Ecological Engineering 58 (2013) 149–155

Contents lists available at ScienceDirect

Ecological Engineering

journal homepage: www.elsevier.com/locate/ecoleng

The allelopathic capacity of submerged macrophytes shapes the microalgal assemblages from a recently restored coastal wetland

C. Rojo*, M. Segura, M.A. Rodrigo

Integrative Ecology Group, Cavanilles Institute for Biodiversity and Evolutionary Biology, University of Valencia, Catedrático José Beltrán 2, E-46980 Paterna, Valencia, Spain

ARTICLE INFO

Article history:
Received 30 January 2013
Received in revised form 29 April 2013
Accepted 8 June 2013
Available online 25 July 2013

Keywords:
Phytoplankton
Passive-competition
Diatoms
Chlorophyceae
Cyanobacteria
Characeae
Semi-arid wetlands

ABSTRACT

We have tested the efficiency of isolated and combined submerged macrophyte cultures to inhibit, through allelopathy, the natural phytoplankton growth. Both plants and microalgae come from the same wetland, a recently restored area in Albufera de València Natural Park (Spain). The need to replant the area under restoration with submerged macrophytes makes this information essential for wetland management. The selection and culture of the submerged macrophytes used in that restoration (four charophytes: *Chara hispida*, *Chara vulgaris*, *Chara baltica*, *Nitella hyalina*, and one angiosperm: *Myriophyllum spicatum*) provided a good opportunity to test in the laboratory the allelopathic effect of macrophyte assemblages on environmental phytoplankton communities. Three experiments were carried out using spring communities: in Experiment 1, a diverse phytoplankton assemblage (29% of biomass Chlorophyceae, 26% Cryptophyceae, 19% diatoms and 9% cyanobacteria) was cultivated with exudates from monocultures of *C. hispida*, *N. hyalina* and *M. spicatum*. Experiment 2 proved the allelopathic effect of a macrophyte assemblage exudate (*C. hispida*, *C. baltica*, *C. vulgaris*, *N. hyalina* and *M. spicatum* in a mixed culture) on two different phytoplankton communities (one diverse: 53% biomass of diatoms, 27% cyanobacteria, 18% chlorophytes and another dominated by small chlorophytes). The response variables were Chl *a* concentration, phytoplankton biovolume and main algal groups' biovolumes. When phytoplankton grew in water with exudates from monocultures, microalgal biomass was from 4 to 6 times lower than in the control after 5 days and Chl *a* concentration was up to 4 times lower. The inhibitory effect of *C. hispida* was greater than that of *M. spicatum*. Mixed macrophyte assemblages resulted in even stronger allelopathic effects than monocultured macrophytes; the biomass was reduced by 7 fold after 5 days using the mixed exudates and Chl *a* concentration was between 3 and 5 times lower. The experiments demonstrate that macrophytes are particularly effective in inhibiting the growth of both small diatoms and the least desirable phytoplankton component in these wetlands (filamentous cyanobacteria), but not chlorophytes (reduction by 37–69, 7–14 and 1–7 fold for diatoms, cyanobacteria and chlorophytes, respectively). The predictions are that spring macrophytes might enhance microalgae that are suitable for grazing (mostly small chlorophytes) and will decrease non-edible filamentous taxa. Thus, restoration managers should replant with the mixture of submerged native macrophytes that provide the most harmful allelopathic effects to promote benefits on aquatic communities in two synergistic ways: by directly reducing microalgal biomass and by indirectly enhancing grazing, which in turn, would promote a clear-water phase. We present replanted macrophytes as ecosystem engineers, *i.e.* organisms that directly or indirectly modulate the availability of resources for the wetland food webs, by causing changes in primary producer assemblages.

1. Introduction

Shallow aquatic ecosystems in semi-arid areas, such as the Mediterranean where the shortage of large bodies of water makes wetlands, lagoons or coastal ponds essential aquatic ecosystems, have been highly threatened during recent decades by deterioration and issues associated with climate change (Álvarez-Cobelas et al., 2005). Then, their restoration and the increase of our knowledge regarding factors that can mitigate phytoplankton growth linked to these new scenarios are necessary (Rojo et al., 2012). This restoration entails the reconstruction of submerged macrophyte prairies in an attempt to recover their beneficial functions (Wang et al., 2009; Hu et al., 2010). Thus, an urgent research topic

* Corresponding author. Tel.: +34 963543656.
E-mail addresses: carmen.rojo@uv.es (C. Rojo), m.matilde.segura@uv.es (M. Segura), maria.a.rodrigo@uv.es (M.A. Rodrigo).

0925-8574/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ecoleng.2013.06.019

would be to target macrophytes limiting the undesirable effects of eutrophication, such as massive phytoplankton growth causing a lack of transparency, biodiversity losses and changes in aquatic food webs and ecosystem function (Burks et al., 2006). An effort in this arena has been made in the coastal wetland Albufera de València Natural Park (hereafter termed AVNP) since 2008. It is a protected area with a large eutrophic shallow lagoon, marshes and rice fields. A 40 ha area of the latter is being restored and converted to "naturalized" wetlands (Rodrigo et al., 2013). Restoration by replantation favoured the growth of the angiosperm *M. spicatum* L. and the charophytes *C. hispida* L., *C. vulgaris*, *C. baltica* A.Bruzelius and *N. hyalina* (De Candolle) C. Agardh. All of these taxa occurred in the wetland prior to eutrophication (Rodrigo et al., 2010). It is in that ecosystem restoration context where allelopathic potential of macrophytes on microalgal growth (Gross et al., 2007) can be extremely significant and then, more knowledge about allelopathic potential of macrophytes on coexisting phytoplankton assemblages will improve management. Our main goal here is to test the capacity of macrophytes used in that replantation (Rodrigo et al., 2013) to reduce the total microscopic primary producer concentrations and to provide a more appropriate microalgal food for herbivores, for example reducing toxic and harmful filamentous and colonial cyanobacteria (Chen et al., 2012) observed in AVNP (Romo et al., 2012) and favouring zooplankton grazing (Gross et al., 2007; Hilt and Gross, 2008). Broadly, we would use replanted macrophytes as ecosystem engineers (Wright and Jones, 2006), *i.e.* as organisms that directly or indirectly modulate the availability of resources for the wetland food web, by causing changes in primary producer assemblage. Summarizing, the challenge here is to recommend what set of populations form the macrophyte assemblage that best promotes clear water conditions in the restored wetland.

Until now, the negative effect of certain macrophytes on microalgal growth has often been tested in bioassays, most administering extracts or exudates of a potentially allelopathic plant to a mono-cultured microalgae (Gross et al., 2007; Chen et al., 2012) and few studies in field conditions (Hilt et al., 2012). However, despite the effort applied to biochemical analyses aimed at identifying allelopathic compounds and the increase in bioassays demonstrating their effect on some plankton populations in monocultures, the complexity of this relationship affecting natural assemblages is still poorly understood (Vanderstukken et al., 2011). Monoculture assays have demonstrated that the allelopathic effects of a macrophyte can be species-specific (Hilt and Gross, 2008) and that a combination of secondary metabolites can increase their allelopathic efficiency (Chen et al., 2012). Therefore, the resistance or vulnerability of phytoplankton to macrophytes in the field will depend on the composition of both microalgae and macrophyte assemblages. In this context, a specific goal of the present study is to demonstrate how a set of macrophytes, either isolated or co-existing, can affect the growth of phytoplankton communities differently depending on their diverse structures or compositions.

To achieve these goals, we have undertaken laboratory experiments addressing the allelopathic effects of the aforementioned macrophytes on natural phytoplankton communities collected from areas inhabited by such macrophytes. As we are testing the allelopathic efficiency of species that have not previously been studied in this way, we will also test the synergistic effects of mixed cultures and the vulnerability of phytoplankton from shallow and eutrophic water bodies to these effects. This study will enable us to determine whether combination of macrophytes is the most suitable solution to continue the restoration with submerged macrophyte assemblages in Mediterranean wetlands and what predictable changes might occur throughout the aquatic community.

2. Materials and methods

Our experiments tested the effect of macrophyte exudates on different phytoplankton communities collected directly from connected shallow lagoons located within AVNP. The experimental design aimed to improve previous laboratory experiments following some recommendations made by Hilt et al. (2012). The following experimental conditions were therefore applied: (1) the use of exudates from macrophytes in growth phase, (2) a density of plants in the cultures similar to that observed in the field, (3) plants collected during springtime, (4) no nutrient limitation for macrophytes and target phytoplankton species, and (5) frequent replenishment of cultures with water containing macrophyte exudates.

We decided to use the exudates from cultivated macrophytes as allelopathic compounds because (i) the results of recent laboratory experiments show they inhibit monocultures of microalgae to a greater extent than extracts (Zhao et al., 2012) and (ii) they reflect better the natural conditions than an isolated or pure allelopathic compound, allowing to observe the allelopathic effect of complex exudates of substances or secondary metabolites (Whittaker and Feeny, 1971).

2.1. Field site for the collection of cultured materials and pre-experimental conditions

A 40 ha area of Mediterranean coastal wetlands in AVNP known as "Tancat de la Pipa" (hereafter termed TPipa) has been restored, transforming former rice fields into a wetland area. An effort to reintroduce submerged macrophytes was made in the transformed area. These macrophytes, which have previously been collected as adult specimen from ponds and lagoons within AVNP, have been grown in the laboratory and later transferred and replanted in the field (Rodrigo et al., 2013). The same species have been used in the present study.

Five macrophyte species (the charophytes *C. hispida*, *C. baltica*, *C. vulgaris* and *N. hyalina* and the higher plant *M. spicatum*) were collected from TPipa in the spring of 2012 (Table 1), the growing season (Rodrigo et al., 2013) and when the production of allelopathically active compounds is usually the highest (Bauer et al., 2009). The macrophytes were planted in different plastic containers filled with de-chlorinated tap water and wetland sediment; the containers were placed in a culture room (conditions shown in Table 1). The plant cultures were the stock used to initiate the experiments. For more details on the culturing process see Rodrigo et al. (2013).

Because one goal of this work is to compare the strength of the allelopathic effects of different species of macrophytes and their combinations, the experiments accounted for: (i) 4 species of charophytes and 1 submerged angiosperm; (ii) the charophytes belonged to 2 different genera (*Chara* and *Nitella*) of 2 different tribes within the monophyletic group of Characeae (McCourt et al., 1996) for which there is little or no information about allelopathic effects; (iii) all of macrophytes inhabited the same wetland system (TPipa); and iv) these macrophyte populations could be observed together in different assemblages depending on the water quality (Blindow, 1992; Rodrigo et al., 2010).

Water samples containing the phytoplankton communities were carefully collected (without sediment disturbance) in 1 l bottles from different sites in TPipa. The samples were filtered through a 45 μm pore size filter to remove zooplankton. Sub-samples of these samples were observed with an inverted microscope to determine which communities exhibited different phytoplankton compositions that were suitable for the applied experimental approach.

Table 1
Water and sediment quality parameters and conditions in the collection area of macrophytes and phytoplankton assemblages (TPipa, Albufera de Valencia Natural Park, Spain) and in cultures of microalgae and macrophytes during the trials. Mean values ± standard error. PAR: photosynthetically active radiation.

Parameter	Wetland April 2012	Microalgae medium	Macrophyte medium
Nitrogen (mg TN l^{-1}); (g TN kg^{-1} sediment)	1.67; 1.01	0.57	0.16 ± 0.01; 1.06 ± 0.01
Phosphorus (mg TP l^{-1}); (g TP kg^{-1} sediment)	0.02; 0.01	0.24	0.024 ± 0.002; 0.15 ± 0.01
Temperature (°C)	25.5–17.8	20.0	
pH	7.4–7.7	8.2 ± 0.1	
PAR (μmol photons m^{-2} s^{-1})	509 ± 71	102 ± 4	

2.2. Phytoplankton community structural variables

In vivo Chl *a*, as a proxy of microalgal concentrations, was determined with an Aquafluor fluorimeter (Turner, CA, USA). Lugol-fixed phytoplankton was counted and measured and the biovolume (mm^3 l^{-1}) was calculated as in Rojo et al. (2000). The biovolume of each taxonomic group (Reynolds, 1997) was also reported.

2.3. Experimental design 1 (Exp-1A): allelopathic effect of different individual macrophytes on the same phytoplankton community

The macrophytes used for this experiment were *N. hyalina*, *C. hispida* and *M. spicatum*. Young selected plants had initiated growth when they were transferred to the experimental setups, and the total length varied from 5 to 10 cm. 20–25 of each charophyte and 10–15 *Myriophyllum* plants were planted as monocultures in 3 transparent cylindrical containers (16 cm diameter, 25 cm high, 4.5 l and 0.023 m^2) with a 3-cm layer of sediment in the bottom, using a similar setup than in the pre-experimental conditions. A control container was also placed in the same culture room with sediment and water but without macrophytes. *M. spicatum*, *C. hispida* and *N. hyalina* were grown in the experimental medium for 4 weeks, until they had almost reached the water surface, attaining biomass of approximately 182, 79 and 75 g WW l^{-1} (473, 464 and 135 g DW m^{-2} in 20 cm-deep water, respectively). The observed average annual biomass in the field (± standard deviation) was 400 ± 200 g DW m^{-2} for *M. spicatum* and 1000 ± 600 g DW m^{-2} for *C. hispida*; additionally, *N. hyalina* reached 150 ± 40 g DW m^{-2} in the first month of growth in the field (Rodrigo et al., 2013). Therefore, these values suggest that, as predicted by the concentrations of nutrients (Table 1), there were no limiting conditions for the macrophyte growth in the experimental cultures. The apparent nutrient availability was important in this experiment because (i) there is a relationship between resources and the allelopathic substances produced by macrophytes (Bauer et al., 2009); (ii) the macrophytes in the experiment reached densities that were considerably higher than those that generally occur in laboratory experiments according to data provided by Hilt and Gross (2008); and (iii) these densities are similar to the densities observed in the field, which was a goal of the experiments involving exudate concentrations.

After four weeks, 1 l of macrophyte culture water was obtained from each cylindrical container holding each of the species (*C. hispida*: CH, *N. hyalina*: NI and *M. spicatum*: MY) and from the control (C). Each litre was filtered through a Millipore filter (0.45 μm) to exclude protozoa and microbes and other particulate material. The filtrates were stored in a refrigerator at 4 °C until further use (no more than half an hour after filtering). The solutions were enriched with Jaworski's culture medium (Table 1) which is optimal for the growth of microalgae (Rodrigo et al., 2009). It is deemed important to fertilize exudate water so that limitation of microalgal growth does not mask allelopathic effects (Gross et al., 2007).

Once the water containing the exudates was fertilized, it was inoculated with the wetland phytoplankton community (hereafter termed assemblage A). This phytoplankton assemblage was composed of 19% (total biomass) of diatoms, which were mainly small individuals of *Cyclotella* and *Nitzschia* species, 29% Chlorophyceae (small cells of Sphaeropleales), 26% Cryptophyceae and 6% cyanobacteria (mainly filamentous of *Pseudanabaena* spp.). The initial phytoplankton biovolume was 0.1 mm^3 (2.0 μg Chl *a* l^{-1}). Five flasks (replicates) were filled with 80 ml of fertilized and inoculated water from each exudate treatment (CH, NI, MY) and the control (C). The twenty flasks were placed randomly in the culture room and held under the conditions described above.

Total phytoplankton biovolume, the biovolume of the different taxonomic groups and chlorophyll *a* concentrations were recorded at this initial time (*t*0). Samples (2 ml) from each flask were used to quantify the Chl *a* concentration and total phytoplankton biovolumen. The biovolume of the taxonomic groups were determined at the sixth day (*t*1) and the tenth day (*t*2) of culture. We decided on this schedule in accordance to the Zhao et al. (2012) study which cited these timings when allelopathic effect is more pronounced and declining, respectively; more than ten days might imply phytoplankton culture ageing. Water from the macrophyte (with exudates) and control (without it) cultures was added every day to prevent loss of inhibitory effects arising from either adsorption to non-target surfaces or the volatility of some allelopathic substances (Gross et al., 2007), since such a loss of effect can be significant for fast-growing microalgae (Zhao et al., 2012).

To determine the degree of vulnerability to allelopathy of each taxonomic group of phytoplankton, the ratio between the mean biovolume in the control and in the exudate treatments was calculated.

2.4. Experimental design 2 (Exp-2B and Exp-2C): allelopathic effects of an assemblage of macrophytes on two different phytoplankton communities

This experiment on allelopathic effects of an assemblage of macrophytes has been performed on two different phytoplankton communities to highlight the different results when the target is either a monoculture or a diverse assemblage of phytoplankton. It is probable to find both types of communities in the same water body.

A rectangular plastic container tray (90 cm long × 50 cm wide × 30 cm high), filled with 30 l of dechlorinated tap water, was used to hold 15 small pots supporting plants of five macrophyte species (*C. hispida*, *C. baltica*, *C. vulgaris*, *N. hyalina* and *M. spicatum*). Plants in the container were allowed to grow outdoors during four weeks (average monthly temperature in April of 26 °C, natural photoperiod of 15:9 h light:darkness; State Meteorological Agency: http://www.aemet.es, 2012). A similar tray containing pots without plants was used to obtain control water samples. Water from the mixed culture (hereafter the Treatment) and control (hereafter the Control) was filtered and enriched as in Exp-1A. Five replicates (flasks of 25 ml) were set up for the Control and Treatment. In experiment called Exp-2B hereafter, the target phytoplankton (hereafter termed assemblage B) was composed by 53%

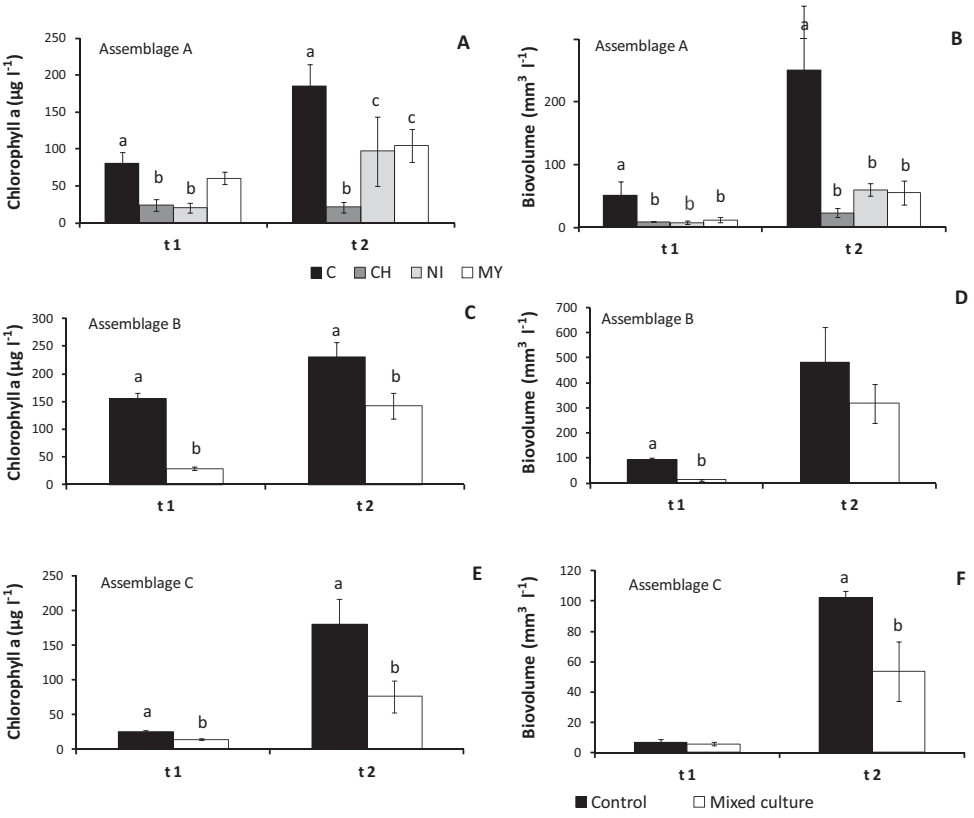

Fig. 1. Phytoplankton abundance reached the sixth and the tenth day after the start of the experiments ($t1$ and $t2$, respectively). Different assemblages (A–C) cultured under treatments without exudates (C) and with exudates from *C. hispida* (CH), *N. hyalina* (NH), *M. spicatum* (MY) and a mixed culture of macrophytes. Bars represent standard deviation. Lower case letter show significant differences ($p < 0.01$) within treatments in each time.

(total biomass) of diatoms, 27% cyanobacteria and 18% Chlorophyceae (the dominant species were the same as in assemblage A). A sample of the phytoplankton community was used to inoculate the flasks (0.1 $mm^3 l^{-1}$ initial biovolume, 1.7 µg Chl $a\,l^{-1}$). The design and material of the third experiment (Exp-2C) is exactly the same as in Exp-2B but the target phytoplankton inoculated (0.1 $mm^3 l^{-1}$ initial biovolumen, 0.8 µg Chl $a\,l^{-1}$) was different. It was composed by 98% Chlorophyceae, mainly cells of Sphaeropleales (hereafter termed assemblage C). All of the flasks were placed randomly in the culture room under the conditions described above. The sampling and analytical methods used to obtain the values of the dependent (response) variables (chlorophyll *a* and biovolume of total phytoplankton and taxonomic groups) were the same as in Exp-1A.

2.5. Statistical analyses

The influence of the macrophyte exudates on the growth of the target phytoplankton communities was examined using the Chl *a* concentration, the total phytoplankton biovolume and the biovolumes of Chlorophyceae, cyanobacteria and diatoms as dependent variables. One-way ANOVA tests were used to check differences in the response variables between the control and treatments, in both $t1$ and $t2$. Prior to the ANOVA tests, all of the data were tested for normality (Shapiro–Wilk test, $p = 0.05$) and homoscedasticity (Levene's test, $p = 0.05$). When ANOVA revealed significant effects, and there were more than two treatments a Bonferroni multiple comparisons test was applied to group homogeneous means. All analyses were performed with the software package SPSS-19 package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Were macrophytes harmful to the phytoplankton assemblages?

In Exp-1A, Chl *a* concentration was between 1 and 4 times lower when phytoplankton grew in water with macrophyte monoculture exudates (*C. hispida*, *N. hyalina*, *M. spicatum*) than in control conditions without any exudates ($F = 44.5$, $p < 0.0001$) at $t1$ (Fig. 1A). At $t2$ Chl *a* concentration was between 2 and 8 times lower in the treatments ($F = 25.5$, $p < 0.0001$). The microalgal biovolume increased from the initial value of 0.1–50 $mm^3 l^{-1}$ in the Control at $t1$. Phytoplankton abundance in the Treatments was significantly lower ($F = 16.8$, $p = 0.000$), and only 1/4 or 1/6 of biovolume reached in the Control was observed, depending on the origin of the exudates (Fig. 1B). Additionally, the 314 $mm^3 l^{-1}$ of phytoplankton reached at $t2$ in the Control was reduced between 4 and 10 times by Treatments ($F = 5.3$, $p = 0.010$; Fig. 1B).

When the source of exudates was the mixed culture of *C. hispida*, *C. baltica*, *C. vulgaris*, *N. hyalina* and *M. spicatum* (Exp-2B and Exp-2C), the values obtained for both Chl *a* and biovolume were also statistically lower in the Treatment than in the Control (Fig. 1C and D). Chl *a* concentration of assemblage B was 155 ± 11 µg Chl $a\,l^{-1}$ at $t1$ in the Control (Fig. 1C), significantly higher than in the Treatment (29 ± 3 µg Chl $a\,l^{-1}$; $F = 406.8$, $p < 0.0001$). Chl *a* concentration was also lower at $t2$ in the Treatment than in the Control ($F = 19.3$, $p = 0.012$).

A similar pattern was observed with regard to biovolume (Fig. 1D), which was 7 and almost 2 times lower in the Treatment than in the Control at $t1$ ($F = 222.6$, $p < 0.0001$) and $t2$ (statistically

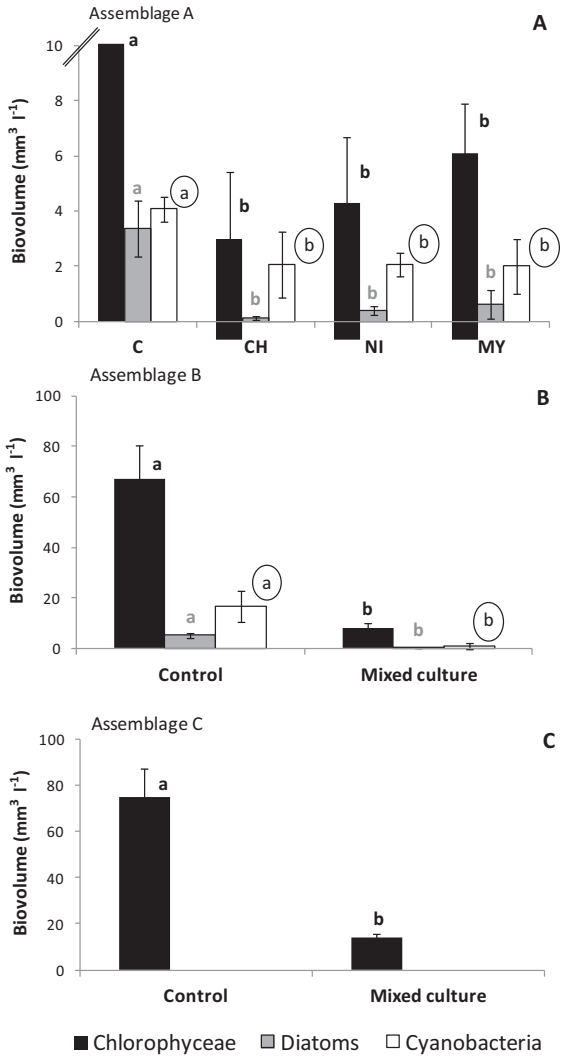

Fig. 2. Taxonomic groups' biovolume of each target assemblage for Exp-1A (A), Exp-2B (B) and Exp-2C (C). Results from A and B are at *t*1, on assemblage C the effect of the treatment was at *t*2. Assemblage C was mainly composed by Chlorophyceae since the beginning of experiment, more information in the text and Fig. 1.

no significant), respectively. Assemblage C growth in Exp-2C was slower than that exhibited by assemblage B. Assemblage C Chl *a* concentration increased until 25 ± 1.4 μg Chl *a* l^{-1} at *t*1 in the Control (Fig. 1E). One half of it was observed in the Treatment ($F = 121.2$, $p < 0.0001$) and it reached 180 ± 36 μg Chl *a* l^{-1} and 76 ± 20 μg Chl *a* l^{-1} at *t*2 in the Treatment and the Control, respectively ($F = 17.9$, $p = 0.013$). Biovolume was not statistically different at *t*1 between the Control and the Treatment (Fig. 1F), but the biovolume in the Treatment at *t*2 was a half of that in the Control (102 ± 1.4 mm^3 l^{-1}) and statistically different ($F = 17.5$, $p = 0.014$).

3.2. Which phytoplankton taxonomic groups were the most vulnerable? And which macrophytes were the most allelopathically harmful?

ANOVA tests on the biovolume of taxonomic groups of assemblage A at *t*1 (Fig. 2A) showed statistically differences between treatments: diatoms in the Control was 29, 8 and 6 times more abundant than in *C. hispida*, *N. hyalina* and *M. spicatum* treatments ($F = 15.3$, $p < 0.0001$; $\text{Bonferroni}_{\text{C-Ch,Ni,MY}} < 0.0001$); Chlorophyceae was 10, 8 and 6 times higher in the Control than in the treatments from *C. hispida*, *N. hyalina* and *M. spicatum* respectively ($F = 19.5$, $p < 0.0001$; $\text{Bonferroni}_{\text{C-Ch,Ni,MY}} < 0.0001$). Finally, differences in cyanobacteria concentrations were double in the Control compared to each treatment ($F = 4.5$, $p = 0.019$). The vulnerability of the different taxonomic groups to exudates from the mixed macrophytes culture was higher than under exudates from monocultures. In Exp-2B (at *t*1) when target was assemblage B (Fig. 2B), diatoms, cyanobacteria and Chlorophyceae were 90, 20 and 8 times higher in the Control than with the Treatment ($F = 99$, $p = 0.001$; $F = 57.6$, $p = 0.002$; $F = 19.3$, $p = 0.012$ respectively). On assemblage C the effect of the Treatment was evident only at *t*2 (Fig. 2C), as mentioned above, and Chlorophyceae biovolume was 5 times lower in the Treatment ($F = 70.7$, $p < 0.001$).

In summary: (i) Chlorophyceae showed a constant degree of vulnerability to allelopathy whatever the origin of the exudate is, (ii) *C. hispida* exudates were the most harmful between macrophytes and (ii) exudates from a mixed culture of macrophytes are clearly more harmful on phytoplankton (mainly on diatoms and cyanobacteria) than those from monocultures.

4. Discussion

From a practical standpoint, in terms of wetland restoration and the establishment of a clear-water state, the main result of this study is that exudates from the macrophytes set collected within a coastal wetland were able to significantly reduce the growth of phytoplankton living there. This result suggests a new aspect of the macrophyte–phytoplankton relationship to be explored in laboratory conditions, the interference interaction between species found in natural assemblages inhabiting the same restored system. In Mediterranean coastal wetlands, as in other shallow systems, submerged macrophyte meadows do not exist as monocultures, but rather as either mosaic landscapes or mixed assemblages of submerged plants (Mouillot et al., 2005). Thus, macrophytes distribution in mosaic is considered in ecological remediation of eutrophic shallow lakes (Wang et al., 2009). Our results stress that the examined combination of macrophytes, widely distributed in Mediterranean wetlands (Cirujano et al., 2008), produced even better results than monocultures with respect to inhibiting phytoplankton growth of similar assemblages. This fact suggests the idea that there are accumulative effects of substances exuded by a combination of macrophyte species and promotes the notion that the synergy among exudates of different macrophytes may represent an interesting new topic to be examined in applied ecology (Dandelot et al., 2008; Chen et al., 2012).

Here, we investigated the allelopathic potential of other charophytes, widely distributed in Mediterranean wetlands (Cirujano et al., 2008), that have not been studied previously: *C. hispida*, *C. baltica*, *C. vulgaris*, *N. hyalina*. In this study, similar densities of *C. hispida* and *N. hyalina* observed in the field showed an inhibitory effect on "natural" phytoplankton growth significantly more intense than that observed for the higher plant *M. spicatum*. This result complements the classification of macrophytes regarding their allelopathic potential (Hilt and Gross, 2008), in which it appears that the allelopathic effects of *M. spicatum* are stronger compared to the hitherto tested charophyte. Moreover, *N. hyalina* can be cited as less harmful than *C. hispida* from our results. These two species, which evidently have morphological and physiological differences (Rodrigo et al., 2011), are members of the same monophyletic group but are widely separated within it (McCourt et al., 1996). Therefore, it could be of interest to add this functional difference between these species arising from the molecular arena. These gradients of allelopathic interference effectiveness should be

taken into account in efforts associated to restoration plans involving replantation.

Moreover, the mixed culture of macrophytes, more precisely of the charophyte mixture, tested in our experiments was highly effective in inhibiting phytoplankton growth, revealing the effectiveness of the macrophyte mixture. In any case, additional information on charophyte secondary metabolites appears to be necessary (Macías et al., 2007), especially comparisons of their effects with those of other macrophytes, as were performed here using the same methods. In this way, all charophytes used in this study, and also *Chara aspera* Dethard. ex Willd., were analyzed and both their tissues and exudates were found to contain different allelopathic phenolic compounds (Palma et al., unpublished results); this lack of similarity in their allelopathic phenolic compounds supports our functional results of higher potential interference when combinations of macrophytes are acting, as has been recently suggested by Chen et al. (2012).

On the other hand, experiments investigating macrophyte allelopathic interaction on microalgae have mainly addressed their effects in monoculture from collections and not on whole phytoplankton assemblages collected from the field. Our results examining natural assemblages reveal more severe inhibition in phytoplankton assemblages with diversity of taxonomic groups than in mono-specific (Chlorophyceae) compositions. The most strongly affected taxa were small planktonic diatoms (*Cyclotella* spp.) and filamentous cyanobacteria (*Pseudanabaena* sp.), whereas the most resistant species were small Chlorophyceae (*i.e.*, *Scenedesmus* spp., *Monoraphidium* spp.). The taxonomic groups, within a natural assemblage, more affected by macrophytes were in accordance to what is known from some comparative monoculture assays: more vulnerability of Oscillatoriales and diatoms than for Chlorophyceae, which can even be favourably impacted (Kufel et al., 2007).

Moreover, according to Reynolds (1997), in shallow eutrophic polymictic lakes, such as those in Mediterranean environments, dominance of filamentous, non-heterocystous cyanobacteria, as *Pseudanabena* spp. are, can be expected, though alternating with centric *Cyclotella* spp. diatoms and other small, fast-growing Chlorophyceae algae. The results obtained in the present study using three different natural assemblages of spring phytoplankton collected from a coastal wetland mimicking Reynolds' models demonstrate that macrophytes are particularly effective in inhibiting the growth of the least desirable component of phytoplankton in these wetlands, *i.e.*, filamentous cyanobacteria, as well as small diatoms. Therefore, we can suggest that spring macrophyte growth enhances microalgae, mostly small Chlorophyceae, that are suitable for grazing and decreases non-edible filamentous taxa (Gilbert and Durand, 1990), thereby promoting larger cladoceran herbivores, whose grazing activity results in cleaner water states in these environments (Sahuquillo et al., 2007). This allelopathic selective effects could be very important in Mediterranean coastal wetlands, such as in the restored areas of AVNP, Sicily, the South of France or Greek, where *Pseudanabaena* spp. can produce frequent blooms and represent a common biomass component, causing turbidity in an alternative state to a macrophyte-dominated environment when deterioration of water quality occurs (Montesanto et al., 2000; Villena and Romo, 2003; Chomerat et al., 2007; Barone et al., 2009).

To conclude, our results demonstrate the stronger effect of charophytes, such as *C. hispida*, compared to the angiosperm *M. spicatum*, which belongs to a genus known for its effective allelopathy against phytoplankton. Moreover, mixed meadows of macrophytes can act as an even better inhibitor of microalgae than monospecific prairies. Hence, assemblages of macrophytes appear to produce synergistic allelopathic effects, which in turn, impact aquatic communities in two ways: by directly reducing microalgal biomass and by indirectly enhancing grazing by large cladocerans, consequently promoting the occurrence of a clear-water phase. In accordance to these results we recommend in restoration replantation with macrophytes to assay the interactions between macrophyte and microalgal assemblages to propose the most effective set of macrophytes based on the phytoplankton inhabiting the system.

Acknowledgements

This study was funded by projects CGL 2009 10292 (Spanish Ministry of Economy and Competitiveness) and ACOMP/2012/236 (Autonomic Administration "Generalitat Valenciana"). The authors thank Fidel Rubio, Sara Calero and Fran Cortés (ICBIBE, University of Valencia, Spain) for their assistance in the laboratory work. We are grateful to Professor Miguel Palma (Cadiz University, Spain) for confirm the existence of polyphenols from the macrophytes used in this study. The authors also appreciate the assistance provided by the staff of the "Tancat de la Pipa" nature reserve in AVNP. American Journal Experts reviewed the English of the manuscript.

References

Álvarez-Cobelas, M., Rojo, C., Angeler, D.G., 2005. Mediterranean limnology: current status, gaps and the future. J. Limnol. 64, 13–29.

Barone, R., Castelli, G., Naselli-Flores, L., 2009. Red sky at night cyanobacteria delight: the role of climate in structuring phytoplankton assemblage in a shallow Mediterranean lake (Biviere di Gela, southeastern Sicily). Hydrobiologia 639, 43–53.

Bauer, N., Blaschke, U., Beutler, E., Gross, E.M., Jenett-Siems, K., Siems, K., Hilt, S., 2009. Seasonal and interannual dynamics of polyphenols in *Myriophyllum verticillatum* and their allelopathic activity on *Anabaena variabilis*. Aquat. Bot. 91, 110–116.

Blindow, I., 1992. Decline of charophytes during eutrophication; comparison to angiosperms. Freshw. Biol. 28, 9–14.

Burks, R.L., Mulderij, G., Gross, E., Jones, I., Jacobsen, L., Jeppesen, E., Van Donk, E., 2006. Center stage: the crucial role of macrophytes in regulating trophic interactions in shallow lake wetlands. Wetlands: functioning, biodiversity conservation, and restoration. Ecol. Stud. Anal. Synth. Ser. 191, 37–59.

Chen, J., Zhanga, H., Hana, Z., Yea, J., Liub, Z., 2012. The influence of aquatic macrophytes on *Microcystis aeruginosa* growth. Ecol. Eng. 42, 130–133.

Chomerat, N., Garnier, R., Bertrand, C., Cazaubon, A., 2007. Seasonal succession of cyanoprokaryotes in a hypereutrophic oligo-mesohaline lagoon from the South of France. Estuar. Coast. Shelf Sci. 72, 591–602.

Cirujano, S., Cambra, J., Sánchez Castillo, P.M., Meco, A., Flor Arnau, N., 2008. Carófitos (Characeae). In: Algas continentales Flora ibérica. CSIC, Madrid, pp. 340.

Dandelot, S., Robles, C., Pech, N., Cazaubon, A., Verlaque, R., 2008. Allelopathic potential of two invasive alien *Ludwigia* spp. Aquat. Bot. 88, 311–316.

Gilbert, J.G., Durand, M.W., 1990. Effect of *Anabaena flos-aquae* on the abilities of *Daphnia* and *Keratella* to feed and reproduce on unicellular algae. Freshw. Biol. 24, 577–596.

Gross, E.M., Hilt, S., Lombardo, P., Mulderij, G., 2007. Searching for allelopathic effects of submerged macrophytes on phytoplankton—state of the art and open questions. Hydrobiologia 584, 77–88.

Hilt, S., Gross, E.M., 2008. Can allelopathically active submerged macrophytes stabilise clear-water states in shallow eutrophic lakes? Basic Appl. Ecol. 9, 422–432.

Hilt, S., Beutler, E., Bauer, N., 2012. Comparison of methods to detect allelopathic effects of submerged macrophytes on green algae. J. Phycol. 48, 40–44.

Hu, L.M., Hu, W.P., Deng, J.C., Li, Q.Q., Gao, F., Zhu, J.G., Han, T., 2010. Nutrient removal in wetlands with different macrophyte structures in eastern Lake Taihu, China. Ecol. Eng. 36, 1725–1732.

Kufel, L., Pasztaleniec, A., Czapla, G., Strzalek, M., 2007. Constitutive allelochemicals from *Stratiotes alcides* L. affect both biomass and community structure phytoplankton. Pol. J. Environ. Stud. 55, 387–393.

Macías, F.A., Molinillo, J.M.G., Varela, R.V., Galindo, J.C.G., 2007. Review: allelopathy—a natural alternative for weed control. Pest. Manage. Sci. 63, 327–348.

McCourt, R.M., Karol, K.G., Guerlesquin, M., Feist, M., 1996. Phylogeny of extant genera in the family Characeae (Charales, Charophyceae) based on rbcL sequences and morphology. Am. J. Bot. 83, 125–131.

Mouillot, D., Gaillard, S., Aliaume, C., Verlaqueb, M., Belsherc, T., Trousselliera, M., Do Chi, T., 2005. Ability of taxonomic diversity indices to discriminate coastal lagoon environments based on macrophyte communities. Ecol. Indic. 5, 1–17.

Montesanto, B., Ziller, S., Danielidis, D., Economou-Amilli, A., 2000. Phytoplankton community structure in the lower reaches of a Mediterranean river (Aliakmon, Greece). Archiv. Hydrobiol. 147, 171–191.

Reynolds, C.S., 1997. Vegetation processes in the pelagic: a model for ecosystem theory. In: Excellence in Ecology. Ecology Institute, Oldendorf.

Rodrigo, M.A., Rojo, C., Segura, M., Larrosa, J., 2009. Mechanisms of microalgae selection during the assembly of a planktonic community. Aquat. Ecol. 43, 61–72.

Rodrigo, M.A., Alonso-Guillén, J.L., Soulié-Märsche, I., 2010. Reconstruction of the former charophyte community out of the fructifications identified in Albufera de València lagoon sediments. Aquat. Bot. 92, 14–22.

Rodrigo, M.A., Sendra, J., Rubio, F., 2011. Enhanced UV-B radiation effects on *Chara* and *Nitella* species. Int. Res. Group Charophytes News 22, 2–5.

Rodrigo, M.A., Rojo, C., Alonso-Guillén, J.L., Vera, P., 2013. Restoration of two small Mediterranean lagoons: the dynamics of submerged macrophytes and factors that affect the success of revegetation. Ecol. Eng. 54, 1–15.

Rojo, C., Ortega-Mayagoitia, E., Rodrigo, M.A., Álvarez-Cobelas, M., 2000. Phytoplankton structure and dynamics in a semiarid wetland, the National Park Las Tablas de Daimiel (Spain). Archiv. Hydrobiol. 148, 397–419.

Rojo, C., Álvarez-Cobelas, M., Benavent-Corai, J., Barón-Rodríguez, M.M., Rodrigo, M.A., 2012. Trade-offs in plankton species richness arising from drought: a long-term study in a National Park wetland (Central Spain). Biodivers. Conserv. 21, 2453–2476.

Romo, S., Soria, J., Fernández, F., Ouahid, Y., Barón-Solá, A., 2012. Water residence time and the dynamics of toxic cyanobacteria. Freshw. Biol. 58, 513–522.

Sahuquillo, M., Melaõ, M.G.G., Miracle, M.R., 2007. Low filtering rates of *Daphnia magna* in a hypertrophic lake: laboratory and in situ experiments using synthetic microspheres. Hydrobiologia 594, 141–152.

Vanderstukken, M., Mazzeo, N., Colen, W., Declerck, S.A.J., Muylaert, K., 2011. Biological control of phytoplankton by the subtropical submerged macrophytes *Egeria densa* and *Potamogeton illinoensis*: a mesocosm study. Freshw. Biol. 56, 1837–1849.

Villena, M.J., Romo, S., 2003. Phytoplankton changes in a shallow Mediterranean lake (Albufera of Valencia, Spain) after sewage diversion. Hydrobiologia 506–509, 281–287.

Wang, G.X., Zhang, L.M., Chua, H., Li, X.D., Xia, M.F., Pu, P.M., 2009. A mosaic community of macrophytes for the ecological remediation of eutrophic shallow lakes. Ecol. Eng. 35, 582–590.

Whittaker, R.H., Feeny, P.P., 1971. Allelochemicals: chemical reactions between species. Science 171, 757–770.

Wright, J.P., Jones, C.G., 2006. The concept of organisms as ecosystem engineers ten years on: progress, limitations, and challenges. BioScience 56, 203–209.

Zhao, J.G., He, F.F., Chen, Z.H., Li, H.J., Hu, J.M., Lin, F.P., 2012. Effect of culture and extract solutions of macrophytes on the growth of three common algae. J. Freshw. Ecol., http://dx.doi.org/10.1080/02705060.2012.661936.