	0.72	1.14	1.12	1.20
Consumers	Richness	6	2	3	6	4	5	3	3	3	5
	Dominance	0.59	0.99	0.53	0.90	0.41	0.55	0.60	0.57	0.75	0.82
	Shannon	0.86	0.04	0.73	0.26	1.01	0.89	0.65	0.70	0.46	0.43

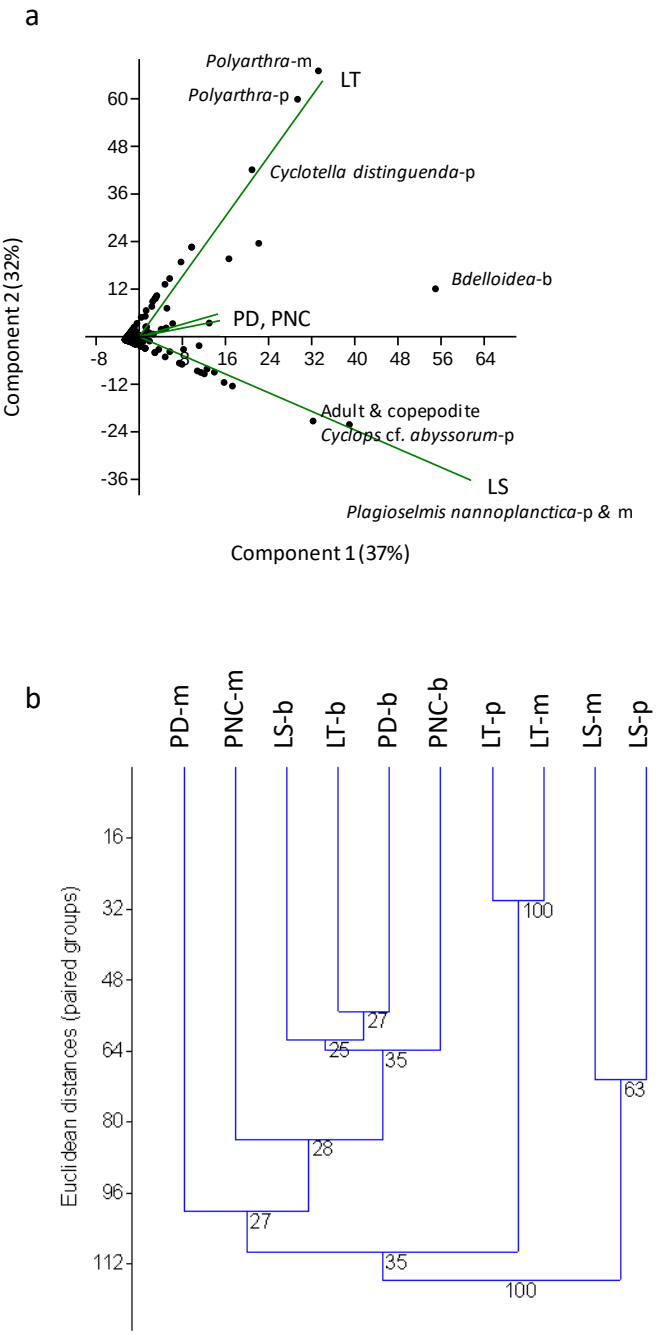

Fig. 2. a) Principal Component Analysis (PCA) of relative abundance of taxa and biplot with origin of assemblage (PD, PNC, LS and LT). Explained variance of each component is shown in brackets. b) Cluster of site origin of taxa assemblages; numbers in the dendrogram are % of replicates in the bootstrap analysis. Abbreviations of ecosystems as in Table 1; -p pelagic habitat; -m within-meadow habitat and –b benthic habitat

times more populations by nodes in the benthic than in the pelagic habitat (Table 3). The redundancy of consumer nodes followed the same trend as that of primary producers, with more similar average values between within-meadow and benthic habitats in the ponds, and an increase from pelagic to benthic habitats in the lakes (Table 3).

Table 3. Descriptors of the four multi-interaction networks (two ponds and two lakes named as in Table 1; Fig. 3). Averaged redundancy and its standard deviation calculated from nodes of primary producers (PP) and consumers (CS) inhabiting pelagic, within-meadow and benthic habitats (p, m, and b, respectively). S is the number of nodes (charophytes and bacteria from the different habitats are considered in these global parameters), L is the number of links, C is the directed connectance, M is the modularity coefficient, thanks to which different modules have been highlighted (their number in parentheses), and N is the nestedness of the network with the associated p-value.

	PD	PNC	LS	LT
PP_p			2.5±1.5	
PP_m	7.3±3.8		3.8±2.2	
PP_b	6.8±3.9		8.8±6.2	
CS_p			1.3±0.8	
CS_m	1.9±1.8		2.5±3.5	
CS_b	2.8±3.4		4.9±5.5	
S	25	26	32	36
L	121	122	150	157
C	0.20	0.19	0.15	0.12
Nº modules (M)	3 (0.19)	3 (0.17)	2 (0.17)	2 (0.26)
N (p)	11.9 (<0.001)	12.2 (<0.001)	9.3 (<0.001)	7.8 (<0.001)

3.2. Aquatic multi-interaction networks in systems with macrophyte meadows

The multi-interaction networks of the four ecosystems (Fig. 3) differed in global structure parameters. In the lakes there were, on average, 25% more nodes (S) than in the ponds (due to the exclusive presence of the pelagic habitat on the former). This was not accompanied by a proportional increase in the number of links (L; 21% increase; Table 3) mainly due to a lower number of links per node in LT. This fact

resulted in a 44% decrease, on average, in connectance (C) in the lakes compared to the ponds (Table 3). All the networks were significantly nested, but nestedness (N) in the lakes was 41% lower than in the ponds (Table 3).

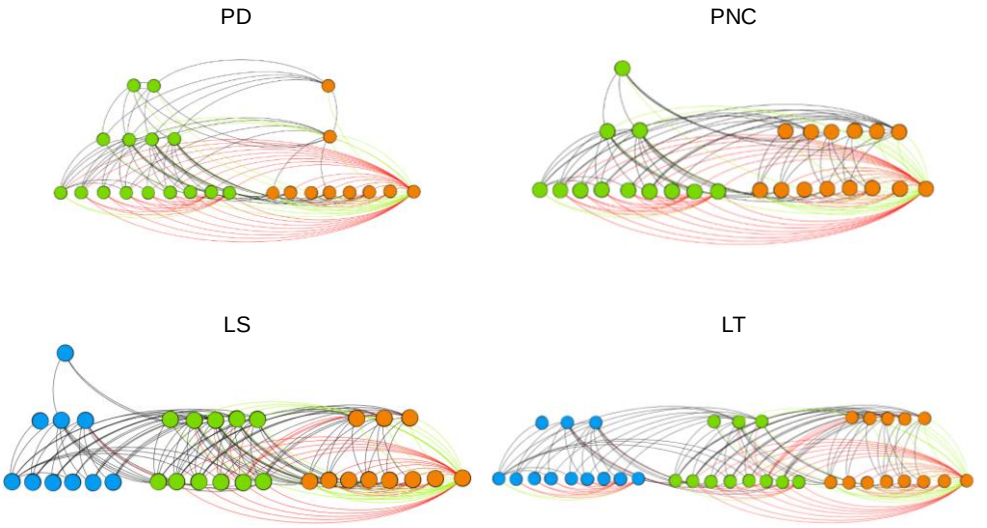

Fig. 3. Graphical representation of the multi-interaction functional network of the four studied ecosystems. The colour of the nodes represents the habitat to which they belong: pelagic (blue), within-meadow (green) and benthic (orange). Nodes are horizontally distributed in groups according to which compartment they belong to. The vertical distribution corresponds to the trophic position of the nodes (primary producers at the base, herbivores at the second level and carnivores at the top). The line colours represent the different types of interactions: trophic (black), non-trophic negative (red) and non-trophic positive (green). The curvature of lines connecting the nodes represents the directionality of the interaction, with lines arcing clockwise from the source to the target nodes. Abbreviations of ecosystems as in Table 1.

Considering the spread of the node effects in the indirect neighbourhood (TI), the within-meadow herbivores were top in the four ecosystems (Fig. 4 nodes 26-29). Benthic consumers also had high values of TI but only in the ponds (Fig. 4 nodes 38-43). With respect to the proximity of a node to others in the network by means of shortest paths (CC), within-meadow herbivores were also top in all the ecosystems (Fig. 4 nodes 26-28). The high CC values of the within-meadow small phytoplankton only in the lakes (Fig. 4 nodes 15-20) is worth noting. These patterns were similar when it comes to the intermediation capacity of the nodes in the connections between other nodes in the network (BC). When all the direct and indirect relationships of the

network were considered, it was found that the benthic consumers were highly sensitive to the disturbance of other nodes of the network in all the ecosystems (Sens.; Fig. 4 nodes 38-44). Phytobenthos had a high capacity of affecting other nodes in the network when disturbed (E), regardless of the ecosystem (Fig. 4 nodes 32-36), and within-meadow phytoplankton (and bacteria) were more effective in the lakes (Fig. 4 nodes 14-18). Considering all these indices in a non-parametric multivariate (NPMANOVA) cross-ecosystem analysis, the ponds and lakes were clearly distinguished (distance measured with Bray Curtis; $F=4.3$, $p=0.006$). A *post hoc* analysis revealed significant differences between the ponds and LS ($p<0.030$), and between the ponds and LT ($p<0.002$).

Based on the modularity analyses, zooplanktonic herbivores (such as cladocerans), mixotrophic algae and charophytes were classified as connectors, according to their within-module z score and participation coefficient P, between the modules of the ponds' multi-interaction networks (Fig. 5). These modules corresponded to: i) a microbial loop module composed of small consumers (mixotrophs and herbivores) plus bacteria, inhabiting within-meadow and benthic habitats; ii) a module of an autotrophic food chain, formed by phytoplankton and large consumers, and iii) a benthos module, with benthic primary producers (charophytes, microalgae and cyanobacteria; Fig. 6). In the lakes, none of the nodes was framed within this connector role (Fig. 5), and two disconnected modules emerged: i) a planktonic module, with the pelagic autotrophic chain including within-meadow herbivores, and ii) a benthos module, with macrophytes and the benthic autotrophic chain which includes some within-meadow primary producers (Fig. 6).

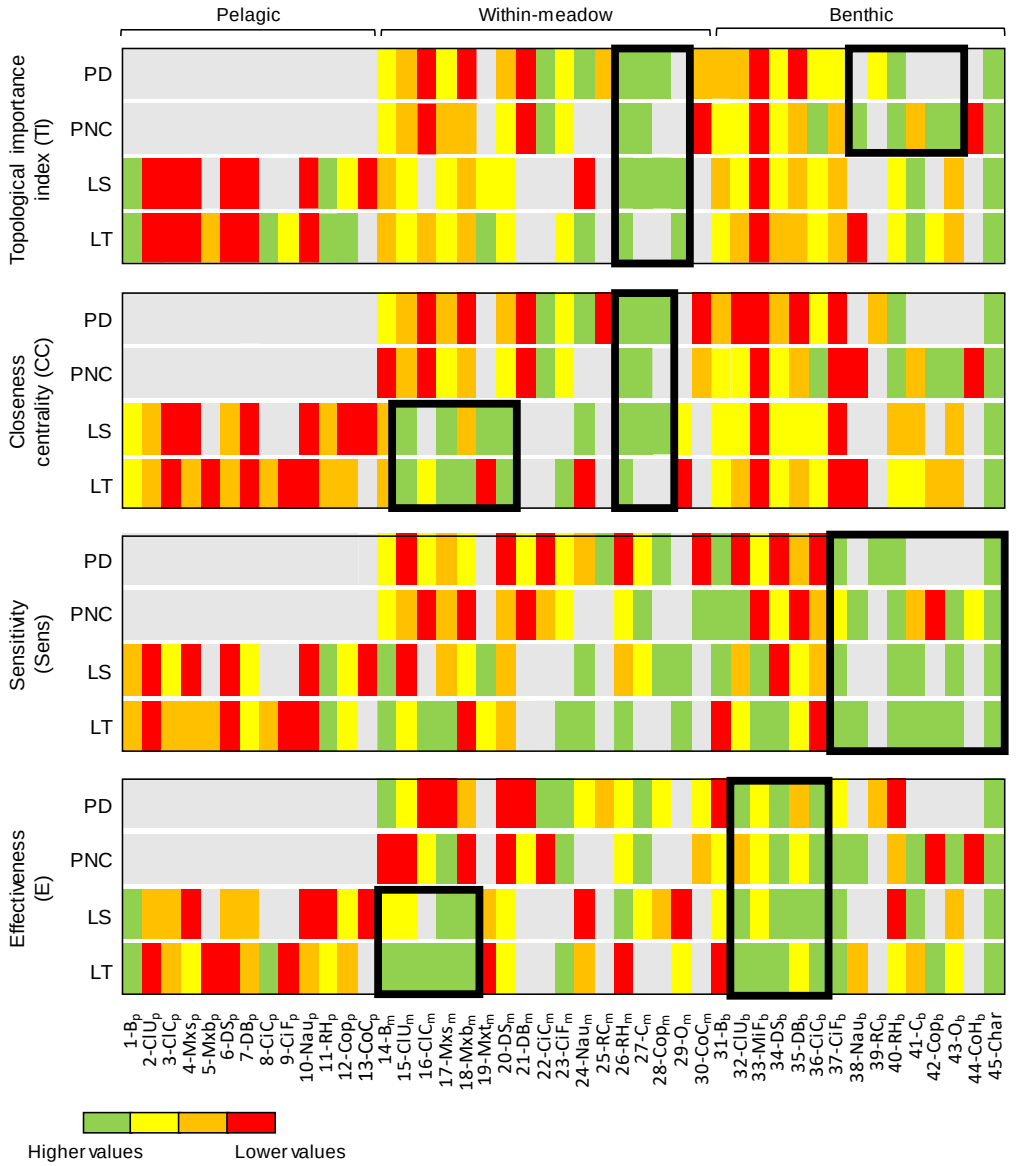

Fig. 4. Representation of the values of the measured node indices in the four studied ecosystems (abbreviations as in Table 1). The nodes were assigned to quartiles based on the values of each index separately, with nodes with lowest value occupying quartile one (red) and those with higher values occupying quartiles two (orange), three (yellow), and four (green; the highest values). The groups that are commented on in the text are marked with black boxes.

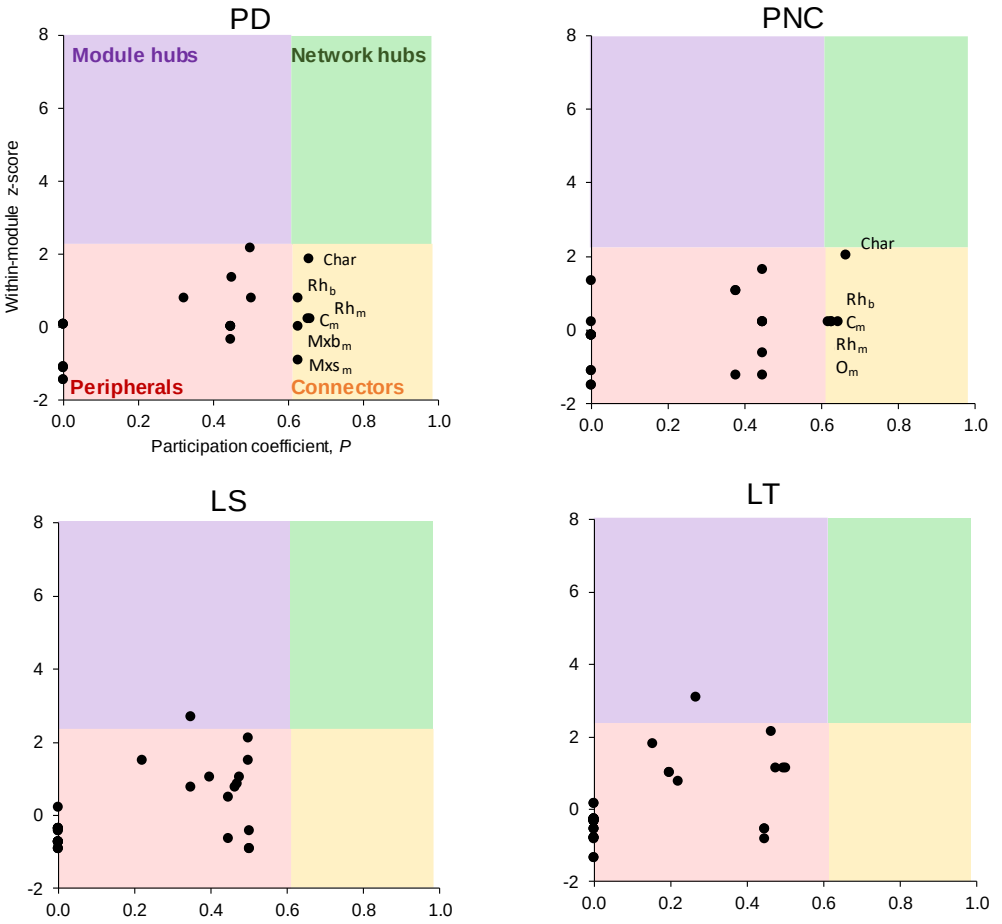

Fig. 5. Roles of the nodes in the network of each ecosystem according to their within-module z (y-axis) and their participation coefficient P (x-axis). Four regions (roles) are considered following Olesen *et al.* (2007). Each dot is a node of the network. Only the connector nodes are named. Abreviations and more explanations in Fig. 1 and Table S2 Supplementary material Chapter 7.

4. Discussion

4.1. Assemblages and their habitats in systems with macrophyte meadows

The communities used here to detect sets of keystone species in the coupling of different habitats within an aquatic ecosystem have clearly characterized the area and depth gradients in the studied ecosystems (Søndergaard *et al.* 2005). This result supports the idea that the distance between pelagic and benthic habitats has crucial

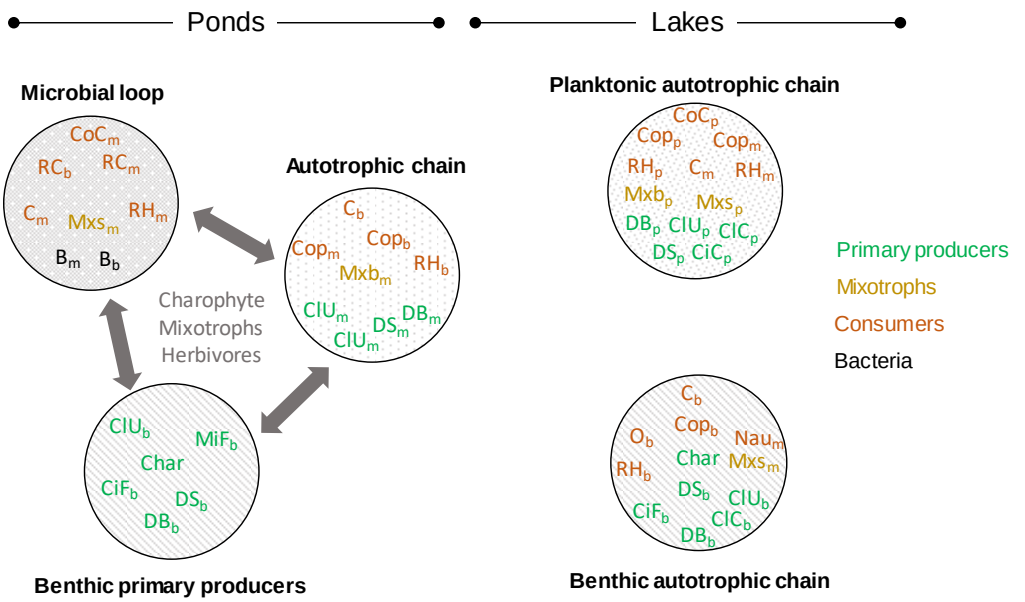

Fig. 6. Representation of the modules defined in pond and lake networks by the modularity algorithm (Guimerà and Amaral 2005) and named based on their ecological function. The nodes of each module are shown (abbreviations as in Table S1 Suuplementary material Chapter 7). Thick arrows represent a connection between modules, and nodes with a connector role (*i.e.* connecting modules) are represented in grey.

implications for the structure and function of aquatic communities (Schindler and Scheuerell 2002, Dolson *et al.* 2009).

Overall, macrophytes have been attributed a central role in all types of aquatic ecosystems (Carpenter and Lodge 1986, Rodrigo *et al.* 2015, Rojo *et al.* 2017); however, we have disentangled, through a descriptive approach of assemblages from different habitats (pelagic, within-meadow and benthic), different patterns of connections in the ponds compared to those in the lakes. The connection between within-meadow and benthic habitats occurred only in the ponds (sharing 50% of taxa), supporting what Rojo *et al.* (2017) and Antón-Pardo and Armengol (2016) stated. This corroborates the expected higher benthic-pelagic coupling in shallow lakes and ponds compared to deep lakes (Schindler and Scheuerell 2002, Vander Zanden and Vadeboncoeur 2002, McCann *et al.* 2005). The morphometric characteristics of ponds

enable mixing by factors such as wind and the spatial proximity between habitats, which would point to a *totum revolutum* of populations from both habitats (Søndergaard *et al.* 2005), as shown by the higher taxonomic overlap.

We also found similar evidence of compositionally coupled habitats in the lakes. However, in these ecosystems the coupling occurred between the pelagic (water column) and the within-meadow habitats. Therefore, it seems that the water is the vehicle connecting the epilimnion and the free water at the bottom of the lake, at least during the spring vertical mixing of the water column in the lakes (Wetzel 2001), and then, planktonic populations were more common between the epilimnetic and the within-meadow free water than with the benthic habitat. In addition, there were no shared populations between the within-meadow and the benthic habitat from the isolated (disconnected) meadow at the bottom of the lakes, and the composition of this benthic habitat of the lakes was more similar to the benthic habitat of both ponds. These facts could be relevant for at least two reasons: i) from a biodiversity point of view, the greatest diversity and exclusivity of species occurring in the benthic habitat of the lakes, and ii) from a functional point of view it means that the important production of benthic meadows can be disconnected from the trophic network (matter and energy flow) of the lake (Schindler and Scheuerell 2002).

The species characterizing the ponds belonged to the benthic group of Bdelloidea rotifers, which are commonly attached to substrates (Kutikova 2003). However, the most characteristic taxa of the lakes were planktonic rotifers of the genus *Polyarthra*, which are capable of migrating both in spring and summer throughout the water column, even during the day (Zhou *et al.* 2007), as well as different stages of cyclopoid copepods (Ludovisi *et al.* 2008, Tiberti and Barbieri 2011) plus two small cosmopolitan and ubiquitous microalgae, such as *Cyclotella distinguenda* and the flagellated cryptophyte *Plagioselmis nannoplanctica*. The latter can also migrate throughout the water column (Clegg *et al.* 2007) even to anoxic layers (Camacho *et al.* 2001). This cross-habitat connection ensures the matter and energy flow from deep habitats to

the water column, and this is generated by highly mobile organisms such as zooplanktonic organisms as well as fish (Vander Zanden *et al.* 2006, Adamczuk 2014).

The relationship of plankton with the periphyton and its host seems to be dependent on both waterbody morphometry and meadow site conditions (whether it has a continuum extension in the waterbody or disconnected at the bottom). In a previous study, Rojo *et al.* (2017) related these conditions to the microalgae and cyanobacteria strategy of distribution in habitats within lakes and ponds, using a metacommunity approach. Shallower sites, like the ponds studied here, shared more periphytic and planktonic species, suggesting a mass-effect (Leibold *et al.* 2004). In fact, the shared species between the benthic and within-meadow habitats in our study were not strictly attached to a substrate, but appeared equally on the substrate as in the free water (*e.g.* microalgae and cyanobacteria such as *Cyclotella* spp., *Pseudanabaena* spp., *Chroococcus* spp. or rotifer species of *Lecane* genus and cyclopoid nauplii). The mass-effect perspective implies that overabundant species, with good dispersal possibilities, can enhance its occurrence in many different assemblages such as those we observed in our study (*e.g.* cryptophytes and dinoflagellates). On the other hand, in the lakes the isolated meadows at the bottom of these systems shared very few benthic species with the water column assemblages, adjusting better to the species-sorting paradigm, arguing that habitats (patch types) cause the differences in the local presence and demography of species (Leibold *et al.* 2004). Therefore, morphometric features, which favour or do not favour physical dispersion, can control these mechanisms, structuring the distribution of populations in the different assemblages (*e.g.* in metacommunity structure; Heino *et al.* 2014, Shoemaker and Melbourne 2016), but also the dispersion capacity of individuals, for example, zooplankters which are good swimmers were responsible for the coupling between habitats (*e.g. Cyclops* cf. *abyssorum*, *Polyarthra* spp., or microalgae of the dinoflagellate group).

4.2. Functional perspective of aquatic communities in systems with macrophyte meadows

The functional approach (*i.e.* considering the multi-interaction network) has disentangled modules in the four ecosystems, clearly characterizing different patterns for the ponds and lakes. The emerged modules corresponded to specialized functions in the ecosystem, corroborating that the elements shaping the communities tend to form subsystems with a specific function in the waterbody (Proulx *et al.* 2005, Kéfi *et al.* 2016).

In both studied ponds, there were three modules clearly related to the main paths of matter and energy flows: the microbial loop, the autotrophic food chain (Stockner and Porter 1988, Pomeroy *et al.* 2007) and the benthic primary producers (Vadeboncoeur and Jeppesen 2003). In the ponds and shallow lakes, the microbial loop may have even more relevance than the autotrophic chain, mainly during blooms of almost non-edible organisms by herbivores such as cyanobacteria and filamentous algae (Kisand and Nõges 2004). Mixotrophic organisms (*e.g.* cryptophytes and dinoflagellates mentioned in the previous section) were part of the microbial loop and, surely, they enhanced its complexity by alternating autotrophy and heterotrophy (consuming bacteria; Roberts and Laybourn-Parry 2001). These organisms are also highly mobile and easily eaten by both planktonic and benthic herbivores (Medina-Sánchez *et al.* 2004), thus they are in a topologically central position in the network (Puche *et al.* 2020b, c) and act as a bypass of carbon flux toward the autotrophic food web (Medina-Sánchez *et al.* 2004). From the module formed by the autotrophic food chain, the cladocerans were the main connectors due to their capacity to shift between phytoplankton and periphyton as food resources (Burks *et al.* 2002, Siehoff *et al.* 2008) and the non-trophic interactions they establish with macrophytes, thus coupling the planktonic autotrophic chain with the benthic primary producers' module (Puche *et al.* 2020a, b, c). In this vein, it seems that more than 80% of the benthic primary production in shallow lakes could be transferred to the water column if there are large

herbivores (whether they are fish or cladocerans; Vadeboncoeur *et al.* 2002, Adamczuk 2014). Moreover, the connector role, either from the microbial loop or from the autotrophic chain, confered high values of centrality to the edible nodes (*e.g.* edible mixotrophs, diatoms or chlorophytes), since they were food resources for both planktonic and benthic herbivores, and they might also be allelopathycally interacting with macrophytes (Rojo *et al.* 2013a, b).

For their part, in the lakes, despite considering three habitats for the definition of the nodes in their multi-interaction network, only two functional and disconnected modules emerged. The planktonic autotrophic chain module included nodes such as copepodites, rotifers and cladocerans (good swimmers) that are trophically linked to highly edible planktonic microalgae (such as unicellular chlorophytes, diatoms), and even bacteria (Alva-Martínez *et al.* 2007, Burian *et al.* 2014). Moreover, the within-meadow consumers were included in this autotrophic chain module because of their higher number of connections with planktonic elements than with the benthic module (*e.g.* refuge provided by macrophytes against predators or even radiation; Schriver *et al.* 1995). These mobile predator-edible prey combinations in the water column (from the epilimnetic layer to the bottom layer within the meadows) may be responsible for the fusion of pelagic and within-meadow habitats in a functional module, and suggests the role of meadows as a source of highly diverse food for pelagic organisms (Declerck *et al.* 2011).

The emerged benthic autotrophic chain module in the lakes was, mainly, the charophyte meadow, with all the benthic predator-prey interactions occuring on the surface of these macroalgae. The network analyses included in this benthic module the within-meadow primary producers (*e.g.* mixotrophs or cyanobacteria) only due to the likely allelopathic relationships between them and charophytes (Rojo *et al.* 2013a, b). The cladocerans-macrophytes connector tandem (Puche *et al.* 2020a, b, c) mentioned above in the ponds, was not observed in the lakes, nor did any other connector node between modules arise in these ecosystems. In this regard, we support the idea that,

in the lakes, the coupling between the benthic autotrophic chain at the bottom of the lake and the pelagic autotrophic chain should be mainly attributed to larger and mobile vertebrates such as fish (Vadeboncoeur *et al.* 2001, Schindler and Scheuerell 2002).

Hence, considering these contrasting models between the ponds and lakes, we highlight the relative importance of benthic elements for both types of ecosystem. Benthic nodes inhabiting meadows are very influential and sensitive to changes in other nodes of the network (Vadeboncoeur *et al.* 2002). Thus, benthic elements can be considered as good spreaders of the disturbance effects in the ecosystems (Puche *et al.* 2020c). Their changes would compromise the structure and dynamics of the overall network in the ponds (Puche *et al.* 2020a, b, c). However, this performance can not be extended to the lakes, as the pelagic and benthic modules were disconnected. Another difference between the lakes and ponds was the higher redundancy of species in benthic producer nodes of lakes. This could be explained by the higher redundancy in the benthic consumer nodes, also. A wide, varied diet favours the richness of the consumers and, in turn, any consumer can have a higher consumption efficiency over any group of algae or cyanobacteria (Rakowski *et al.* 2020). Thus, the effect of benthic species loss, mainly in the lakes, on the functional integrity of the entire community (Wellnitz and Poff 2001) would also be minimized by this high redundancy. Furthermore, Olesen *et al.* (2007) demonstrated a loss of community stability due to the greater vulnerability of the different modules when they are disconnected (as occurred in our studied lakes), since the negative effects in a module cannot be buffered by connections to other modules in the network. Hence, macrophyte meadows contribute to the community with elements that can promote its stability, but also transmit the effects of the disturbances. The importance of this trade-off for the community lies in the coupling of the modules that ultimately emerges from the type of waterbody.

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|BLOCK 3| FUNCTIONAL ECOLOGY: THE MACROPHYTE ROLE

(LIMNOCORRAL SCALE)

|CHAPTER 8|

Macrophyte meadows mediate the response of the sediment microbial community to global change-related factors

Rojo, C., Segura, M., Puche, E., Rodrigo, M.A. Macrophyte meadows mediate the response of the sediment microbial community to global change-related factors. Ready for submission. *Biodiversity and Conservation*.

Abstract

Macrophyte meadows serve as a refuge and support for a great richness of aquatic organisms, and have a relevant role in biogeochemistry and water quality. These meadows are especially relevant in Mediterranean wetlands where an increase in ultraviolet radiation (UVR) penetration and pollution by nitrates are two global change-related factors which can affect sediment communities and, hence biogeochemical cycles. Considering these facts, our objectives were to establish how a sediment microbial community (SMC) of a nitrate-enriched system is affected by UVR, and how such effects can be mitigated by the presence of macrophyte meadows. We also tested if the SMC changes alter their functions (*i.e.* carbon sink and denitrification processes). We carried out a factorial experiment based on limnocorrals located in a Mediterranean protected wetland, with two radiation qualities (sunlight and filtered UVR) and the presence or absence of charophytes (*Chara hispida*). The abundance and composition of microbial communities in the superficial and sub-superficial layers of the sediment were analyzed. The methods included inverted microscopy, flow-cytometry and genetic studies of sediment microbial diversity and C:N stoichiometry. All our hypotheses were confirmed: incident UVR on sediments reduces the biomass and richness of microorganisms in the periphytic biofilm; charophyte meadows fuel the periphytic biofilm and sub-superficial bacterial community; and denitrifying bacteria and chlorophytes microalgae are enhanced with UVR reduction and the presence of meadows. We consider that these results help to understand the sensibility of SMC to global change-related factors, and also encourage proactive management in favour of macrophyte meadows in vulnerable shallow ecosystems.

Keywords: C:N stoichiometry; charophytes; denitrifying bacteria; Mediterranean wetland; periphyton biofilm; UVR

Resum

*Les praderes de macròfits serveixen com a refugi i suport per a una elevada riquesa d'organismes aquàtics i tenen un paper rellevant en la biogeoquímica i la qualitat de l'aigua. Aquestes praderes són especialment importants en els aiguamolls mediterranis on l'increment de la penetració de la radiació ultraviolada (RUV) i la contaminació per nitrat són dos dels factors de canvi global que afecten a les comunitats del sediment i, per tant, als cicles biogeoquímics. Considerant aquests fets, els nostres objectius foren establir com la comunitat microbiana del sediment (CMS) d'un sistema enriquit en nitrat es veu afectada per la RUV, i com aquests efectes poden ser mitigats per la presència de praderes de macròfits submergits. També hem comprovat si aquests canvis sobre la CMS alteren les funcions d'aquests ecosistemes (*i.e. *els processos de retenció de carboni i desnitrificació). Hem dut a terme un experiment factorial basat en limnocorrals localitzats en un aiguamoll mediterrani protegit: dues qualitats de radiació (radiació solar natural i filtrant la RUV) i presència o absència de caròfits (*Chara hispida*). Es va analitzar l'abundància i la composició de les comunitats microbianes en les capes superficial i sub-superficial del sediment. Els mètodes inclogueren microscòpia invertida, citometria de flux, estudis genètics sobre la diversitat microbiana i estequiometria C:N. Totes les nostres hipòtesis van ser confirmades: la RUV incident sobre el sediment redueix la biomassa i la riquesa dels microorganismes del biofilm perifític; les praderes de caròfits afavoreixen el biofilm perifític i la comunitat bacteriana sub-superficial del sediment; i els bacteris desnitrificants així com les microalgues clorofícies van ser beneficiades per la reducció de la RUV i la presència de praderes. Creguem que aquests resultats ajuden a comprendre la sensibilitat de la CMS front a factors de canvi global, però també fomenten una gestió proactiva a favor de les praderes de macròfits submergits en els vulnerables ecosistemes aquàtics somers.*

Paraules clau: *estequiometria C:N; caròfits; bacteris desnitrificants; aiguamoll mediterrani; biofilm perifític; RUV.*

1. Introduction

The aquatic microbial community is an assemblage of microbes that plays an important role in aquatic ecosystems, which turns out to be the engine of biogeochemical cycles in inland waters. The aquatic microbial community includes both the periphyton biofilm inhabiting the water-sediment interface where active photosynthetic radiation arrives, and the group of bacteria (including cyanobacteria) plus Archaea in different compartments of the aquatic ecosystem (*e.g.* pelagic, benthic; Callieri *et al.* 2019). The aquatic microbial community is involved, for example, in the fixation and transfer of carbon, or the elimination of nitrogen into the atmosphere (Eyre and Ferguson 2002; Canfield *et al.* 2010; Callieri *et al.* 2019). Therefore, in recent years, the likely effect of global change on such a community is a topic of concern which needs to be studied in depth (Baveye *et al.* 2019; Orland *et al.* 2020; Yang *et al.* 2020).

Differences in the abundance and composition of microbial community of sediment (SMC hereafter) are expected in the different layers, and therefore different main functions can be assigned in each layer (Baveye *et al.* 2019). The periphyton biofilm is a variety of autotrophic and heterotrophic microbes, mostly benthic microalgae, cyanobacteria, bacteria and Archaea (Rysgaard *et al.* 1995; Song *et al.* 2016). This assemblage plays significant roles in the primary productivity, energy flow, and nutrient cycling in aquatic ecosystems (Azim *et al.* 2005; Canfield *et al.* 2010), and it is used to analyse water quality (Sabater *et al.* 2007; Wu *et al.* 2018). In addition to this complex structure in the superficial sediment where photosynthetically active light arrives, the microbial community also shows a relevant biogeochemical activity in the sub-superficial anoxic sediment (Morina *et al.* 2018).

The aquatic microbial community in general, and the SMC in particular, are sensitive to environmental conditions, something which can be seen throughout its geographical distribution (Gugliandolo *et al.* 2016). And it is well known that some of the microbial community components are sensitive to both biotic and abiotic environmental conditions on a local scale; for example, being affected by light quality,

temperature and N pollution (Navarro *et al.* 2009; Baron *et al.* 2013), or the presence of macrophytes (Rojo *et al.* 2017). Thus, it is predictable that this sensibility will result in changes in abundance and composition in every sediment layer when some stressors (*i.e.* global change-related factors) act.

These changes in the SMC will be especially worrying if they occur in shallow lakes or wetlands, because these ecosystems are highly vulnerable to global change (Jeppesen *et al.* 2014). The SMC in a shallow lake will have very close relationships with macrophytes (Dai *et al.* 2019). Moreover, the alteration undergone by the macrophytes due to changes in the environment spreads to the trophic web including benthic and planktonic habitats (Puche *et al.* 2020). The most studied foreseeable environmental changes are, among others, warming, eutrophication, salinization, loss of water column depth and changes in light quality (Carrillo *et al.* 2002; Jeppesen *et al.* 2014; Rojo *et al.* 2019). In this regard, shallow Mediterranean aquatic ecosystems represent a paradigmatic case. In this climatic region, shallow lakes or wetlands are already suffering the effects of drought, namely the loss of depth due to evaporation, the lack of precipitation and water overexploitation. These conditions result in an increase in nutrient concentrations and changes in the quality of light (Parcerisas *et al.* 2012; IPCC 2014). These changes, which can occur in a few days, make shallow lakes a type of temporary waterbodies, where the SMC will have to respond in the short-term (Rojo *et al.* 2017a).

One of the consequences of the loss of water column depth in aquatic ecosystems will be that the ultraviolet radiation (UVR hereafter) reaching the bottom will be able to affect the benthic community, affecting both the macrophytes and the microorganisms from the SMC (Rojo *et al.* 2019). The harmful effect of UVR on the photosynthetic metabolism and DNA of aquatic primary producers, such as microalgae and cyanobacteria, reduces their production (Barrado-Moreno *et al.* 2017). Moreover, UVR triggers a loss in their diversity towards more resistant taxa (Harrison and Smith 2009; Rojo *et al.* 2012a). Furthermore, UVR could also directly affect the concentration

and composition of the aquatic bacterial community (Manrique *et al.* 2012), and indirectly affects it by altering its matter and energy sources (Mayer *et al.* 2006). Therefore, our first hypothesis is that incident UVR on sediments of shallow aquatic systems will reduce the biomass and richness of microorganisms (bacteria, Archaea, microalgae and cyanobacteria) in the periphyton biofilm.

Submerged macrophytes, vascular plants as well as charophytes (green macroalgae), through their contribution of organic matter to the sediment, exudates of compounds and the morphological architecture provide a landscape to develop the SMC (Hilt and Gross 2008; Morina *et al.* 2018; Dai *et al.* 2019). These macrophytes are a source of nutrients for sediment; nutrients that these plants have incorporated from the water column (Rodrigo *et al.* 2013; Rojo *et al.* 2020). A high concentration of an organic particulate source of C and N could promote bacterial development and processes such as carbon incorporation into the aquatic web and nitrogen loss, both of which are beneficial to the ecosystem (Rabalais 2002). Moreover, the presence of macrophyte meadows could imply beneficial shading for the SMC, minimizing the harmful UVR effect in shallow waterbodies. Therefore, we can establish as a second hypothesis that under charophyte meadows, the nutrient enriched sediment, free from UVR, fuels the SMC (Navarro *et al.* 2009).

On the other hand, the high nitrogen concentration found in ecosystems sited in areas with abusive anthropogenic nitrogen inputs (*i.e.* the coastal Mediterranean area where there is an intensive agricultural fertilization) is considered a serious pollution problem (Jeppessen *et al.* 2011). This problem might become even more dramatic due to water evaporation caused by global warming (Giorgi and Lionello 2008). Therefore, the biotic communities involved in the N biogeochemical cycle, particularly those involved in denitrifying processes in the sediments, are of main interest (Canfield *et al.* 2010). Coastal lagoons and wetlands are ecosystems undergoing intense biogeochemical transformations, where, for example, nitrogen gas resulting from denitrification is lost to the atmosphere (Jordan *et al.* 2011). The role of submerged

macrophytes in this process is crucial, both directly and indirectly (Veraart *et al.* 2011). Directly, denitrification rates may be strongly affected by the presence of macrophytes due to their effects on oxygen conditions in the water column and the sediment, and by providing a surface area for attached biofilms (both in the roots and the shoots), where the heterogeneous oxygen conditions may affect both nitrification and denitrification. In an indirect way, macrophytes affect denitrification rates by changing the nutrient concentrations by uptake and release during growth and senescence, and, moreover, by influencing oxygen levels, pH, and organic carbon availability in the sediment and the water column.

Therefore, it is expected that aquatic plant meadows affect not only the physical and chemical properties of sediment (Neubauer *et al.* 2005), but also the structure and function of the microbial communities in the periphyton biofilm and sub-superficial layers (Morina *et al.* 2018). The presence of higher concentrations of organic matter, rich in nitrogen compounds, should be accompanied by greater density and activity of denitrifying bacteria if anaerobic conditions are established (Rodrigo *et al.* 2007; Morina *et al.* 2018). All these ideas give rise to a third hypothesis: the presence of charophytes (carbon-rich macroalgae; Rojo *et al.* 2020), promotes a sediment with a higher bacterial density related to the N metabolism, and thus, a different C:N ratio in the stoichiometry of the sediment underneath the charophyte meadows compared to the bare sediment is expected.

Hence, our goal is to disentangle the effect of charophyte meadows on the SMC (both the periphytic microbial community and that of sub-superficial sediment layers) of highly illuminated wetland sediments, particularly on the primary producers and the denitrifying bacteria. To do this, and by means of an outdoor experiment with mesocosms (*i.e.* limnocorrals) in a protected Mediterranean coastal wetland, we have compared the structure of the microbial communities of the sediment (superficial and sub-superficial layers), from unvegetated areas and from *Chara hispida* meadows under sunlight and under reduced UVR conditions.

2. Material and methods

2.1. Experimental design

2.1.1. Obtaining the charophytes and the preparation of cultures

The charophyte chosen for the study was *Chara hispida* (Characeae family), a freshwater benthic macroalga which is anchored to the substrate by means of rhizoids. This is a cosmopolitan species, naturally present in freshwater ecosystems in the Mediterranean region, and it has previously been used in studies related to global change (Rojo *et al.* 2017b). The original plant material was collected from a small Mediterranean coastal lagoon (39°12'29.2''N and 0°14'4.7''W) close to where the experiment took place. Using this collected material, small plants of *C. hispida* were cultivated in a chamber and when the roots had sprouted they were planted in the limnocorrals (more detailed in Supplementary material Chapter 8).

2.1.2 Global design

The experiment followed a two-way ANOVA design: i) the presence or absence of charophytes (CH or NCH, respectively), and ii) sunlight or sunlight with reduced UVR (hereinafter termed PAB and PAR, respectively). Therefore, the experiment had four conditions (CHPAB, CHPAR, NCHPAB, NCHPAR) with three replicates of each of them, which meant a total of 12 limnocorrals were needed. The 12 limnocorrals were located in a protected wetland, El Tancat de la Pipa (39°21'51"N and 0°20'47"W) a restored area from former rice fields belonging to the Albufera de València Natural Park (Fig. 1A). The limnocorrals were quadrangular cages anchored to the sediment; the sides and tops were covered with plastic mesh and plastic sheets, respectively, to prevent animal incursions (Rodrigo *et al.* 2013, Fig. 1B-C). For the PAB treatment, the limnocorrals were covered with polyethylene sheets which transmitted 90% PAR (400-700 nm) and the majority of UVR [100% UVB (280-320 nm) and 92% UVA (320-400) nm)].

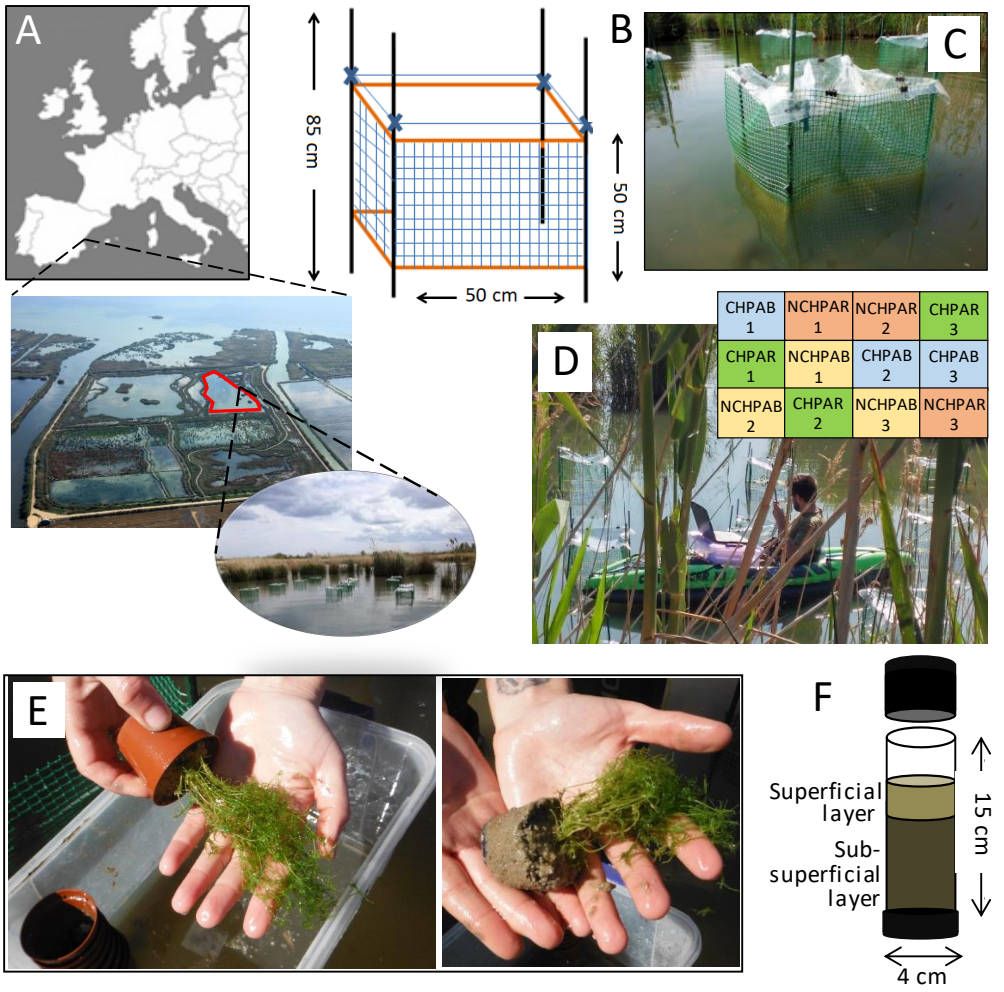

Fig. 1. A) Location map with an enlargement of the area where the experiment took place (Albufera de València Natural Park). The lagoon of interest (the Educative lagoon in Tancat de la Pipa protected area) can be seen along with a detail of the set-up of the limnocorrals. B, C) Sketch of the limnocorrals specifying their dimensions and a photograph of one of them. D) Photograph showing the way of approaching to the limnocorrals (with a one-person inflatable kayak) and in this occasion measuring the underwater radiation in each limnocorral. In the right-up corner: diagram showing the allocation of treatments (CH: charophyte meadows; NCH: without charophytes meadows; PAB: limnocorrals subjected to sunlight; PAR: limnocorrals subjected to reduced UVR; the number is the replicate for each condition). E) A charophyte culture with and without the pot, ready to be planted. F) Diagram of a sediment core specifying its dimensions, the visually differentiable superficial and sub-superficial layers correspond to the more oxic and more anoxic zones, respectively.

The UVR filter sheet for the PAR treatment transmitted 80% of the PAR radiation, 48% of the UVB and 56% of the UVA. The underwater radiation doses were measured in each limnocorral with a JAZ system spectrometer (Ocean Optics, Inc.) (details in Supplementary material Chapter 8). The limnocorrals were placed at a distance of approximately one meter from each other; the treatments corresponding to each limnocorral were established randomly (Fig. 1D). For the CH treatment, 16 small cultures of charophytes belonging to the laboratory stock (after removing the plastic pot; Fig. 1E) were planted in each limnocorral (96 cultures in total). Thus, approximately 80% of the surface of these limnocorrals was covered with charophytes. In the NCH-treatment limnocorrals, 16 sediment units (from pots containing the same sediment as for charophyte cultures, treated in the lab exactly the same as the formers) were also placed in each limnocorral.

At the beginning of the experiment, a sediment core (4 cm in diameter by 15 cm in height; Fig. 1F) was extracted from each limnocorral as spatial heterogeneity was not expected. However, at the end of the experiment, five cores were extracted per limnocorral in order to include the possible spatial heterogeneity due to the treatments. In each core, two parts of the substrate were distinguishable according to their coloration and the presence of primary producers (Fig. 1F); a lighter surface part corresponding to the more oxygenated zone where the periphytic biofilm was located (hereafter the superficial layer), and a deeper part, darker in colour, corresponding to the more anoxic part (hereafter the sub-superficial layer). In the field, these layers were separated and kept in sterile plastic pots. Once in the laboratory, the superficial parts of the five cores of each limnocorral were homogenized; the same procedure was carried out with the five sub-superficial layers.

At the end of experiment, the limnological environment in the limnocorrals was recorded (Table S1 Supplementary material Chapter 8). There was no difference in the biomass of the charophyte meadows based on the radiation treatments (Table S2

Supplementary material Chapter 8), and the charophyte chlorophyll a concentration was higher under PAB treatments (Table S2).

2.2. Response variables

2.2.1. Bacteria: counting and density estimation

The preparation of the samples for counting by flow cytometry was carried out from an adaptation (see Supplementary material Chapter 8 for more details) of the dilution / fixation / staining protocol to analyse freshwater bacteria in lake sediments proposed by Duhamel and Jacquet (2006). Once the sample was stained, it was put into the cytometer (Cytomics FC 500 Beckman Coulter) and a high flow rate for 120 seconds was programmed; this process was repeated 3 times per sample in three different sessions. These results were analysed with the specific program Flowing Software 2. A dot plot was made with channels FL1 and FL4, which discriminate bacteria from other particles since bacteria stained with SYBR Green II have a maximum emission collected by channel FL1, and a minimum collected by FL4; from this graph, the region corresponding to the bacteria was delimited (more details in Fig. S1 Supplementary material Chapter 8).

In parallel, the water content of the sediment was assessed by weighing aliquots of this sediment (fresh weight (FW) initially and dry weight (DW) after 24h at 70˚C); the relationship between the FW and DW of the sediment was calculated by measuring them from aliquots of all samples (DW=0.179xFW; R^2=0.99). Thus, bacterial counting was normalized by the grams of DW of sediment considered for the sample, and, in this way, the number of bacteria per gram of DW of sediment for each layer and each limnocorral was obtained.

2.2.2. Bacteria and Archaea: composition

For DNA analyses, 0.25 g of sediment was used following the PowerSoil® DNA Isolation Kit (Qiagen) manufacturer's protocol. 16S rRNA gene sequencing and bioinformatic data analyses were carried out at the Genomics core facility of the SCSIE-*Universitat*

de València. Variable V3 and V4 regions of the 16S rDNA were amplified following the 16S rRNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). Gene-specific primers (PCR1_f: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'; PCR1_r: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') containing Illumina adapter overhang nucleotide sequences were selected according to Klindworth *et al.* (2013). After 16S rDNA gene amplification for each sample, the multiplexing step was performed using the Nextera XT Index Kit. Amplicon libraries were sequenced using a 2 × 300 pb paired-end run on a MiSeq Sequencer according to the manufacturer's instructions (Illumina). Sequencing data were demultiplexed using the Illumina bcl2fastq© program. Forward and reverse raw reads were checked for quality, adapter trimmed and filtered using AfterQC (Chen *et al.* 2017) and FastQC v0.11.8 (http://www.bioinformatics.babraham.ac.uk) tools.

Sequence analysis was conducted using the 16S-based metagenomics workflow of MiSeq Reporter v2.5 (Illumina), including forward and reverse read joining, data filtering and taxonomic annotation. OTUS clustering and classification at several taxonomic levels were performed using a high-performance implementation of the Ribosome Database Project (RDP) Classifier algorithm, described in Wang *et al.* (2007). Taxonomic classification was carried out using an Illumina-curated version of the Greengenes database (http://greengenes.secondgenome.com/downloads/database/13.5)

The results were reported as the number of sequences (hits) obtained on the different sample sizes analyzed and the percentage of sequences from each OTU. To obtain a measure of abundance of each OTU, these percentages were used on total bacteria abundance (cells/gDW of sediment), obtaining an approximation to hits of each OTU per g DW of sediment; this is an abundance measurement which is more easily comparable to the abundance of other organisms inhabiting the sediment, such as microalgae and cyanobacteria. For each sample, we selected OTUs with more than 300 hits, or 0.1% of total hits; and, although some OTUs are at the species level, we

considered the matrix of the genera more trustworthy (Fox *et al.* 1992; Azua-Bustos *et al.* 2018). For the comparative analysis of sample composition, we used phyla that had more than 1% of hits. Archaea sequences, despite being detected in all the samples, did not result in more than 1% of hits of each sample.

2.2.3 Abundance and composition of microalgae and cyanobacteria (MC)

To study the MC assemblages, a fraction of the sediment from the superficial layer of each core was weighed (FW). Then, this fraction was washed by stirring in 50 ml of deionized water; this water preserved with a Lugol´s iodine solution was the sample used to observe the organisms.

Taxonomic classification (at the finest possible resolution), counts and measurements of MC were conducted using Utermöhl chambers under an inverted microscope at 400× and 1000× magnification following standard protocols (cited in Rojo *et al.* 2012a). To determine whether most of the periphytic species richness of each assemblage was covered, a species accumulation curve was plotted as a saturating criterion (Rojo *et al.* 2017b). Counted individuals were single cells, colonies and filaments; their biovolume was calculated following Hillebrand *et al.* (1999). Therefore, the biomass of MC for each species and site was expressed as mm^3/gDW of sediment.

2.2.4 Diversity analysis

We also calculated the diversity for each assemblage in the sediment as the richness (S), the effective number of species and the evenness value. We used the Shannon-Wiener index (H, using natural logarithms), which is sensitive to less frequent species (Shannon and Weaver 1949), and determined the departure from the maximal value of this index with the evenness value (expH/S). The effective number of species was calculated following Jost *et al.* (2010) as expH. These diversity indicators were calculated on both biomass (mm^3/gDW of sediment) of MC species and density of hits (hits/gDW of sediment) of bacterial phyla.

2.2.5. Limnological conditions and sediment of limnocorrals

Limnological conditions, including physical and chemical variables and the charophyte state were reported for each limnocorral at the end of experiment (Tables S1 and S2).

Moreover, three replicated samples of sediment were collected from each treatment. From the collected sediment cores from each limnocorral, a homogenate belonging to the most superficial layer and another belonging to the sub-superficial layer were obtained (previously detailed in section 2.1). These homogenates were kept in tubes in the freezer at -20°C until proceeding with the stoichiometric analyses. Carbon and nitrogen contents of these samples were measured using a Perkin-Elmer CHSN-2400 elemental analyser. The precision (reproducibility) of all measurements were 0.22% and 0.06% for carbon and nitrogen, respectively. The limits of detection were 0.10% and 0.05% for carbon and nitrogen, respectively.

2.3. Statistical analyses

The normality of the residuals and the homoscedasticity of the variances, the criteria necessary to be able to apply an analysis of variance (ANOVA), were verified by means of Shapiro-Wilk and Levene tests, respectively. When both conditions were met, one- or two-way ANOVAs were performed to study the effect of charophyte and radiation factors, as well as their interaction on the response variables. When the requirements for the ANOVA were not met, non-parametric tests were used (*i.e.* Mann-Whitney test). Statistically significant differences were considered from a probability $p<0.05$. When considered helpful, correlations (Pearson coefficient) between variables were carried out.

SIMPER analyses, based on Euclidean distances and considering the set of comparisons between conditions, were performed for superficial and sub-superficial samples to highlight which phyla of bacteria were the most relevant for characterizing the conditions. Principal component analyses were performed to order the samples based on main bacterial phyla. To order samples based on their SMC composition, a cluster analysis (Euclidean distances and UPGM) was performed. Statistical analyses were carried out using the PAST 3.14 software (Hammer *et al.* 2001; ohammer@nhm.uio.no) and software SPSS Statistics v.22 (IBM Corp, Armonk, NY).

3. Results

3.1. Bacterial (and archaeal) communities under different experimental conditions

There was a weak relationship between the number of bacteria in superficial samples (periphytic biofilm) and the bacteria from the sub-superficial sediment (R^2=0.35; p=0.04). In fact, the average density of bacteria in the superficial and sub-superficial layers of sediment were different (one-way ANOVA, F= 20.3 and p< 0.0001), periphytic bacterial density being almost double ($6 \cdot 10^9 \pm 4 \cdot 10^8$ cells/g DW superficial sediment *versus* $3 \cdot 10^9 \pm 3 \cdot 10^8$ cells/gDW sub-superficial sediment; Fig. 2). UVR reduced bacteria density in the surface of the sediment; when UVR was removed, the density was significantly higher, not taking into account the presence or absence of charophytes (average density was $7 \cdot 10^9 \pm 5 \cdot 10^8$ cells/gDW sediment under PAR conditions, and $5 \cdot 10^9 \pm 4 \cdot 10^8$ cells/g DW sediment under PAB conditions; Fig. 2A; Table 1). The mean density was 35% higher in CHPAR compared to CHPAB conditions, and 27% higher in NCHPAR compared to NCHPAB. The highest density was reached in the limnocorrals with charophytes and filtered UVR; however, this synergic interaction was not statistically significant (Table 1). The tested factors did not significantly affect the number of bacteria in the sub-superficial layer of sediment, although it was under the CHPAR conditions where the highest density was again observed, and the density in

the meadow sediment (whatever type of light received) was 25% greater than in the sediments of the limnocorrals without charophytes (Fig. 2B).

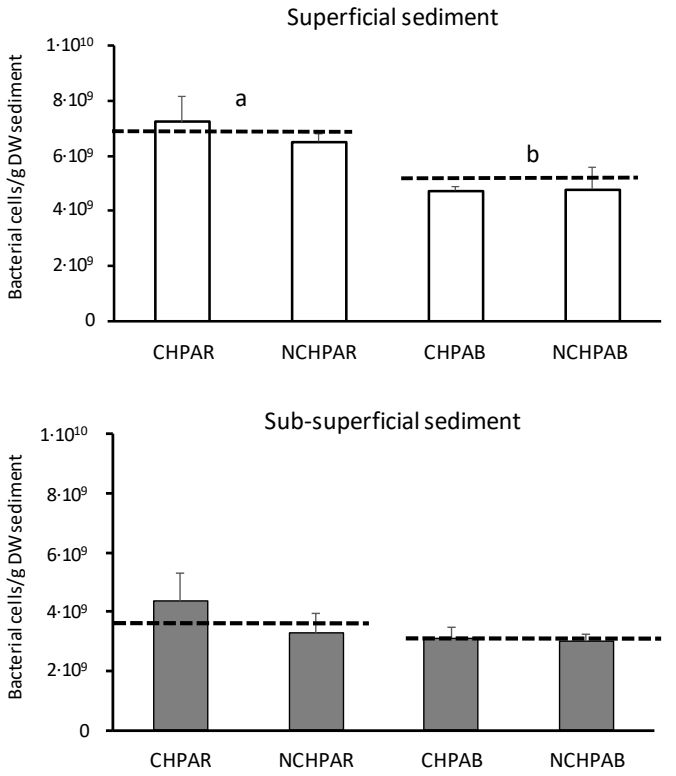

Fig. 2. A) Average bacterial density in the superficial layer of the sediment. Lowercase letters are in accordance with the result of a *post hoc* Tukey analysis of density variance; the dashed line indicates the average value. B) The same representation of bacteria density in the sub-superficial layer of sediment. Bars are standard errors; abbreviations of the four environmental conditions as in Fig. 1.

Table 1. Two-way ANOVA parameters (F and probability p) calculated on total bacterial density and density of main phyla from superficial layers of sediment. 1 freedom degrees for the two factors (presence or not of charophytes and filtered or unfiltered UVR) and their interaction, and 8 freedom degrees within groups. In bold significant values of p (p<0.05). *Gamma-* (*γ-*), *Delta-* (*δ-*) and *Beta-* (*β-*) *proteobacteria*, *Bacteroid* (*Bacteroidetes*).

	Total density		*γ-proteobacteria*		*Verrucomicrobia*		*δ-proteobacteria*		*β-proteobacteria*		*Bacteroid*	
Factor	F	p	F	p	F	p	F	p	F	p	F	p
UVR	1.10	**0.010**	31.60	**<0.001**	17.67	**0.003**	27.16	**0.001**	20.85	**0.002**	79.30	**<0.001**
Charophytes	0.27	0.610	6.11	**0.039**	0.65	0.442	3.41	0.102	1.89	0.206	4.66	0.063
Interaction	0.37	0.560	0.07	0.801	2.26	0.172	0.31	0.594	0.09	0.768	1.43	0.266

Of the total number of analysed DNA sequences (including all the samples), no more than 6% were unclassified, 30% were bacteria but not classified, 0.4% were Archaea and the rest were bacteria classified in the following 16 phyla (Table 2): *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Caldithrix*, *Cyanobacteria*, *Chlorobi*, *Chloroflexi*, *Firmicutes*, *Nitrospira*, *Planctomycetes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Spirochaetes* and *Verrucomicrobia*. Only nine of them accounted for more than 1% of each superficial and sub-superficial sample and *Delta-* and *Gammaproteobacteria* were the most abundant (Table 2).

The diversity of the bacterial community did not differ depending on UVR, in either the superficial or the sub-superficial layer (Table S3 Supplementary material Chapter 8). The number of phyla only resulted somewhat lower in the superficial layer of the limnocorral with meadows compared to the unvegetated ones (15.3±0.2 and 16.0±0.0 respectively; Table S3). The richness of phyla was higher in the superficial compared to the sub-superficial layer (15.7±0.1 and 10.9±0.0 respectively; Mann Whitney test p<0.001).

In the periphytic communities (*i.e.* superficial ones), *Gamma-* and *Deltaproteobacteria*, *Bacteroidetes*, *Betaproteobacteria* and *Verrucomicrobia* were the phyla that contributed most to the difference between the four experimental conditions (SIMPER analysis showed a contribution of 38, 37, 10, 9 and 5%, respectively).

Table 2. Average and standard error of hit percentage of the most represented (>1%) phyla and the most representative species. Data included samples from the superficial and sub-superficial sediment layers.

	Mean	Standard error
Bacterial phyla	64.3	1.4
its main species		
Actinobacteria	1.8	0.1
Bifidobacterium bombi	*0.6*	*0.0*
Bacteroidetes	7.9	0.2
Pedobacter kwangyangensis	*1.3*	*0.0*
Chlorobi	2.5	0.1
Ignavibacterium sp.	*2.2*	*0.1*
Chloroflexi	3.5	0.1
Longilinea arvoryzae	*1.1*	*0.0*
Firmicutes	4.1	0.2
Clostridium sp.	*0.7*	*0.0*
Betaproteobacteria	5.9	0.3
Thiobacillus sp.	*2.7*	*0.1*
Deltaproteobacteria	13.3	0.5
Desulfococcus sp.	*2.8*	*0.1*
Gammaproteobacteria	17.4	0.8
Steroidobacter denitrificans	*4.1*	*0.5*
Marichromatium gracile	*2.1*	*0.1*
Verrucomicrobia	7.4	0.4
Luteolibacter sp.	*3.0*	*0.5*
Candidatus Methylacidiphilum	*1.4*	*0.0*
Archaea	0.4	0.0
Unclassified	5.8	0.1
Other bacteria	29.9	1.3

These phyla were more abundant when UVR was filtered (Table 1). Among phyla, only the *Gammaproteobacteria* density was also statistically higher when charophytes were present (Table 1). However, there was no interactive effect of the two factors (Table 1). Principal component analysis arranged samples of superficial sediment based on the bacterial density of the five mentioned selected phyla (Fig. 3A). The samples obtained from the limnocorrals with no UVR were in the positive part of axis 1 (87% explained variance), which also corresponds to the main bacterial phyla. Axis 2 (11% explained variance) separated the samples into ones with *Deltaproteobacteria* dominance or *Gammaproteobacteria* dominance.

SIMPER analysis on sub-superficial samples from the four conditions highlighted *Gamma*- and *Deltaproteobacteria* plus *Bacteroidetes* (41, 23 and 12%, respectively).

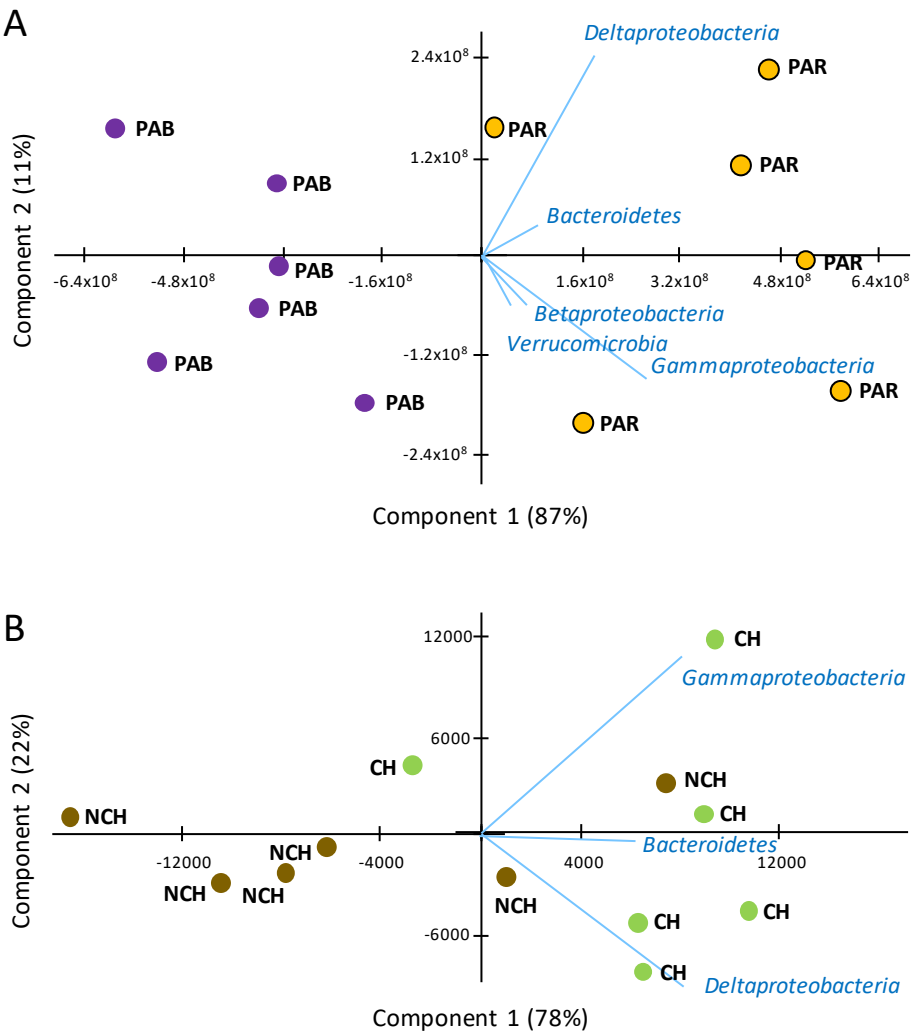

Fig. 3. Principal component analysis (% of explanation) which arrange the sediment microbial community of the limnocorrals (abbreviations as in Fig. 1) based on their main bacterial phyla density; the order of the main phyla is also indicated. A) from superficial layer, B) from sub-superficial layer.

Principal components analysis of the sub-superficial layer arranged samples without any relationship with light quality, and most of the samples of sediment with charophytes were located in the most positive part of axis 1 (78%; Fig. 3B), together with the main bacterial phyla. In addition, again, it was the dominance of *Delta*- or *Gammaproteobactera* which established the division in axis 2 (22%; Fig. 3B).

3.2. Microalgae and cyanobacteria communities (MC) under different experimental conditions

The biomass of MC was greater when UVR was removed (Fig. 4A; Table 3). Its average and standard error was 0.053±0.007 mm^3/gDW of superficial sediment, double the biomass that was found in sediment with UVR penetration (0.027±0.003 mm^3/gDW). The charophyte meadows did not exert a positive effect on MC abundance (Table 3). However, there was a significant interactive effect of both factors since under the CHPAR condition the MC biomass was the highest (Fig. 4A).

The biomass of all the taxonomic groups was affected by the tested factors (Table 3), but in different ways. Diatoms (dominant group) and cyanobacteria (smaller proportion) appeared in all the conditions (Fig. 4B-C). The biomass of diatoms was, not considering the presence or absence of charophytes, more abundant when there was no UVR (0.041±0.003 mm^3/gDW) compared to the treatment with the complete light spectrum (0.032±0.003 mm^3/gDW; Fig. 4B, Table 3). Cyanobacteria, which did not reach more than 0.018 mm^3/gDW in any limnocorral sediment, were more abundant when charophytes were present and UVR was filtered out, yet, there was no interactive effect of these factors (Fig. 4B-C; Table 3). Chlorophytes were only observed when UVR was filtered (Fig. 4B-C) and appeared in a greater biomass under the charophyte meadows (0.10±0.02 mm^3/gDW in front to 0.03 ± 0.01 mm^3/gDW), thus, an interactive effect of factors occurred (Table 3).

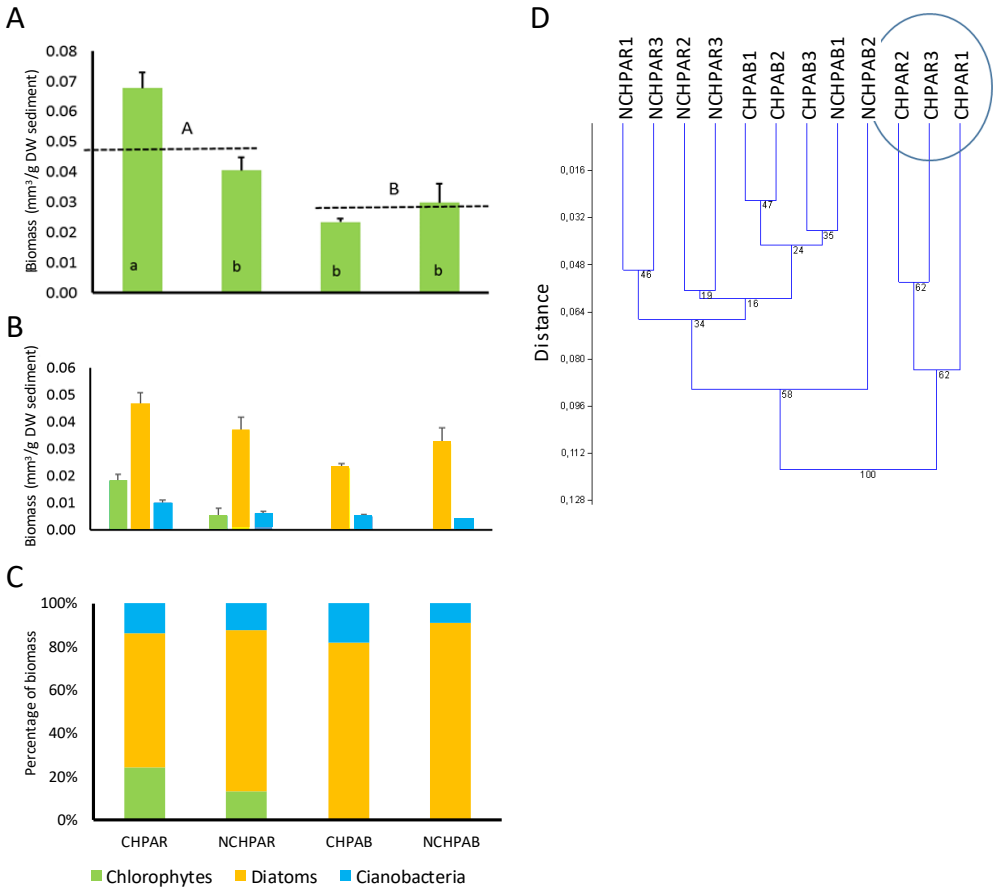

Fig. 4. Structural differences in periphytic microalgae and cyanobacteria communities (MC) inhabiting the sediment superficial layer under the four experimental conditions (presence or not of charophytes, and filtered, or not filtered UVR; abbreviations as in Fig. 1). A) Average and standard error (thin bars) of the biomass; the capital letters indicate statistically significant differences due to the radiation treatment; the lowercase letters are in accordance with the result of a *post hoc* Tukey analysis of variance on the biomass. B) Average and standard error (thin bars) of the biomass of main MC groups in each experimental condition; C) Percentage of total biomass for the main taxonomic groups. D) Dendrogram made based on the biomass of MC species of the 12 limnocorrals; the cluster-tree is based on the unweighted pair-group method with arithmetic mean (UPGMA) calculated on Euclidean distance similarity; the numbers in the nodes are the 1000 bootstrap results.

Table 3. Two-way ANOVA parameters (F and probability p) applied on species diversity indicators and total biomass of microalgae and cyanobacteria and the main taxonomic groups. 1 freedom degree for the two factors (presence or not of charophytes and filtered or unfiltered UVR) and their interaction, and 8 freedom degrees within groups. In bold significant values of p ($p<0.05$); only response variable with significant differences are shown.

	Richness		Total biomass		Chlorophytes		Diatoms		Cyanobacteria	
Factor	F	p	F	p	F	p	F	p	F	p
UVR	6.3	**0.036**	37.8	**<0.001**	43.2	**<0.001**	9.7	**0.010**	21.7	**0.001**
Charophytes	0.4	0.549	4.2	0.075	13.1	**0.007**	0.0	0.951	11.1	**0.010**
Interaction	0.8	0.407	11.4	**0.009**	13.1	**0.007**	3.9	0.084	0.4	0.523

The specific composition of MC communities was also sensitive to the tested factors, and it was different under the CHPAR conditions compared to the other treatments. A multivariate analysis of ordination and classification based on specific composition clustered CHPAR samples separately from the others (Fig. 4D); CHPAB and NCHPAR limnocorrals were also clustered, and NCHPAB limnocorrals showed the highest variability in their composition. The filamentous chlorophyte *Oedogonium* sp. explains almost 60% of the dissimilarity between CHPAR and the other conditions (SIMPER analysis). When UVR was filtered, the difference between CHPAR and NCHPAR was mainly due to two filamentous chlorophytes *Oedogonium* sp. and *Spirogyra* sp., each being present only in one of these conditions. In addition, amongst the limnocorrals containing charophytes (CHPAR and CHPAB), the main difference was due not only to the absence of chlorophytes in the latter, but also to the disappearance of diatoms, such as *Nitzschia sigmoidea* and *N. tryblionella* when affected by UVR.

3.3. C:N stoichiometry in the sediment

Both the %C and %N in the superficial sediment were significantly and positively affected by the absence of UVR; elemental proportions were 7.0±0.2 %C under PAB *vs* 7.7±0.2 %C under PAR and 0.052±0.002 %N under PAB *vs* 0.056±0.001 %N under PAR (Fig. 5; Table 4). The presence of charophytes did not show statistically significant differences in the percentage of both elements, and the interaction between UVR and charophytes was only significant in the case of %C (Fig. 5; Table 4). The average C:N

molar ratio was significantly higher in the treatments with the presence of charophytes, however, there is only a small difference (9.00±0.04 *vs* 8.80±0.05, respectively; Fig. 5; Table 4). Regarding the sub-superficial sediment layer, the %C and %N were favoured by the presence of charophytes, while the C:N was lower in the treatment with charophytes (Fig. 5; Table 4). In this layer, the interaction between UVR and charophytes was significant regarding %C and C:N (Table 4).

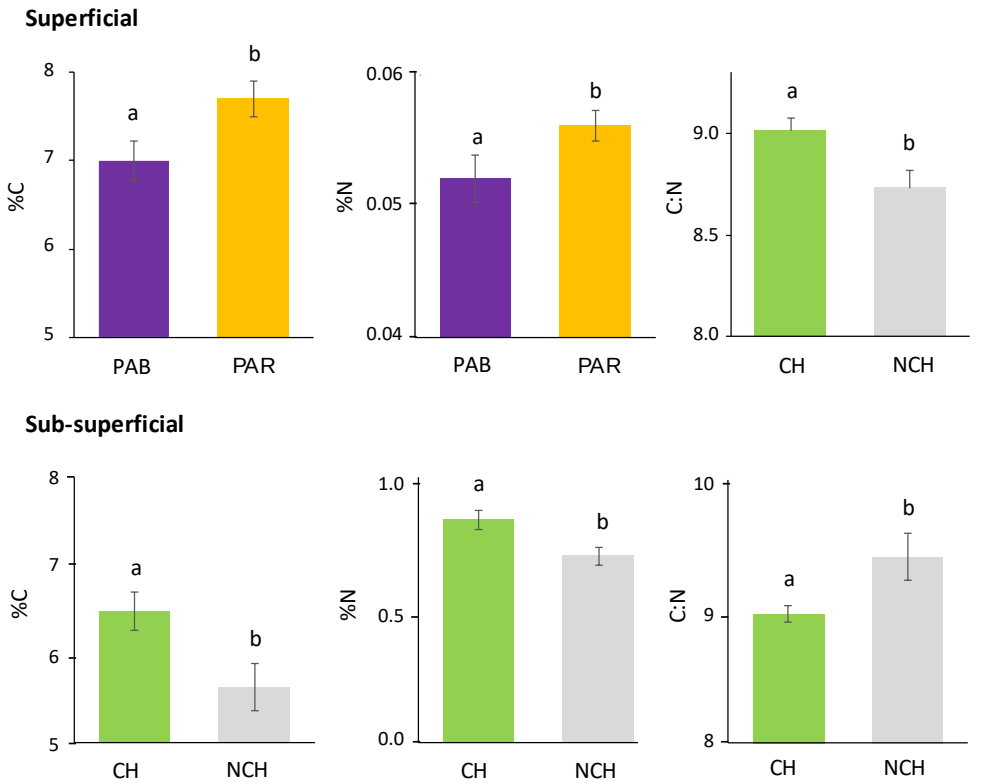

Fig. 5. Average percentatge of carbon (%C), nitrogen (%N) and C:N ratio in the superficial (upper panels) and sub-superficial layer of the sediment (lower panels). Lowercase letters are in accordance with the result of a *post hoc* Tukey analysis of variance. Abbreviations of the environmental conditions as in Fig. 1.

Table 4. Two-way ANOVA parameters (F and probability p) applied on percentage of carbon (%C), nitrogen (%N) and C:N ratio in superficial and sub-superficial layers of sediment. 1 freedom degree for the two factors (presence or not of charophytes and filtered or unfiltered UVR) and their interaction, and 8 freedom degrees within groups. In bold significant values of p ($p<0.05$).

	%C		%N		C:N	
	F	p	F	p	F	p
Superficial						
UVR	11.1	**0.010**	9.6	**0.015**	1.92	0.203
Charophytes	0.01	0.917	0.46	0.517	13.00	**0.007**
Interaction	5.40	**0.049**	4.13	0.077	0.08	0.789
Sub-superficial						
UVR	0.48	0.509	0.5	0.499	3.04	0.120
Charophytes	5.93	**0.041**	7.21	**0.028**	11.35	**0.009**
Interaction	10.2	**0.013**	0.71	0.425	10.17	**0.013**

4. Discussion

4.1 Bacterial response to UVR and the presence ofmeadows

On the superficial layer, 12 phyla made up over 63% of read sequences from the sediment samples. This fact reveals the largely known bacterial biosphere hidden in the wetland sediments and its distribution in very diverse habitats, *e.g.* similar data were observed in a mesocosm experiment with macrophytes and sediment inoculum from a river flowing in Shanghai (Dai *et al.* 2019). However, these concentrations of sequences in a few phyla are especially high if we compare them with other published data, for example, in sediment from a deep cold lake (Fang *et al.* 2015). Furthermore, only nine phyla accounted for more than 1% of sequences in each sample, and around 36% of the sedimentary reads could not be grouped into any known phyla, revealing the largely unknown bacterial biosphere which is masked in the bottom sediments of wetlands, as has been highlighted in very different lentic systems (Fang *et al.* 2015). The highly diverse rare biosphere might be of ecological significance in the evolution of the system (Pedrós-Alió 2012) and be a depository of relevant roles for the functioning of the system (Fang *et al.* 2015).

Phyla as well as genera with different metabolic functions were found to co-dominate in the limnocorral sediments. We found the genus *Desulfococcus* (*Deltaproteobacteria*), to which chemoorganotrophic and sulphate-reducing bacteria belong, and also *Thiobacillus* (*Betaproteobacteria*), chemolithoautotrophic and sulphur-oxidizing bacteria (Barton and Hamilton 2007; Lamers *et al.* 2012). *Steroidobacter denitrificans* (*Gammaproteobacteria*) was the most abundant anaerobic nitrate-reducing species, and some strains of *Marichromatium gracile* have been described to efficiently remove nitrite and ammonium under aerobic or anaerobic conditions, along with motility in the upper layer of sediment (Thar and Kühl 2001; Jiang *et al.* 2015; Hong *et al.* 2017). Representative genera of the phylum *Verrucomicrobia* such as *Luteolibacter* and *Candidatus Methylacidiphilum*, with aerobic heterotrophic species can use polysaccharides, including those produced during degradation of algal biomass (Zemskaya *et al.* 2018) or are methane-oxidizing (Yun *et al.* 2013; Kalyuzhnaya *et al.* 2019). *Bacteroidetes* (*i.e. Pedobacter* genus) are aerobic and chemoorganotrophic bacteria with an oxidative type of metabolism (Margesin and Shivaji 2015) involved, for example, in the degradation of aromatic compounds, or in denitrification processes, and they are highly relevant in wetlands (Sánchez 2017). Finally, is noteworthy that Archaea, which are involved in a variety of biogeochemical processes (methanogenesis, sulphate reduction or ammonia oxidation; Zhang *et al.* 2015), were in a very low proportion.

No great difference was found in the bacterial composition of the different limnocorrals with regard to the treatments; therefore, the predicted or potential functions based on the taxonomic composition showed overlapping between treatments. Something similar has been observed in wetland sediment communities with very different salinity and vegetation, but within the same geographical area (Menéndez-Serra *et al.* 2019). In the limnocorrals with filtered UVR, an exception can be observed between *Deltaproteobacteria* along with *Bacteroidetes* and *Gammaproteobacteria* together with, for example, *Verrucomicrobia*; that is, two

different combinations of bacteria containing reducers of compounds of sulfur and nitrogen with methane oxidizers. The abundance of bacteria did vary with the treatments and was greater when UVR was reduced. Here, we demonstrate that UVR, although not very intense in these latitudes and at sea level, can negatively affect the abundance of bacteria. The negative effect of UVR on freshwater aquatic microorganisms has been known for a long time (Rojo *et al.* 2012a; Carrillo *et al.* 2017). But when primary producers and bacteria are part of the community under UVR conditions, the results can follow different patterns.

The predictable reduction in bacterial growth can be offset by a greater availability of the excretion of organic carbon by microalgae (Carrillo *et al.* 2002); this mechanism will be evident in those oligotrophic ecosystems where the carbon source for bacteria is very scarce, such as oligotrophic high mountain lakes (Carrillo *et al.* 2002). But this is not the case of the shallow lagoon considered here, which is a highly enriched environment so that no compensation effect is observed, and the abundance of both groups of organisms is reduced.

Hence, with regard to bacteria and Archaea, the first hypothesis raised is partially fulfilled: greater UVR reduces the abundance but does not modify the composition, that is, it does not reduce the bacterial richness.

The presence of macrophyte meadows improves the conditions for bacteria in the superficial sediment, as it is in the sediment of the limnocorrals with meadows and filtered UVR where the greatest abundance was observed. This fact would agree with the recent results of Dai *et al.* (2019) who observed a greater abundance of some bacterial groups in environments with macrophytes (angiosperms). However, this relationship is not conclusive and the effect of the macrophyte (*e.g.* light mitigation, nutrient supply, allelopathies, etc.) on the abundance of the bacterial community, which was our second hypothesis, remains untested and this opens up an interesting research line (Dai *et al.* 2019; Morina *et al.* 2018).

A different pattern can be seen in the sub-surface bacterial community where, as might be expected, a direct effect of the presence or absence of UVR, or the presence meadows, is not observed. Only a higher bacterial density in the limnocorrals with meadows and no UVR was found, but these relationships are not conclusive. However, in terms of composition, a greater presence of *Delta*- and *Gammaproteobacteria* (removers of sulfur and nitrogen compounds, respectively) is established in the limnocorrals with meadows and, as was observed in the superficial sediment layers, their distribution seems exclusive, *i.e.* either one or the other. So, we can suggest an effect of the existence of meadows on sub-surface communities. We have not found studies that have dealt with this issue, and we believe it is another interesting relationship which is worth checking.

4.2. Microalgae and cyanobacteria responses to UVR and the presence of meadows

The harmful effect of UVR on microalgae and cyanobacteria has been demonstrated again. Their biomass increased when this radiation was mostly removed, even if, as we have already mentioned, the level of UVR is not very high in the studied system.

The relevance of this affirmation is due to the fact that it has been demonstrated in the periphyton from the bottom of the aquatic system, a habitat which is generally less studied habitat than plankton, and much less studied in relation to UVR, because it is generally assumed that UVR does not reach the bottom.

There were two groups in the periphyton biofilm that make up the greatest proportion of biomass: diatoms and chlorophytes (cyanobacteria were rare). And these groups presented a different pattern: diatoms, as a group, turned out to be UVR resistant but chlorophytes (*i.e.* the filamentous *Oedogonium* and *Spirogyra* genera) only appeared when UVR was filtered. Benthic diatoms were present in all treatments, and their abundance did not significantly vary between them; however, their composition did. Their resistance to UVR is a trait that is considered evolutionary, and

is related to the screen-protection provided by the frustule against UVR (Aguirre *et al.* 2018).

The abundances of diatoms were maintained but their composition changed; thus, when UVR was present, two large species of the genus *Nitzschia* did not appear, yet they were observed in nearby limnocorrals with filtered UVR. These large pennate diatoms can play an important role in the generation of biofilms, which provide a suitable microenvironment for bacterial communities (Landoulsi *et al.* 2011; Hou 2020), so that their loss is relevant to the entire microbial community.

The presence charophytes had a positive effect on the biomass of MC, which was greater with the combination of factors: UVR filtering and the presence of meadows. In addition, the presence of meadows also affected the specific composition. The most favorable condition (CHPAR) had its own composition, different from the other conditions, and the periphyton biofilm composition with natural light and charophyte meadows was similar to that of the sediment without meadows, but with filtered UVR, which suggests a charophyte shading effect. Regarding microalgae and cyanobacteria from the periphyton biofilm the first two hypotheses are fulfilled: they are damaged in the sediment, by even low amounts of UVR, and they are favored by the presence of meadows.

4.3. Stoichiometric implications of UVR and meadows presence

The increase of %C and %N in the sediment of the limnocorrals with charophyte meadows was expected since these organisms are a main source of organic matter for the sediment (either through exudates or by senescence), with compounds rich in these elements (Hilt and Gross 2008; Dai *et al.* 2019). In addition, the aforementioned favouring of the denitrifying bacteria by charophytes could explain the increase in the C:N ratio in the sediment with charophytes compared to the limnocorrals without meadows. It is remarkable that UVR (even being lower than in other ecosystems such as high mountain lakes) has effects on the stoichiometry of the most superficial

sediment. Specifically, we observed an increase in %C and %N when filtering UVR, regardless of whether or not the sediment was covered by charophytes. Regarding the sub-superficial sediment layer stoichiometry, it was not affected by the light environment, as expected, since this layer is not exposed to it. What is remarkable is how the presence of charophytes in the superficial sediment has effects on the deeper sediment layer stoichiometry, probably due to the microbial activity associated with the rhizoidal system of these organisms (Cedergreen and Madsen 2003; Vermeer *et al.* 2003). All these results point to a complex set of processes acting simultaneously (*e.g.* decomposition processes, fixation of elements, photochemical transformations) which have repercussions on the C:N stoichiometry of the sediment (Hansson *et al.* 2005; Zhang *et al.* 2013) and whose effects need to be addressed in depth in future research. From our results, it seems that mainly UVR drives stoichiometry of superficial layer of sediment, while the presence of charophytes meadows has implications in the stoichiometry of sub-superficial layers of the sediment.

4.4. Conclusion

The periphyton and, in general, the sediment microbial community, which is so relevant for the biogeochemical functioning of a wetland, is altered even by the small doses of UVR which reach the superficial sediment in the Mediterranean shallow systems. Due to their shading effect, the meadows should be conserved, but also because their presence favors a greater growth of the microbiota that, including the superficial and sub-superficial sediment, increases the C:N ratio. Thus, the sediment retains more carbon than nitrogen and this improves the health of the ecosystem.

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|GENERAL DISCUSSION|

This thesis has allowed to promote the relevance of the vulnerable Mediterranean aquatic ecosystems in the current global change scenario. We have demonstrated that this relevance is closely linked to submerged macrophyte meadows. These organisms have not been commonly considered in ecological works, despite dominating the majority of shallow waterbodies in this region and fulfilling important functions. All this has been demonstrated from a multiscale experimental framework in which the simulated conditions have not been extreme, but realistic, and that have led to differential responses, even in the short-term both in the macrophytes themselves and in their associated communities. We consider it critically important to combine knowledge about organisms-populations (*e.g.* tolerance ranges, phenotypic plasticity, adaptation –ecotypes–) with that regarding the ecological interactions occurring among them in aquatic ecosystems, as if they were pieces of a complex puzzle. In this way, it is possible to better understand the roles played by the different elements that make up these systems as well as their vulnerability to environmental disturbances and their implications for ecosystem functioning. Furthermore, we have highlighted the potential applicability of our results for the conservation efforts made in these threatened ecosystems.

We have demonstrated that, behind the response of charophyte populations to changes in some of the main global change drivers, both phylogeny (species-specific responses) and adaptation to the local environment inhabited by the populations (population-specific responses) are involved.

Regarding eutrophication, we have found that charophytes have a high tolerance threshold to increased nitrate concentrations in the water. As far as we know, this is the first attempt made in this regard on these macroalgae and, in this way, we are helping to fill the gap with respect to the environmental thresholds of these organisms that has recently been requested (Martínez *et al.* 2014, Auderset Joye and Rey-Biossezon 2015). According to our results, nitrate, *per se*, was not toxic for the metabolism and growth of charophytes, even at concentrations much higher than

those considered as harmful for these organisms in previous works (*e.g.* Lambert and Davy 2011). In this way, we have been able to discern that the ecological reasons, and not so much the physiological ones, linked to the increase of nutrients in the water (such as the explosion of phytoplankton growth) would be the main causes of the decline of the charophyte meadows in aquatic ecosystems. Furthermore, we have observed species-specific differences in the response of charophytes to eutrophication. Clearly, the populations of *C. vulgaris* had a greater growth and a greater capacity of nitrogen uptake than those of *C. hispida* throughout the tested threshold of nitrate concentrations (Fig. 1A). This supports the pioneering character attributed to *C. vulgaris* (Moore 1986, Rojo *et al.* 2015) and reinforces the phylogenetic reasons regarding the responses to nutrients in water that has been described for microalgae (*i.e.* phytoplankton; Dortch 1990). We have also been able to confirm that the coastal populations of this species are the best adapted to the highest concentrations of nitrate, highlighting the effect of the local environment on the response of these organisms to disturbances.

Along with nutrients, temperature is another of the main drivers of global change (Lake *et al.* 2000). We have experimentally demonstrated that populations of charophytes cohabiting in the same system, present different reaction norms to warming. Again, the populations of *C. vulgaris* both from the mountain and the coastal system were the ones that benefited the most from the increase in temperature (in terms of increased growth), showing a wide phenotypic plasticity in this species and its ability to outcompete the co-occurring populations of *C. hispida* in a global change scenario. This supports the results recently offered by Rojo *et al.* (2015, 2017b) who investigated the intraspecific responses of *C. vulgaris* populations from systems in an altitudinal gradient.

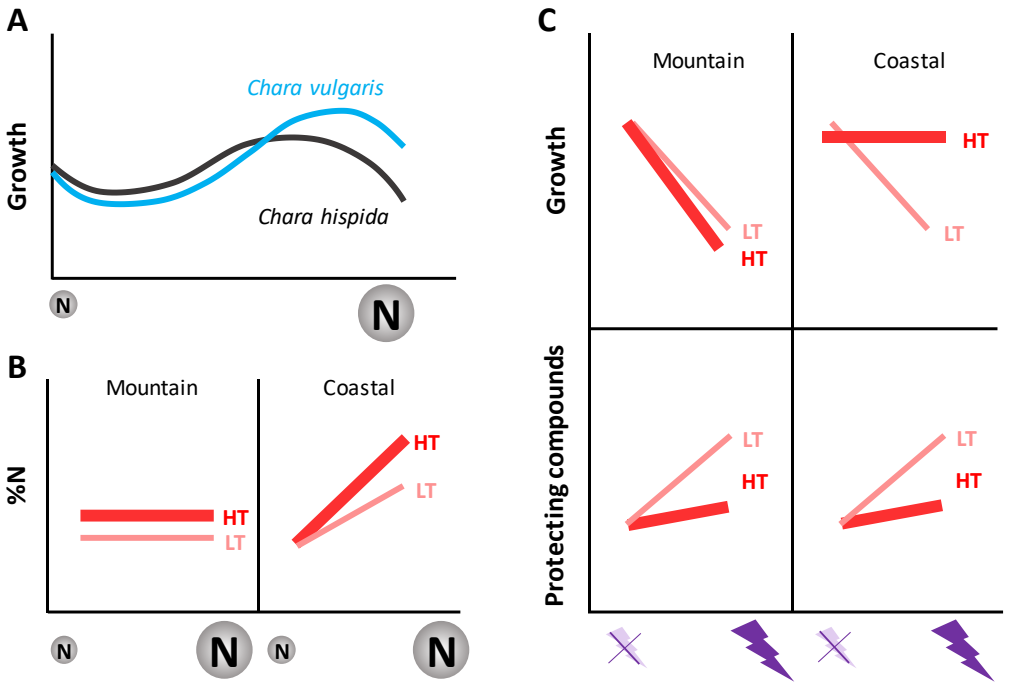

Fig. 1. Summary-graphs depicting the main results of the microcosm experiments. A) Changes in the growth of *Chara hispida* and *C. vulgaris* populations subjected to a gradient of nitrate concentrations in the water. B) Changes in the percentage of nitrogen in the biomass of charophytes from a mountain lake and coastal lagoon populations subjected to two levels of nitrate concentration (LN, low nitrate and HN, high nitrate) and two levels of temperature (LT, low temperature and HT, high temperature) C) Changes in the production of UVR-protecting compounds and in the growth of the same charophytes populations as in B, subjected to two levels of UVR (presence or absence) and two levels of temperature.

Another of the remarkable novelties that this thesis contributes to the knowledge of the ecology of charophytes is the interactive effect of the global-change drivers on these organisms. In this vein, we have tested the warming x eutrophication effect on charophytes populations. The most relevant of this interaction occurred in reference to the assimilation and accumulation of nitrogen in the tissues of these organisms (Fig. 1B). The coastal populations demonstrated that, faced with an abrupt increase in the concentration of nitrate in the water, they were able to capture and store more nitrogen in their tissues. Furthermore, specifically in the population of *C. vulgaris* from the coast, this nitrogen uptake was favored by the increase in temperature. Mountain

populations, for their part, did not show this capacity to accumulate more nitrogen in their tissues in the face of eutrophication (Fig. 1B). In this way, it is evidenced that the local environment in which the charophyte populations inhabit imposes physiological and metabolic mechanisms related to nitrogen uptake, being those populations of the environmentally more variable systems, such as coastal shallow lakes, the most reactive against concomitant warming and eutrophication. These findings reinforce the described relationships between ranges of environmental factors and the adaptation of local populations to them (Peipoch *et al.* 2014) that was recently tested in charophytes regarding temperature (Rojo *et al.* 2015).

The UVR is another important factor related to global change, the increase of which has deleterious effects on aquatic organisms, mainly through genetic damage (Beardall and Raven 2004, Wolf and Heuschele 2018). In the shallow aquatic ecosystems of the Mediterranean region, the reduction of the water column due to global change causes the loss of the UVR-filtering effect it exerts, and the organisms linked to the sediment, such as submerged macrophytes, receive high doses of UVR that affects their growth and metabolism (Rubio *et al.* 2015). But, again, in nature everything happens at the same time and the increase in the incident doses of UVR is accompanied and/or caused by the aforementioned warming and/or the input of nutrients (Cabrerizo *et al.* 2014, Carrillo *et al.* 2017). Despite the interesting results obtained regarding the interaction of warming and eutrophication on the damage caused by UVR in both marine and freshwater phytoplankton (Gao *et al.* 2008, Marcoval *et al.* 2008, Carrillo *et al.* 2017), there are few studies addressing these interactions in macroalgae (Cabello-Pasini *et al.* 2011, Heinrich *et al.* 2015). Thus, we have experimentally tackled this interactive effects on different charophytes populations. Our results highlight that the increase in temperature has a more efficient mitigating effect of the damage caused by UVR on the charophytes than the increase in nitrate concentration. Responses at the molecular level, such as the production of UVR-absorbing compounds (UVACs) are given by more conservative, ancient mechanisms, considered as adaptation of cellular

stress (Pierce *et al.* 2005, Vágnerová *et al.* 2017). Due to this, we did not observe differences in the production of these compounds in the interactive UVR x temperature effect neither in the studied species nor in the populations of charophytes (Fig. 1C). In addition, we suggest some trade-off in the production of UVACs, since the increase in temperature favours the growth of charophytes but prevents the production of this type of molecules in pursuit of less energetically expensive DNA photorepair mechanisms. This is in accordance with the cellular strategies facing stressors described in plants (Pierce *et al.* 2005) and recently assessed in filamentous algae (Vágnerová *et al.* 2017). Despite this uniformity in terms of the molecular response to an increase in UVR modulated by the increase in temperature, the populations of the most variable system (the coastal system) are those that possess sufficient phenotypic plasticity to respond morphologically and in growth to these concomitant environmental changes (Fig. 1C). In this way, a greater protective-restorative capacity against concomitant UVR x warming or eutrophication of the populations of the highly-variable coastal systems is revealed compared to the more-stable mountain ecosystems.

Despite the simplification of microcosm-scale experiments with respect to natural systems and the limitation to extrapolate conclusive assertions to the real world (Beyers and Odum 2012), these results, based on a common garden approach (Santamaría *et al.* 2003, Vitasse *et al.* 2009), allowed us to investigate the species-specific responses of charophytes as well as the phenotypic plasticity of their populations in the face of foreseeable changes in global change-related factors. Undoubtedly, these differential responses, whether due to phylogenetic reasons or to the local environment inhabited, will affect the distribution of these important organisms in freshwater ecosystems. Thus, a set of winning populations will be established with sufficient capacity to face the expected environmental changes (such as the populations inhabiting coastal systems), to the detriment of losing populations that will not be able to face them (such as the mountain populations). Moreover, in

this thesis we praise the importance of tackling concomitant changes in environmental factors as a realistic approach of the effects of global change on freshwater ecosystems, as it has been claimed in recent years (Jackson *et al.* 2016, Villar-Argaiz *et al.* 2018). The study of these interactions is even more necessary in organisms such as charophytes that form the structural basis of aquatic systems and, therefore, have an influence on the entire aquatic community associated to them.

After analysing the responses of charophytes to realistic and predictable changes in the environment, we have zoomed out to shift the focus to the aquatic community linked to the meadows formed by these organisms in shallow freshwater ecosystems. Thus, we are considering not only the differential responses of the populations of these ecosystems to environmental changes, and the habitats they occupy, but also the connections and feedbacks established between them in the form of matter and energy flows (Berlow *et al.* 2004). Under this premise, we have applied the network approach to the study of these communities, considering a wide range of organisms (from bacteria to macroinvertebrates) connected to each other by a group of trophic and non-trophic links.

In fact, the incorporation of non-trophic interactions in ecological models has been on the rise in recent years mainly regarding host-parasitoid networks (Jordán *et al.* 2003), plant-animal mutualistic networks (Bascompte *et al.* 2003) or plant-pollinator networks (Ramos-Jiliberto *et al.* 2020). In aquatic ecosystems the consideration of this type of connections is less frequent (Kéfi *et al.* 2015). This gap is more noticeable regarding the benthic habitat and specially for submerged macrophytes. Implementing these networks is complex, hence the shortage of works that consider both types of interactions. One of the most critical tasks is the one we have addressed in this thesis and consists of establishing the taxonomic-functional criteria that allowed us to define the nodes and links to construct the multi-interaction network, in our case, from a recreated macrophyte-dominated shallow freshwater ecosystem. In addition, we have gone one step further by subjecting the communities of these experimental systems

to different global change scenarios and we have analysed the changes that occur in the multi-interaction network, emphasizing the role played by non-trophic interactions.

Charophytes were the most central node, in the sense of being the best connected in the network with the rest of the elements. In fact, charophytes are the main contributors of non-trophic relationships in the system. This heterogeneous distribution of non-trophic interactions has been studied in aquatic systems (Kéfi *et al.* 2012, 2015), and recently Ellison (2019) and Borst *et al.* (2018) have defined the foundational role for those species that are located at the base of the ecological network (*e.g.* corals or sponges in marine systems or trees in forests), dominate in terms of biomass and centralize non-trophic relationships. Thus, we propose charophytes as foundational species in freshwater ecosystems (Fig. 2). Another interesting result was the emergence of zooplankton herbivores as good connectors in the network. This can be explained through ecological underpinnings: these organisms (such as cladocerans and copepods) have high mobility and a broad-spectrum diet (Rodrigo *et al.* 2015, Stewart *et al.* 2017, Meyer *et al.* 2019), which makes them establishing connections with the different habitats (both planktonic and benthic) of these ecosystems. Joining these results, we suggest a structurally and functionally important macrophyte-herbivore tandem in freshwater ecosystems (Fig. 2). Indeed, when harmful effects (*e.g.* by a disturbance) impact on these elements, the entire structure of the network was affected. In the case of damage to charophytes (as expected due to environmental changes related to global change), the benthic elements (benthic habitat) followed by the planktonic elements most linked to the meadow (within-meadow habitat), will be the most harmed. Damage to zooplanktonic herbivores would cause greater isolation between the planktonic and benthic environments in the system by losing this bridging role that they exert.

Combining these findings from the network approach, with mesocosm experimentation, allowed us to empirically investigate the behaviour of these

networks facing environmental disturbances. The fact of analysing the carbon biomass contributed by each node in the network, led to check which ones were favored or harmed by the tested disturbances. Faced with an increase in UVR, the "winning" nodes were the planktonic mixotrophs and the heterotrophic bacteria (Rojo *et al.* 2012, Carrillo *et al.* 2017, González-Olalla *et al.* 2019; Fig. 2). The “losers” were macrophytes and zooplanktonic herbivores and carnivores. This result points to a higher prevalence of the microbial loop under this scenario, resulting in a phytoplankton dominance due to the lack of top-down effects by organisms at higher trophic levels. However, faced with a warming scenario, the resulting configuration of the system was the opposite. Charophytes reached the highest biomass and herbivores and diatoms were favored leading to a macrophyte-dominated configuration (Fig. 2). These contrasting configurations in the tested scenarios remind of the alternative states defined for shallow aquatic systems (Scheffer *et al.* 1993) and evidence the pivotal role of macrophyte meadows in their achievement (Su *et al.* 2019).

Furthermore, to consolidate our argument about the need to include this type of interactions in the study of aquatic ecosystems, we analysed how the relevance of a node changes when it is considered within a purely trophic or a multi-interaction network and how environmental changes influence these changes. What we observed was that the structural influence of the nodes changed dramatically when incorporating non-trophic relationships into a trophic model. This highlights the overestimation of the top-down control at the expense of masking the structural importance that other elements which, despite not participating much as a food source, have an important role in the functioning of these ecosystems, such as submerged macrophytes or filamentous algae. In addition, by incorporating these non-trophic interactions, the benthic habitat (where this type of interactions is condensed) is praised as crucial for the functioning of aquatic ecosystems. With our results, we support and contribute to the demands of certain authors (Vadeboncoeur and

Steinman 2002, Vadeboncoeur *et al.* 2002) who defended the need to consider benthic habitat as well as to reconcile the functional plankton-benthos connections to achieve a less skewed and more realistic view of the structure and function of aquatic ecosystems, especially in the face of the current global change to which they are subjected.

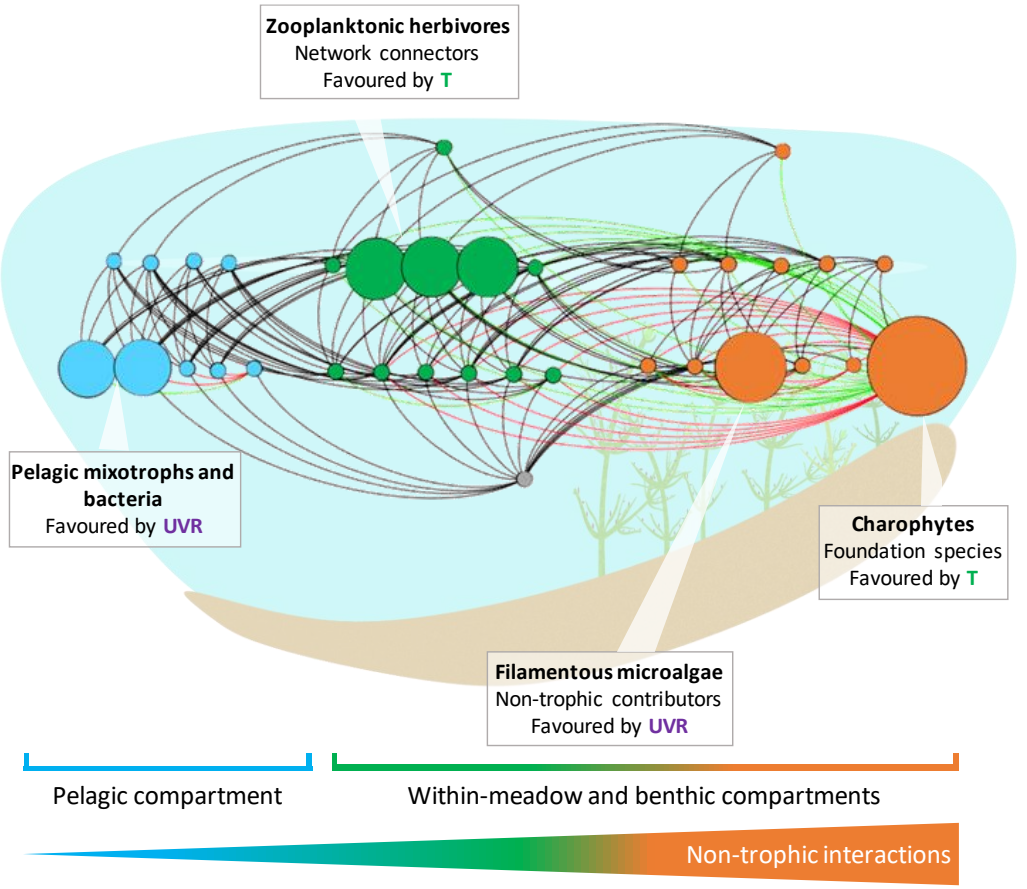

Fig. 2. Scheme-summary of the main outcomes of our studies on the multi-interaction network of a macrophyte-dominated shallow freshwater ecosystem. The nodes involved in these outcomes are enlarged. The network is overlapped on a representation of a shallow freshwater ecosystem to visually link the model to the system it represents. UVR is ultraviolet radiation, T is temperature.

The effort of modelling, building and testing the benefits of our multi-interaction network approach, together with the emerged pattern regarding the interaction between habitats thanks to the role played by macrophyte meadows, has led us to analyze these connections, so relevant to the functioning of the ecosystem, in natural environments (ponds and lakes). In fact, we have found that the benthic-pelagic coupling differs between ponds and lakes. The morphometric differences between these two contrasted types of ecosystems made that, when analyzing their communities from a taxonomic-functional perspective, different patterns appeared. In this vein, Schindler and Scheuerell (2002) remarked that the benthic-pelagic coupling depends on the perimeter:area (or depth) ratios, small lakes or ponds being those with greater coupling. In our ponds, despite the high sharing of taxa between their habitats (which could point to an apparent jumble), their multi-interaction networks were divided into functional modules that point to a complexity beyond what could be expected. Among these modules, the microbial loop stands out, with special mention of the mixotrophs, which serve as a carbon bypass towards the autotrophic chain (Medina-Sánchez *et al.* 2004, Carrillo *et al.* 2017). In addition, the herbivores that inhabit the charophyte meadow served as connectors between the benthic and planktonic habitats (Stewart *et al.* 2017), as we had demonstrated in our simulated systems. Both the degree of taxa sharing and the connections established between the functional modules indicate that there is an effective benthic-pelagic coupling in this type of system. However, in the lakes this pattern did not occur, emerging a planktonic and a benthic module, clearly disconnected. This implies that the important production of benthic meadows can be disconnected from the trophic network (matter and energy flow) of the lake. Despite this, we have observed that there was a greater taxonomic redundancy in the benthos of the lakes than in that of the ponds. Supporting what was stated by Wellnitz and Poff (2001) we suggest that the effect of benthic species loss on the functional integrity of the entire community would be minimized mainly in the lakes, due to this redundancy found in the benthos.

Continuing with the previous development, and taking a step further, we analysed the effect of submerged macrophytes meadows on some aspects of the functioning of a shallow ecosystem by means of a field experiment, focussing on the microbial community in the sediment. This microbial community has been recognized as the engine of biogeochemical cycles in wetlands (Callieri *et al.* 2019). In addition, it has been demonstrated that this is affected by environmental changes with strong implications on the functioning of these ecosystems (Orland *et al.* 2020). However, the relationship of this community with another key piece for the functioning of these ecosystems, such as the macrophyte meadows, has been less investigated (Zhao *et al.* 2013). Our work has shed light on the great diversity housed by the sediment of these ecosystems, and the important implications of the relationship of this with the macrophytes meadows in a global change context. In this vein, we have demonstrated that UVR negatively affects the biomass and richness of the microorganisms that make up the periphytic biofilm (both bacteria-Archaea and photosynthetic microorganisms –microalgae and cyanobacteria–) supporting the outcomes revealed for aquatic microorganisms (Rojo *et al.* 2012, Carrillo *et al.* 2017). However, this effect was minimized by the presence of charophyte meadows. It should be noted that the occupation of the sediment by these macroalgae favored denitrifying bacteria, which transform nitrate into nitrogen gas (N_2) which abandon the waterbody, and this is very beneficial for ecosystems such as the often highly eutrophicated Mediterranean shallow lakes (Fig. 3). These results support what we have previously stated regarding the importance of the benthic habitat and the effects that global change will have on this. Furthermore, we open the gate for future research that deeply addresses the relationship between submerged macrophytes and the underneath sediment microbial community, since the mix of simultaneous processes acting at this water-sediment interface is complex but crucial.

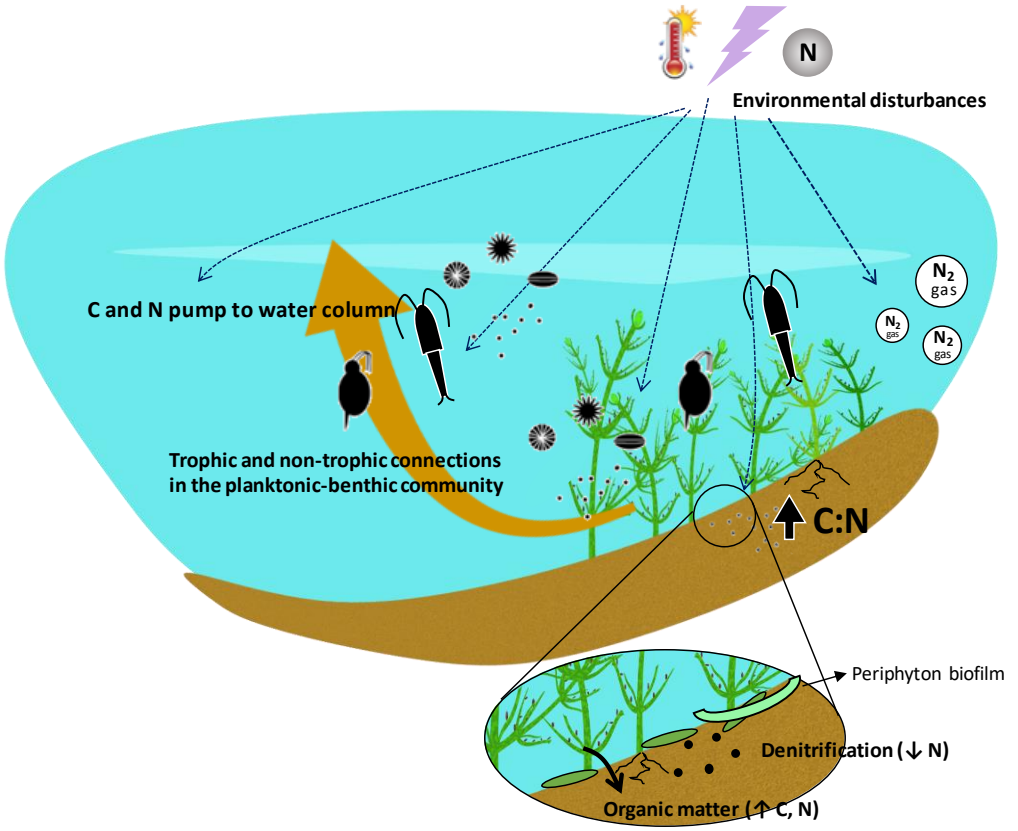

Fig. 3. Representation of the main impacts of macrophyte meadows on the sediment microbial community and the implications for the water column of these systems. Dashed lines show the main targets of the environmental effects.

In this way, we have depicted a complex puzzle in which charophytes meadows are a central piece that host the main connectors of the system, provide habitat to a wide range of organisms tightly linked to them, favour the non-toxic easy-edible primary producers, contribute with C and N and promote the growth of denitrifiers, thus becoming enormously involved in the functioning of these ecosystems and subjugating their response facing environmental changes. Provided this central position in the freshwater ecosystems, and based on the outcomes delivered by this thesis, the gates for future research regarding submerged macrophytes meadows are fully opened. As an example, the responses of submerged macrophytes to concomitant environmental changes on a regional and even continental scale, should be addressed. Other studies

should implement the strength of the relationships in the multi-interaction network in order to get models that allow to quantify, in a more realistic way, the energetic transference in aquatic ecosystems. Thus, finding a common currency between trophic and non-trophic interactions emerged as crucial. Through all these advances, it would be possible to delve into the mechanisms that promote the performance of vulnerable aquatic ecosystems in the face of global change and a proactive management that favors submerged macrophyte meadows would be praised.

|FINAL REMARKS AND CONCLUSIONS|

1. The responses of charophyte organisms and populations to the tested environmental changes are driven by both phylogeny and adaptation to the inhabited local environment.

2. Charophytes have a high tolerance threshold to nitrate concentration in the water. Thus, nitrate *per se* is not toxic for these organisms and the decline of their meadows in eutrophicated systems should be attributed to ecological reasons derived from the nutrient increase.

3. The organisms of coastal populations (mainly of the species *Chara vulgaris*) are those that present a greater phenotypic plasticity and have the capacity to react and overcome global change-related disturbances with respect to water warming, eutrophication and their interactive effects.

4. The deleterious effect of UVR on charophyte populations is mainly minimized by warming. This amelioration is more evident in coastal populations, thus demonstrating their greater responsiveness than their high-mountain counterparts.

5. These responses with species- and population-specific patterns will compromise the distribution of these organisms in freshwater ecosystems, establishing a set of winning populations (i.e. coastal populations) to the detriment of other losers (i.e. mountain populations).

6. The incorporation of non-trophic relationships in the study of aquatic systems dominated by charophyte meadows is crucial to establish more realistic ecological models that allow us to better understand the functioning of these systems.

7. In the experimental multi-interaction network here studied, the charophyte's node is the best connected with the rest of the elements. These organisms can be considered as foundational species since they centralize non-trophic relationships, are the basis of these networks (*i.e.* primary producers) and dominate in biomass.

8. Large meadow-related zooplanktonic herbivores emerge as efficient connector between functional modules of the network.

9. A macrophyte-herbivores tandem rise as crucial for the structure and function of these systems.

10. When subjecting the aquatic community to global change-related scenarios, two contrasting configurations are reached: phytoplankton dominance in the face of an increase in UVR and charophyte dominance in the face of a warming scenario. The performance of macrophyte meadows are pivotal in achieving these configurations.

11. The application of the network approach in natural systems leads to the emergence of a different pattern of habitat coupling between ponds and lakes with macrophyte meadows. The benthic-pelagic coupling occurs in ponds while in the lakes, the functional modules remain disconnected.

12. The presence of macrophyte meadows protects the sediment microbial community from the harmful effects of UVR and promotes the growth of denitrifying bacteria. This is beneficial for reducing the internal loading of eutrophicated shallow Mediterranean ecosystems.

13. Combining knowledge about the ecology of charophytes together with that about the implications at the community level in a context of global change allows us to bring closer to the complexity of Mediterranean aquatic systems and to better understand their response to the environmental disturbances to which they are subjected.

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|ANNEXES / *ANNEXOS*|

Annex I Supplementary material

Chapter 1. On the tolerance of charophytes to high-nitrate concentrations

Table S1. Results of descriptive statistics for the main analysed variables in the four populations of charophytes. The abbreviations are: minimum and maximum values (Min, Max); 95% confidence intervals for the mean (Lower and Upper conf.), standard error (Std. error), standard deviation (Stand. dev).

	Relative Growth Rate (RGR) based on Dry Weight (/d)													
	Exp. I (unplanted)							Exp. IIa-IIb (planted)						
	Nitrate dose (mg NO_3-N/l)							Nitrate dose (mg NO_3-N/l)						
	0.5	1.5	3	7.5	15	30	50	0.5	1.5	3	7.5	15	30	50
Chara hispida Somolinos														
Min	0.042	0.069	0.027	0.072	0.076	0.110	0.079	0.048	0.032	0.038	0.028	0.068	0.012	0.004
Max	0.059	0.094	0.053	0.097	0.101	0.114	0.120	0.172	0.074	0.079	0.147	0.106	0.153	0.045
Mean	0.053	0.084	0.040	0.081	0.085	0.113	0.100	0.089	0.062	0.051	0.076	0.082	0.073	0.026
Lower conf.	0.046	0.075	0.027	0.065	0.069	0.111	0.081	0.052	0.051	0.041	0.050	0.059	0.033	0.009
Upper conf.	0.063	0.100	0.053	0.090	0.094	0.115	0.122	0.120	0.083	0.059	0.098	0.097	0.108	0.043
Std. error	0.005	0.008	0.008	0.008	0.008	0.001	0.012	0.019	0.010	0.005	0.013	0.012	0.021	0.010
Stand. dev	0.009	0.013	0.013	0.014	0.014	0.002	0.021	0.048	0.020	0.014	0.037	0.020	0.052	0.020
Median	0.057	0.090	0.040	0.074	0.078	0.114	0.102	0.075	0.072	0.051	0.064	0.074	0.057	0.028
Chara vulgaris Somolinos														
Min	0.059	0.045	0.046	0.001	0.022	0.016	0.026	0.060	0.11	0.03	0.03	0.08	0.08	0.09
Max	0.104	0.077	0.060	0.031	0.086	0.048	0.048	0.170	0.160	0.150	0.150	0.140	0.160	0.130
Mean	0.074	0.066	0.053	0.013	0.052	0.039	0.038	0.135	0.138	0.085	0.110	0.100	0.121	0.103
Lower conf.	0.054	0.056	0.049	0.002	0.030	0.031	0.030	0.117	0.120	0.058	0.091	0.080	0.106	0.085
Upper conf.	0.087	0.080	0.058	0.023	0.073	0.054	0.046	0.157	0.155	0.110	0.132	0.114	0.137	0.115
Std. error	0.010	0.007	0.003	0.007	0.014	0.008	0.005	0.011	0.011	0.014	0.011	0.010	0.008	0.009
Stand. dev	0.021	0.014	0.006	0.013	0.027	0.015	0.010	0.035	0.022	0.045	0.035	0.023	0.026	0.019
Median	0.067	0.070	0.054	0.010	0.051	0.046	0.039	0.150	0.140	0.080	0.120	0.090	0.130	0.095
Chara hispida Quartons														
Min	0.056	0.052	0.050	0.069	0.105	0.111	0.052	0.070	0.070	0.070	0.020	0.070	0.070	0.050
Max	0.090	0.087	0.075	0.076	0.114	0.121	0.090	0.140	0.100	0.110	0.110	0.090	0.130	0.090
Mean	0.075	0.074	0.063	0.073	0.108	0.116	0.065	0.118	0.083	0.095	0.060	0.077	0.089	0.075
Lower conf.	0.060	0.062	0.051	0.070	0.103	0.111	0.040	0.104	0.070	0.083	0.037	0.063	0.073	0.060
Upper conf.	0.094	0.097	0.076	0.077	0.112	0.121	0.780	0.134	0.095	0.108	0.081	0.083	0.101	0.090
Std. error	0.010	0.011	0.007	0.002	0.003	0.003	0.013	0.008	0.008	0.007	0.012	0.007	0.008	0.010
Stand. dev	0.017	0.019	0.013	0.004	0.005	0.005	0.022	0.024	0.015	0.016	0.032	0.012	0.021	0.019
Median	0.079	0.084	0.064	0.074	0.106	0.116	0.053	0.125	0.080	0.100	0.060	0.070	0.080	0.080
Chara vulgaris Quartons														
Min	0.039	0.043	0.048	0.029	0.009	0.062	0.098	0.100	0.120	0.010	0.050	0.020	0.060	0.050
Max	0.085	0.114	0.117	0.069	0.084	0.108	0.123	0.160	0.180	0.140	0.150	0.130	0.150	0.110
Mean	0.057	0.074	0.070	0.048	0.046	0.085	0.113	0.132	0.155	0.074	0.098	0.090	0.110	0.090
Lower conf.	0.039	0.049	0.039	0.034	0.018	0.065	0.104	0.120	0.135	0.045	0.082	0.062	0.095	0.073
Upper conf.	0.072	0.100	0.089	0.063	0.074	0.105	0.122	0.145	0.180	0.104	0.113	0.123	0.126	0.118
Std. error	0.010	0.016	0.016	0.009	0.015	0.012	0.006	0.007	0.013	0.016	0.008	0.017	0.008	0.014
Stand. dev	0.020	0.032	0.032	0.018	0.031	0.023	0.011	0.022	0.025	0.051	0.027	0.042	0.027	0.027
Median	0.053	0.070	0.058	0.047	0.046	0.084	0.115	0.140	0.160	0.075	0.100	0.095	0.115	0.100

Table S1. continuation.

	Daily elongation (cm/d)													
	Exp. I (unplanted)							Exp. IIa-IIb (planted)						
	Nitrate dose (mg NO_3-N/l)							Nitrate dose (mg NO_3-N/l)						
	0.5	1.5	3	7.5	15	30	50	0.5	1.5	3	7.5	15	30	50
Chara hispida Somolinos														
Min	0.03	0.05	0.01	0.01	0.02	0.12	0.06	0.11	0.17	0.10	0.17	0.20	0.03	0.04
Max	0.10	0.08	0.07	0.04	0.06	0.21	0.16	0.72	0.55	0.46	0.83	0.39	0.69	0.32
Mean	0.07	0.06	0.03	0.02	0.05	0.17	0.12	0.31	0.39	0.26	0.37	0.31	0.32	0.15
Lower conf.	0.04	0.05	0.00	0.01	0.03	0.14	0.08	0.14	0.26	0.17	0.23	0.23	0.08	0.03
Upper conf.	0.09	0.07	0.05	0.04	0.06	0.21	0.15	0.44	0.54	0.34	0.48	0.42	0.55	0.23
Std. error	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.09	0.08	0.05	0.07	0.06	0.14	0.06
Stand. dev	0.03	0.01	0.03	0.02	0.02	0.04	0.05	0.22	0.16	0.13	0.20	0.10	0.31	0.12
Median	0.07	0.06	0.02	0.02	0.05	0.18	0.12	0.27	0.42	0.21	0.32	0.34	0.16	0.11
Chara vulgaris Somolinos														
Min	0.01	0.01	0.01	0.01	0.02	0.04	0.07	0.25	0.55	0.08	0.16	0.27	0.10	0.21
Max	0.31	0.06	0.04	0.03	0.13	0.09	0.18	2.12	1.06	1.47	1.78	1.24	1.89	0.83
Mean	0.12	0.03	0.02	0.02	0.07	0.05	0.10	1.13	0.82	0.59	0.97	0.83	1.12	0.42
Lower conf.	0.02	0.01	0.02	0.01	0.03	0.04	0.06	0.67	0.65	0.33	0.64	0.62	0.73	0.16
Upper conf.	0.20	0.04	0.03	0.02	0.10	0.07	0.13	1.58	0.99	0.83	1.29	1.09	1.54	0.62
Std. error	0.05	0.01	0.00	0.00	0.02	0.01	0.02	0.25	0.11	0.14	0.17	0.13	0.22	0.14
Stand. dev	0.12	0.02	0.01	0.01	0.04	0.02	0.05	0.74	0.22	0.43	0.55	0.32	0.68	0.29
Median	0.07	0.03	0.02	0.02	0.07	0.05	0.08	0.89	0.83	0.51	0.87	0.88	1.29	0.32
Chara hispida Quartons														
Min	0.05	0.09	0.05	0.14	0.08	0.20	0.01	0.17	0.18	0.39	0.23	0.43	0.29	0.26
Max	0.15	0.17	0.18	0.56	0.36	0.49	0.16	1.46	0.42	1.12	0.89	1.18	1.33	0.50
Mean	0.12	0.14	0.10	0.30	0.24	0.36	0.11	0.83	0.29	0.76	0.50	0.81	0.83	0.38
Lower conf.	0.08	0.11	0.05	0.12	0.14	0.27	0.05	0.44	0.20	0.55	0.33	0.43	0.56	0.30
Upper conf.	0.16	0.17	0.15	0.43	0.34	0.48	0.17	1.23	0.37	0.97	0.66	1.18	1.11	0.47
Std. error	0.02	0.02	0.03	0.09	0.06	0.06	0.04	0.21	0.05	0.12	0.09	0.38	0.15	0.05
Stand. dev	0.05	0.03	0.06	0.18	0.13	0.12	0.07	0.60	0.10	0.29	0.24	0.53	0.41	0.10
Median	0.13	0.15	0.09	0.25	0.26	0.39	0.13	0.83	0.28	0.75	0.41	0.81	0.85	0.39
Chara vulgaris Quartons														
Min	0.01	0.01	0.00	0.01	0.00	0.05	0.17	0.56	0.78	0.51	0.21	0.73	0.23	0.20
Max	0.07	0.12	0.05	0.06	0.02	0.19	0.35	2.16	1.04	1.14	2.11	2.07	1.84	0.73
Mean	0.04	0.05	0.02	0.04	0.01	0.12	0.26	1.32	0.90	0.77	1.00	1.54	1.21	0.47
Lower conf.	0.02	0.02	0.00	0.02	0.00	0.07	0.20	0.98	0.80	0.66	0.64	1.07	0.86	0.86
Upper conf.	0.05	0.09	0.03	0.05	0.01	0.17	0.32	1.68	1.01	0.87	1.34	2.05	1.58	1.58
Std. error	0.01	0.02	0.01	0.01	0.00	0.03	0.03	0.19	0.06	0.06	0.19	0.31	0.20	0.11
Stand. dev	0.02	0.04	0.02	0.02	0.01	0.06	0.07	0.60	0.13	0.19	0.60	0.61	0.62	0.22
Median	0.04	0.04	0.01	0.04	0.01	0.11	0.26	1.56	0.89	0.77	1.02	1.68	1.34	0.47

Table S1. continuation.

	Percentage of Nitrogen in charophyte biomass (%N)													
	Exp. I (unplanted)							Exp. IIa-IIb (planted)						
	Nitrate dose (mg NO_3-N/l)							Nitrate dose (mg NO_3-N/l)						
	0.5	1.5	3	7.5	15	30	50	0.5	1.5	3	7.5	15	30	50
Chara hispida Somolinos														
Min	0.65	0.73	0.88	0.98	0.91	0.72	0.78	0.86		0.82	0.85	0.85	0.74	0.74
Max	0.67	0.77	0.94	1.02	0.94	0.79	0.82	0.89		0.86	0.89	0.93	0.79	0.77
Mean	0.66	0.75	0.91	0.99	0.93	0.77	0.80	0.87		0.84	0.87	0.88	0.77	0.76
Lower conf.	0.64	0.73	0.88	0.97	0.91	0.74	0.77	0.86		0.83	0.84	0.83	0.75	0.74
Upper conf.	0.66	0.77	0.94	1.01	0.94	0.81	0.81	0.89		0.87	0.88	0.91	0.80	0.77
Std. error	0.01	0.01	0.02	0.01	0.01	0.02	0.01	0.01		0.01	0.01	0.03	0.02	0.01
Stand. dev	0.01	0.02	0.03	0.02	0.02	0.04	0.02	0.02		0.02	0.02	0.04	0.03	0.02
Median	0.65	0.75	0.91	0.98	0.93	0.79	0.79	0.87		0.85	0.86	0.86	0.78	0.76
Chara vulgaris Somolinos														
Min	0.81	0.95	1.33	1.50	1.39	1.19	1.18	0.78		0.66	0.90	0.79	0.92	0.92
Max	0.84	1.02	1.36	1.61	1.45	1.22	1.22	0.80		0.71	0.91	0.84	0.99	0.98
Mean	0.83	0.99	1.34	1.56	1.42	1.20	1.20	0.79		0.69	0.90	0.82	0.97	0.94
Lower conf.	0.82	0.96	1.32	1.52	1.39	1.18	1.19	0.78		0.66	0.90	0.79	0.94	0.90
Upper conf.	0.85	1.03	1.35	1.63	1.45	1.21	1.23	0.80		0.71	0.91	0.84	1.01	0.96
Std. error	0.01	0.02	0.01	0.03	0.02	0.01	0.01	0.01		0.01	0.00	0.01	0.02	0.02
Stand. dev	0.02	0.04	0.02	0.06	0.03	0.02	0.02	0.01		0.03	0.01	0.03	0.04	0.03
Median	0.84	1.00	1.33	1.58	1.42	1.19	1.21	0.79		0.69	0.90	0.82	0.99	0.92
Chara hispida Quartons														
Min	0.66	0.62	0.71	1.00	0.99	0.98	0.72			0.80	1.02	0.87	0.73	0.80
Max	0.76	0.70	0.74	1.05	1.03	1.02	0.76			0.83	1.04	0.89	0.77	0.86
Mean	0.70	0.65	0.72	1.02	1.01	1.00	0.73			0.82	1.03	0.88	0.74	0.82
Lower conf.	0.65	0.61	0.01	0.99	1.00	0.97	0.71			0.80	1.02	0.88	0.72	0.78
Upper conf.	0.75	0.69	0.01	1.04	1.04	1.01	0.75			0.83	1.04	0.90	0.76	0.84
Std. error	0.03	0.02	0.01	0.02	0.01	0.01	0.01			0.01	0.01	0.01	0.01	0.02
Stand. dev	0.05	0.04	0.02	0.03	0.02	0.02	0.02			0.02	0.01	0.01	0.02	0.03
Median	0.69	0.64	0.71	1.01	1.02	0.99	0.72			0.82	1.03	0.89	0.73	0.80
Chara vulgaris Quartons														
Min	1.33	1.27	1.52	1.16	1.83	1.15	0.95	0.87		0.98	0.96	0.94	1.00	1.04
Max	1.42	1.36	1.59	1.27	1.93	1.18	1.00	0.93		1.05	0.99	1.07	1.05	1.14
Mean	1.37	1.32	1.56	1.23	1.88	1.17	0.98	0.90		1.03	0.98	1.01	1.03	1.09
Lower conf.	1.31	1.29	1.53	1.19	1.83	1.16	0.96	0.88		1.00	0.97	0.96	1.02	1.03
Upper conf.	1.40	1.38	1.60	1.30	1.93	1.19	1.01	0.94		1.07	1.00	1.09	1.07	1.13
Std. error	0.03	0.03	0.02	0.04	0.03	0.01	0.02	0.02		0.02	0.01	0.04	0.02	0.03
Stand. dev	0.05	0.05	0.04	0.06	0.05	0.02	0.03	0.03		0.04	0.02	0.07	0.03	0.05
Median	1.35	1.34	1.57	1.26	1.88	1.18	0.99	0.91		1.05	0.99	1.03	1.05	1.08

Table S1. continuation.

	Nitrate-reductase (nmoles NO_2/mgFW h)							Respiration Rate (mgO_2/gDW h)				
	Exp. I (unplanted)							Exp. IIa (planted)				
	Nitrate dose (mg NO_3-N/l)							Nitrate dose (mg NO_3-N/l)				
	0.5	1.5	3	7.5	15	30	50	0.5	7.5	15	30	50
Chara hispida Somolinos												
Min								0.57	0.78	0.61	0.69	0.78
Max								0.82	0.81	0.85	0.90	1.02
Mean								0.72	0.79	0.73	0.77	0.89
Lower conf.								0.62	0.78	0.62	0.65	0.76
Upper conf.								0.87	0.81	0.86	0.86	1.01
Std. error								0.08	0.01	0.07	0.06	0.07
Stand. dev								0.13	0.02	0.12	0.11	0.13
Median								0.77	0.79	0.74	0.73	0.87
Chara vulgaris Somolinos												
Min								0.88	1.07	0.86	0.96	0.96
Max								1.41	1.48	1.62	1.57	1.37
Mean								1.03	1.27	1.21	1.14	1.17
Lower conf.								0.83	1.14	1.01	0.96	1.07
Upper conf.								1.14	1.41	1.39	1.26	1.27
Std. error								0.10	0.08	0.11	0.09	0.06
Stand. dev								0.22	0.18	0.27	0.22	0.14
Median								0.96	1.21	1.20	1.08	1.17
Chara hispida Quartons												
Min								0.98	0.77	0.57	1.00	1.06
Max								1.17	1.15	0.65	1.33	1.36
Mean								1.10	0.94	0.62	1.14	1.22
Lower conf.								1.03	0.73	0.58	0.95	0.95
Upper conf.								1.22	1.12	0.66	1.28	1.28
Std. error								0.06	0.11	0.02	0.10	0.09
Stand. dev								0.11	0.19	0.04	0.17	0.15
Median								1.15	0.91	0.62	1.09	1.24
Chara vulgaris Quartons												
Min								0.68	0.69	0.64	0.90	0.84
Max								1.00	0.88	0.94	1.07	1.11
Mean								0.81	0.78	0.75	1.00	1.01
Lower conf.								0.73	0.72	0.66	0.96	0.95
Upper conf.								0.89	0.83	0.81	1.05	1.09
Std. error								0.04	0.03	0.04	0.02	0.04
Stand. dev								0.11	0.08	0.10	0.06	0.10
Median								0.79	0.76	0.74	1.01	1.03

Table S2. Values of the linear-curve fittings of the diferent analysed variables.

	Relative Growth Rate (RGR) based on Dry Weight (/d)													
	Exp. I (unplanted)							Exp. II (planted)						
	Constant	a1	a2	a3	F	p	R^2	Constant	a1	a2	a3	F	p	R^2
Chara hispida Somolinos	0.0566	0.0029	-0.000040		11.2	<0.001	0.56	0.0803	-0.0035	0.00022	-0.00000345	2.4	0.080	0.18
Chara vulgaris Somolinos	0.0701	-0.0060	0.000273	-0.00000331	3.0	0.050	0.05	0.1380	-0.0072	0.00036	-0.00000468	3.0	0.040	0.15
Chara hispida Quartons	0.6226	0.0039	0.000077		13.7	<0.001	0.60	0.1190	-0.0076	0.00034	-0.00000417	6.5	0.001	0.36
Chara vulgaris Quartons	0.0642	-0.0010	0.000041		8.0	0.002	0.39	0.1403	-0.0089	0.00043	-0.00000538	4.4	0.007	0.21
	Daily elongation (cm/d)													
	Exp. I (unplanted)							Exp. II (planted)						
	Constant	a1	a2	a3	F	p	R^2	Constant	a1	a2	a3	F	p	R^2
Chara hispida Somolinos	0.0724	-0.0134	0.000963	-0.00001355	26.7	<0.001	0.78							
Chara vulgaris Somolinos	0.0387	-0.0005	0.000052	-0.00000034	4.3	0.012	0.30							
Chara hispida Quartons	0.0996	0.0195	-0.000383		5.7	0.001	0.43							
Chara vulgaris Quartons	0.0366	-0.0016	0.000124		57.5	<0.001	0.79	1.2534	-0.0771	0.005	0.000	3.6	0.019	0.19
	Percentage of Nitrogen in charophyte biomass (%N)													
	Exp. I (unplanted)							Exp. II (planted)						
	Constant	a1	a2	a3	F	p	R^2	Constant	a1	a2	a3	F	p	R^2
Chara hispida Somolinos	0.6891	0.0545	-0.002819	0.0000355	19.5	<0.001	0.77	0.8760	-0.0025			30.2	<0.001	0.65
Chara vulgaris Somolinos	0.8835	0.1137	-0.005516	0.0000675	20.1	<0.001	0.78	0.7794	0.0040			16.1	0.001	0.50
Chara hispida Quartons	0.6259	0.0543	-0.001961	0.0000184	37.1	<0.001	0.87	0.9144	-0.0026			3.5	0.080	0.21
Chara vulgaris Quartons	1.2724	0.0640	-0.003318	0.0000383	7.2	0.003	0.56	0.9620	0.0026			13.4	0.002	0.46
	Respiration Rate (mgO_2/gDW h)													
	Exp. I (unplanted)							Exp. II (planted)						
	Constant	a1	a2	a3	F	p	R^2	Constant	a1	a2	a3	F	p	R^2
Chara hispida Quartons								1.0932	-0.0491	0.00187		8.5	0.005	0.59
Chara vulgaris Quartons								0.8006	-0.0061	0.00048		13.1	<0.001	0.49
	Elongation (LMAV) (cm)							**Number of ramifications**						
	Exp. I (unplanted)							Exp. I (unplanted)						
	Constant	a1	a2	a3	F	p	R^2	Constant	a1	a2	a3	F	p	R^2
Chara hispida Somolinos	1.3178	-0.2574	0.017992	-0.00025112	29.2	<0.001	0.78							
Chara vulgaris Somolinos	0.8219	0.0146			2.0	0.172	0.10							
Chara hispida Quartons	1.7733	0.3427	-0.006700		9.2	0.001	0.42							
Chara vulgaris Quartons	0.6561	-0.0330	0.002300		57.7	0.001	0.78	0.8443	0.0473			14.1	0.001	0.30

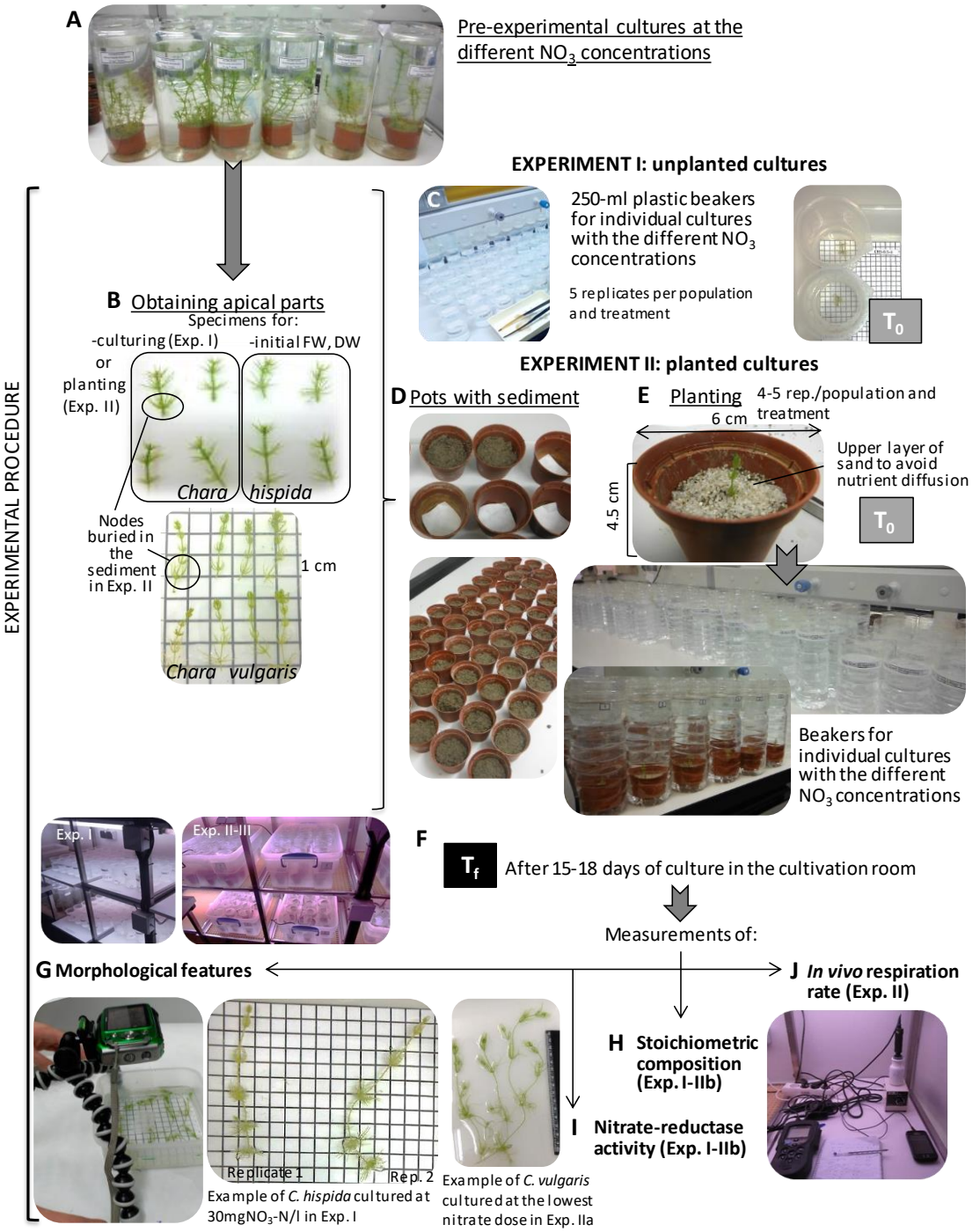


Fig. S1. Outlines of the experimental setup, for the experiments with unplanted (Exp. I) and planted specimens (Exp. II).

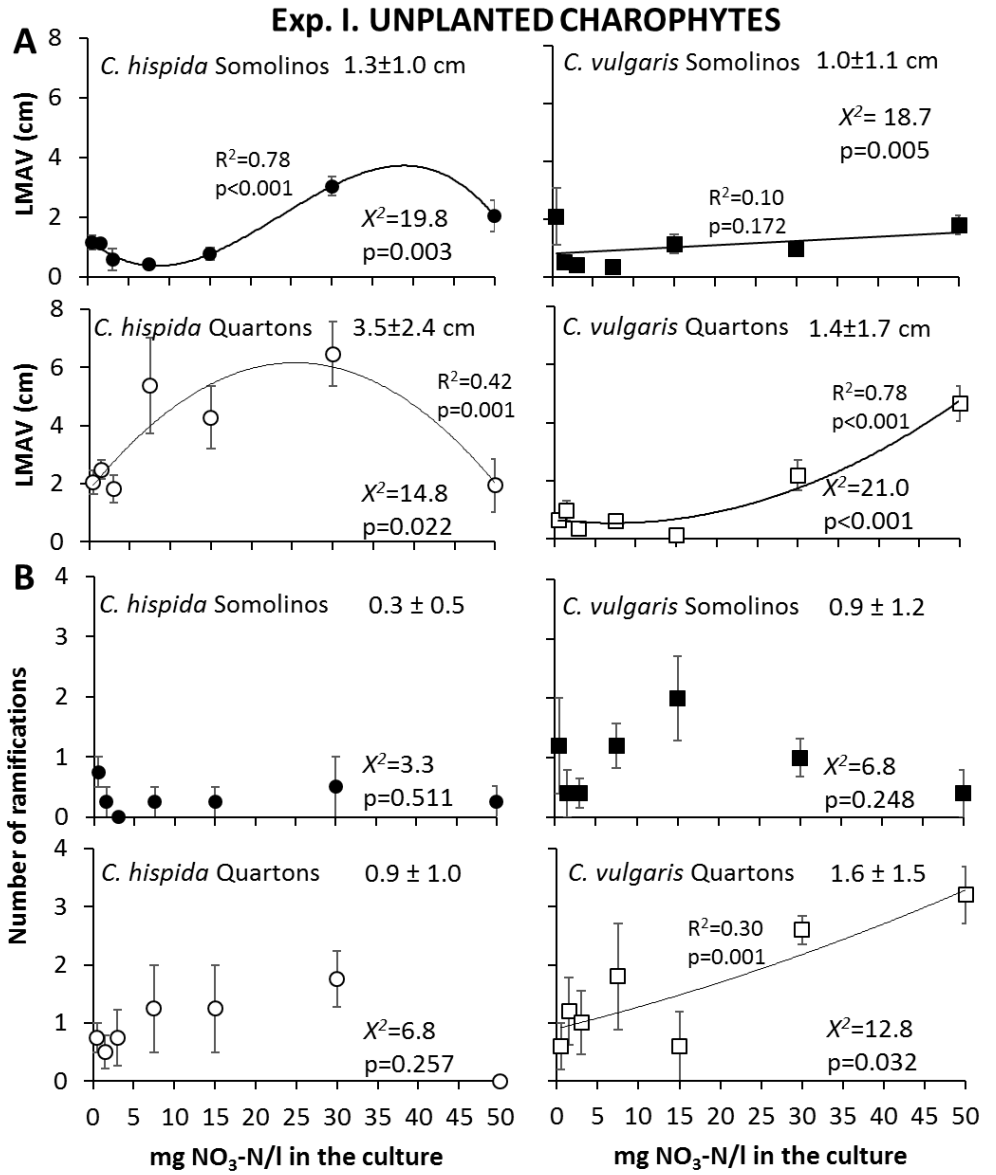


Fig. S2. Average values of elongation of the main axis (A) and number of lateral ramifications (B) for the two populations of *Chara hispida* and *C. vulgaris* (Somolinos lake and Quartons spring) cultivated unplanted under seven nitrate concentrations. Bars show standard errors. Results of Kruskal-Wallis tests (X^2 and probability), R^2 and probabilities of the curve fittings are presented when there were significant differences among nitrate doses. Average values for all the doses ± standard deviation are also indicated.

Table S1. Mean and standard error of the mean (SEM) of all analyzed variables for each population. Sets of data from the two different experimental temperatures, the two different experimental nitrate concentrations and for each of four resulting conditions in the experiment. CHS and CHQ are *Chara hispida* populations from Somolinos Lake and Quartons Spring, respectively. CVS and CVQ are *Chara vulgaris* populations from Somolinos Lake and Quartons Spring, respectively. LT (low temperature 20°C); HT (high temperature 24°C); LN (low nitrate concentration); HN (high nitrate concentration). Abbreviations used are: length variability of the main axis (LMAV), normalized dry weight (NDW), dry weight per centimeter of main axis (DW/LMA), internodal distance (LMA/N), number of nodes (N), number of branches (B), braches per node (B/N), relative growth rate (RGR), respiratory rate (RR), concentration of chlorophylls a and b (Chl-a, Chl-b), percentage of carbon, nitrogen and phosphorus in plant dry weight (%C, %N and %P), carbon *vs* nitrogen and nitrogen *vs* phosphorus molar ratio (C:N and N:P, respectively) and percentage of calcium carbonate incrustation (% $CaCO_3$).

	CHS															
	TEMPERATURE				NITRATE				CONDITION							
	LT		HT		LN		HN		LTLN		LTHN		HTLN		HTHN	
Variable	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
LMAV (cm)	7.64	0.89	11.05	0.63	9.92	0.64	8.86	1.07	8.58	0.75	6.85	1.50	11.26	0.63	10.87	1.09
NDW	17.60	2.45	26.26	1.43	23.17	1.74	20.90	2.74	20.10	2.42	15.52	4.01	26.25	1.74	26.27	2.33
DW/LMA (mg cm^{-1})	8.50	0.49	9.51	0.22	9.17	0.31	8.87	0.46	9.05	0.56	8.04	0.76	9.29	0.33	9.69	0.29
LMA/N (cm $node^{-1}$)	1.59	0.12	2.05	0.11	1.89	0.11	1.77	0.16	1.72	0.12	1.49	0.20	2.06	0.15	2.05	0.18
N	5.55	0.21	6.09	0.21	6.00	0.15	5.67	0.26	5.80	0.20	5.33	0.33	6.20	0.20	6.00	0.37
B	2.27	0.43	3.27	0.30	2.90	0.31	2.67	0.45	2.40	0.40	2.17	0.75	3.40	0.40	3.17	0.48
B/N	0.40	0.07	0.54	0.05	0.49	0.05	0.46	0.07	0.41	0.06	0.39	0.12	0.56	0.08	0.53	0.08
RGR (d^{-1})	0.11	0.01	0.13	0.00	0.12	0.00	0.12	0.01	0.12	0.00	0.10	0.01	0.13	0.00	0.13	0.00
RR (mg O_2 g^{-1} DW h^{-1})	0.84	0.10	1.61	0.67	0.79	0.11	1.52	0.56	0.71	0.08	0.94	0.15	0.87	0.22	2.10	1.09
Chl-a (µg g^{-1} org DW)	4.37	0.31	5.01	1.49	3.86	0.27	5.16	0.79	4.13	0.07	4.60	0.65	3.06	0.00	5.99	1.95
Chl-b (µg g^{-1} org DW)	1.07	0.06	1.72	0.43	0.97	0.05	1.57	0.28	1.00	0.06	1.14	0.10	0.87	0.00	2.00	0.45
Carotenoids (µg g^{-1} org DW)	1.21	0.20	1.51	0.30	0.98	0.07	1.57	0.23	1.05	0.00	1.38	0.41	0.78	0.00	1.76	0.25
%C	33.69	0.18	36.10	0.10	34.72	0.58	35.07	0.53	33.47	0.32	33.91	0.05	35.97	0.01	36.24	0.18
%N	2.10	0.02	2.23	0.02	2.15	0.04	2.18	0.03	2.07	0.03	2.13	0.00	2.23	0.03	2.23	0.05
%P	0.16	0.01	0.16	0.00	0.16	0.00	0.16	0.01	0.16	0.01	0.16	0.01	0.16	0.00	0.15	0.00
C:N	18.77	0.09	18.90	0.18	18.87	0.12	18.79	0.16	18.91	0.11	18.62	0.05	18.82	0.25	18.97	0.30
N:P	29.04	0.83	31.86	0.78	29.77	0.79	31.13	1.14	29.19	1.52	28.89	1.06	30.35	0.68	33.38	0.55
$CaCO_3$ (%)	39.66	1.37	45.25	1.46	40.91	1.01	44.00	2.27	38.74	0.61	40.57	2.86	43.08	0.09	47.42	2.43

Table S1. continuation.

	CHQ															
	TEMPERATURE				NITRATE				CONDITION							
	LT		HT		LN		HN		LTLN		LTHN		HTLN		HTHN	
Variable	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
LMAV (cm)	9.94	0.86	10.82	0.84	10.49	0.87	10.28	0.85	9.54	1.59	10.27	1.00	11.44	0.66	10.30	1.48
NDW	21.61	2.25	24.52	2.25	23.12	2.70	23.01	1.95	19.27	4.50	23.55	1.82	26.97	2.26	22.47	3.65
DW/LMA (mg cm^{-1})	14.02	1.12	14.44	0.82	13.94	1.04	14.47	0.93	12.51	1.66	15.28	1.45	15.38	1.06	13.65	1.19
LMA/N (cm $node^{-1}$)	1.81	0.10	2.03	0.10	1.98	0.10	1.87	0.11	1.80	0.14	1.81	0.15	2.16	0.11	1.93	0.15
N	6.27	0.24	6.00	0.27	6.00	0.21	6.25	0.28	6.00	0.45	6.50	0.22	6.00	0.00	6.00	0.52
B	3.45	0.28	4.00	0.40	3.60	0.43	3.80	0.30	3.00	0.55	3.83	0.17	4.20	0.58	3.83	0.60
B/N	0.55	0.04	0.66	0.06	0.60	0.07	0.61	0.04	0.49	0.08	0.59	0.03	0.70	0.10	0.62	0.09
RGR (d^{-1})	0.13	0.01	0.13	0.01	0.13	0.01	0.13	0.01	0.12	0.01	0.13	0.00	0.14	0.00	0.13	0.01
RR (mg O_2 g^{-1} DW h^{-1})	1.30	0.30	0.62	0.08	1.00	0.33	0.86	0.22	1.35	0.63	1.26	0.39	0.65	0.12	0.60	0.13
Chl-a (µg g^{-1} org DW)	1.62	0.18	2.74	0.27	2.51	0.43	2.00	0.26	1.69	0.51	1.57	0.15	3.05	0.40	2.43	0.35
Chl-b (µg g^{-1} org DW)	0.39	0.05	0.72	0.07	0.62	0.11	0.53	0.09	0.42	0.14	0.37	0.03	0.74	0.13	0.70	0.10
Carotenoids (µg g^{-1} org DW)	0.51	0.09	0.85	0.08	0.82	0.14	0.59	0.06	0.56	0.26	0.48	0.03	1.00	0.08	0.69	0.07
%C	34.69	0.27	33.75	0.81	33.02	0.48	35.41	0.14	34.09	0.05	35.28	0.01	31.96	0.11	35.54	0.27
%N	2.16	0.10	2.06	0.15	1.83	0.05	2.39	0.02	1.94	0.00	2.39	0.02	1.73	0.00	2.39	0.05
%P	0.12	0.01	0.13	0.01	0.12	0.01	0.13	0.01	0.11	0.01	0.14	0.00	0.13	0.01	0.12	0.00
C:N	18.89	0.75	19.47	0.94	21.05	0.23	17.31	0.12	20.55	0.06	17.22	0.13	21.55	0.07	17.39	0.22
N:P	39.00	1.42	37.06	3.75	33.65	2.55	42.41	1.50	38.34	2.90	39.66	1.09	28.97	1.49	45.15	1.58
$CaCO_3$ (%)	42.96	2.83	47.11	1.63	42.15	2.76	47.91	1.20	39.19	4.97	46.72	1.11	45.11	2.17	49.10	2.13

Table S1. continuation.

	CVS															
	TEMPERATURE				NITRATE				CONDITION							
	LT		HT		LN		HN		LTLN		LTHN		HTLN		HTHN	
Variable	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
LMAV (cm)	13.00	1.60	16.27	1.36	14.49	1.61	14.77	1.53	12.76	2.22	13.22	2.46	16.22	2.30	16.32	1.80
NDW	58.41	7.33	71.56	5.56	64.89	6.95	65.07	6.69	60.92	11.96	56.32	9.98	68.86	8.13	73.82	8.18
DW/LMA (mg cm^{-1})	5.67	0.20	6.98	0.27	6.38	0.31	6.28	0.32	5.95	0.27	5.44	0.29	6.81	0.53	7.12	0.28
LMA/N (cm $node^{-1}$)	2.06	0.24	2.25	0.20	2.36	0.22	1.98	0.21	2.45	0.37	1.74	0.27	2.28	0.26	2.23	0.31
N	7.09	0.46	8.09	0.28	6.80	0.36	8.25	0.33	5.80	0.20	8.17	0.48	7.80	0.20	8.33	0.49
B	4.55	0.78	6.18	0.30	4.20	0.66	6.33	0.45	2.40	0.40	6.33	0.84	6.00	0.45	6.33	0.42
B/N	0.60	0.08	0.77	0.04	0.59	0.07	0.77	0.05	0.41	0.06	0.76	0.09	0.77	0.05	0.77	0.07
RGR (d^{-1})	0.16	0.01	0.20	0.00	0.18	0.01	0.18	0.01	0.16	0.01	0.15	0.01	0.19	0.01	0.20	0.01
RR (mg O_2 g^{-1} DW h^{-1})	0.88	0.30	1.28	0.17	1.03	0.22	1.11	0.27	0.69	0.05	1.00	0.53	1.37	0.23	1.21	0.28
Chl-a (µg g^{-1} org DW)	3.45	0.40	4.00	0.57	3.52	0.38	3.80	0.49	3.19	0.24	3.72	0.81	4.52	0.00	3.87	0.71
Chl-b (µg g^{-1} org DW)	0.83	0.09	1.00	0.13	0.96	0.14	0.88	0.09	0.83	0.06	0.82	0.18	1.37	0.00	0.91	0.12
Carotenoids (µg g^{-1} org DW)	1.06	0.12	1.35	0.22	0.94	0.05	1.33	0.17	0.91	0.06	1.20	0.23	1.02	0.00	1.43	0.27
%C	34.04	0.20	36.77	0.51	34.98	0.29	35.83	0.94	34.34	0.10	33.74	0.32	35.62	0.01	37.92	0.02
%N	2.13	0.02	2.28	0.05	2.14	0.02	2.27	0.06	2.11	0.01	2.15	0.04	2.17	0.02	2.39	0.05
%P	0.20	0.00	0.25	0.01	0.22	0.02	0.22	0.01	0.19	0.01	0.20	0.01	0.26	0.01	0.24	0.01
C:N	18.70	0.18	18.86	0.24	19.09	0.08	18.46	0.22	19.03	0.07	18.36	0.19	19.50	0.15	18.57	0.44
N:P	23.89	0.41	20.30	0.95	21.33	1.30	22.86	0.66	24.14	0.67	23.65	0.57	18.52	0.30	22.08	1.11
$CaCO_3$ (%)	24.00	1.12	23.48	1.88	21.08	1.02	26.40	0.98	22.34	1.55	25.66	1.08	19.81	1.11	27.15	1.74

Table S1. continuation.

	CVQ															
	TEMPERATURE				NITRATE				CONDITION							
	LT		HT		LN		HN		LTLN		LTHN		HTLN		HTHN	
Variable	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
LMAV (cm)	16.15	1.13	19.82	1.41	16.82	1.39	19.15	1.31	14.52	1.67	17.52	1.41	18.73	1.89	21.12	2.18
NDW	80.43	4.55	100.90	7.52	93.77	8.66	87.56	4.61	83.75	8.99	77.66	4.31	102.12	13.79	99.44	4.94
DW/LMA (mg cm^{-1})	9.25	0.69	10.65	0.74	10.77	0.72	9.13	0.69	10.50	1.13	8.21	0.63	11.00	1.00	10.23	1.20
LMA/N (cm $node^{-1}$)	1.92	0.13	2.45	0.13	2.11	0.16	2.25	0.15	1.71	0.17	2.09	0.17	2.45	0.17	2.44	0.24
N	9.27	0.27	8.82	0.40	8.82	0.42	9.27	0.24	9.40	0.51	9.16	0.31	8.33	0.61	9.40	0.40
B	9.64	0.91	8.09	0.53	8.64	0.70	9.09	0.85	9.20	1.31	10.00	1.34	8.17	0.75	8.00	0.84
B/N	1.03	0.09	0.93	0.07	0.98	0.07	0.98	0.09	0.97	0.12	1.08	0.14	0.99	0.09	0.87	0.12
RGR (d^{-1})	0.18	0.00	0.21	0.00	0.20	0.01	0.20	0.01	0.18	0.00	0.18	0.00	0.21	0.01	0.21	0.00
RR (mg O_2 g^{-1} DW h^{-1})	1.65	0.27	0.94	0.09	1.02	0.21	1.56	0.26	1.29	0.52	1.89	0.30	0.85	0.12	1.07	0.04
Chl-a (µg g^{-1} org DW)	1.40	0.22	2.49	0.28	1.86	0.40	2.02	0.29	1.28	0.35	1.51	0.31	2.44	0.58	2.53	0.23
Chl-b (µg g^{-1} org DW)	0.42	0.14	0.65	0.05	0.47	0.10	0.64	0.12	0.29	0.08	0.61	0.36	0.64	0.09	0.66	0.06
Carotenoids (µg g^{-1} org DW)	0.46	0.09	0.95	0.11	0.68	0.17	0.72	0.12	0.38	0.11	0.53	0.16	0.99	0.20	0.91	0.12
%C	33.67	0.25	37.86	0.40	35.05	0.86	36.48	1.02	33.14	0.04	34.21	0.15	36.97	0.04	38.76	0.07
%N	2.54	0.09	2.97	0.14	2.50	0.08	3.01	0.12	2.34	0.03	2.74	0.01	2.67	0.02	3.27	0.04
%P	0.17	0.01	0.19	0.02	0.15	0.00	0.21	0.01	0.16	0.00	0.18	0.00	0.15	0.01	0.22	0.01
C:N	15.56	0.45	14.99	0.53	16.36	0.13	14.20	0.18	16.56	0.16	14.56	0.03	16.15	0.12	13.83	0.15
N:P	32.90	0.32	36.20	1.68	35.92	1.71	33.19	0.61	32.27	0.27	33.53	0.22	39.56	1.11	32.85	1.30
$CaCO_3$ (%)	30.45	2.01	30.82	0.97	30.44	2.08	30.83	0.83	31.09	4.43	29.81	0.48	29.79	1.23	31.84	1.47

Table S2. F or U values of either two-way parametric ANOVA or non-parametric Mann–Whitney tests, respectively for the four populations: *Chara hispida* from the Somolinos mountain Lake (CHS) and the Quartons Spring (CHQ) and *C. vulgaris* from the same sites (CVS and CVQ). Degrees of freedom: 1. Factors are temperature and nitrate. p < 0.05:*, p <0.01:**, p <0.001:***. Abbreviations are as in Table S1.

	TEMPERATURE								NITRATE								TEMPERATURE x NITRATE							
	CHS		CHQ		CVS		CVQ		CHS		CHQ		CVS		CVQ		CHS		CHQ		CVS		CVQ	
Variable	F/U	p	F/U	p	F/U	p	F/U	p	F/U	p	F/U	p	F/U	p	F/U	p	F/U	p	F/U	p	F/U	p	F/U	p
LMAV (cm)	14.0	0.002 **	48.5	0.431	2.2	0.156	4.7	0.043 *	53.0	0.644	58.0	0.895	0.0	0.902	2.2	0.152					0.0	0.936	0.0	0.867
NDW	8.5	0.009 **	44.0	0.278	1.7	0.205	20.0	0.080 **	0.6	0.442	55.0	0.742	0.0	0.986	44.0	0.279	0.6	0.437			0.2	0.627		
DW/LMA (mg cm^{-1})	3.2	0.093	0.2	0.654 **	13.2	0.002 **	1.6	0.221	0.3	0.576	0.1	0.706	0.1	0.782	2.4	0.139	1.7	0.203	2.7	0.116	1.4	0.255	0.6	0.455
LMA/N (cm $node^{-1}$)	22.0	0.018 *	2.7	0.117	0.3	0.598	8.9	0.008 **	49.0	0.480	0.6	0.464	1.5	0.230	1.0	0.329			0.7	0.431	1.2	0.292	1.1	0.308
NDW	38.0	0.095	50.5	0.478	38.0	0.128	52.5	0.583	46.0	0.296	45.0	0.285	22.0	0.010 **	49.5	0.451								
B	33.5	0.066	34.5	0.066 **	9.4	0.007 **	36.0	0.103	56.5	0.811	55.5	0.749	13.2	0.002 **	52.0	0.572					9.4	0.007 **		
B/N	2.4	0.136	33.5	0.072	34.5	0.086	0.7	0.423	0.1	0.760	56.0	0.789	35.0	0.097	0.0	0.963	0.0	0.990					1.0	0.331
RGR (d^{-1})	14.0	0.002 **	44.0	0.278 ***	28.1	0.000 ***	11.0	0.001 ***	59.0	0.947	55.0	0.742	0.0	0.917	50.0	0.491					0.3	0.594		
RR (mg O_2 g^{-1} DW h^{-1})	7.0	0.251	2.0	0.050	1.3	0.304	4.9	0.069	6.0	0.201	10.0	1.000	0.0	0.854	2.1	0.202					0.4	0.565	0.4	0.535
Chl-a	8.0	0.796	10.0	0.016	0.8	0.408	7.7	0.024 *	5.0	0.221	1.1	0.329	0.0	0.942	0.2	0.687			0.5	0.495	0.5	0.505	0.0	0.870
Chl-b	9.0	0.522	9.9	0.016	3.7	0.094	6.0	0.100	4.0	0.088	0.2	0.646	2.0	0.201	9.0	0.273			0.0	0.952	1.9	0.209		
Carotenoids	9.0	0.522	3.0	0.028	9.0	0.273	10.2	0.013 *	4.0	0.088	6.0	0.100	6.0	0.131	0.5	0.826							0.6	0.459
%C	167.8	0.000 ***	15.0	0.631 **	0.0	0.004 **	0.0	0.004 **	3.6	0.093	0.0	0.004 **	17.0	0.873	9.0	0.150	0.2	0.660						
%N	17.8	0.004 **	16.2	0.420 **	17.3	0.003 **	260.3	0.150	0.9	0.520	452.6	0.004 **	12.1	0.008 **	351.3	0.004 ***	0.9	0.560	15.2	0.007 **	5.7	0.044 *	13.2	0.007 **
%P	7.0	0.462	10.0	1.000 ***	42.2	0.001 ***	6.4	0.045 *	7.0	0.462	10.0	1.000	0.6	0.481	64.5	0.000 ***					2.8	0.143	19.0	0.005 **
C:N	0.4	0.542	12.0	0.337	0.4	0.536	20.1	0.002 **	0.1	0.727	0.0	0.004 **	6.2	0.037 *	290.1	0.000 ***	1.2	0.312			0.0	0.866	1.7	0.234
N:P	7.6	0.025 *	1.1	0.335 ***	24.6	0.001 ***	14.3	0.005 **	1.8	0.220	21.4	0.002 **	4.5	0.067	9.8	0.014 *	2.7	0.142	15.4	0.004 **	7.8	0.023 *	20.9	0.002 **
$CaCO_3$ (%)	8.0	0.109	2.0	0.199	0.1	0.720	13.0	0.423	7.0	0.078	3.8	0.088	14.5	0.005 **	13.0	0.423			0.4	0.568	2.1	0.188		

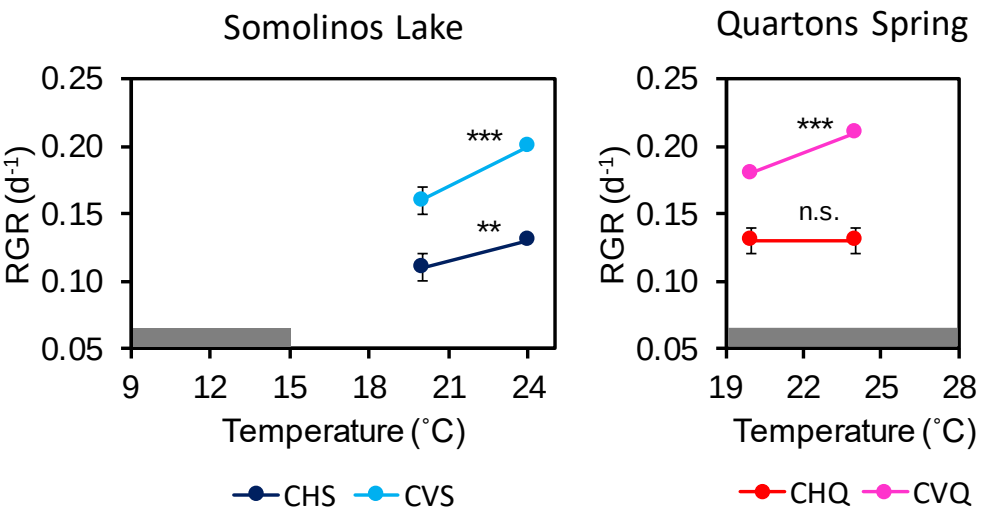


Fig. S1. Relative growth rate measured in laboratory cultures at 20°C and 24°C of two populations of *Chara hispida* and *Chara vulgaris*. These populations cohabit in two thermally different ecosystems. The colder Somolinos mountain Lake and the warmer Quartons coastal Spring. CVS and CHS are populations of *C. vulgaris* and *C. hispida* from Somolinos Lake, CVQ and CHQ are their corresponding populations from Quartons Spring. Grey bars show the range of temperatures in the origin sites during the charophyte vegetative period. p <0.01:**, p <0.001:*** and n.s. means no significant variation (see Table 2 for more statistical information).

Chapter 3. The antagonistic effect of UV radiation on warming or nitrate enrichment depends on ecotypes of freshwater macroalgae (charophytes)

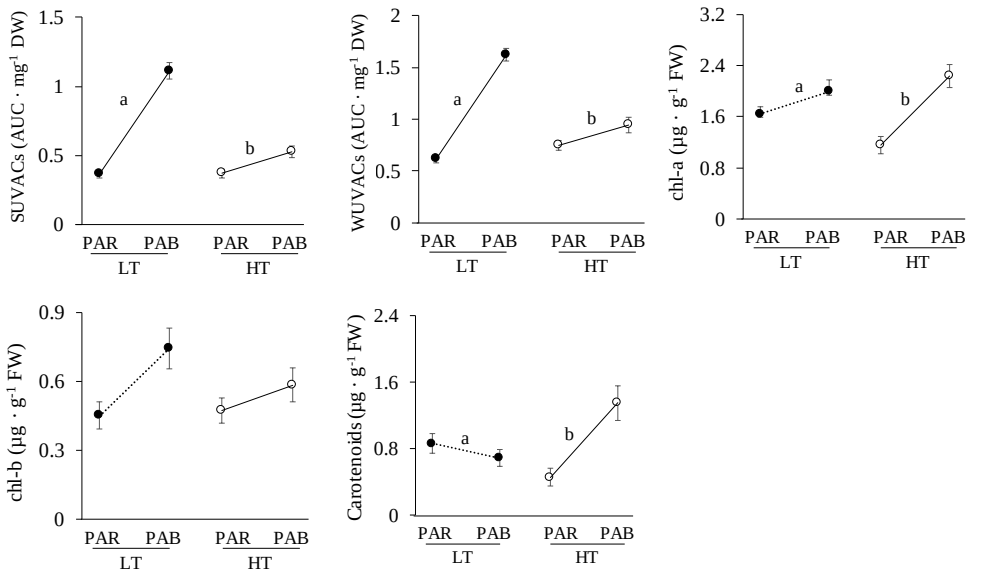


Fig. S1. UV-absorbing compounds concentration (methanol-soluble and insoluble, SUVACs and WUVACs, respectively) and photosynthetic pigments concentration (chl-a, chl-b, and carotenoids) in charophytes (all the populations together) of the UVR x T experiment cultivated under four experimental conditions: photosynthetic active radiation (PAR) and PAR plus UVBR and UVAR radiation (PAB), and low temperature (LT, black dots) and high temperature (HT, white dots). Variation of data between two radiation levels were lineally fitted; a continuous line indicates significant differences ($P < 0.05$) whereas a dotted one shows that the adjustment is not significant. When letters above the lines appear, an interactive effect between the two factors (radiation and temperature or nitrate) is significant. Bars show standard error.

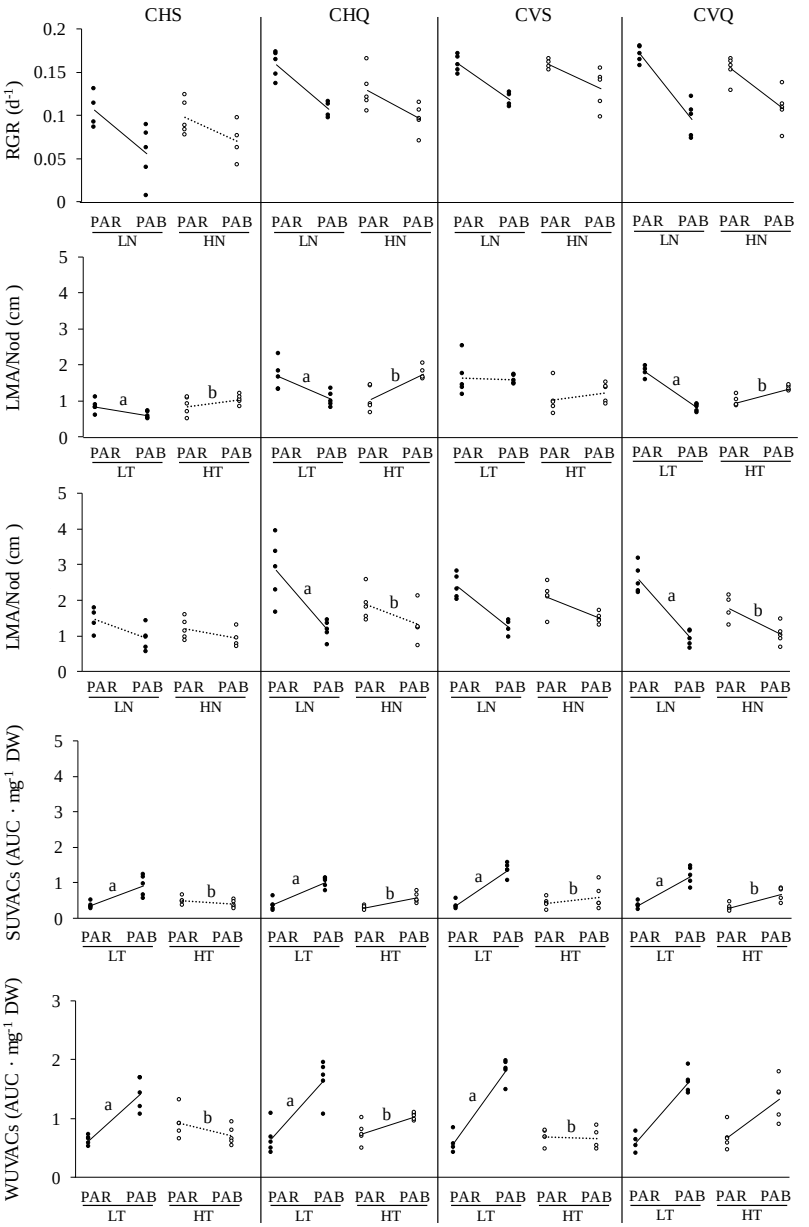


Fig. S2. Relative growth rate (RGR), internodal distance (LMA/Nod) and methanol-soluble and -insoluble fractions of UV-absorbing compounds concentration (SUVACs and WUVACs, respectively) in charophytes in the UVR x T or UVR x N experiments, in the two populations of *Chara hispida* and *Chara vulgaris* from the Somolinos mountain Lake (CHS and CVS) and the Quartons coastal Spring (CHQ and CVQ), cultivated under four experimental conditions. Details of experimental conditions in Fig. 2. Abbreviations as in Table 2. Variation of data between two radiation levels were lineally fitted; a continuous line indicates significant differences ($P < 0.05$) whereas a dotted one shows that the adjustment is not significant. When letters above the lines appear, an interactive effect between the two factors (radiation and temperature) is significant. Each dot represents a replicate.

Chapter 4. Structure and vulnerability of the multi-interaction network in macrophyte-dominated lakes

Table S1. Main physical and chemical variables measured in the experimental mesocosm. Mean of two months (measured weekly) and standard deviation (mean±SD) are shown. For photosynthetic active radiation (PAR) the average dose was calculated from measurements made at depths of 0, 10, 20 and 30 cm in the mesocosm. Abbreviations: TN total nitrogen, TP total phosphorus.

Variable	Mean±SD
Temperature (˚C)	21.1 ± 0.8
Conductivity (µS cm^{-1})	1575±60
pH	8.4±0.2
Nitrate (mg N-NO_3 l^{-1})	1.3±0.8
TN (mg N l^{-1})	2.9±1.1
TP (mg P l^{-1})	0.2±0.05
PAR (mol photons m^{-2} d^{-1})	2.2
Sediment %C	10.5±3.2
Sediment %N	0.12±0.08
Sediment %P	0.02±0.00

Table S2. Complete list of the identified taxa in each compartment, noting if they are autotrophic (Aut.) or heterotrophic organisms (Het.). Order is alphabetical within each compartment.

Taxon	Compartment	Autotroph (Aut.) / Heterotroph (Het.)
Achnanthidium minutissimum	*Pelagic*	Aut.
Bacteria	*Pelagic*	Het.
Ceriodaphnia	*Pelagic*	Het.
Chlamydomonas sp.	*Pelagic*	Aut.
Chroococcus aphanocapsoides	*Pelagic*	Aut.
Chydorus	*Pelagic*	Het.
Ciliate sp. 1	*Pelagic*	Het.
Ciliate sp. 2	*Pelagic*	Het.
Cyclopoid copepodite	*Pelagic*	Het.
Cyclopoid copepod	*Pelagic*	Het.
Cyclotella meneghiniana	*Pelagic*	Aut.
Lecane bulla	*Pelagic*	Het.
Lecane cf. *furcata*	*Pelagic*	Het.
Lecane cf. *hastata*	*Pelagic*	Het.
Lecane closterocerca	*Pelagic*	Het.
Lecane hamata	*Pelagic*	Het.
Lecane luna	*Pelagic*	Het.
Lepadella	*Pelagic*	Het.
Nauplii	*Pelagic*	Het.
Oedogonium sp.	*Pelagic*	Aut.
Oscillatoria sp.	*Pelagic*	Aut.
Pleuroxus	*Pelagic*	Het.
Rhopalodia gibba	*Pelagic*	Aut.
Scenedesmus aculeolatus	*Pelagic*	Aut.
Simocephalus	*Pelagic*	Het.
Tetraedron minimum	*Pelagic*	Aut.
Achnanthidium minutissimum	*Meadow*	Aut.
Bacteria	*Meadow*	Het.
Bdelloidea	*Meadow*	Het.
Carteria sp.	*Meadow*	Aut.
Chlorella sp.	*Meadow*	Aut.
Ciliate sp. 1	*Meadow*	Het.
Ciliate sp. 2	*Meadow*	Het.
Coelastrum microporum	*Meadow*	Aut.
Cyclopoid copepodite	*Meadow*	Het.
Cyclopoid copepod	*Meadow*	Het.
Cyclotella meneghiniana	*Meadow*	Aut.
Diploneis parma	*Meadow*	Aut.

Table S2. continuation.

Taxon	Compartment	Autotroph (Aut.) / Heterotroph (Het.)
Encyonopsis microcephala	*Meadow*	Aut.
Geitlerinema amphibium	*Meadow*	Aut.
Gomphosphaeria aponina	*Meadow*	Aut.
Lecane bulla	*Meadow*	Het.
Lecane cf. *pyriformis*	*Meadow*	Het.
Lecane closterocerca	*Meadow*	Het.
Lecane hamata	*Meadow*	Het.
Lecane luna	*Meadow*	Het.
Lepadella	*Meadow*	Het.
Lophocanis	*Meadow*	Het.
Nauplii	*Meadow*	Het.
Navicula sp.	*Meadow*	Aut.
Oedogonium sp.	*Meadow*	Aut.
Oscillatoria sp.	*Meadow*	Aut.
Phormidium sp.	*Meadow*	Aut.
Pleuroxus	*Meadow*	Het.
Scenedesmus aculeolatus	*Meadow*	Aut.
Scenedesmus acutus	*Meadow*	Aut.
Scenedesmus sp.	*Meadow*	Aut.
Simocephalus	*Meadow*	Het.
Tetraedron minimum	*Meadow*	Aut.
Ulnaria ulna var. *acus*	*Meadow*	Aut.
Achnanthidium minutissimum	*Periphyton*	Aut.
Aphanocapsa elachista	*Periphyton*	Aut.
Aphanothece stagnina	*Periphyton*	Aut.
Bacteria	*Periphyton*	Het.
Bdelloidea	*Periphyton*	Het.
Ceriodaphnia	*Periphyton*	Het.
Chara hispida	*Periphyton*	Aut.
Chlorella sp.	*Periphyton*	Aut.
Chroococcus aphanocapsoides	*Periphyton*	Aut.
Chroococcus obliteratus	*Periphyton*	Aut.
Chroococcus sp.	*Periphyton*	Aut.
Chroococcus turgidus	*Periphyton*	Aut.
Chydorus	*Periphyton*	Het.
Coelastrum microporum	*Periphyton*	Het.
Colurella	*Periphyton*	Het.
Copepodite	*Periphyton*	Het.
Copepod	*Periphyton*	Het.
Cyclotella meneghiniana	*Periphyton*	Aut.

Table S2. continuation.

Taxon	Compartment	Autotroph (Aut.) / Heterotroph (Het.)
Cymbella sp.	*Periphyton*	Aut.
Diploneis parma	*Periphyton*	Aut.
Encyonopsis microcephala	*Periphyton*	Aut.
Fragilaria biceps	*Periphyton*	Aut.
Geitlerinema amphibium	*Periphyton*	Aut.
Komvophoron sp.	*Periphyton*	Aut.
Lecane bulla	*Periphyton*	Het.
Lecane cf. *furcata*	*Periphyton*	Het.
Lecane cf. *hastata*	*Periphyton*	Het.
Lecane cf. *pyriformis*	*Periphyton*	Het.
Lecane closterocerca	*Periphyton*	Het.
Lecane hamata	*Periphyton*	Het.
Lecane luna	*Periphyton*	Het.
Lecane sp. 2	*Periphyton*	Het.
Lepadella	*Periphyton*	Het.
Merismopedia sp.	*Periphyton*	Aut.
Nauplii	*Periphyton*	Het.
Navicula sp.1	*Periphyton*	Aut.
Navicula sp.2	*Periphyton*	Aut.
Navicymbulla pusilla	*Periphyton*	Aut.
Nitzschia sp.1	*Periphyton*	Aut.
Oedogonium sp	*Periphyton*	Aut.
Oscillatoria curviceps	*Periphyton*	Aut.
Ostracod	*Periphyton*	Het.
Phormidium cf. *formosum*	*Periphyton*	Aut.
Phormidium sp.	*Periphyton*	Aut.
Physella acuta	*Periphyton*	Het.
Pleuroxus	*Periphyton*	Het.
Pseudanabaena biceps	*Periphyton*	Aut.
Pseudanabaena sp.	*Periphyton*	Aut.
Simocephalus	*Periphyton*	Het.
Snowella lacustris	*Periphyton*	Aut.
Spirulina sp.	*Periphyton*	Aut.
Ulnaria ulna var. acus	*Periphyton*	Aut.
Ulothrix sp.	*Periphyton*	Aut.

Table S3. Degree, closeness and betweenness centrality measures (C_D, C_C and C_B, respectively) for each node in the network.

ID	Compartment	Node	C_D	C_C	C_B
1		Nutrients	0.537	0.661	0.000
2	*Pelagic*	Bacteria	0.195	0.494	0.008
3	*Pelagic*	Unicellular chlorophytes	0.220	0.513	0.005
4	*Pelagic*	Colonial chlorophytes	0.171	0.488	0.003
5	*Pelagic*	Filamentous chlorophytes	0.073	0.441	0.000
6	*Pelagic*	Small diatoms	0.220	0.513	0.005
7	*Pelagic*	Big diatoms	0.171	0.488	0.003
8	*Pelagic*	Colonial cyanobacteria	0.268	0.539	0.001
9	*Pelagic*	Filamentous cyanobacteria	0.171	0.482	0.000
10	*Pelagic*	Ciliates	0.098	0.402	0.000
11	*Pelagic*	Rotifers	0.195	0.456	0.002
12	*Pelagic*	Cladocerans	0.341	0.526	0.005
13	*Pelagic*	Copepodites	0.244	0.471	0.000
14	*Pelagic*	Copepods	0.146	0.456	0.000
15	*Meadow*	Bacteria	0.268	0.539	0.020
16	*Meadow*	Unicellular chlorophytes	0.341	0.586	0.014
17	*Meadow*	Colonial chlorophytes	0.268	0.554	0.006
18	*Meadow*	Filamentous chlorophytes	0.122	0.500	0.000
19	*Meadow*	Small diatoms	0.341	0.586	0.014
20	*Meadow*	Big diatoms	0.268	0.554	0.006
21	*Meadow*	Colonial cyanobacteria	0.366	0.603	0.022
22	*Meadow*	Filamentous cyanobacteria	0.220	0.539	0.017
23	*Meadow*	Ciliates	0.195	0.506	0.013
24	*Meadow*	Rotifers	0.341	0.569	0.029
25	*Meadow*	Cladocerans	0.561	0.683	0.062
26	*Meadow*	Copepodites	0.415	0.603	0.036
27	*Meadow*	Copepods	0.244	0.547	0.000
28	*Periphyton*	Bacteria	0.244	0.526	0.002
29	*Periphyton*	Unicellular chlorophytes	0.293	0.547	0.002
30	*Periphyton*	Colonial chlorophytes	0.244	0.532	0.001
31	*Periphyton*	Filamentous chlorophytes	0.146	0.488	0.000
32	*Periphyton*	Small diatoms	0.293	0.547	0.002
33	*Periphyton*	Big diatoms	0.244	0.532	0.001
34	*Periphyton*	Colonial cyanobacteria	0.341	0.562	0.000
35	*Periphyton*	Filamentous cyanobacteria	0.244	0.513	0.000
36	*Periphyton*	Ciliates	0.146	0.471	0.005
37	*Periphyton*	Rotifers	0.268	0.506	0.006
38	*Periphyton*	Cladocerans	0.390	0.539	0.008
39	*Periphyton*	Copepodites	0.268	0.500	0.000
40	*Periphyton*	Copepods	0.171	0.506	0.000
41	*Periphyton*	Charophyceae	1.195	0.745	0.447
42	*Periphyton*	Gastropoda	0.220	0.471	0.000

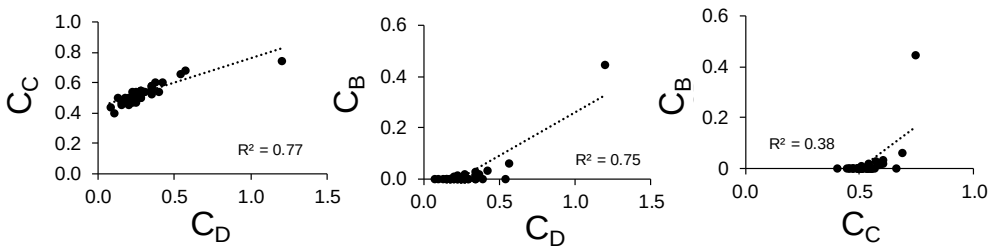


Fig. S1. Significant linear correlations ($p < 0.05$) between a) degree centrality (C_D) and closeness centrality (C_C), b) C_D and betweenness centrality (C_B) and c) C_C and C_B. Pearson's R coefficient is indicated on each graph.

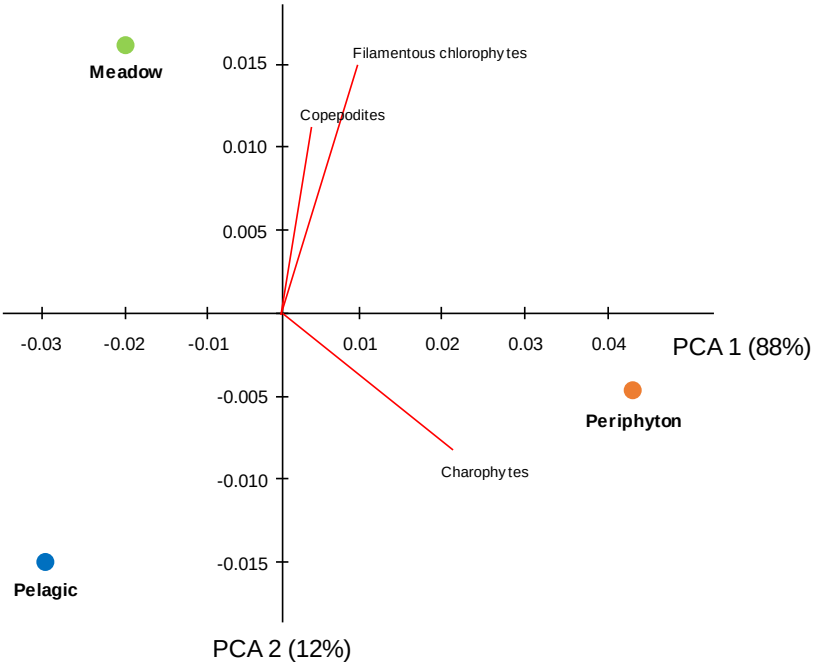


Fig. S2. Biplot of the two first principal components generated by the principal components analysis (PCA) considering the nodes of the network from the three compartments (pelagic, meadow and periphyton). Lines in red show the variables (nodes) with the highest principal component coefficients.

Chapter 5. Multi-interaction network performance under global change: a shallow ecosystem experimental simulation

Table S1. Results of repeated measures analysis of variance (ANOVAR) on mesocosms' temperature. The probabilities (P) of the significant effects of Time (5 levels), Scenario (3 levels: TPAR, TUVR, +TPAR) and their interaction are shown as well as F values and degrees of freedom (df). Effects were considered significant at $P < 0.05$.

	df	F	*P*
Time	4	70.3	<0.001
Scenario	2	952.5	<0.001
Time x Scenario	8	2.7	0.07

The significant effect of time on the temperature of the mesocosms was not due to a trend, but rather to a specific change: a decrease of 0.5 degrees in all mesocosm during the day 46. This small drop in temperature was probably due to a change in the room temperature for climatic reasons. Furthermore, these changes over time were not significantly different among scenarios (interaction Time x Scenario). The temperature of the mesocosms was 21.8 ± 0.1 ˚C (mean ± SEM) in TPAR, 22.3 ± 0.1 ˚C in TUVR, and 25.9 ± 0.1 ˚C in +TPAR.

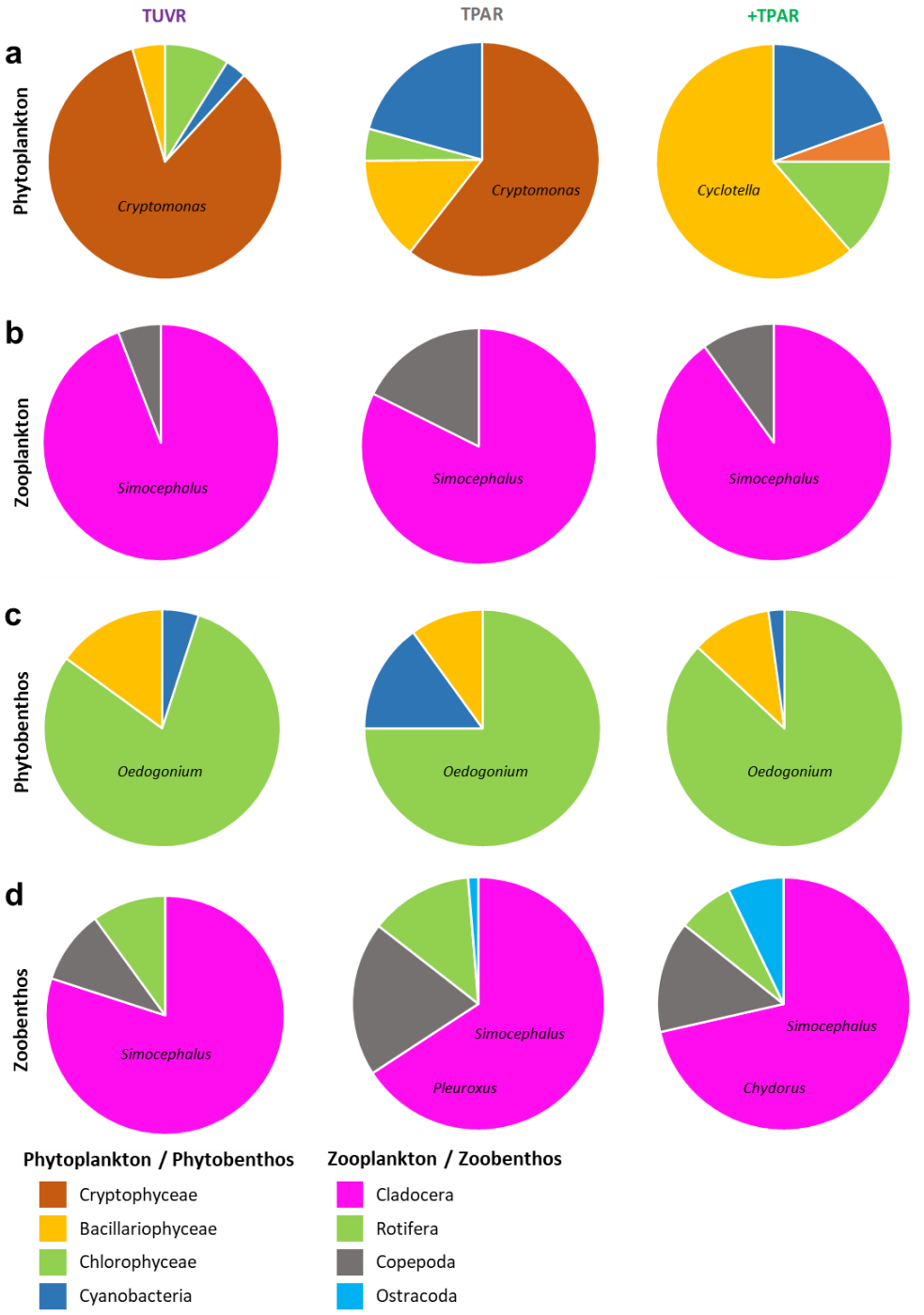


Fig. S1. Pie charts of the percentage of carbon biomass in the different taxonomic groups at the middle of the experiment (33 days) within a) Phytoplankton, b) Zooplankton, c) phytobenthos and d) zoobenthos under the tested scenarios (TUVR, TPAR and +TPAR). The dominant genus is shown in each graph.

Chapter 6. Non-trophic key players in aquatic ecosystems: a mesocosm experiment

Table S1. List of the nodes considered in the networks of the experimental mesocosms. Each scenario (TUVR, TPAR and +TPAR) has four replicates.

ID	Node	TPAR1	TPAR2	TPAR3	TPAR4	+TPAR1	+TPAR2	+TPAR3	+TPAR4	TUVR1	TUVR2	TUVR3	TUVR4
Pelagic compartment													
1	B_p	X	X	X	X	X	X	X	X	X	X	X	X
2	ClU_p	X	X	X	X	X	X	X	X	X	X	X	X
3	ClC_p		X	X	X	X	X	X	X		X	X	
4	DS_p					X	X	X	X				
5	DB_p					X	X	X	X				
6	CiC_p		X		X		X	X					
7	CiF_p				X	X	X	X	X	X	X	X	
8	Cr_p										X	X	X
9	Cil_p									X	X	X	X
10	R_p	X	X	X	X	X	X	X	X			X	X
11	C_p	X	X	X	X	X	X	X	X				
12	O_p	X				X	X		X	X			
13	Cop_p	X	X	X	X	X	X	X	X	X	X	X	X
14	Co_p	X	X	X	X								
Meadow compartment													
15	B_m	X	X	X	X	X	X	X	X	X	X	X	X
16	ClU_m	X	X	X	X	X	X	X	X	X	X	X	X
17	ClC_m		X		X		X	X	X			X	
18	DS_m					X	X	X	X				
19	DB_m					X	X	X	X				
20	Cr_m										X	X	X
21	CiC_m	X	X	X			X		X		X		

Table S1. continuation.

ID	Node	TPAR1	TPAR2	TPAR3	TPAR4	+TPAR1	+TPAR2	+TPAR3	+TPAR4	TUVR1	TUVR2	TUVR3	TUVR4
Meadow compartment													
22	CiF_m	X	X	X	X	X	X	X			X		X
23	Cil_m	X	X	X			X	X	X	X	X	X	X
24	R_m	X	X	X	X	X	X	X	X	X	X	X	X
25	C_m	X	X	X	X	X	X	X	X	X	X	X	X
26	O_m					X	X	X	X				
27	Cop_m	X	X	X	X	X	X	X	X	X	X	X	X
28	Co_m	X	X	X	X	X	X	X	X	X	X	X	X
Periphyton compartment													
29	B_b									X	X	X	X
30	ClF_b	X	X	X	X	X	X	X	X	X	X	X	X
31	DS_b	X	X	X	X	X	X	X	X	X	X	X	X
32	DB_b	X	X	X	X	X	X	X	X	X	X	X	X
33	CiC_b	X	X	X	X	X	X	X	X	X	X	X	X
34	CiF_b	X	X	X	X	X	X	X	X	X	X	X	X
35	R_b	X	X	X	X	X	X	X	X	X	X	X	X
36	C_b		X	X	X	X	X	X	X	X	X	X	X
37	O_b	X	X	X		X	X	X				X	X
38	Cop_b	X	X		X	X	X			X		X	X
39	Co_b		X	X	X	X	X	X		X		X	X
40	Char	X	X	X	X	X	X	X	X	X	X	X	X
41	G	X	X	X	X	X	X	X	X	X	X	X	X

Table S2. Pearson correlation coefficients (r) between the rankings of nodes by the four considered indices in the three experimental scenarios (TUVR, TPAR and +TPAR) in trophic networks (TN) and multi-interaction networks (IN). Abbreviations as in Tables 1 and 2.

Trophic networks (TN)

	TUVR			
	TI	TO	CC	BC
TI				
TO	**0.8**			
CC	**0.8**	**0.9**		
BC	**0.9**	**0.6**	**0.6**	

	TPAR			
	TI	TO	CC	BC
TI				
TO	**0.8**			
CC	**0.8**	**0.9**		
BC	**0.9**	**0.5**	**0.6**	

	+TPAR			
	TI	TO	CC	BC
TI				
TO	**0.8**			
CC	**0.7**	**0.8**		
BC	**0.9**	**0.5**	**0.5**	

Multi-interaction networks (IN)

	TUVR			
	TI	TO	CC	BC
TI				
TO	**0.7**			
CC	**0.9**	**0.9**		
BC	**0.9**	**0.7**	**0.8**	

	TPAR			
	TI	TO	CC	BC
TI				
TO	**0.7**			
CC	**0.8**	**0.9**		
BC	**0.9**	**0.7**	**0.9**	

	+TPAR			
	TI	TO	CC	BC
TI				
TO	**0.7**			
CC	**0.8**	**0.9**		
BC	**0.9**	**0.8**	**0.8**	

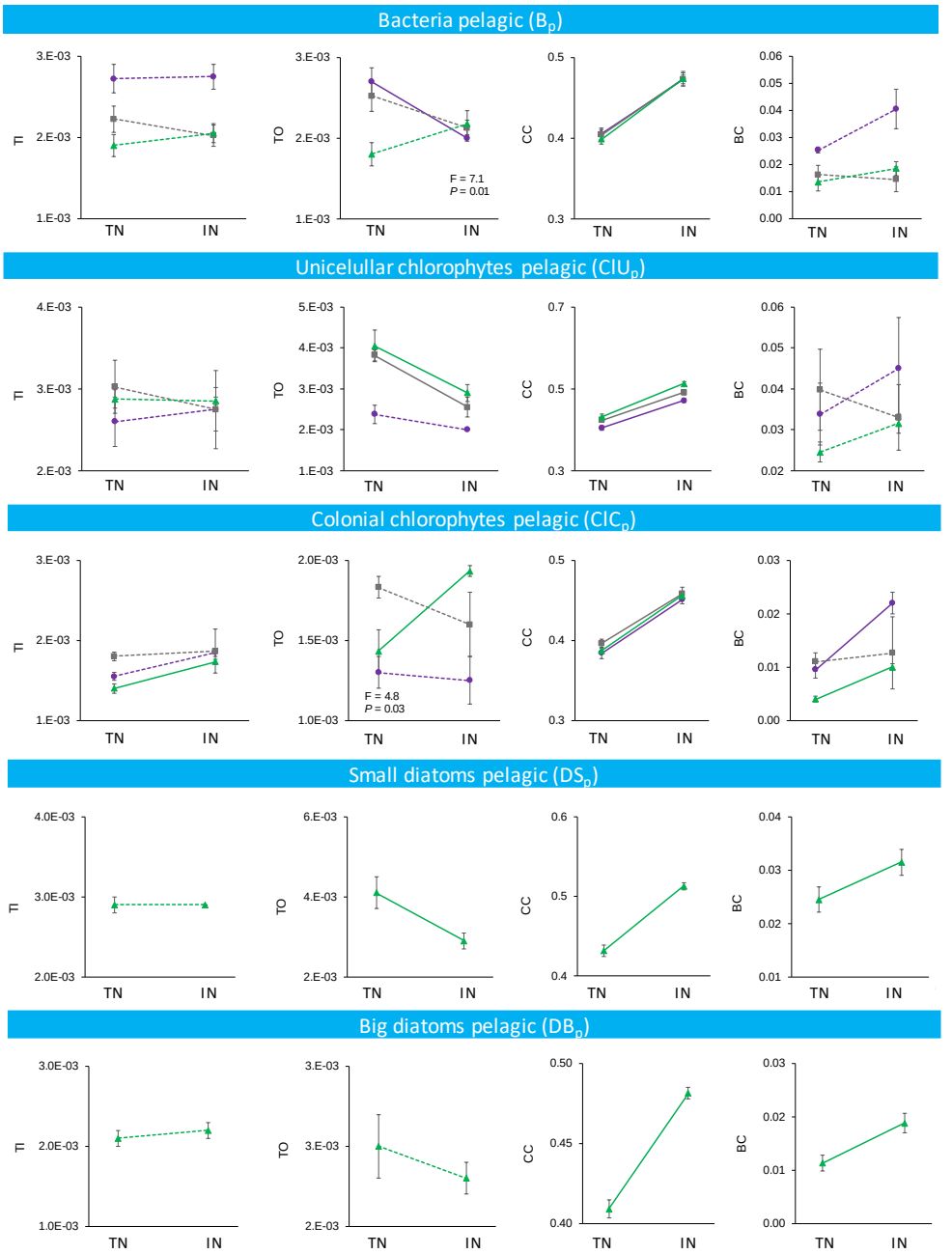


Fig. S1. Index values of nodes in the three environmental scenarios (TUVR: purple lines, TPAR: grey lines and +TPAR: green lines) for trophic networks (TN) and multi-interaction networks (IN). Abbreviations as in Tables 1 and 2. Values' variations between the two versions of the network were lineally fitted; a continuous line indicates significant differences ($P < 0.05$), whereas a dotted one shows that the adjustment is not significant. When F statistic and P appear in the graph, an interactive effect (type of network x environmental scenario) is significant. Bars show standard error. (Figure continues in next pages).

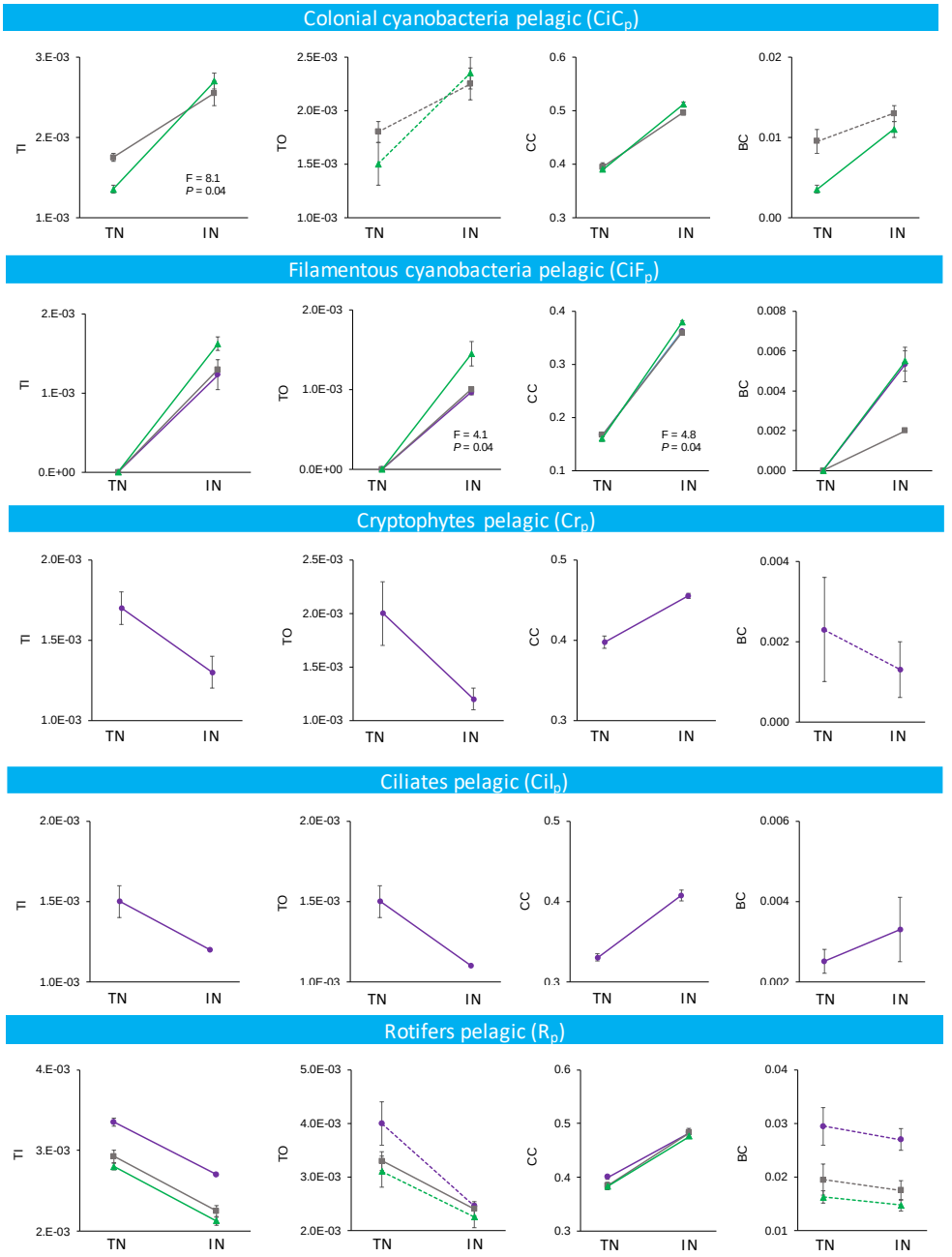


Fig. S1. continuation.

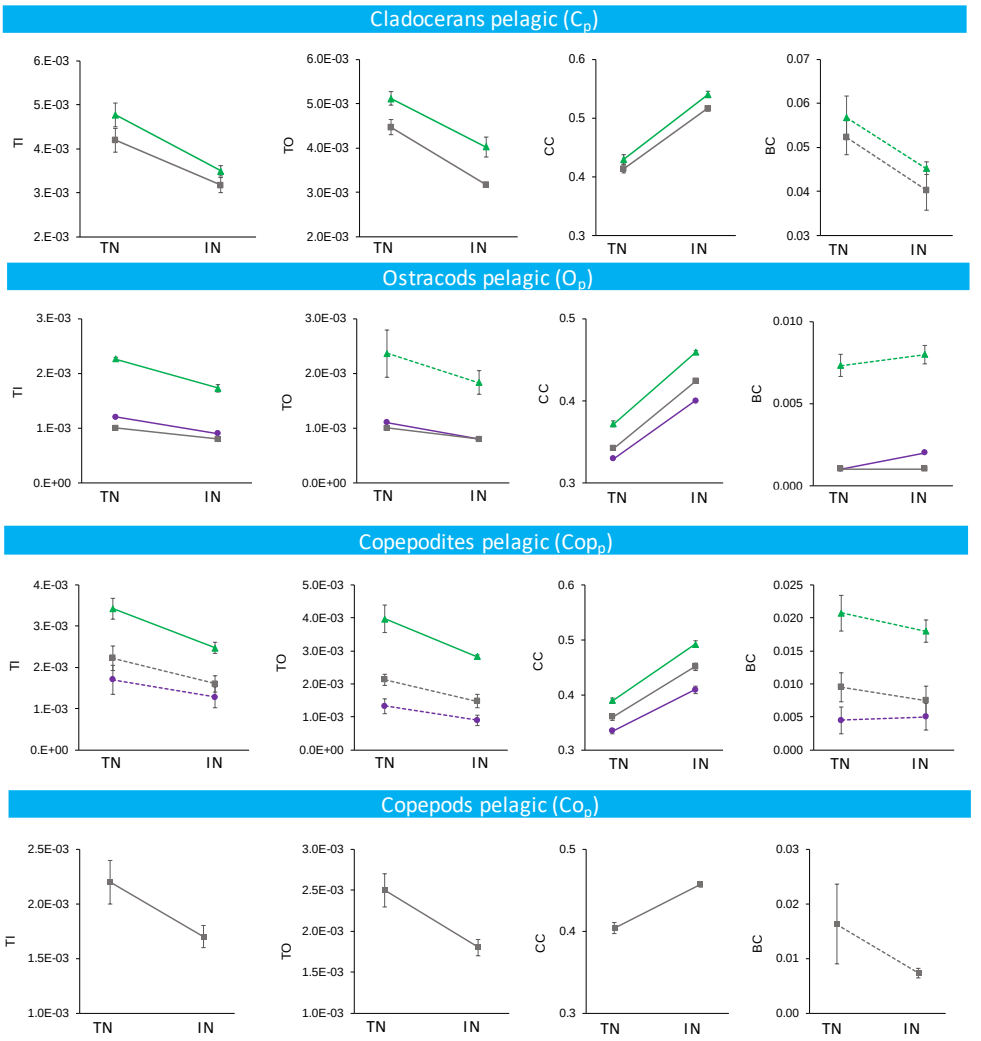
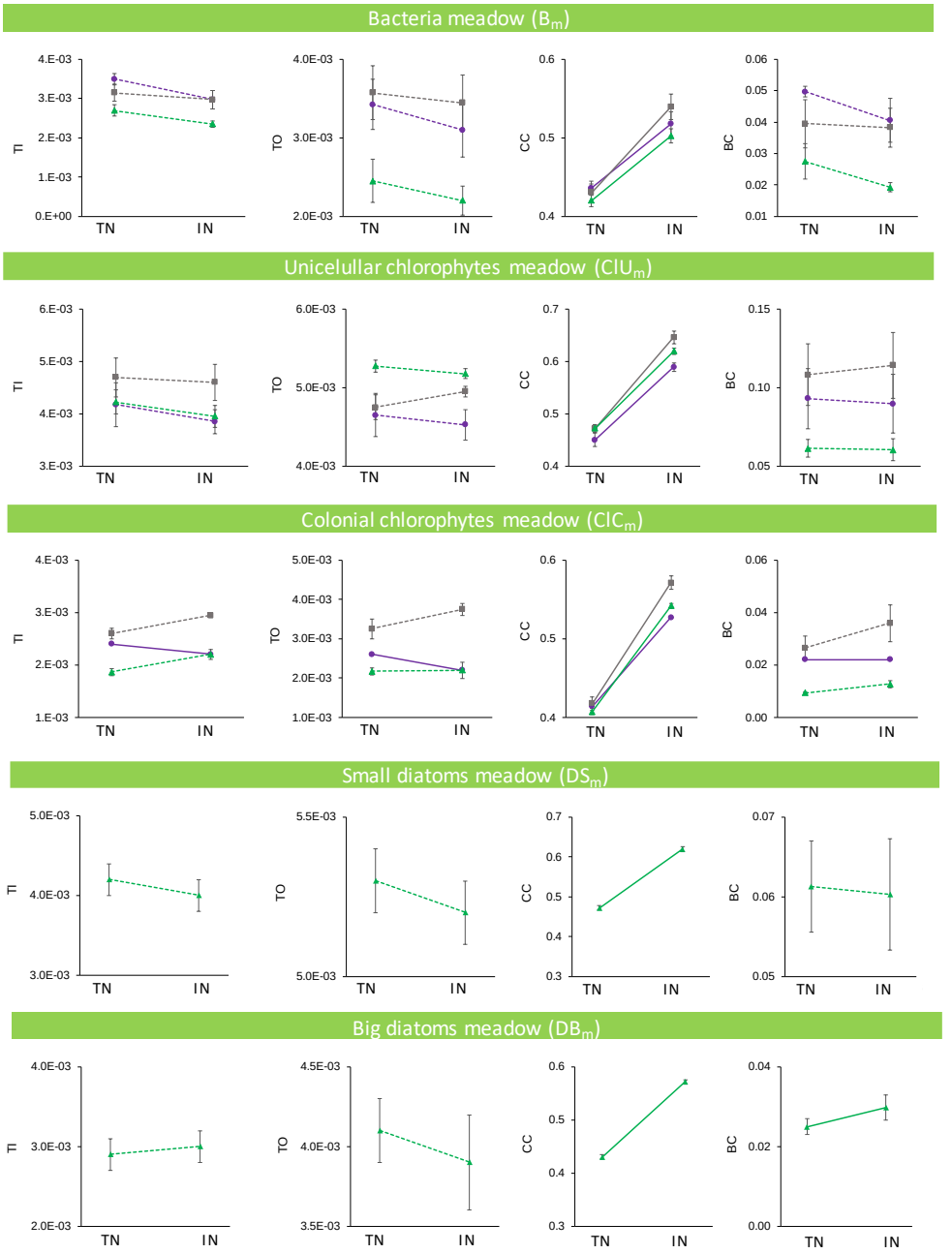


Fig. S1. continuation.



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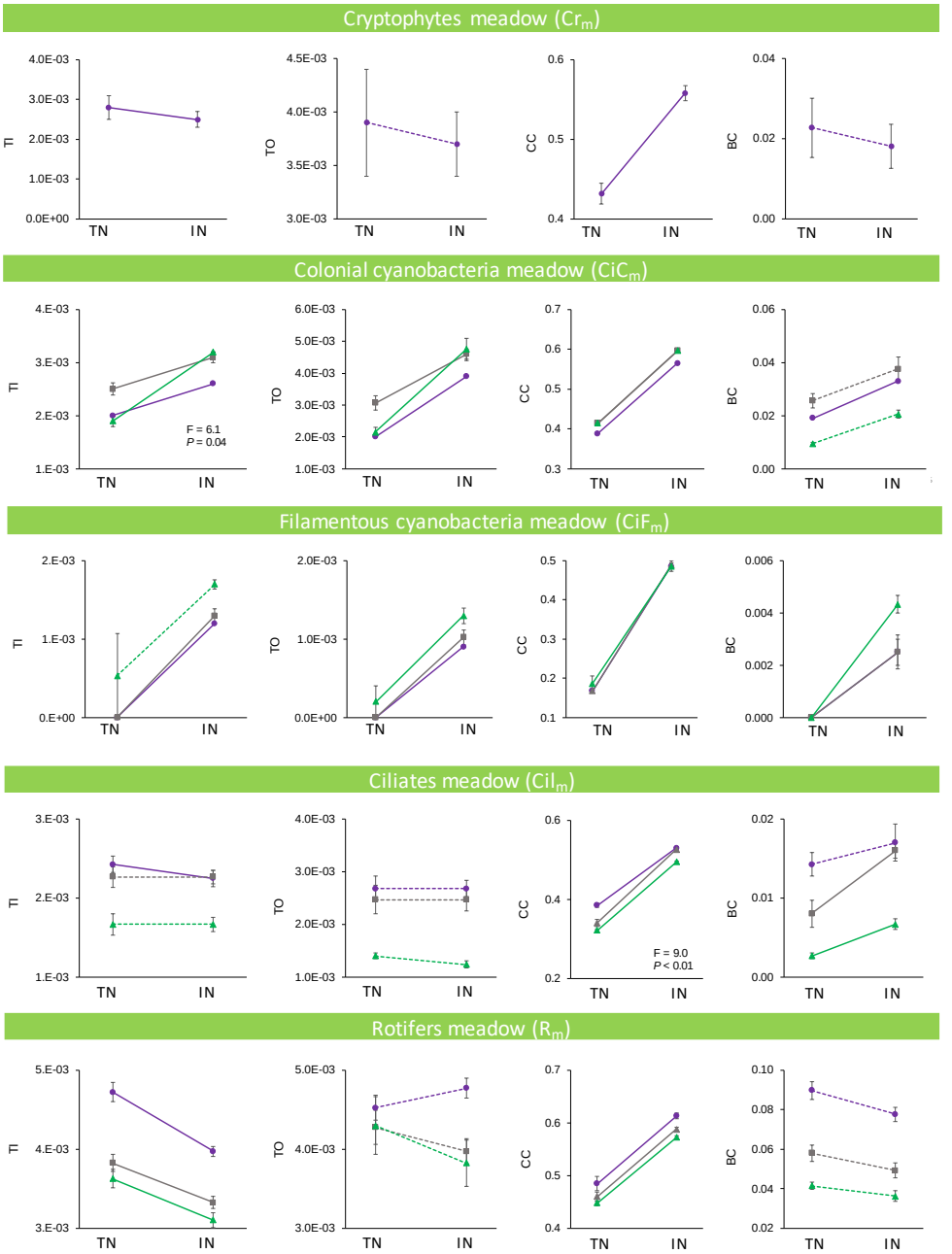
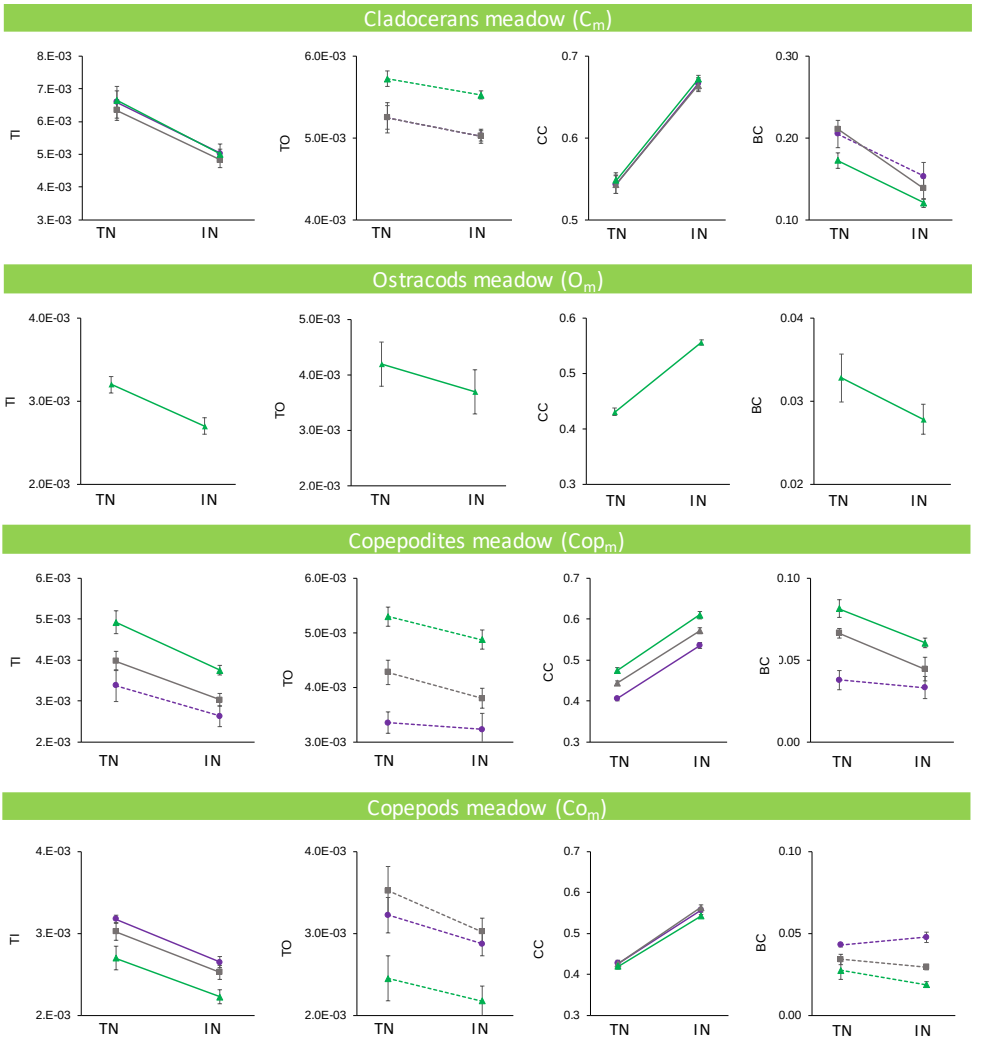


Fig. S1. continuation.



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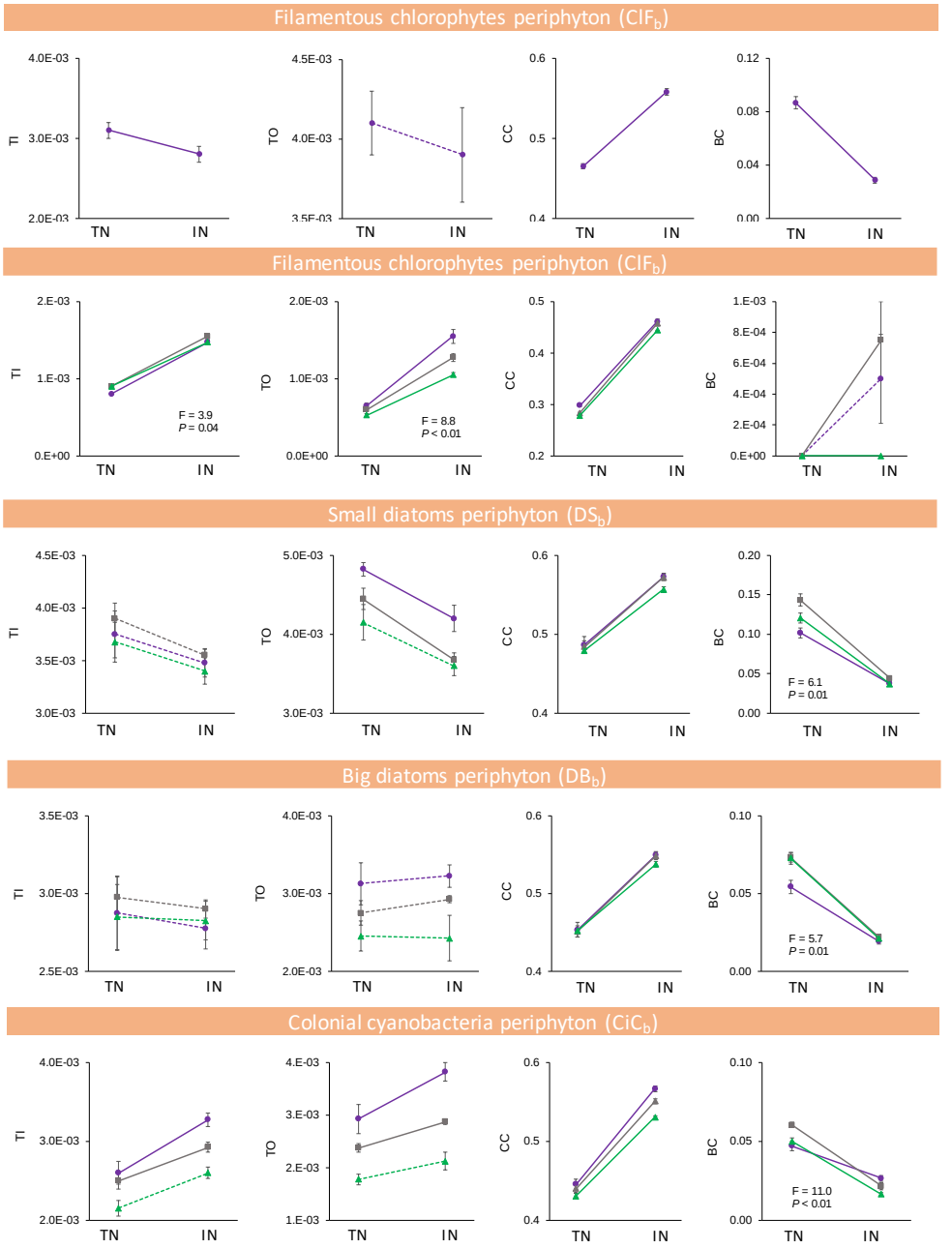

Fig. S1. continuation.

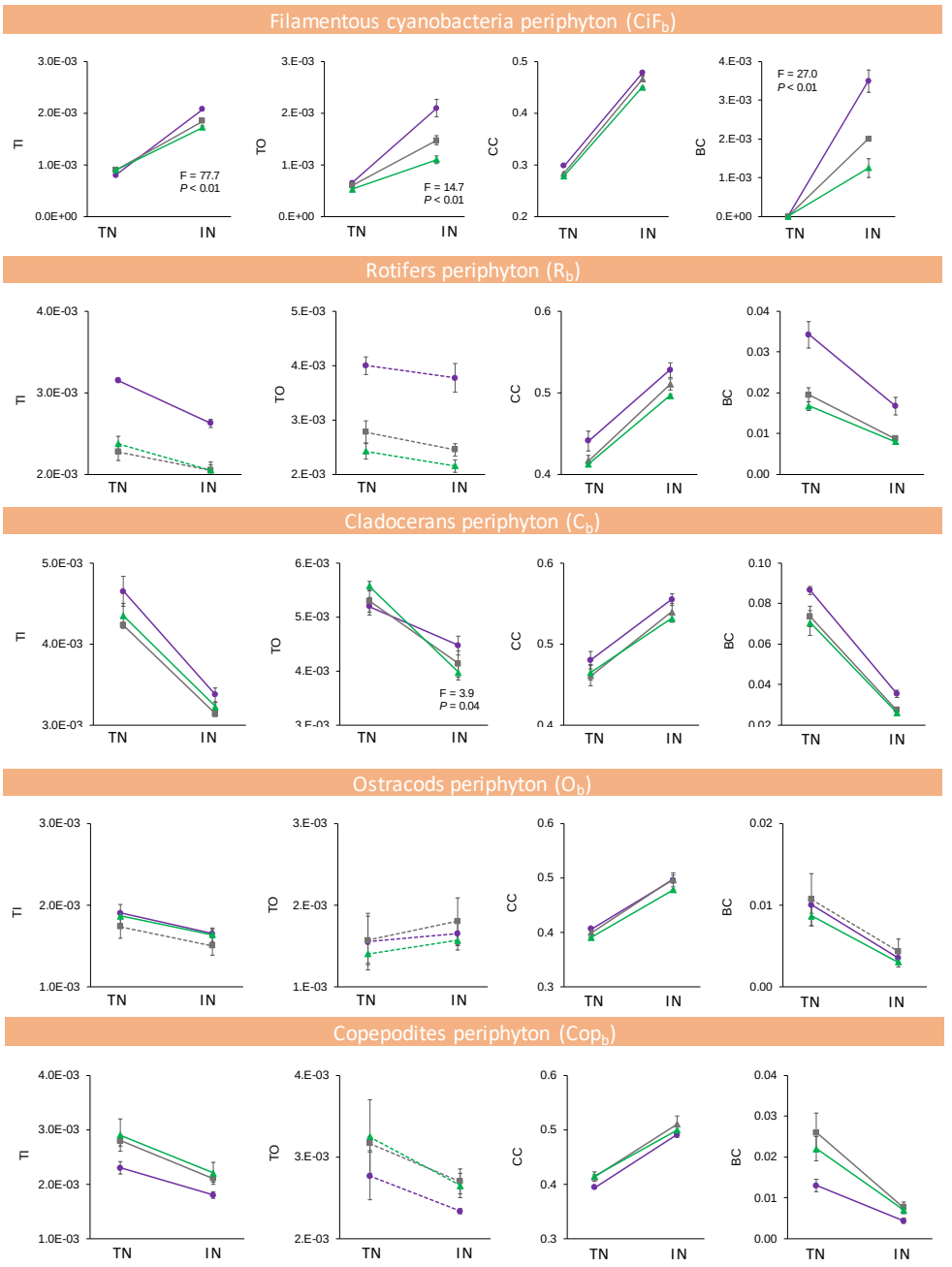

Fig. S1. continuation.

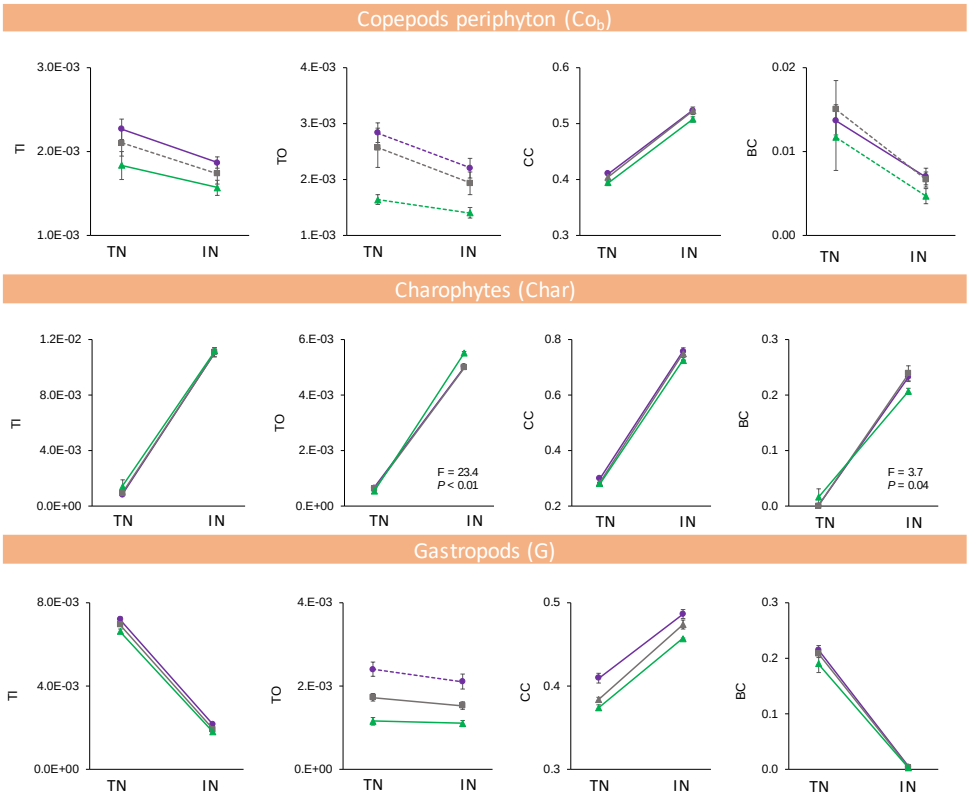

Fig. S1. continuation.

Chapter 7. Habitat coupling mediated by the multi-interaction network linked to macrophyte meadows: ponds *versus* lakes

Table S1. Taxonomical composition of the assemblages sampled in pelagic (-p), within-meadow (-m) and benthic habitats (-b) in the four studied ecosystems: PD, Pond Dossel; PNC, Pond Nova Canyar; LS, Lake Somolinos; LT, Lake Tinaja. The node to which each population belongs is indicated to its left. The abbreviations of nodes have a subscript (p, m or b) depending on the habitat the node belongs to. For node abbreviations see Table S2 Supplementary material Chapter 7.

	PD			PNC			LS			LT	
Node	Taxa	%	Node	Taxa	%	Node	Taxa	%	Node	Taxa	%
CiC_m	*Aphanothece stagnina*-m	0.24	CiC_m	*Aphanocapsa elachista*-m	0.43	ClC_p	*Quadrigula lacustris*-p	3.33	CiC_p	*Aphanocapsa elachista*-p	0.32
CiC_m	*Chroococcus aphanocapsoides*-m	0.47	CiC_m	*Aphanocapsa* sp.-m	0.18	ClU_p	*Eutetramorus* sp.-p	0.83	CiF_p	*Jaaginema minimum*-p	1.59
CiC_m	*Chroococcus obliteratus*-m	0.71	CiC_m	*Chroococcal* col. undertermined-m	0.07	ClU_p	*Monoraphidium komarkovae*-p	12.50	CiF_p	*Pseudanabaena biceps*-p	0.32
CiC_m	*Chroococcus* sp.-m	0.47	CiC_m	*Chroococcus minutus*-m	0.46	Mxs_p	*Cryptomonas rostratiformis*-p	2.50	CiF_p	*Pseudanabaena* sp.-p	0.32
CiC_m	*Coelomoron* sp.-m	1.42	CiC_m	*Chroococcus* sp.-m	0.09	Mxs_p	*Plagioselmis nannoplanctica*-p	77.08	ClC_p	*Chlorococcal* col. undetermined-p	0.32
CiC_m	*Microcystis* sp.-m	0.71	CiC_m	*Chroococcus turgidus*-m	0.07	Mxs_p	*Dinobryon divergens*-p	0.42	ClC_p	*Oocystis* sp.-p	0.95
CiF_m	*Aphanizomenon* sp.-m	0.24	CiC_m	*Johannesbaptistia pellucida*-m	0.09	DB_p	*Navicula* sp.-p	0.42	ClU_p	*Cosmarium tinctum*-p	0.63
CiF_m	*Cylindrospermum skujae*-m	0.71	CiC_m	*Microcystis* sp.-m	0.35	DS_p	*Cyclotella distinguenda*-p	0.83	Mxs_p	*Cryptomonas erosa*-p	0.63
CiF_m	*Jaaginema* sp.-m	0.94	CiC_m	*Snowella lacustris*-m	0.07	DS_p	*Cymbella* sp.-p	0.42	Mxs_p	*Cryptomonas marssonii*-p	0.32
CiF_m	*Komvophoron* sp.-m	0.71	CiC_m	*Synechocystis aquatilis*-m	1.55	DS_p	*Nitzschia acicularis*-p	1.67	Mxs_p	*Cryptomonas rostratiformis*-p	2.22
CiF_m	*Phormidium* sp.-m	0.71	CiF_m	*Cylindrospermum* sp.-m	0.71	ClU_m	*Chlamydomonas* sp.-m	0.51	Mxs_p	*Plagioselmis nannoplanctica*-p	2.86
CiF_m	*Pseudanabaena biceps*-m	0.94	CiF_m	*Geitlerinema acutissimum*-m	0.25	ClU_m	*Closteriopsis aciculare*-m	0.11	Mxs_p	*Dinobryon sertularia*-p	4.76
CiF_m	*Pseudanabaena catenata*-m	0.47	CiF_m	*Geitlerinema amphibium*-m	0.29	ClU_m	*Eutetremorus* sp.-m	0.26	DB_p	*Gomphonema angustum*-p	0.32
CiF_m	*Pseudanabaena* sp.-m	1.42	CiF_m	*Geitlerinema* sp.-m	3.10	ClU_m	*Monoraphidium komarkovae*-m	2.32	Mxb_p	*Ceratium hirundinella*-p	0.32
CiF_m	*Spirulina* sp.-m	0.24	CiF_m	*Glaucospira* sp.-m	0.07	Mxs_m	*Cryptomonas erosa*-m	0.13	Mxb_p	*Gymnodinium uberrimum*-p	12.06
ClC_m	*Botryococcus braunii*-m	1.18	CiF_m	*Limnothrix redekei*-m	1.43	Mxs_m	*Cryptomonas marssonii*-m	0.26	Mxb_p	*Gymnodinium wawrikae*-p	0.95
ClC_m	*Coelastrum astroideum*-m	0.24	CiF_m	*Limnothrix* sp.-m	3.22	Mxs_m	*Plagioselmis nannoplanctica*-m	81.61	Mxb_p	*Peridinium umbonatum*-p	16.19
ClC_m	*Granulocystopsis* sp.-m	0.47	CiF_m	*Planktolyngbya contorta*-m	1.64	Mxs_m	*Kephyrion* sp.-m	0.90	DS_p	*Cyclotella cyclopuncta*-p	3.49
ClC_m	*Oocystis solitaria*-m	2.83	CiF_m	*Planktolyngbya limnetica*-m	0.07	Mxs_m	*Mallomonas akrokomos*-m	0.39	DS_p	*Cyclotella distinguenda*-p	50.16
ClC_m	*Oocystis* sp.-m	1.89	CiF_m	*Planktothrix agardhii*-m	0.06	Mxs_m	*Mallomonas* sp.-m	0.13	DS_p	*Cymbella affinis*-p	0.32
ClC_m	*Scenedesmus aculeolatus*-m	2.36	CiF_m	*Pseudanabaena* cf. *skujae*-m	0.07	Mxb_m	*Gymnodinium* sp.-m	0.13	DS_p	*Encyonopsis cesatii*-p	0.32
ClC_m	*Scenedesmus disciformis*-m	0.24	CiF_m	*Pseudanabaena galeata*-m	1.14	Mxb_m	*Peridinium willei*-m	0.13	DS_p	*Navicula* sp.-p	0.63
ClU_m	*Euastrum insulare*-m	0.94	CiF_m	*Pseudanabaena minima*-m	0.43	DS_m	*Cyclotella distinguenda*-m	10.81	CiF_m	*Jaaginema minimum*-m	2.34

Table S1. continuation.

PD			PNC			LS			LT		
Node	Taxa	%	Node	Taxa	%	Node	Taxa	%	Node	Taxa	%
ClU_m	*Tetraedron minimum*-m	0.24	CiF_m	*Pseudanabaena papillaterminata*-m	0.36	DS_m	*Cymbella* sp.-m	0.13	CiF_m	*Pseudanabaena biceps*-m	0.26
ClU_m	*Tetraselmis* sp.-m	2.12	CiF_m	*Pseudanabaena* sp.-m	1.79	DS_m	*Nitzschia acicularis*-m	0.13	CiF_m	*Pseudanabaena* sp.-m	0.52
Mxs_m	*Cryptomonas erosa*-m	18.63	CiF_m	*Spirulina* sp.-m	0.36	DS_m	*Nitzschia* sp.-m	0.13	ClC_m	Chlorococcal undetermined-m	0.52
Mxs_m	*Cryptomonas marssonii*-m	4.72	ClC_m	*Ankistrodesmus fusiformis*-m	11.36	Mxt_m	*Chrysochromulina parva*-m	1.93	ClC_m	*Quadrigula lacustris*-m	0.26
Mxs_m	*Cryptomonas rostratiformis*-m	1.89	ClC_m	*Coenocystis* sp.-m	0.27	CiC_b	*Aphanocapsa elachista*-b	0.17	ClC_m	*Scenedesmus ecornis*-m	0.26
Mxs_m	*Cryptomonas* sp.-m	0.24	ClC_m	*Nephrocytium agardhianum*-m	0.37	CiC_b	*Aphanothece stagnina*-b	0.08	ClU_m	*Monoraphidium contortum*-m	0.26
Mxs_m	*Plagioselmis nannoplanctica*-m	9.67	ClC_m	*Oocystis solitaria*-m	19.91	CiC_b	*Chroococcus aphanocapsoides*-b	2.51	ClU_m	*Monoraphidium tortile*-m	0.26
Mxs_m	*Chromulina* sp.-m	11.79	ClC_m	*Scenedesmus aculeolatus*-m	1.68	CiC_b	*Chroococcus* sp.1-b	0.30	ClU_m	*Tetraselmis* sp.-m	0.52
Mxs_m	*Kephyrion* sp.-m	9.20	ClC_m	*Scenedesmus* cf. *grahneisii*-m	1.93	CiC_b	*Chroococcus* sp.2-b	0.21	Mxs_m	*Cryptomonas marssonii*-m	0.26
Mxs_m	*Pseudokephyrion pseudospirale*-m	6.37	ClC_m	*Scenedesmus obtusus*-m	0.16	CiC_b	*Rhabdoderma* sp.-b	0.08	Mxs_m	*Cryptomonas ovata*-m	0.78
DB_m	*Fragilaria biceps*-m	0.24	ClC_m	*Scenedesmus* sp.-m	0.14	CiC_b	*Ophiocytium* sp.-b	0.25	Mxs_m	*Cryptomonas rostratiformis*-m	2.60
DB_m	*Mastogloia braunii*-m	0.24	ClU_m	*Chlamydomonas* sp.-m	0.42	CiF_b	*Geitlerinema* sp.-b	0.13	Mxs_m	*Plagioselmis nannoplanctica*-m	1.04
DB_m	*Mastogloia smithii*-m	0.47	ClU_m	*Chlorella* sp.-m	0.61	CiF_b	*Leptolynbya* sp.-b	1.57	Mxs_m	*Dinobryon sertularia*-m	0.78
DB_m	*Rhopalodia gibba*-m	0.24	ClU_m	*Closterium aciculare*-m	1.86	CiF_b	*Limnothrix redekei*-b	0.04	Mxb_m	*Gymnodinium* sp.-m	0.52
Mxb_m	*Gymnodinium* sp.-m	1.42	ClU_m	*Closterium acutum*-m	1.62	MiF_b	*Mougeotia* sp.-b	0.08	Mxb_m	*Gymnodinium uberrimum*-m	27.08
Mxb_m	*Peridinium umbonatum* var. *goslaviense*-m	0.94	ClU_m	*Closterium dianae*-m	14.32	MiF_b	*Oedogonium* sp. 1-b	0.47	Mxb_m	*Gymnodinium wawrikae*-m	0.52
Mxb_m	*Peridinium umbonatum* var. *umbonatum*-m	5.19	ClU_m	*Cosmarium laeve*-m	0.14	MiF_b	*Oedogonium* sp. 2-b	0.64	Mxb_m	*Peridinium umbonatum*-m	11.98
DS_m	*Achnanthidium minutissimum*-m	0.24	ClU_m	*Didymocystis comasii*-m	0.27	ClU_b	*Closterium kuetzingii*-b	0.04	DS_m	*Cyclotella cyclopuncta*-m	6.51
DS_m	*Cyclotella meneghiniana*-m	0.71	ClU_m	*Didymocystis* sp.-m	0.25	ClU_b	*Cosmarium granatum*-b	0.13	DS_m	*Cyclotella distinguenda*-m	34.38
DS_m	*Encyonopsis microcephala*-m	2.83	ClU_m	*Euastrum* sp.-m	0.02	ClU_b	*Cosmarium* sp.-b	0.04	DS_m	*Cymbella affinis*-m	0.52
DS_m	*Navicymbulla pusilla*-m	2.12	ClU_m	*Franceia* sp.-m	0.07	ClU_b	*Staurodesmus* sp.-b	0.04	DS_m	*Cymbella cymbiformis*-m	0.26
CiC_b	*Aphanocapsa elachista*-b	1.06	ClU_m	*Monoraphidium circinale*-m	1.18	DB_b	*Fragilaria capucina*-b	2.12	DS_m	*Encyonopsis cesatii*-m	2.34
CiC_b	*Aphanothece stagnina*-b	0.31	ClU_m	*Monoraphidium contortum*-m	0.09	DB_b	*Fragilaria nanana*-b	1.36	DS_m	*Encyonopsis minuta*-m	0.52
CiC_b	Chroococcal undetermined-b	0.22	ClU_m	*Tetraedron minimum*-m	0.07	DB_b	*Fragilaria pinnata*-b	1.23	DS_m	*Navicula cryptotenella*-m	0.26
CiC_b	*Chroococcus aphanocapsoides*-b	4.02	DB_m	*Entomoneis alata*-m	0.18	DB_b	*Gomphonema acuminatum*-b	0.08	DS_m	*Navicula* sp.-m	0.78
CiC_b	*Chroococcus obliteratus*-b	4.99	DB_m	*Fragilaria* cf. *nanana*-m	0.14	DB_b	*Gomphonema affine*-b	1.78	DS_m	*Nitzschia* sp.1-m	2.86
CiC_b	*Chroococcus* sp.-b	0.57	DB_m	*Fragilaria dilatata*-m	0.11	DB_b	*Gomphonema angustum*-b	6.80	Mxt_m	*Chrysochromulina parva*-m	0.78
CiC_b	*Coelomoron* sp.-b	0.57	DB_m	*Fragilaria* sp.-m	0.49	DB_b	*Gomphonema cistula*-b	0.04	CiC_b	*Aphanocapsa clathrata*-b	0.45
CiC_b	*Merismopedia tenuissima*-b	0.13	DB_m	*Mastogloia smithii*-m	0.06	DB_b	*Gomphonema gracile*-b	0.51	CiC_b	*Aphanocapsa elachista*-b	0.40
CiC_b	*Microcystis* sp.-b	0.66	DB_m	*Navicula radiosa*-m	1.10	DB_b	*Gomphonema* sp.-b	0.21	CiC_b	*Aphanothece nidulans*-b	2.77
CiC_b	*Synechocystis aquatilis*-b	0.13	DB_m	*Navicula* sp. 1-m	0.67	DB_b	*Mastogloia braunii*-b	0.04	CiC_b	*Aphanothece stagnina*-b	1.11
CiF_b	*Aphanizomenon* sp.-b	0.13	DB_m	*Rhopalodia gibba*-m	0.03	DS_b	*Achnanthes flexella*-b	0.13	CiC_b	*Chroococcus aphanocapsoides*-b	0.60
CiF_b	*Cylindrospermum skujae*-b	17.40	DS_m	*Achnanthidium minutissimum*-m	1.81	DS_b	*Achnanthidium minutissimum*-b	8.50	CiC_b	*Chroococcus dispersus*-b	0.30
CiF_b	*Geitlerinema amphibium*-b	0.27	DS_m	*Cyclotella meneghiniana*-m	1.26	DS_b	*Brachysira neoexilis*-b	1.66	CiC_b	*Chroococcus minimus*-b	1.36
CiF_b	*Jaaginema* sp.-b	7.77	DS_m	*Cymbella cesatii*-m	0.14	DS_b	*Cocconeis* sp.-b	0.04	CiC_b	*Chroococcus obliteratus*-b	2.06
CiF_b	*Komvophoron* sp.-b	2.34	DS_m	*Cymbella minuta*-m	1.87	DS_b	*Cymbella affinis*-b	17.25	CiC_b	*Chroococcus* sp.-b	0.05

Table S1. continuation.

PD			PNC			LS			LT		
Node	Taxa	%	Node	Taxa	%	Node	Taxa	%	Node	Taxa	%
CiF_b	*Leptolynbya* sp.-b	0.80	DS_m	*Cymbella* sp.-m	0.21	DS_b	*Cymbella helvetica*-b	0.47	CiF_b	*Borzia* sp.-b	0.35
CiF_b	*Limnothrix redekei*-b	0.13	DS_m	*Nitzschia gracilis*-m	0.09	DS_b	*Cymbella pusilla*-b	3.48	CiF_b	*Cylindrospermum* sp.-b	0.05
CiF_b	*Oscillatoria curviceps*-b	1.50	DS_m	*Nitzschia microcephala*-m	0.40	DS_b	*Cymbella* sp.-b	1.10	CiF_b	*Jaaginema minimum*-b	22.71
CiF_b	*Phormidium* sp.-b	2.43	DS_m	*Nitzschia* sp.-m	0.46	DS_b	*Cymbella timidula*-b	1.83	CiF_b	*Komvophoron* sp.-b	0.55
CiF_b	*Planktolyngbya limnetica*-b	0.09	DS_m	*Sellaphora pupula*-m	0.02	DS_b	*Denticula tenuis*-b	0.68	CiF_b	*Leptolynbya* sp.-b	2.37
CiF_b	*Planktothrix* sp.-b	0.22	Mxb_m	*Peridinium umbonatum*-m	0.86	DS_b	*Encyonopsis subminuta*-b	15.85	CiF_b	*Limnothrix redekei*-b	1.01
CiF_b	*Pseudanabaena biceps*-b	0.53	Mxb_m	*Peridinium willei*-m	2.17	DS_b	*Epithemia argus*-b	0.13	CiF_b	*Oscillatoria* undetermined-b	1.06
CiF_b	*Pseudanabaena catenata*-b	3.36	Mxs_m	*Cryptomonas* sp.-m	0.35	DS_b	*Eunotia arcus*-b	0.42	CiF_b	*Phormidium okenii*-b	0.70
CiF_b	*Pseudanabaena* sp.-b	1.02	Mxs_m	*Cryptomonas erosa*-m	0.14	DS_b	*Navicula cryptotenella*-b	6.03	CiF_b	*Planktolyngbya limnetica*-b	0.81
CiF_b	*Spirulina* sp.-b	1.06	Mxs_m	*Cryptomonas marssonii*-m	0.46	DS_b	*Navicula radiosa*-b	0.13	CiF_b	*Pseudanabaena biceps*-b	0.55
MiF_b	*Mougeotia* sp.-b	1.72	Mxs_m	*Cryptomonas phaseolus*-m	0.35	DS_b	*Navicula* sp.-b	18.86	CiF_b	*Pseudanabaena galeata*-b	0.86
MiF_b	*Oedogonium* sp.-b	0.27	Mxs_m	*Cryptomonas rostratiformis*-m	11.37	DS_b	*Nitzschia* cf. *normannii*-b	1.87	CiF_b	*Pseudanabaena* sp.-b	1.36
ClU_b	*Closterium aciculare*-b	0.04	Mxs_m	*Ochromonas* sp.-m	0.14	DS_b	*Nitzschia linearis*-b	0.04	CiF_b	*Spirulina* sp.-b	0.25
ClU_b	*Cosmarium granatum*-b	0.27	CiC_b	*Aphanocapsa elachista*-b	2.06	DS_b	*Sellaphora pupula*-b	0.55	MiF_b	*Mougeotia* sp.-b	0.40
ClU_b	*Euastrum insulare*-b	0.97	CiC_b	*Aphanocapsa delicatissima*-b	1.72	CoC_p	Adult *Cyclops* cf. *abyssorum*-p	41.30	MiF_b	*Oedogonium* sp-b	0.35
DB_b	*Fragilaria biceps*-b	0.18	CiC_b	*Aphanothece stagnina*-b	3.65	Cop_p	Copepodite *Cyclops* cf. *abyssorum*-p	47.83	ClU_b	*Cosmarium granatum*-b	0.10
DB_b	*Fragilaria tenera*-b	0.04	CiC_b	*Chroococcus aphanocapsoides*-b	0.55	Nau_p	Nauplii *Cyclops* cf. *abyssorum*-p	2.17	ClU_b	*Cosmarium* sp.-b	0.10
DB_b	*Mastogloia braunii*-b	0.27	CiC_b	*Chroococcus minutus*-b	0.14	RH_p	*Keratella cochlearis*-p	4.35	ClU_b	*Cosmarium tinctum*-b	0.05
DB_b	*Mastogloia smithii*-b	7.51	CiC_b	*Chroococcus* sp.-b	0.00	RH_p	*Lepadella patella*-p	2.17	DB_b	*Amphora ovalis*-b	0.05
DB_b	*Navicula halophila*-b	0.18	CiC_b	*Johannesbaptistia pellucida*-b	2.19	RH_p	*Notholca acuminata*-p	2.17	DB_b	*Diploneis oblongella*-b	0.05
DB_b	*Pinnularia* sp.-b	0.04	CiC_b	*Microcystis* sp.-b	2.89	C_m	*Acroperus neglectus*-m	2.17	DB_b	*Diploneis ovalis*-b	0.05
DB_b	*Rhopalodia gibba*-b	4.15	CiC_b	Chroococcal col. undetermined-b	0.00	Cop_m	Copepodite *Cyclops* cf. *abyssorum*-m	2.17	DB_b	*Epithemia adnata*-b	0.15
DB_b	*Ulnaria ulna* var. *acus*-b	0.13	CiF_b	*Geitlerinema acutissimum*-b	0.00	Nau_m	Nauplii *Cyclops* cf. *abyssorum*-m	17.39	DB_b	*Eucocconeis flexella*-b	0.15
DS_b	*Achnanthidium minutissimum*-b	1.33	CiF_b	*Geitlerinema* sp.-b	2.92	O_m	Ostracod 1-m	6.52	DB_b	*Eunotia arcus*-b	1.31
DS_b	*Encyonopsis microcephala*-b	25.44	CiF_b	*Glaucospira* sp.-b	0.00	RH_m	Bdelloidea-m	13.04	DB_b	*Fragilaria tenera*-b	0.65
DS_b	*Navicymbulla pusilla*-b	4.86	CiF_b	*Leptolyngbya* sp.-b	6.94	RH_m	*Colurella adriatica*-m	6.52	DB_b	*Gomphonema angustum*-b	1.06
DS_b	*Nitzschia* sp.-b	0.62	CiF_b	*Limnothrix* sp.-b	8.22	RH_m	*Dicranophorus* sp.-m	2.17	DB_b	*Gomphonema helveticum*-b	1.61
DS_b	*Sellaphora pupula*-b	0.22	CiF_b	*Lyngbya* cf. *stagnina*-b	0.61	RH_m	*Euchlanis dilatata*-m	4.35	DB_b	*Hantzschia* sp.-b	0.05
MiF_b	*Tribonema* sp.-b	0.04	CiF_b	*Oscillatoria* sp.-b	0.00	RH_m	*Keratella cochlearis*-m	21.74	DB_b	*Mastogloia baltica*-b	0.35
C_m	*Ceriodaphnia*-m	0.67	CiF_b	*Pseudanabaena minima*-b	4.38	RH_m	*Lecane* cf. *hornemanni*-m	4.35	DB_b	*Mastogloia smithii*-b	2.77
CoC_m	Adult cyclopoid-m	5.99	CiF_b	*Pseudanabaena* sp.-b	0.71	RH_m	*Lecane luna*-m	8.70	DB_b	*Pinnularia microstauron*-b	0.05
Cop_m	Copepodite cyclopoid-m	12.99	CiF_b	*Spirulina* sp.-b	1.89	RH_m	*Lecane* sp.-m	2.17	DB_b	*Rhopalodia gibba*-b	0.20
Nau_m	Nauplii cyclopoid-m	75.44	ClU_b	*Closterium acutum*-b	0.51	RH_m	*Notholca acuminata*-m	4.35	DB_b	*Ulnaria ulna* var. *acus*-b	0.05
RC_m	*Asplanchna*-m	2.27	ClU_b	*Closterium dianae*-b	0.67	RH_m	*Platyias quadricornis*-m	2.17	DS_b	*Achnanthidium minutissimum*-b	0.55
RH_m	Bdelloidea-m	0.51	ClU_b	*Cosmarium* sp.-b	0.01	RH_m	*Trichotria pocillum*-m	2.17	DS_b	*Brachysira neoexilis*-b	0.30

Table S1. continuation.

	PD			PNC			LS			LT	
Node	Taxa	%	Node	Taxa	%	Node	Taxa	%	Node	Taxa	%
RH_m	*Lecane bulla*-m	0.24	ClU_b	*Cosmarium laeve*-b	0.00	C_b	*Acroperus neglectus*-b	0.08	DS_b	*Caloneis latiuscula*-b	0.25
RH_m	*Lecane luna*-m	1.28	ClU_b	*Euastrum lacustre*-b	0.29	C_b	*Alona* cf. *rectangula*-b	0.08	DS_b	*Cymbella affinis*-b	1.36
RH_m	*Lecane* sp. 1-m	0.24	DB_b	*Amphora ovalis*-b	0.00	C_b	*Alona quadrangularis*-b	0.93	DS_b	*Cymbella cymbiformis*-b	1.31
RH_m	Polyarthra sp.-m	0.37	DB_b	*Fragilaria* cf. *nanana*-b	0.43	C_b	*Chydorus sphaericus*-b	0.51	DS_b	*Cymbella helvetica*-b	9.97
RC_b	Asplachna sp.-b	0.61	DB_b	*Fragilaria* sp.-b	0.83	C_b	*Pleuroxus truncatus*-b	0.08	DS_b	*Cymbella timidula*-b	1.06
RH_b	Bdelloidea-b	40.34	DB_b	*Gomphonema clavatum*-b	0.00	O_b	Ostracod 1-b	8.78	DS_b	*Denticula kuetzingii*-b	0.55
RH_b	*Colurella* sp.-b	19.08	DB_b	*Mastogloia braunii*-b	0.00	O_b	Ostracod 2-b	16.64	DS_b	*Encyonopsis cesatii*-b	8.51
RH_b	*Lecane bulla*-b	3.40	DB_b	*Mastogloia smithii*-b	5.91	RH_b	Bdelloidea-b	30.74	DS_b	*Encyonopsis minuta*-b	12.64
RH_b	*Lecane luna*-b	10.45	DB_b	*Rhopalodia gibba*-b	1.04	RH_b	*Colurella adriatica*-b	0.51	DS_b	*Navicula cryptocephala*-b	0.30
RH_b	*Lecane* sp. 1-b	6.80	DS_b	*Achnanthidium minutissimum*-b	15.96	RH_b	*Dicranophorus* sp.-b	0.68	DS_b	*Navicula cryptotenella*-b	8.16
RH_b	*Lecane* sp. 2-b	0.61	DS_b	*Cocconeis* sp.-b	0.04	RH_b	*Lecane* cf. *hornemanni*-b	11.91	DS_b	*Navicula radiosa*-b	0.25
RH_b	*Lecane* sp. 3-b	3.52	DS_b	*Cymbella minuta*-b	3.47	RH_b	*Lecane closterocerca*-b	0.42	DS_b	*Navicula* sp.-b	1.36
RH_b	*Lecane* sp. 4-b	4.98	DS_b	*Cymbella* sp.-b	1.86	RH_b	*Lecane flexilis*-b	23.56	DS_b	*Nitzschia linearis*-b	0.70
RH_b	*Lecane* sp. 5-b	9.36	DS_b	*Navicula radiosa*-b	0.37	RH_b	*Lecane furcata*-b	0.42	DS_b	*Nitzschia* sp.1-b	0.30
RH_b	*Trichotria* sp.-b	0.85	DS_b	*Navicula* sp. 1-b	11.59	RH_b	*Lecane luna*-b	1.01	DS_b	*Nitzschia* sp.2-b	0.05
			DS_b	*Nitzschia microcephala*-b	10.83	RH_b	*Lecane lunaris*-b	0.17	DS_b	*Sellaphora pupula*-b	1.06
			DS_b	*Nitzschia* sp.-b	1.83	RH_b	*Lecane ungulata*-b	0.51	Cop_p	Copepodite cyclopoid-p	2.70
			DS_b	*Sellaphora pupula*-b	0.29	RH_b	*Lepadella patella*-b	0.42	Nau_p	Nauplii cyclopoid-p	27.03
			MiF_b	*Bulbochaete* sp.-b	0.13	RH_b	*Lepadella* sp.-b	0.08	RH_p	*Polyarthra* sp.-p	70.27
			MiF_b	*Mougeotia* sp. 1-b	1.07	RH_b	*Trichocerca* sp.-b	2.20	Nau_m	Nauplii cyclopoid-m	12.86
			MiF_b	*Mougeotia* sp. 2-b	0.04	RH_b	*Trichotria pocillum*-b	0.25	O_m	Ostracod-m	1.43
			MiF_b	*Oedogonium* sp. 1-b	3.51				RH_m	Bdelloidea-m	5.71
			MiF_b	*Oedogonium* sp. 2-b	0.46				RH_m	*Lecane luna*-m	1.43
			C_m	*Chydorus* sp. -m	34.26				RH_m	*Polyarthra* sp.-m	78.57
			CoC_m	Adult copepod cyclopoid-m	1.85				C_b	*Alona costata*-b	2.44
			RH_m	*Cephalodella* sp.-m	6.48				C_b	*Alonella excisa*-b	1.05
			RH_m	*Collotheca* sp.-m	38.89				Cop_b	Copepodite cyclopoid-b	0.17
			RH_m	*Colurella* sp.-m	10.19				Nau_b	Nauplii cyclopoid-b	3.66
			RH_m	*Notholca* sp.-m	3.70				O_b	Ostracod-b	2.27
			RH_m	*Testudinella* sp.-m	4.63				RH_b	Bdelloidea-b	24.43
			C_b	*Chydorus* sp.-b	3.73				RH_b	*Cephalodella* sp.-b	17.80
			CoH_b	Adult copepod harpacticoid-b	0.30				RH_b	*Collurella* sp.-b	7.85
			Cop_b	Copepodite harpacticoid-b	0.40				RH_b	*Hexarthra* sp.-b	0.35
			Nau_b	Nauplii harpacticoid-b	0.60				RH_b	*Lecane nana*-b	6.11
			O_b	Ostracod1.-b	0.20				RH_b	*Lecane* sp.2-b	1.57
			RH_b	Bdelloidea-b	62.38				RH_b	*Lecane* sp.3-b	2.79

Table S1. continuation.

	PD			PNC			LS			LT	
Node	Taxa	%	Node	Taxa	%	Node	Taxa	%	Node	Taxa	%
			RH_b	*Cephalodella* sp.-b	0.70				RH_b	*Lecane* sp.4-b	5.58
			RH_b	*Collotheca* sp.-b	4.23				RH_b	*Lecane* sp.5-b	3.66
			RH_b	*Colurella* sp.-b	1.11				RH_b	*Lecane bulla*-b	6.63
			RH_b	*Lecane* sp.2-b	25.84				RH_b	*Lecane hastata*-b	11.17
			RH_b	*Testudinella* sp.-b	0.50				RH_b	*Lecane luna*-b	2.27

Table S2. Description of nodes, their abbreviations and the ID. Nodes from the pelagic assemblage are indicated by the subscript p, from the within-meadow assemblage by subscript m and from the benthic assemblage by subscript b.

ID	Abbrev.	Node	ID	Abbrev.	Node
1	B_p	Bacteria-pelagic	24	Nau_m	Nauplii-within-meadow
2	ClU_p	Unicellular chlorophytes-pelagic	25	RC_m	Carnivores rotifers-within-meadow
3	ClC_p	Colonial chlorophytes-pelagic	26	RH_m	Herbivores rotifers-within-meadow
4	Mxs_p	Small mixotrophs-pelagic	27	C_m	Cladocerans-within-meadow
5	Mxb_p	Large mixotrophs-pelagic	28	Cop_m	Copepodites-within-meadow
6	DS_p	Small diatoms-pelagic	29	O_m	Ostracods-within-meadow
7	DB_p	Large diatoms-pelagic	30	CoC_m	Carnivores copepods-within-meadow
8	CiC_p	Colonial cyanobacteria-pelagic	31	B_b	Bacteria-benthic
9	CiF_p	Filamentous cyanobacteria-pelagic	32	ClU_b	Unicellular chlorophytes-benthic
10	Nau_p	Nauplii-pelagic	33	MiF_b	Filamentous microalgae-benthic
11	RH_p	Herbivores rotifers-pelagic	34	DS_b	Small diatoms-benthic
12	Cop_p	Copepodites-pelagic	35	DB_b	Large diatoms-benthic
13	CoC_p	Carnivores copepods-pelagic	36	CiC_b	Colonial cyanobacteria-benthic
14	B_m	Bacteria-within-meadow	37	CiF_b	Filamentous cyanobacteria-benthic
15	ClU_m	Unicellular chlorophytes-within-meadow	38	Nau_b	Nauplii-benthic
16	ClC_m	Colonial chlorophytes-within-meadow	39	RC_b	Carnivores rotifers-benthic
17	Mxs_m	Small mixotrophs-within-meadow	40	RH_b	Herbivores rotifers-benthic
18	Mxb_m	Large mixotrophs-within-meadow	41	C_b	Cladocerans-benthic
19	Mxt_m	Toxic mixotrophs-within-meadow	42	Cop_b	Copepodites-benthic
20	DS_m	Small diatoms-within-meadow	43	O_b	Ostracods-benthic
21	DB_m	Large diatoms-within-meadow	44	CoH_b	Herbivores copepods-benthic
22	CiC_m	Colonial cyanobacteria-within-meadow	45	Char	Charophytes
23	CiF_m	Filamentous cyanobacteria-within-meadow			

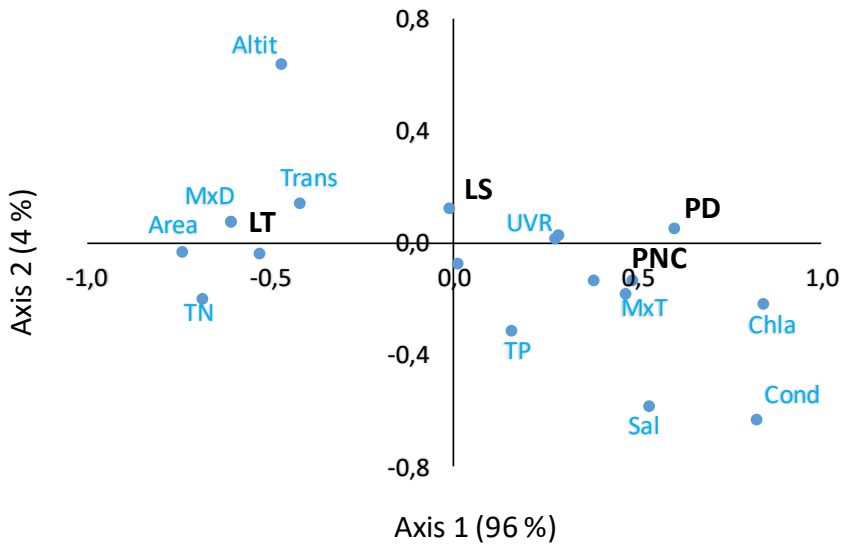

Fig. S1. Correspondence analysis of 15 abiotic variables (geographic position, morphometry, light conditions, physical and chemical water features and biotic ones) ordering the four Mediterranean aquatic ecosystems (two ponds and two lakes). Abbreviations as in Table 1. Explained variances are in brackets.

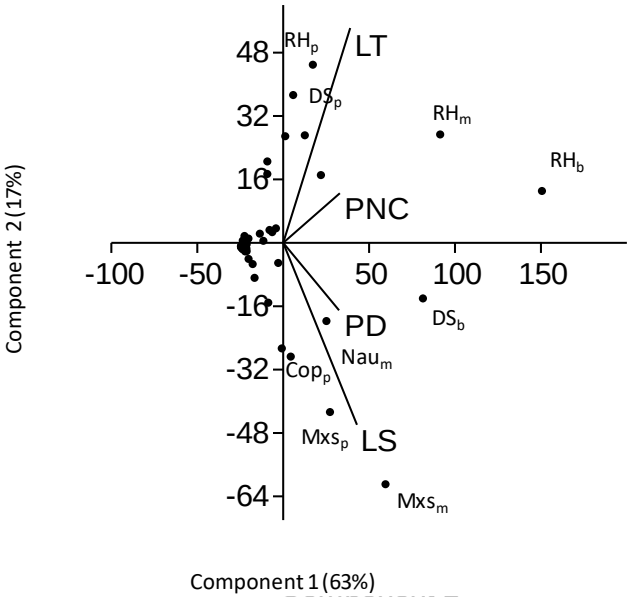

Fig. S2. Principal Component Analysis (PCA) of relative abundance of nodes (abreviations in Table S1 Supplementary material Chapter 7) of four multi-interaction networks; nodes are from different habitats in the ecosystems (pelagic: p; within-meadow: m and benthic: b). Communities were from four Mediterranean aquatic ecosystems (two ponds and two lakes in Spain; abreviations as in Table 1); they are shown in the biplot. Explained variances are in brackets.

Chapter 8. Macrophyte meadows mediate the response of the sediment microbial community to global change-related factors

1. Limnology of limnocorrals at the end of experiment

1.1. Pelagic environment (Table S1)

Some physical and chemical features were measured *in situ* in the water column of each limnocorral site with portable field equipment: a WTW Meter (WTW GmbH, Weilheim, Germany) for temperature, pH and conductivity. Water samples from each limnocorral were collected and transported to the laboratory to analyse total nitrogen (TN) and total phosphorus (TP). Underwater ultraviolet radiation (both UVA and UVB) and photosynthetically active radiation (PAR) doses were measured in each limnocorral with a JAZ system spectrometer (Ocean Optics, Inc.). Water samples from each limnocorral were preserved in 250 ml PVC bottles and fixed immediately with iodine-Lugol solution for phytoplankton classification and counting following the methodology in Rojo *et al.* 2012.

Table S1. Main physical, chemical and biotic variables measured at the end of experiment (July 2018). Light was measured at noon and 5 cm below of water surface. Mean of twelve mesocosm and standard deviation (Mean±SD) are shown.

Variable	Mean±SD
Water level (cm)	22± 0
Temperature (°C)	28.6 ± 0.6
Conductivity (mS cm^{-1})	2.7± 0.5
pH	7.3± 0.4
TN (mg N l^{-1})	2.3± 0.5
TP (mg P l^{-1})	0.20± 0.15
PAR in PAR treatment (Wats m^{-2})	160 ±51
PAR in PAB treatment (Wats m^{-2})	206±21
UVA in PAR treatments(Wats m^{-2})	9.8±1.6
UVA in PAB treatments(Wats m^{-2})	18.0±3.0
UVB in PAR treatments(Wats m^{-2})	0.4±0.1
UVB in PAB treatments(Wats m^{-2})	0.8±0.2
Total phytoplankton (mm^3 l^{-1})	1.9± 1.2

1.2. Chara hispida *development (Table S2)*

For detailed methods about cultivation of charophytes see Rojo *et al.* (2019).

Table S2. Results of one-way ANOVA calculated on *C. hispida* features after growing two months in the experimental limnocorrals with two light qualities (unfiltered or filtered UVR, named PAB and PAR respectively). SE=Standard error; FW=fresh weight; AUC=area under the curve.

	F	p		Mean	SE
Biomass (g FW)	0.031	0.872		35.7	12
UVACs (AUC mg^{-1} FW)	0.041	0.858		7.2	0.6
Chlorophyll a (µg g^{-1} FW)	132.7	**0.007**	PAB	503.4	8.8
			PAR	350.7	80.2
Chlorophyll b (µg g^{-1} FW)	3.04	9.223		222.6	69.2
Carotenoids (µg g^{-1} FW)	4.77	0.161		100.4	21.4

2. Detailed methods

2.1. Flow cytometry (Fig. S1)

The preparation of the samples for counting by flow cytometry was carried out from an adaptation of the dilution / fixation / staining protocol to analyse freshwater bacteria in lake sediments proposed by Duhamel and Jacquet (2006). First, 0.5 ml of sediment from the core was taken and transferred to a test tube where 3 ml of phosphate buffered saline solution was added (as the sediment was quite aqueous, it was pipetted easily from the collected sample). Then, for its fixation, 350 µl of a mixture of 10% formaldehyde + glutaraldehyde (PAGA) was added. Subsequently, for the separation of the biological particles from inert ones in the sediment, 1 ml of 0.01 M sodium pyrophosphate, 5 µl of 10% sodium dodecyl sulphate (SDS) and 4 ml of Milli-Q water were added to the sample. Next, the sample was placed in an Elmasonic S30H ultrasound bath for 3 minutes, interrupting this treatment every minute for 30 seconds of manual shaking. Finally, the sample was incubated on ice for 15 minutes, followed by one minute of manual shaking. After this, the sample was centrifuged in a Sorvall ST 16R centrifuge at 800 x*g* for a minute.

To eliminate any large particles in the sediment (ones greater than 5 µm give erroneous signals in the cytometer), the supernatant obtained after centrifugation was filtered through a 5 µm pore size membrane, and finally the sample was diluted at a 1:400 ratio with Milli-Q water. After processing and dilution of the sediment sample, an aliquot of 0.5 ml was extracted and stained with 20 µl of SYBR Green II and incubated in the dark for 30 min. SYBR Green II is a fluorescent marker that adheres to DNA, exciting at 497 nm (with a secondary excitation peak at 254 nm) and emitting fluorescence at 520 nm (corresponding to the green channel of the cytometer, FL1). Once the sample was stained, it was put into the cytometer (Cytomics FC 500 Beckman Coulter) and a high flow rate for 120 seconds was programmed, after checking that these were the right conditions for these samples (number of events between 700-1000 events s^{-1}). This process was repeated 3 times per sample, in three different sessions, to make the data

more reliable and check the cytometer error margin. These results were analysed with the specific program Flowing Software 2. From the raw results obtained with the cytometer, a dot plot was made with channels FL1 and FL4 in a log scale as axes x and y, respectively. Using these two channels, we can discriminate the bacteria from other particles (for example, those containing pigments such as chlorophylls), since bacteria stained with SYBR Green II have a maximum emission collected by channel FL1, and a minimum collected by FL4. From this graph, the region corresponding to the bacteria was delimited (Fig. S1).

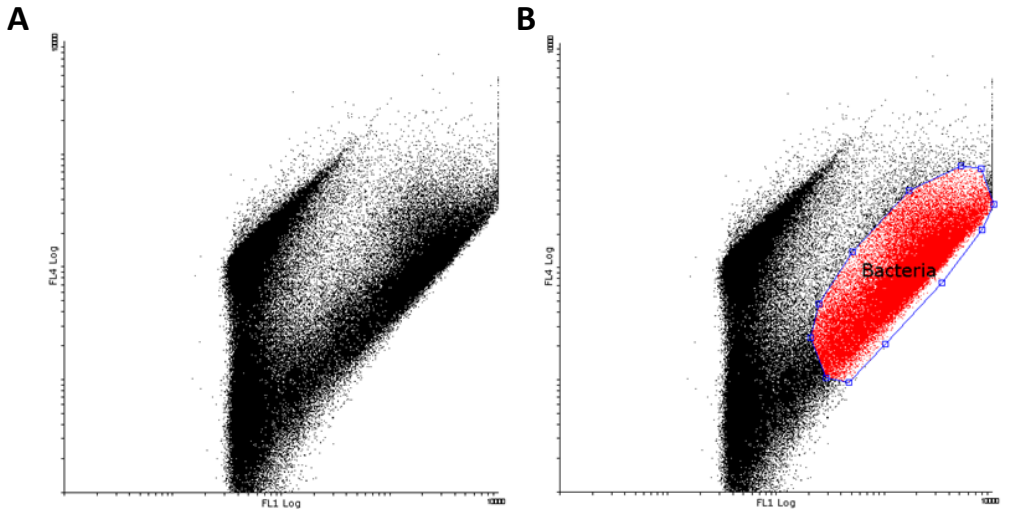

Fig. S1. A) Row results after sediment analysis by flow cytometry where the FL1 and FL4 axes represent channels that collected maximum and minimum emission the green fluorescence, respectively. The points represent the total detected particles. B) The same results where the region considered as bacteria is highlighted in red.

2.2. Preparation of cultures of charophyte and limnocorrals

After obtaining the charophytes from the field, they were quickly transported to the laboratory, less than half an hour from the site of origin. The charophytes were washed and then apical buds with several similar length nodes were selected, to be planted into small pots with a mixture of commercial sand and sediment in a 2:1 ratio. To ensure a good coverage of the mesocosms in the experiment, 96 pots were planted. These pots were immersed in containers with tap water (Rojo *et al.* 2015) in a culture chamber at a constant temperature (20°C), under artificial lighting provided by Sylvania Gro-Lux F58W tubes (photosynthetically active radiation (PAR), 100 micromoles of photons m^{-2} s^{-1}, light: darkness 13:11 h). These growing conditions do not limit the growth of the charophytes (Rodrigo *et al.* 2013). The pots were kept under these conditions for a month; this was to ensure uniform growth and the development of the structures (rhizoids) that anchor them to the sediment. These pots were transplanted to the six limnocorrals of CH treatments keeping the small portion of substrate that held the rhizoids to ensure their stability in the new site. In order to maintain the same conditions, the substrates

of the 16 stock pots without charophytes were added to the sediment in each of the six limnocorrals of the NCH treatment.

The limnocorrals consisted of four rigid plastic rods, each one being 85 cm in length, located as the sides of a quadrilateral measuring 50 cm on each side (2500 cm^2), which were buried in the substrate at a depth of 20 cm. A 50-cm wide plastic mesh with 1-cm pore openings was wrapped around these four rods, preventing the entry of fish and crayfish. The tops of the quadrilaterals were covered with plastic sheets designed to prevent birds from grazing on the macrophytes, and the light filtering properties either allowed the entire spectrum of sunlight to pass through, or eliminated most UVR according to the needs of experiment.

3. Results

3.1. Results of comparing means of diversity of bacteria communities. Data are density of each phylum (Table S3)

Table S3. Mann-Whitney test probability (Mann-W p) and two-way ANOVA (F and p) comparing diversity indicators for bacteria communities (richness of phyla, effective number of phyla –diversity- and evenness) between treatments. Same analysis is due to data from both superficial and from sub-superficial layers of sediment. 1 freedom degree for the two factors (filtered or unfiltered UVR and presence or not of charophytes) and their interaction, and 8 freedom degrees within groups. In bold significant values ($p<0.05$).

	Superficial layer			Sub-superficial layer		
	UVR	Charophyte	Interaction	UVR	Charophyte	Interaction
Richness						
Mann-W p	0.282	**0.025**		0.405	0.405	
Diversity						
F	0.900	0.300	0.100	0.022	0.263	0.783
p	0.366	0.610	0.743	0.886	0.622	0.402
Evenness						
F	0.001	2.198	2.043	1.462	1.887	0.561
p	0.979	0.177	0.191	0.261	0.207	0.475

Annex II Normative/ *Annex I Normativa*

This report summarizes the work performed by the doctorate student between October 2015 and September 2020 in the Cavanilles Institute of Biodiversity and Evolutionary Biology (University of València).

All the legal requirements to obtain the degree of Doctor in Biodiversity by the University of València are now presented in Catalan, one of the official languages of this university. Particularly, they refer to the requirements to conduct a PhD by publications and to obtain an International PhD. In this sense, the thesis is written in English and part of the research was performed abroad: (1) a three-moth stay in Chile under the supervision of Dr. Rodrigo Ramos-Jiliberto (*Universidad Mayor*) and (2) a three-month stay in Hungary under the supervision of Dr. Ferenc Jordán (Balaton Limnological Institute).

La present memòria resumeix el treball realitzat pel doctorand entre els mesos de octubre de 2015 i setembre de 2020 a l'Institut Cavanilles de Biodiversitat i Biologia Evolutiva de la Universitat de València sota el programa de doctorat en Biodiversitat i Biologia Evolutiva regulat pel Reial Decret 99/2011, de 28 de gener.

La normativa de la Universitat de València (Reglament sobre dipòsit, avaluació i defensa de la Tesi Doctoral aprovat al Consell de Govern del 28 de juny de 2016) acull la possibilitat de presentar la Tesi Doctoral com a compendi de publicacions. Per a això, els requisits a complir són:

1) *El doctorand ha de presentar un mínim de tres articles, ja publicats o acceptats en revistes indexades en algun índex internacional, com ara JCR (WoS) i/o SJR (Scopus) i ha de ser el primer signant de tots els treballs que presente.*
2) *La Tesi ha d'incloure un resum global de la temàtica (mínim de 4000 paraules), dels principals resultats i de les conclusions, que justifique l'aportació original de l'autor, redactat en qualsevol de les llengües oficials de la Universitat de València.*
3) *Com a annex, s'ha d'incloure una còpia completa dels treballs publicats o admesos per a la seua publicació, en què figure clarament la referencia completa de la revista.*
4) *Amb la sol·licitud de dipòsit, cal presentar un escrit de les directores de la Tesi sobre el factor d'impacte, o categorització de la revista, de les publicacions que es recullen en la Tesi doctoral. En cas que es presenten un o més treballs fets en coautoria, cal aportar un informe en què s'especifique exhaustivament quina ha sigut la participació del doctorand en cada article i, si és el cas, les circumstàncies justificatives que el doctorand no siga el primer signant d'alguns dels treballs.*

5) *Per poder optar a la menció internacional del títol de Doctor, almenys el resum i les conclusions de la Tesi han d'estar redactades i defensades en un idioma diferent de qualsevol de les llengües oficials a Espanya.*

*Per aquests motius, la Tesi està elaborada en anglès, encara que es pot trobar una versió reduïda i traduïda al català en la secció "Resum en extens". Els resums curts de cada article es presenten també en català a l'inici de cada capítol. Part de la investigació es va realitzar a l'estranger: 1) una estada de tres mesos a Xile sota la supervisió del Dr. Rodrigo Ramos-Jiliberto (*Universidad Mayor*) i 2) una estada de tres mesos a Hongria sota la supervisió del Dr. Ferenc Jordán* (Balaton Limnological Institute).

Annex III Author contribution to the papers

This PhD thesis is based on eight original papers, organized and presented in separated chapters. The doctoral student is integrated into a research group, therefore, the signature as the first author of the articles basically corresponds to the person in charge of preparing and writing the article while the rest of the co-authors have contributed to other necessary tasks such as sampling, data collection and statistical analysis essential for the achievement of scientific manuscripts. The doctoral student meets the requirements indicated in the regulations, being the first author of five of the articles that make up this thesis. The detailed contribution of the author of this thesis (shown underlined), as well as the rest of the authors of the articles compiled in it, was as follows:

Chapter 1 M.A. Rodrigo, E. Puche, C. Rojo. 2017. **On the tolerance of charophytes to high-nitrate concentrations**. *Chemistry and Ecology*, 34: 22-42. MAR and CR designed and planned the experiments. MAR and EP conducted the laboratory work and data gathering. MAR analysed the data and wrote the manuscript, finally reviewed by CR and EP.

Chapter 2 E. Puche, S. Sánchez-Carrillo, M. Álvarez-Cobelas, A. Pukacz, M.A. Rodrigo, C. Rojo. 2018. **Effects of overabundant nitrate and warmer temperature on charophytes: The roles of plasticity and local adaptation**. *Aquatic Botany*, 146: 15-22. MAR, CR and EP planned and designed the experiments. EP performed the laboratory work and data gathering. SSC and MAC conducted the stoichiometric analysis of charophyte and sediment samples. AP performed calcium carbonate analysis of charophytes samples. MAR, CR and EP analysed and interpreted the data. EP wrote the manuscript with the valuable comments and revisions of MAR and CR.

Chapter 3 C. Rojo, E. Puche, M.A. Rodrigo. 2019. **The antagonistic effect of UV radiation on warming or nitrate enrichment depends on ecotypes of freshwater macroalgae (charophytes)**. *Journal of Phycology*, 55: 714-729. All the authors (CR, EP and MAR) designed and planned the experiments. EP performed the laboratory work, analysis of samples and data gathering. All the authors analysed the data. CR and EP wrote the paper. All the authors reviewed the manuscript after each submission to different journals.

Chapter 4 E. Puche, C. Rojo, R. Ramos-Jiliberto, M.A. Rodrigo. 2020a. **Structure and vulnerability of the multi-interaction network in macrophyte-dominated lakes**. *Oikos*, 129:

35-48. RRJ designed the paper from an original idea devised by CR, MAR and EP. EP, MAR and CR performed the laboratory work in the mesocosms. EP analysed the samples and gathered the data. EP, RRJ and CR analysed the data. EP wrote the manuscript with the valuable revisions of RRJ, CR and MAR.

Chapter 5 E. Puche, C. Rojo, M.A. Rodrigo. 2020b. **Multi-interaction network performance under global change: a shallow ecosystem experimental simulation**. *Hydrobiologia*, 847: 3549-3569. All the authors (EP, CR and MAR) designed and planned the complex mesocosm experiment. All the authors conducted the laboratory work. EP performed the samples analysis and data gathering. All the authors analysed and interpreted the data. EP wrote the paper, that was finally reviewed by CR and MAR.

Chapter 6 E. Puche, F. Jordán, M.A. Rodrigo, C. Rojo. 2020c. **Non-trophic key players in aquatic ecosystems: a mesocosm experiment**. *Oikos*, DOI: 10.1111/oik.07476. EP and FJ designed the manuscript from an original idea of CR and MAR. EP gathered and analysed the data by means of the methodological basis provided by FJ. EP wrote a first draft of the manuscript that was reviewed by FJ, MAR and CR and partially rewritten by CR.

Chapter 7 E. Puche, M.A. Rodrigo, M. Segura, C. Rojo. **Habitat coupling mediated by the multi-interaction network linked to macrophyte meadows: ponds versus lakes**. Submitted to *Aquatic Sciences*. EP, MAR and CR designed the manuscript. EP, MAR and MS performed the field work. EP and MS analyse the samples and gathered the data. EP and CR analyses and interpreted the data. EP and CR wrote the manuscript. EP, CR and MAR contributed with valuable comments in the revision process.

Chapter 8 C. Rojo, M. Segura, E. Puche, M.A. Rodrigo. **Macrophyte meadows mediate the response of sediment microbial community to global change-related factors.** Ready for submission to *Biodiversity and Conservation*. CR, EP and MAR designed the manuscript. EP and MAR performed the field work. MS identified and counted the samples of microalgae and cyanobacteria. EP analysed the bacteria samples. CR, EP and MAR analysed and interpreted the data. CR and EP wrote the manuscript that was finally reviewed by EP and MAR.

In accordance with the normative, the first page of each of the published papers is provided below as a proof of publication as well as to show the affiliations of the co-authors:

Chapter 1. Rodrigo, M.A. *et al.* 2017. *Chemistry and ecology*.

CHEMISTRY AND ECOLOGY, 2018
VOL. 34, NO. 1, 22–42
https://doi.org/10.1080/02757540.2017.1398237

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RESEARCH ARTICLE

On the tolerance of charophytes to high-nitrate concentrations

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ABSTRACT

Currently a debate exists about whether the reduced growth of macrophytes with increased nitrogen loading in shallow ecosystems is determined by ecological or physiological factors. To discover whether nitrate in the water is detrimental *per se* to charophytes, we subjected *Chara hispida* and *Chara vulgaris* specimens, collected from two habitats greatly differing in nitrate concentrations (1.5 and 10 mg NO_3-N/L, annual means), to a wide nitrate range (0.5–50 mg NO_3-N/L) in two experiments (with free-floating specimens using nitrate as the sole N source, and with planted specimens, with other N sources in sediment). Charophytes grew both unplanted and planted in all treatments, and growth reductions occurred at the highest concentration in all cases. Some charophyte responses when faced with nitrate increases were different depending on (i) the species and (ii) population origin. Under the most realistic situation, the growth of both planted *C. vulgaris* populations was higher than that of *C. hispida* populations. *C. vulgaris* specimens from the nitrate-rich waterbody adapted best to the highest nitrate concentrations when they grew floating. Despite charophytes being vital and growing under high-nitrate concentrations in short-term laboratory experiments, such a situation in the environment may eventually not be sustainable, since ecological factors act in the field.

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Chara hispida; *Chara vulgaris*; nitrate pollution; NO_3 threshold; nitrate-reductase activity; Mediterranean region

1. Introduction

In the Mediterranean region, traditional intensive agriculture is established and an over-abundance of fertilizers, such as nitrate, in land and freshwater is enhanced [1]. Freshwater ecosystems in this climatic region are often shallow water bodies or small lakes, hence they are particularly sensitive to increases in nutrient concentrations [2,3]. Moreover, the current projections for climate change by the end of the century [4–6] for such a region will worsen this situation: the increase in temperature combined with a decrease in precipitation will lead to a higher rate of evaporation, thus reducing the depth of the water column and concentrating the water in nutrients (e.g. nitrate).

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Chapter 2. Puche, E. *et al.* 2018. *Aquatic botany*.

Aquatic Botany 146 (2018) 15–22

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Effects of overabundant nitrate and warmer temperatures on charophytes: The roles of plasticity and local adaptation

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ABSTRACT

Global change effects, such as warming and increases in nitrogen loading, alter vulnerable Mediterranean aquatic systems, and charophytes can be one of the most affected groups. We addressed the possible interaction between these factors on two populations of the cosmopolitan charophytes *Chara hispida* and *Chara vulgaris*. Populations were taken from two different environments, a nitrate-poor mountain lake and a nitrate-rich Mediterranean coastal spring. The laboratory experiment had a 2 × 2 factorial design based on two nitrate levels (similar to and double the local conditions) and two temperatures. Increased temperatures favoured the growth of the four populations, but an increase in nitrate did not have any effect on their growth or architecture. Both species took up and stored more nitrogen (measured as %N in plant tissue) when more nitrate was supplied, and warming favoured this increase in %N and, consequently, in N:P ratio. The effects of both factors depended on the local conditions where the populations originated and on the species. *Chara vulgaris*, a pioneer species, exhibited more phenotypic plasticity than *C. hispida*, and its ecotype from the coastal spring was better adapted to changes in temperature and nitrate level. These differential responses to warming conditions and nitrate pollution may modify charophyte diversity, which might be reflected in ecosystem performance, a matter of concern in vulnerable Mediterranean water bodies where these species co-occur.

1. Introduction

Global warming caused by current climate change and the increase in nitrogen input, with impacts on the biosphere, are currently well-documented processes (Lake et al., 2000). Their combination is especially noteworthy in the Mediterranean region (Moss et al., 2011), where the increase in temperature will promote higher evaporation rates, which, combined with a decrease in precipitation, will reduce the depth of the water column in freshwater bodies (IPCC, 2014). Such a decrease in water resources will be especially severe in this region, where intensive agriculture and the overabundant use of fertilisers, such as nitrate, have traditionally existed. The interactive effects of climate change and eutrophication in Mediterranean areas have been a matter of concern for a decade (Giorgi and Lionello, 2008; Jeppesen et al., 2011). Dramatic predictions have been made for Mediterranean countries, where freshwater ecosystems are often shallow water bodies or small lakes (Álvarez-Cobelas et al., 2006; Parcerisas et al., 2012).

Charophytes are a group of aquatic organisms that can be strongly affected by nitrate levels and increased temperatures. They play a structuring role in aquatic ecosystems since they directly and indirectly structure the planktonic and benthic food webs (Rojo et al., 2013, 2017a), and they act as nitrate sinks because the amount of nitrate they take up from the water column is higher than that released by decomposition (Kufel and Kufel, 2002; Rodrigo et al., 2007).

The effects of an increase in nitrate levels on charophytes are not fully understood. Some authors linked a reduction in macrophyte (including charophyte) richness to increases in nitrate concentrations of up to 2 mg $N\text{-}NO_3\ l^{-1}$ (Barker et al., 2008; Lambert and Davy, 2011). Yet, Kipriyanova and Romanov (2013), found charophyte species in aquatic systems in western Siberia with nitrogen concentrations much higher than this threshold. Others (Álvarez-Cobelas et al., 2006; Rodrigo and Alonso-Guillén, 2008) reported the healthy growth of *Chara hispida* and *C. vulgaris* in long-lived meadows in different lakes and ponds affected by the seepage of agricultural run-off in Spain, with nitrate concentrations much higher than 2 mg $N\text{-}NO_3\ l^{-1}$. Moreover, we have observed charophyte growth in nitrate threshold microcosm

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J. Phycol. **55,** 714–729 (2019)

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THE ANTAGONISTIC EFFECT OF UV RADIATION ON WARMING OR NITRATE ENRICHMENT DEPENDS ON ECOTYPES OF FRESHWATER MACROALGAE (CHAROPHYTES)[1]

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Increases in ultraviolet radiation (UVR), a negative global change factor, affect aquatic primary producers. This effect is expected to be modulated by other global change factors, and to be different for populations adapted to different environments. A common garden experimental approach using freshwater green macroalgae, the cosmopolitan charophyte species *Chara hispida* and *C. vulgaris*, allowed us to test whether the beneficial increases in water temperature (T) and nitrate concentration (N) mitigate negative UVR effects. Also, whether these interactions would be not only species–specific but also according to the origin of the population; therefore, two populations of each species were used: one from a coastal wetland and the other from a mountain lake. Two factorial-design experiments were performed: (i) the presence and absence of UVR × lower and higher T × four populations, and (ii) the presence and absence of UVR × lower and higher N × four populations. Response variables were: growth, morphometry, UVR-protective compounds, photosynthetic pigments, and stoichiometric composition. There were consistent response patterns in the key variables that represent different organization levels. Our main results showed that both warming and, to a lesser extent, the increase in nutrients ameliorated the negative effects of UVR on the molecular processes involved in acclimation to UVR, and that such a mitigating effect depended on the different phenotypic plasticity of each species and each ecotype. The coastal populations, being from a more variable environment, were more resilient than the mountain populations, mainly because of changes in growth and morphology.

Key index words: **Charophyceae; common garden; global change; local adaptation; Mediterranean region; photoprotection; plasticity**

***Abbreviations*: CHQ, *Chara hispida* from Quartons Spring; CHS, *Chara hispida* from Somolinos Lake; C, total carbon content; CVQ, *Chara vulgaris* from Quartons Spring; CVS, *Chara vulgaris* from Somolinos Lake; DW/LMA, dry weight per unit of length of the main axis; HN, high nitrate concentration; HT, high temperature; LMA/Nod, length of the main axis per node; LMA, length of the main axis; LMAV, variation of the length of the main axis; LN, low nitrate concentration; LT, low temperature; Nod, number of nodes; PAB, photosynthetically active radiation + ultraviolet A radiation + ultraviolet B radiation; RGR, relative growth rate; SUVACs, methanol-soluble ultraviolet radiation absorbing compounds; T, temperature; UVACs, total ultraviolet radiation absorbing compounds; UVAR, ultraviolet A radiation; UVBR, ultraviolet B radiation; UVR, ultraviolet radiation; WUVACs, methanol-insoluble ultraviolet radiation absorbing compounds**

Charophytes (green macroalgae from the Family Characeae, Order Charales, Class Charophyceae, Division Chlorophyta) are benthic primary producers of key relevance in aquatic habitats all over the world (Blindow et al. 2014), and have proven to be highly vulnerable to changes in their environment (i.e., Auderset Joye and Rey-Boissezon 2015, Rojo et al. 2015, Puche et al. 2018). For this reason, they are a key group to predict the effects of global change on the function and structure of freshwater ecosystems (Rodrigo et al. 2010, Pełechata et al. 2015).

Environmental factors, considered drivers of global change, such as eutrophication, drought, increased ultraviolet radiation (UVR), or global warming (IPCC 2014, Williamson et al. 2014, EEA 2015), are receiving increasing attention because they interactively affect the biodiversity and the functioning of aquatic ecosystems (Sala et al. 2000, Jackson et al. 2016). A well-described example of these related factors is the concomitant effect of warm temperatures and low precipitation in the Mediterranean region where freshwater ecosystems are especially vulnerable as they are often shallow water bodies or small lakes (Álvarez-Cobelas et al. 2005, Parcerisas et al. 2012). In this climatic region, it is expected that the average temperature will increase by 4–5°C, due to sudden warm days (Christensen et al. 2007, Giorgi and Lionello 2008) accompanied by a decrease in precipitation by the end of the century (IPCC 2014). Moreover, detailed analyses

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Chapter 4. Puche, E. *et al.* 2020a. *Oikos*.

OIKOS

Research

Structure and vulnerability of the multi-interaction network in macrophyte-dominated lakes

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The network approach is crucial to understand how ecosystems are structured and how they will respond to the disturbances (e.g. the current global change). We have recreated the multi-interaction network of a shallow freshwater lake dominated by submerged macrophytes (Charophytes), a known system very vulnerable to environmental changes, considering both trophic and non-trophic relationships among its elements. To minimize the environmental variability, we established it in an experimental mesocosm, including three habitats: the pelagic, the habitat around the meadow and the periphytic community living on macrophytes. We aimed to study the structure of this network and the roles of its elements, as well as the response of this system to a foreseeable decrease in charophytes due to the global change. Thus, we tested whether there are species in the system that, due to the connections they establish, have central or connecting roles and if the reduction of charophytes affects more the elements that live intimately associated with them. Our results confirm that charophytes are the most central node in the network and that the high-mobility large planktonic herbivores living within the meadow are acting as bridges between the conformant compartments. This suggests a structurally crucial tandem macrophytes-herbivores with the former playing a foundation role (i.e. basal and abundant species centralizing non-trophic interactions) and the latter being connectors in this network. Interestingly, we found that the periphytic elements where those with the highest capacity to affect the other elements of the network when being disturbed. Furthermore, an eventual decrease in the abundance of charophytes will cause a major direct damage to the meadow and periphyton, compartments to which they provide refuge and life support, respectively. Our study highlights the need of approaches encompassing the complex structure of the ecological networks to identify crucial species (such as foundation or connecting species) for their topology and vulnerability geared towards conservation biology.

Keywords: aquatic network, charophyte meadows, foundation species, non-trophic interactions, periphyton, plankton, topology

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Chapter 5. Puche, E. *et al.* 2020b. *Hydrobiologia*.

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PRIMARY RESEARCH PAPER

Multi-interaction network performance under global change: a shallow ecosystem experimental simulation

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Abstract Shallow freshwater ecosystems are structurally complex with different, highly-coupled habitats: the pelagic, the within-macrophyte-meadow, and the benthic. Submerged macrophyte meadows support benthic microorganisms and provide the trophic network with non-trophic relationships. Multi-interaction network analysis disentangles how these systems respond to changes in global-change-related factors. We examined whether (i) populations' responses to such disturbances are habitat-dependent, and (ii) if whole-community configurations are different. We performed an indoor-mesocosm experiment ("control" plus two disturbed scenarios: enhanced ultraviolet radiation (UVR) or temperature), recreating shallow freshwater ecosystems. We assessed the population-nodes' carbon biomass, their resistance and resilience to the disturbances, and global- and node-scale structural parameters of the multi-interaction network. Under the UVR-scenario, the phytoplankton C-biomass (from pelagic and within-meadow habitats) was significantly the highest, with mixotrophs dominating. Warming favoured macrophyte growth and significantly increased the network's size and nestedness, with zooplanktonic herbivores playing a connector role. The within-meadow and benthic habitats' nodes were highly influential for the network, whatever the scenario. The benthic nodes were the most resistant to the disturbances. Therefore, a phytoplankton- and a macrophyte-dominated configuration was attained under UVR and warming scenarios, respectively. The macrophyte meadows, and the community linked to them, were pivotal in the achievement of these contrasting configurations.

Keywords Food web · Non-trophic interactions · Charophytes · Plankton · Benthos

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Introduction

Current global change (GC hereafter) alters the structure of ecosystems around the world by differentially affecting their elements (Steffen et al., 2004)

Chapter 6. Puche, E. *et al.* 2020c. *Oikos*.

OIKOS

Research

Non-trophic key players in aquatic ecosystems: a mesocosm experiment

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The trophic network (TN) has been well stablished, and recently knowledge concerning non-trophic relationships (NTRs) is receiving increasing attention. Although NTRs can influence trophic ones, network models, including both types of interactions (multi-interaction network, IN) and changes in the role of nodes when NTRs are added to TN, are scarce. To evaluate the role of NTRs in freshwater shallow ecosystems, where these interactions are relevant mainly in the benthic habitat, we constructed, from the same communities, the two mentioned networks and compared them focusing on the nodes' topological roles. Our approach is based on empirical data from a mesocosm experiment where aquatic communities inhabited coupled habitats (pelagic, within-meadow and benthic) under three environmental scenarios: warming, increased ultraviolet radiation, plus control conditions. The experiment allowed us to assess: the topological roles of the nodes from different habitats when NTRs were added to the TN, and the relative impact of adding NTRs according to environmental scenarios. We calculated a set of node indices by considering both direct and indirect connections up to an ecologically meaningful number of steps. Our results highlight significant differences in the nodes' roles between both network versions. When NTRs were added: i) pelagic nodes lost relevance in the network; ii) the number of within-meadow relevant nodes increased and iii) the large benthic consumers in TN were substituted by charophytes, plus a chain of small within-meadow predators/preys, as the most relevant to the IN. Furthermore, the scenarios modulated changes in the nodes' roles when including NTRs. The warming scenario promotes the central position of some nodes (e.g. charophytes) and harms others (e.g. benthic cladocerans), and UVR modulates changes in benthic filamentous primary producers' roles. Therefore, the inclusion of NTRs in ecological models seems crucial to better understand the functioning of complex communities and their response to environmental disturbances.

Keywords: centrality, food web, global change, mesoscale indices, multi-interaction network, non-trophic effects

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Annex IV Dissemination of the results

Publications on a science popularisation journal

E. Puche, M.A. Rodrigo, C. Rojo. **2020**. La vegetació submergida: clau de volta per als ecosistemes aquàtics de l'Albufera de València. *L'amfibi*. Awaiting publication.

Communications in international conferences

Oral communications

E. Puche, M.A. Rodrigo, M.B. Carramiñana, I. Parra, C. Rojo. **2016**. Charophytes and climate change: foreseeable responses to several stressors. XVIII Congress of the Iberian Association of Limnology. Tortosa, Spain.

M.A. Rodrigo, E. Puche, A. Pukacz, C. Rojo. **2016**. The experimental approach to study the effects of climate change stressors on charophytes. *7th International Symposium on Extant and Fossil Charophytes (IRGC)*. Astana, Kazakhstan.

E. Puche, M.A. Rodrigo, F. Rubio, C. Rojo. **2017**. Exploring global change stressors on charophytes: does UV-radiation interact with increased nitrate concentration and temperature in affecting charophyte responses? *10th Symposium for European Freshwater Sciences*. Olomouc, Czech Republic.

E. Puche, M.A. Rodrigo, I. Olivares, A. Camarena, C. Rojo. **2017**. Does UV-radiation interact with increased nitrate concentration and temperatura in affecting charophyte responses? *21st Meeting of the Group of European Charophytologists (GEC)*. València, Spain.

M.A. Rodrigo, E. Puche, C. Pérez-García, M. Álvarez-Cobelas, S. Sánchez-Carrillo, C. Rojo. **2017**. Charophytes as influencers of the periphytic-planktonic food web under different environmental scenarios: a mesocosm experimental approach. *21st Meeting of the Group of European Charophytologists (GEC)*. València, Spain.

E. Puche, A. González, R. Martínez, N. Martínez, S. Sánchez-Carrillo, M. Álvarez-Cobelas, M.A. Rodrigo, C. Rojo. **2018**. Modulation of the horizontal interaction web mediated by the response of charophytes to global change stressors: a mesocosm experiment. *XIX Conference of the Iberian Association of Limnology*. Coimbra, Portugal.

E. Puche, N. Martínez, R. Martínez, A. González, Y. Picó, C. Rojo, M.A. Rodrigo. **2018**. Charophyte performance under different environmental scenarios: final outcome from a mesocosm experiment. *22nd Meeting of the Group of European Charophytologists (GEC)*. Palermo, Italy.

E. Puche, C. Rojo, M.A. Rodrigo, E. Sánchez, S. Sánchez-Carrillo, M. Álvarez-Cobelas. **2019**. The sediment of semi-arid wetlands responds to the presence of macrophyte meadows and global change factors. *1st Iberian Ecological Society Meeting*. Barcelona, Spain.

E. Puche, M.A. Rodrigo, C. Rojo. **2019**. Show me who your Friends are… and I'll tell you how you are! Sensitive multi-interaction network of charophytes meadows. *6th Fresh Blood for Freshwater*. Tihany, Hungary. *Award for the Best oral communication in Experimental Ecology*.

E. Puche, M.A. Rodrigo, C. Rojo. **2020**. Charophytes: key players in shallow freshwater ecosystems under global change. *International Workshops in Environment, Universidad Internacional de Andalucía: Temporary wetlands future in drylands under the projected global change scenario*. Baeza, Spain.

E. Puche, M.A. Rodrigo, C. Rojo. **2020**. Charophytes: freshwater key players under global change. 2020. *XX Congress of the Iberian Association of Limnology (AIL)* and *III Iberoamerican Congress of Limnology (CIL)*. Murcia, Spain.

M.A. Rodrigo, E. Puche, M. Segura, E. Sánchez, C. Navarro, A. McAllister, A. Arnal, C. Rojo. **2020**. The charophyte-sediment tándem: effects of charophytes meadows on sedimentary microbial communities and diaspores. *8th International Symposium on Extant and Fossil Charophytes (IRGC)*. Gammarth, Tunisia. Postponed to 2021 due to COVID-19 health crisis.

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B. Murcia, C. Salcedo, E. Puche, Y. Picó, C. Rojo. **2017**. Effect of global change factors (temperature, nitrate concentration and UV radiation) on the production of polyphenols by charophytes. *21st Meeting of the Group of European Charophytologists (GEC)*. València, Spain.

E. Puche, M.A. Rodrigo, R. Ramos-Jiliberto, C. Rojo. **2018**. How important charophytes (submersed macrophytes) are to the planktonic-benthic interaction web? *XIX Conference of the Iberian Association of Limnology*. Coimbra, Portugal. *Award for the 2nd best poster by young researchers*.

M. Segura, E. Puche, M.A. Rodrigo, E. Sánchez, C. Rojo. **2019**. Charophyte meadow performance under the effect of UV radiation alters the benthic community and system functioning. *1st Iberian Ecological Society Meeting*. Barcelona, Spain.

E. Puche, M.A. Rodrigo, C. Rojo. **2020**. Charophytes: key players in shallow freshwater ecosystems under global change. *International Workshops in Environment, Universidad Internacional de Andalucía: Temporary wetlands future in drylands under the projected global change scenario*. Baeza, Spain.

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Other meetings

E. Puche, M.A. Rodrigo, M. Carramiñana, I. Parra, C. Rojo. **2016**. Charophytes and climate change: foreseeable responses to several stressors. Poster at *V Trobada Joves Investigadors de la Facultat de Ciències Biològiques (Universitat de València)*. Burjassot, Spain.

E. Puche, M.A. Rodrigo, C. Rojo. **2017**. Charophytes, good indicators to test interactive effects of global change factors. Oral communication at the *III Annual meeting of PhD students of the Doctorate Program in Biodiversity and Evolutionary Biology*. Paterna, Spain.

E. Puche, M.A. Rodrigo, C. Rojo. **2020**. Charophytes: central species in shallow freshwater ecosystems. Oral communication at *Journal club at the Balaton Limnological Research Institute*. Tihany, Hungary.

Meetings organization

2017. Organizer at the *21st Meeting of the Group of European Charophytologists (GEC)*. València, Spain.

2018. Organizer at the *IV Annual meeting of PhD students of the Doctorate Program in Biodiversity and Evolutionary Biology*. Paterna, Spain.

2020. Organizer at the *7th Fresh Blood for Freshwater meeting*. Bilbao, Spain.

Datasets in digital repositories

C. Rojo, E. Puche, M.A. Rodrigo. **2018**. Growth, morphology and metabolic variables of charophytes (freshwater macroalgae) under different treatments of radiation, temperature and nitrate concentration. RODERIC digital repository from the University of Valencia. http://roderic.uv.es/handle/10550/67467

E. Puche, C. Rojo, R. Ramos-Jiliberto, M.A. Rodrigo. **2019**. Database of an ecological multi-interaction network of a macrophyte-dominated lake. RODERIC digital repository from the University of Valencia. http://roderic.uv.es/handle/10550/70781

E. Puche, C. Rojo, M.A. Rodrigo. **2020**. Carbon biomass of planktonic-periphytic organisms of a mesocosm experiment. RODERIC digital repository from the University of Valencia. http://roderic.uv.es/handle/10550/73066

E. Puche, F. Jordán, M.A. Rodrigo, C. Rojo. **2020**. Database of nodes' topological indices from experimental Aquatic communities. RODERIC digital repository from the University of Valencia. http://roderic.uv.es/handle/10550/75149