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Chara can outcompete *Myriophyllum* under low phosphorus supply

Doreen Richter · Elisabeth M. Gross

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Abstract In the course of re-oligotrophication in Lower Lake Constance, Germany, the tall-growing angiosperm *Myriophyllum spicatum* has been almost replaced by dense and lower-growing charophytes. We hypothesise that *Chara globularis* negatively affects the performance of *M. spicatum* due to density competition and nutrient interference. Intra- and interspecific competition was assessed using a response surface experimental design with different densities of both species in mono and mixed stands in an outdoor mesocosm experiment. After 8 weeks, we measured the growth and various functional traits of both species, including stoichiometry, ash-free dry mass and dry-matter content, and for *M. spicatum,* additionally chlorophyll content, leaf-mass fraction, formation of autofragments, and root/shoot ratio. With increasing density, *C. globularis* reduced the growth of *M. spicatum* much more strongly than that of conspecifics. Increasing density of *C. globularis* led to a lower chlorophyll *a* to *b* ratio and lower nitrogen content based on ash-free dry mass in *M. spicatum* as well as reduced autofragmentation. Established *C. globularis* meadows can negatively affect tall-growing angiosperms such as *M. spicatum* when the environmental conditions, such as low phosphorus availability and high water-clarity, are appropriate. These findings have implications for the management of lakes, specifically those where a nuisance growth of tall macrophytes occurs, e.g. in systems where *M. spicatum* is invasive.

Keywords Competition · Ecological stoichiometry · Functional traits · Re-oligotrophication · Submerged macrophytes

Introduction

Currently, strong re-oligotrophication processes are evident in many deep and shallow lakes (Jeppesen et al. 2005; Koehler et al. 2005; Stich and Brinker 2010), and are associated with an expansion of submerged macrophyte coverage and changes in community structure towards charophytes. Charophytes prosper under oligotrophic conditions and are replaced by tall-growing angiosperms during eutrophication (Chambers 1987; Ozimek and Kowalczewski 1984). Charophytes are generally the "gold standard" in littoral areas, since their bottom-dwelling structure and capacity to act as nutrient traps (Kufel and Kufel 2002) do not generally interfere with anthropogenic use of lakes (van Nes et al. 2002). Factors accounting for their reappearance after re-oligotrophication, however, are not well understood.

Competition for resources such as nutrients, light or space, strongly influence plant–plant interactions, and along with allelopathy or susceptibility to herbivores or pathogens, may affect individual plant performance and thus result in changes in community structure (Bornette and Puijalon 2011; Le Bagousse-Pinguet et al. 2012). Tall- and fast-growing species are considered strong competitors because they may form canopies and interfere with light availability for understory plants. *Myriophyllum spicatum* L. is found in various aquatic systems exhibiting a wide

D. Richter · E. M. Gross (✉)
Limnological Institute, University of Konstanz,
Mainaustraße 252, 78464 Konstanz, Germany
e-mail: gross5@univ-lorraine.fr

E. M. Gross
Laboratoire Interdisciplinaire des Environnements Continentaux
(LIEC) UMR 7360 CNRS, Université de Lorraine, Campus
Bridoux, 8, Rue du Général Delestraint, 57070 Metz, France

range of environmental conditions such as nutrient availability, salinity and light (Barko 1983). We recently showed that the outcome of interactions between *M. spicatum* and two pondweeds strongly depended on the structure and composition of the macrophyte community, the level of herbivory and the presence of chemical defences (Le Bagousse-Pinguet et al. 2012). Factors accounting for the decline of canopy-forming and potentially invasive species such as *M. spicatum* are less well understood (Trebitz et al. 1993).

Several studies report the recolonisation and expansion of charophytes and the replacement of angiosperms after re-oligotrophication (Blindow 1992; van den Berg et al. 1998b). Even early in the season, *Chara globularis* Thuill. showed the highest biomass under low nutrient conditions compared to *Elodea canadensis* (Planch.) H. St. John and *Potamogeton pectinatus* L. (Bakker et al. 2010). While *M. spicatum* is known to take up nutrients via roots and shoots, sediment nutrient uptake for *Chara* has been considered negligible (Kufel and Kufel 2002). However, this capacity might have been underestimated since a new cultivation technique showed good growth of three *Chara* species when only sediment phosphorus was provided (Wuestenberg et al. 2011). Further, recent findings suggest that polar auxin transport is involved in long-distance transport of nutrients from rhizoids to shoots in Charales (Raven 2013). Charophytes may have more efficient nutrient uptake systems at low nutrient availability (Bakker et al. 2010; Wuestenberg et al. 2011), allowing them to outcompete tall-growing aquatic angiosperms. Competition for bicarbonate might further explain the dominance of charophytes. The competitive advantage of *Chara* over *P. pectinatus* was explained by decreased DIC concentrations and increases in pH within *Chara* stands (Hidding et al. 2010; van den Berg et al. 2002).

At present, the littoral zone of Lower Lake Constance is widely covered by well-established charophyte stands (mainly *Chara globularis*, *C. aspera* Deth. ex Willd., also *C. contraria* A. Braun ex Kütz., *C. tomentosa* L.), probably as a result of nutrient depletion in the lake due to water treatment efforts of all adjacent states. Tall-growing angiosperms, among them *M. spicatum,* are declining and presently only occur in sparse stands among charophytes, in contrast to the situation about a decade ago (Gross and Kornijow 2002).

Based on the above-mentioned changes in macrophyte community structure, we hypothesise that high densities of *Chara* negatively affect the physiological performance and thus functional traits of *M. spicatum*. To determine the interaction between both species, we conducted an outdoor mesocosm experiment using the response surface design. The response surface model, also called complete additive design, represents the best choice for investigation if competition strength depends on the density of both the associated and the focal species since densities are varied independently (Inouye 2001). Higher densities of the smaller species are used to compensate for different growth forms or plant size (Connolly 1987). We measured standard growth parameters, leaf chlorophyll content and plant stoichiometry in both species, and for *M. spicatum* additionally biomass allocation in roots or shoots and autofragmentation.

Methods

Experimental design

To investigate the impact of variable plant density on intra- and interspecific interactions of *C. globularis* and *M. spicatum*, we conducted a response surface design experiment in the outdoor mesocosm area at the Limnological Institute, University of Konstanz, Germany (47°41′42 N, 9°11′37E). Both species were planted at two densities, in mono and mixed cultures yielding eight treatments. Twenty mesocosms were distributed among the eight treatments, with three replicates each for the mixed cultures, and two for each density of the monocultures. We allowed for both above- and below-ground interaction, given that *Chara* might also be able to compete for sediment nutrients.

Each mesocosm consisted of a 1-m-tall Perspex® tube (diameter = 14.5 cm, total volume 16 L). Tubes were arranged side-by-side at a southeast facing, 2-m-high wall in a 2-m-wide passage between larger concrete mesocosms, and tied with ropes to a wooden frame. Every tube was connected to a flow-through system (20 L h^{-1}) consisting of a water reservoir (120 L), a circulating pump (Universal pump 1250A, Eheim, Deizisau, Germany), filters (coarse- and fine-pored polyurethane panels, diameter = 31 cm), a UV lamp (Aqua Cristal UV-C 5 W Series II, JBL, Neuhofen, Germany), a heater (T+H EHK CE 1108, Gerstetten, Germany) and the regulated inflow of water from the institutes' Lake Constance water circulating system. The control unit (Technical workshop, University of Constance) maintained a constant temperature of 19 °C.

We chose this set-up to reflect in situ in the calcareous conditions in Lake Constance as closely as possible. The flow-through system avoided strong photosynthesis-driven changes in pH and alkalinity often found in closed containers (Hidding et al. 2010). We measured inorganic carbon a few times during the experiment, yielding values between 1.8 and 2.2 mM bicarbonate. Our sampling sites are located in "Rheinsee", the part of Lower Lake Constance where the river Rhine flows through, and water exchange is high (Rossknecht 2006). Minimum bicarbonate concentrations are 1.8 mM, reaching up to 2.8 mM in winter. The pH ranges from 8.0 to 8.6. Thus our mesocosms represented the field situation in terms of seasonal inorganic carbon availability. The chosen water temperature reflected values recorded in early summer in the littoral zone of Lower Lake Constance. To reduce the effects of lateral incident light, we covered the lateral open site of all tubes with black gauze, yielding irradiances at approx. 20 cm water depth comparable to that in the lake (150–1,200 μmol photons m^{-2} s^{-1}, depending on sunshine).

Each mesocosm consisted of a 20-cm-high base tube, on which an 80-cm extension was placed, connected by an O-ring seal and bolts. This allowed placing of pre-cultured pots (see below) into the base before adding the extension. Pots had a diameter of 13 cm and were two-thirds filled with a mixture of sand, clay (10 %) and long-term substrate fertiliser-concentrate (5 %, TetraPlant® CompleteSubstrate, Tetra, Melle, Germany), covered by a 4-cm layer of original sediment from Lake Constance. The pots remained in the tubes throughout the experiment.

At our field sites *Chara* reappears during May before *M. spicatum* and rapidly establishes high biomass densities of 400–600 g m^{-2} (ash-free dry mass; AFDM), while only individual tufts of *M. spicatum* with maximum shoot length of 20 cm are visible by the end of June (Richter, unpub. data). Thus, we planted *C. globularis* 3 weeks in advance mimicking field conditions. We used apical shoots from an established stand, originally from Lower Lake Constance, grown in one of the permanent outdoor mesocosms connected to continuous lake water through-flow. Shoots of approx. 4 cm and 3 nodes were planted one-third into the sediment, and pots were precultured in their tube bases at a water depth of 80 cm. The final starting densities were 0, 6 and 18 shoots per pot (corresponding to 0, 500 and 1,500 plants m^{-2}), but we planted two-thirds surplus to allow for mortality or weak growth. On 1 July we removed excess shoots; established shoots had a height of 11.2 ± 0.7 cm (mean ± SE), but had not yet formed a dense mat.

Myriophyllum spicatum originated from Lower Lake Constance and was transferred to the outdoor mesocosm in order to adapt to the experimental conditions 3 days before the experiment was started. Due to the low mortality of *M. spicatum* observed in previous experiments, we planted no surplus shoots. Apical shoots were cut into four nodes (about 4 cm long) and planted at densities of 0, 1 and 4 shoots per pot (0, 100 and 300 plants m^{-2}). Additional shoots of both species were taken to determine the initial dry weight for further growth analyses. Unfortunately, at the beginning of the experiment, four *M. spicatum* plants were added to both *C. globularis* pure stands with the highest density resulting in two additional replicates of the highest mixed culture combination. Therefore, only seven treatments were finally used in the experiment.

Water analysis

Conductivity, pH, dissolved oxygen concentration and temperature in the tubes were measured weekly at midday 20 cm below the water surface with a Universal Pocket Meter (MultiLine® P4, WTW, Weilheim, Germany) in one random replicate per mono and mixed culture of high density (four tubes). Water samples for the analyses of nitrogen and phosphorus were taken at the end of the experiment from one replicate per treatment ($n = 7$) and stored in acid-washed glass bottles in darkness at 4 °C overnight. We filtered twice, 50 mL per sample through Tuffryn® Membrane Filter disks (0.2 μm, diameter = 25 mm, Pall Corporation, Ann Arbor, USA). Soluble reactive phosphorus (SRP) and nitrate were determined from the filtrates and the particulate fractions (P_{part} and N_{part}) from the respective filter disks; the sum of soluble and particulate P or N yielded total P or N. Samples for P_{part} were digested in potassium peroxydisulphate/sodium hydroxide at 121 °C for 60 min, and SRP was measured with the molybdate-ascorbic acid method. Determination of P and N followed standard procedures (Greenberg et al. 1995), and were performed with a Technicon® AutoAnalyzer II (SEAL Analytical, Norderstedt).

Plant growth analysis

On August 24, 2009, we harvested the plants, 54 days after the start. We determined the maximum height of the *C. globularis* stands, but could not discern individual plants. The species were placed separately in plastic bags and transported on ice to the laboratory. We carefully washed the plants in tap water to remove all loose deposits on their surface. We recorded the number and length of main and lateral shoots and autofragments of *M. spicatum*. After recording the fresh weight of roots and shoots of *M. spicatum*, roots were dried at 80 °C and shoots were lyophilised. Dry shoots were separated into leaves and stems. We calculated the leaf-mass fraction of *M. spicatum* (LMF) by separately measuring leaves, stems and roots (Cornelissen et al. 2003). The root/shoot mass ratio for *M. spicatum* indicates resource allocation to above- or below-ground biomass. We analysed the dry-matter content (DMC) by dividing the aboveground dry mass by the fresh mass of each sample.

The relative growth rate (RGR) was calculated with the equation

$$\mathrm{RGR} = (\ln \mathrm{dm}_{t2} - \ln \mathrm{dm}_{t1})/\mathrm{d}$$

where dm_{t1} is the mean aboveground dry mass of individuals (g dm) at the beginning of the experiment, dm_{t2} the dry mass of individuals at the end of the experiment, and d the duration of the experiment (Hunt 1982). It was impossible to separate *C. globularis* into individual plants at the end of the experiment, thus the results were calculated based on the initial number of planted shoots to reflect the individual growth increase. The RGR remains the same, irrespective of whether we calculate with individual plants or the total number.

We ground leaves of *M. spicatum* and entire *C. globularis* shoots to a fine powder in a ball mill (Fritsch, Idar-Oberstein, Germany). Ash-free dry mass (AFDM) was determined after incineration for 5 h at 550 °C. We determined the tissue content of carbon, nitrogen and phosphorus (C, N, P) and calculated the molar stoichiometry for C/N and N/P. The C and N contents were measured with an elemental analyser EURO EA (series 3000, HEKAtech, Wegberg, Germany) and the content of P was determined by digesting 1 mg plant powder as mentioned above (see "Water analysis"). Leaf nitrogen concentration (LNC) and leaf phosphorus concentration (LPC) are the total amounts of N and P per unit of dm or AFDM of *M. spicatum* leaves, and per unit of AFDM of *C. globularis*. For *Chara,* dm is not suitable as a reference because of lime accumulation on the surface: the dry mass of charophytes may consist of about 60 % $CaCO_3$, much higher than usually found in other submerged macrophyte species (van den Berg et al. 2002). We present results for C, N and P in *M. spicatum* based on dm and AFDM. Usually *M. spicatum* shows less-firm carbonate encrustations on their surface, and most references show the results based on dm. We additionally used AFDM to have comparable results to *C. globularis*. We also determined the leaf chlorophyll content of *M. spicatum* (Porra et al. 1989).

Statistical analysis

Data were analysed with Microsoft Excel and IBM SPSS Statistics 19 (SPSS Inc., USA). Among the numerous ways to calculate the outcome of competition via indices (Weigelt and Jolliffe 2003), competition coefficients measuring interspecific relative to intraspecific effects are considered valuable (Jensen 1987). Multiple regression analyses are commonly used to elucidate the main factors of competition (Spencer and Rejmanek 1989; van den Berg et al. 1998a). We chose to analyse our data with stepwise linear regression to assess the effects of the response surface treatments on the tested parameters for both species at each density combination. The effects of increasing densities were calculated for the RGR of both species, the shoot length of *M. spicatum* and maximum height of *C. globularis*.

We used the model:

$$\mathrm{Pe}_{\mathrm{indMs}} = \mathrm{Pe}_0 - \mathrm{D}_{\mathrm{Ms}} \times \mathrm{f}_{\mathrm{Ms}} - \mathrm{D}_{\mathrm{Cg}} \times \mathrm{f}_{\mathrm{Cg}}$$

Spencer and Rejmanek (1989) had used the reciprocal equation. Pe indicates the performance, D the planting density of *M. spicatum* (Ms) and *C. globularis* (Cg), and the regression coefficients f are measures of intra- and interspecific competition. The coefficients provide quantitative estimates of the reduction in growth of an individual of *M. spicatum* due to the addition of conspecifics or of individuals of *C. globularis* (Spencer and Rejmanek 1989). The same model was used analogously for *C. globularis*.

In the stepwise regression analysis, density was used as explanatory variable, with AFDM, C, N, P contents, chl *a* and *b* content and ratios as response variables, and also to examine the changes in biomass allocation patterns of *M. spicatum* (LMF, root/shoot ratio and DMC) with and without competition. We used the partial *F*-test to accept or reject a predictor variable. Density was entered into the equation when the probability of the *F*-value was lower than 0.05 and removed when greater. The significance of the partial regression coefficients was tested by the Student's *t* test, corrected by the sequential Bonferroni test, using a base significance level of 0.05. The relation between chlorophyll concentration and N or P content in *M. spicatum* leaves was analysed using Pearson′s correlation. We used a regression 3D panel of RGR of *C. globularis* and of RGR, length and number of autofragments of *M. spicatum* for graphical visualisation (SigmaPlot 11.2.0; Systat, Chicago, USA).

Results

Water chemistry

The flow-through system accounted for stable physico-chemical water parameters during the experiment. Weekly measurements at midday showed a conductivity of 276.3 ± 0.3 µS cm^{-1}, pH 8.24 ± 0.01, oxygen concentration 8.7 ± 0.1 mg L^{-1} and temperature 19.0 ± 0.1 °C, with no differences between mono and mixed treatments or over time. We also did not observe any difference in total phosphorus and nitrogen concentrations (*F*-test: $F_{2,6} = 1.81$, $P = 0.276$ and $F_{2,6} = 2.694$, $P = 0.182$, respectively), which were in the range of present-day values for Lake Constance. Total P was 8 ± 0.2 µg L^{-1} and total N 757 ± 11 µg L^{-1} at the end of the experiment. The low variance of approx. 2 % indicates the homogeneity of macronutrients in all mesocosms, even though we only measured one replicate per treatment. Also, soluble nutrients did not differ between treatments (SRP 1 ± 0.3 µg L^{-1}, nitrate 716 ± 1 µg L^{-1}; *F*-test, $F_{2,6} = 1.071$, $P = 0.424$ and $F_{2,6} = 0.202$, $P = 0.825$, respectively). We performed no analyses of the sediment nutrient concentrations, but analysed the nutrient content of the plants as a proxy of nutrient uptake (see below).

Table 1 Results of stepwise linear regression of growth parameters and functional traits of *Chara globularis* based on the model $Pe_{indCg} = Pe_0$ (± 1 SE) $- D_{Cg} \times f_{Cg}$ (± 1 SE) $- D_{Ms} \times f_{Ms}$ analogous to the model presented in "Methods" section, and with Pe representing the respective performance parameters as listed below

Data	Linear regression equation[a]	R^2	*df*	*F*	*P*	*P* for regression coefficients		Mean values
						Intraspecific	Interspecific	
RGR_{Cg}	$0.083 (\pm 0.002) - 1.5 \times 10^{-5} (\pm 2.2 \times 10^{-6}) \times D_{Cg}$	0.794	1,13	46.286	**<0.001**	**<0.001**	r.v.	$0.074 \pm 0.001/0.060 \pm 0.001$[b]
l_{Cg}	–	0.249	2,13	1.819	0.208	–	–	52.79 ± 1.77[c]
ash_{Cg}	–	0.160	2,13	1.050	0.383	–	–	73.0 ± 0.4[c]
C_{Cg}	–	0.152	2,13	0.982	0.405	–	–	718.7 ± 8.0[c]
N_{Cg}	–	0.183	2,13	1.229	0.330	–	–	34.1 ± 0.6[c]
P_{Cg}	$2.809 (\pm 0.095) - 0.001 (\pm 4.6 \times 10^{-4}) \times D_{Ms}$	0.340	1,13	6.169	0.029	r.v.	0.029	$2.8 \pm 0.1/2.5 \pm 0.1$[d]
C/N_{Cg}	–	0.110	2,13	0.680	0.527	–	–	24.7 ± 0.3[c]
N/P_{Cg}	–	0.369	2,13	3.220	0.079	–	–	712.1 ± 17.1[c]
DMC_{Cg}	$0.22 (\pm 0.004) + 4.8 \times 10^{-5} (\pm 1.9 \times 10^{-5}) \times D_{Ms}$	0.359	1,13	6.713	0.024	r.v.	0.024	$0.23 \pm 0.02/0.24 \pm 0.002$[d]

Equations are not shown when not significant. Bold *P*-values indicate significant results after sequential Bonferroni correction. See text for details. N = 14

D_{Cg} plant density (plants m^{-2}), RGR_{Cg} relative growth rate (d^{-1}), l_{Cg} stand height (cm), ash_{Cg} ash content (% dm), C_{Cg}, N_{Cg}, P_{Cg} carbon, nitrogen and phosphorus content (mg g^{-1} AFDM), C/N_{Cg} carbon to nitrogen molar ratio, N/P_{Cg} nitrogen to phosphorus molar ratio, DMC_{Cg} dry-matter content, *r.v.* rejected variable

[a] Coefficients are presented as means (± SE)

[b] Low/high density

[c] Mean value for all treatments

[d] Pure stand/highest mixture

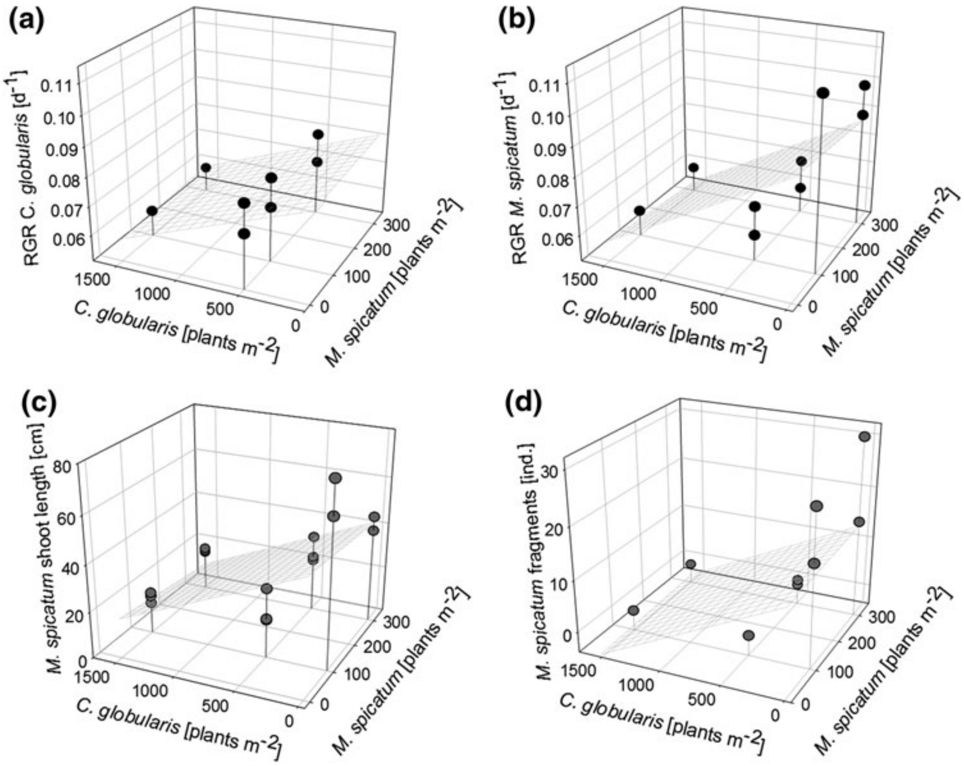

Fig. 1 Effect of *Chara globularis* density on **a** the RGR of *C. globularis* (d^{-1}), **b** the RGR of *Myriophyllum spicatum* (d^{-1}), **c** the length of *M. spicatum* main shoots (cm) and **d** the number of *M. spicatum* autofragments. The coefficients of determination (R^2) and statistical evaluation of the surface planes for the different variables can be found in Tables 1 and 2, respectively. N = 14 for panel a, and N = 16 for all other panels

Chara globularis

We separately analysed plant performance and functional traits for both species. The results for *C. globularis* are summarised in Table 1. The final biomass was 1041 ± 52 g dm m^{-2} at low and 1456 ± 85 g dm m^{-2} at high density. The RGR (Fig. 1a) declined with increasing density by approx. 23 %, documented by the negative intraspecific competition coefficient. *Myriophyllum spicatum* had no influence on *C. globularis* biomass development; therefore this variable was rejected in the stepwise linear regression. The stand heights reached 50–55 cm and did not differ between treatments. Ash and dry-matter content did not vary among treatments. Also, the C, N and P contents and resulting C/N and N/P molar ratios were unaffected.

Myriophyllum spicatum

The results for *M. spicatum* are summarised in Table 2. Growth was strongly affected by *C. globularis*, even at low densities, indicated by the negative interspecific competition coefficients. We found a much lower biomass at high *C. globularis* density with reductions in RGR of 42 % (Fig. 1b), and reductions in shoot length of up to 70 % (Fig. 1c). No intraspecific negative effects were observed. All monospecific treatments produced autofragments, while this dispersal mechanism was almost completely inhibited in the presence of *C. globularis* (Fig. 1d).

Based on dry mass, the C and N content of leaves at high densities of *C. globularis* decreased by 11 and 30 %, respectively, resulting in an increase of the C/N ratio by 33 %. The leaf phosphorus content was low (0.21–1.40 mg g^{-1} dm), but not different between treatments. Thus, the molar N/P ratio of *M. spicatum* leaves was not affected. The lower chl *a* content resulted in a slight decline of the chl *a/b* ratio by 9 % at high *C. globularis* densities. Total chlorophyll concentration correlated with leaf nitrogen content (Pearson′s correlation: $r = 0.498$, $P = 0.025$, n = 16), but not with leaf phosphorus content ($r = 0.025$, $P = 0.464$, n = 16).

The ash content was exceptionally high, ranging from 71 % in monocultures to 75 % at high densities of *C. globularis*, but differences among treatments were not significant. If we calculate the C, N, P and chl content based on the AFDM, the effects on C and chl disappear, but the decline in N remains. Neither high density of *M. spicatum* nor *C. globularis* influenced the leaf-matter fraction or root/shoot ratio of *M. spicatum*. In contrast, the dry-matter content of *M. spicatum* increased dramatically by up to 43 % at high *C. globularis* densities.

Leaves of the autofragments had high C, N and P contents when compared to main shoots, with no difference among treatments (327 ± 8 mg C g^{-1} dm; 24 ± 2 mg N g^{-1} dm; 2 ± 0.2 mg P g^{-1} dm; n = 4). We could not determine differences between mono and mixed cultures because only one single, small fragment was found at low *C. globularis* density, with C, N and P concentrations of 425, 44 and 4 mg g^{-1} dm, respectively.

Table 2 Results of stepwise linear regression for growth parameters and functional traits of *M. spicatum* based on the model $Pe_{indMs} = Pe_0\ (\pm 1\ SE) - D_{Ms} \times f_{Ms}\ (\pm 1\ SE) - D_{Cg} \times f_{Cg}$ analogous to the model presented in "Methods" section, and with Pe representing the respective performance parameters as listed below

Data	Linear regression equation[a]	R^2	*df*	*F*	*P*	*P* for regression coefficients		Mean values
					(P^b)	Intraspecific	Interspecific	
RGR_{Ms}	$0.089\ (\pm 0.005) - 2.3 \times 10^{-5}\ (\pm 5.2 \times 10^{-6}) \times D_{Cg}$	0.573	1,15	18.796	**0.001**	r.v.	0.001	$0.103 \pm 0.005/0.06 \pm 0.000^d$
l_{Ms}	$46.10\ (\pm 5.23) - 0.02\ (\pm 0.01) \times D_{Cg}$	0.539	1,15	16.361	**0.001**	r.v.	0.001	$57.3 \pm 8.5/17.1 \pm 0.8^d$
Fr no.	$13.84\ (\pm 3.16) - 0.011\ (\pm 0.003) \times D_{Cg}$	0.450	1,15	11.476	**0.004**	r.v.	0.004	$22 \pm 4/0 \pm 0^d$
ash_{Ms}	$71.6\ (\pm 0.9) + 0.003\ (\pm 0.001) \times D_{Cg}$	0.329	1,15	6.853	0.020	r.v.	0.020	$71.0 \pm 1.5/75.3 \pm 0.3^d$
C_{Ms}	$213.12\ (\pm 3.49) - 0.015\ (\pm 0.004) \times D_{Cg}$	0.542	1,15	16.601	**0.001** (0.119)	r.v.	0.001	$214.91 \pm 5.41/191.69 \pm 1.53^d$
N_{Ms}	$13.67\ (\pm 0.65) - 0.003\ (\pm 0.001) \times D_{Cg}$	0.576	1,15	19.035	**0.001** (0.007)	r.v.	0.001	$12.88 \pm 0.47/9.03 \pm 0.18^d$
P_{Ms}	–	0.169	2,15	1.323	0.300	–	–	0.59 ± 0.07^c
C/N_{Ms}	$18.24\ (\pm 0.81) + 0.004\ (\pm 0.001) \times D_{Cg}$	0.644	1,15	25.372	**<0.001**	r.v.	<0.001	$19.64 \pm 0.48/25.03 \pm 0.36^d$
N/P_{Ms}	–	0.032	2,15	0.216	0.808	–	–	51.84 ± 6.62^c
chl a_{Ms}	$2.65\ (\pm 0.13) - 3.1 \times 10^{-4}\ (\pm 1.4 \times 10^{-4}) \times D_{Cg}$	0.266	1,15	5.086	0.041 (0.682)	r.v.	0.040	$2.60 \pm 0.21/2.17 \pm 0.09^d$
chl b_{Ms}	–	0.134	2,15	1.006	0.393	–	–	1.10 ± 0.04^c
chl a/b_{Ms}	$2.31\ (\pm 0.03) - 1.3 \times 10^{-4}\ (\pm 2.6 \times 10^{-5}) \times D_{Cg}$	0.658	1,15	26.893	**<0.001**	r.v.	<0.001	$2.31 \pm 0.04/2.11 \pm 0.02^d$
LMF_{Ms}	–	0.221	2,15	1.845	0.197	–	–	0.72 ± 0.01^c
r/s	–	0.150	2,15	1.144	0.349	–	–	0.15 ± 0.01^c
DMC_{Ms}	$0.20\ (\pm 0.01) + 4.8 \times 10^{-5}\ (\pm 8.4 \times 10^{-6}) \times D_{Cg}$	0.702	1,15	32.934	**<0.001**	r.v.	<0.001	$0.19 \pm 0.01/0.27 \pm 0.01^d$

Equations are not shown when not significant. Bold *P*-values indicate significant results after sequential Bonferroni correction. See text for details. N = 16

RGR_{Ms} relative growth rate (d^{-1}), l_{Ms} main shoot length (cm $plant^{-1}$), *Fr no.* number of fragments, ash_{Ms} ash content (% dm), C_{Ms}, N_{Ms}, P_{Ms} carbon, nitrogen and phosphorus content (mg g^{-1} dm), C/N_{Ms} carbon to nitrogen molar ratio, N/P_{Ms} nitrogen to phosphorus molar ratio, *chl* a_{Ms} and *chl* b_{Ms} chlorophyll *a* and *b* content (mg g^{-1} dm), *chl* a/b_{Ms} chlorophyll *a* to *b* ratio, LMF_{Ms} leaf-mass fraction, *r/s* root to shoot ratio, DMC_{Ms} dry-matter content

[a] Coefficients presented as means (± SE)

[b] For results based on AFDM

[c] Mean value for all treatments

[d] Pure stand/highest mixture

Discussion

Predicting changes in submerged macrophyte community composition following changes in environmental conditions is challenging, but also valuable for several reasons. The knowledge about plant community structure allows us to assess possible ecological interactions and ecological functions of the species present, and aids in the decision on possible management options, specifically if the lake offers economic and recreational benefits for the region. Fortunately, at least some lakes have returned to a good ecological status, which provide good growth conditions for submerged macrophytes (Jeppesen et al. 2005; Koehler et al. 2005; Stich and Brinker 2010). Usually, tall-growing macrophytes outcompete charophytes (Blindow 1992), but recent efforts in nutrient reduction offered new opportunities for charophytes (Stewart and Lowe 2008; Schmieder et al. 2006), which are more competitive under clearwater and hardwater conditions than in turbid waters.

We started this project because our field observations indicated strong nutrient limitation in *M. spicatum* (Gross 2009), associated with an increase in charophytes, particularly *C. globularis*. We developed an experimental set-up mimicking field conditions as closely as possible, and were able to elucidate key factors and effects of the interaction among this tall-growing macrophyte and *Chara*. Although both species are well known for their allelopathic effects on phytoplankton (Wium-Andersen et al. 1982; Gross et al. 1996), allelopathic interactions are very unlikely both in the lake and in our experiment due to the high water-exchange rates. Water chemistry and nutrient concentrations remained constant during the course of the experiment, and did not differ between treatments. Similar conditions occur in our field sites, because this part of Lower Lake Constance is strongly influenced by the through-flow of the river Rhine. Using the response surface experimental design (Inouye 2001), we revealed that not only the mere presence of *C. globularis* but also its density is important for the observed *M. spicatum* decline. We thus conclude that in addition to the re-oligotrophication of Lake Constance, the decline of *M. spicatum* in the littoral is significantly enhanced when *Chara* is present.

Chara globularis

Growth of *C. globularis* was never affected by the presence of *M. spicatum*, even at the highest densities of the angiosperm. However, we observed strong intraspecific competition, comparable to that observed for *C. aspera* in a similar competition study with *Potamogeton pectinatus,* where nutrient depletion in the water column may have been the limiting factor (Hidding et al. 2010). Although in our study nutrients in the water column were very low, down to the detection limit for SRP, the constant water turnover provided about 25 mg SRP per mesocosm during the experiment, which is sufficient to account for the phosphorus content found in *C. globularis* tissue. Although competition via sediment-based nutrients was previously considered negligible for *Chara* (Kufel and Kufel 2002), recent findings indicate that *Chara* can grow when only sediment phosphorus is provided (Wuestenberg et al. 2011). Good growth at low nutrient availability indicates that *Chara* might have more efficient nutrient uptake systems than many tall-growing angiosperms (Bakker et al. 2010; this study).

This is further supported by the fact that the biomass development of *C. globularis* in our study was comparable to field data in Lower Lake Constance (Schmieder et al. 2006; Richter and Gross, unpub. data) and that ash content as well as and N and P concentrations were similar to different natural systems (Blindow 1992; Kufel and Kufel 2002). Thus the environmental conditions were appropriate for *C. globularis* in our system. A possible reason for the decline in RGR at higher *C. globularis* densities could have been competition for space, since lateral growth was limited by the tubes and longitudinal growth interferes with sufficient light availability for the lower shoots in the charophyte mats.

Myriophyllum spicatum

As predicted, and expected from our field observations, interspecific competition resulted in strong negative effects on *M. spicatum*. High densities of *C. globularis* strongly reduced the RGR and total length of *M. spicatum* and basically prevented dispersal by autofragmentation. In addition to these indications of reduced fitness, we also observed significant effects on plant structural quality, specifically its toughness and nutrient content. We noticed an exceptionally high ash content of 71 % in the mono- and 75 % in the mixed cultures, although this slight increase was only marginally significant. A possible effect of *C. globularis* on the ash content could have been masked by the already high ash-content of the plants used in the experiment. During recent years, we also observed an increase in the ash content in *M. spicatum* from Lower Lake Constance from 40 to 70 % (Gross, unpub. data). Unfortunately, only a few studies report ash contents for *M. spicatum*; normal values seem to be well below 30 % (Grace and Wetzel 1978). *Myriophyllum alterniflorum* in three Spanish ponds had ash contents between 12 and 23 % (Fernandez-Alaez and Fernandez-Alaez 2011), but this species does not use bicarbonate like *M. spicatum,* making a comparison difficult. What exactly caused the increase in ash content remains unclear. We did not observe carbonate encrustations on *M. spicatum* plants comparable to those on

C. globularis, and also other loose deposits should have been washed away during the cleaning process. Rather, we consider that it may be due to changes within the cell wall of the plant, perhaps changes in silica content, but this remains to be verified by further studies.

The proportion of dry to wet biomass (dry-matter content, DMC) is considered an important plant trait, both for terrestrial and aquatic plants (Garnier et al. 2004; Elger and Lemoine 2005). The DMC of *M. spicatum* increased significantly by 43 % in the presence of *C. globularis*, while intraspecific competition had no effect. A higher DMC is associated with higher tissue toughness, and can also be caused by herbivory (Elger and Lemoine 2005). The higher DMC may reflect the need for more structural tissue (Hyldgaard and Brix 2011), which supports our findings on the ash content. We consider that leaf DMC is comparable to the DMC of the entire shoot for submerged macrophytes, because the DMC consists of structural components such as cell-wall structure and mineral inclusions (Wilson et al. 1999). Growth in water requires minimal investment in structural components, thus resulting in a rather low DMC of freshwater macrophytes compared with terrestrial leaves (Elger and Willby 2003). Thus, the difference between leaf and stem DMC in *M. spicatum* should be negligible. The functional trait leaf DMC is used as an approximation of leaf tissue density, mostly leaf constituents such as lignin, fibre and silica contents, and negatively correlated with nutrient content and thus also RGR (Elger and Willby 2003; Garnier et al. 2004). All measured traits for *M. spicatum* indicate that this macrophyte is severely stressed in Lower Lake Constance.

We calculated C, N and P as well as chlorophyll content for *M. spicatum* both on dry mass (dm) and ash-free dry mass (AFDM), because only a few studies explicitly refer to the latter. Values for C, N and P were in agreement or even lower than those found previously in *M. spicatum* in Lower Lake Constance (Gross 2009; Hempel et al. 2009). The very low leaf carbon content of 19–21 % based on dm was even reduced at high *C. globularis* densities. Yet, carbon content did not differ when calculated on AFDM. This suggests that carbon content based on organic biomass (AFDM) is a fixed value of approx. 40 %. Thus, the proportion of physiological active cell components to structural components (ash content, DMC) was negatively affected by *C. globularis*. In general, the elemental composition of organisms reflects the macromolecular composition of cells (Elser et al. 2011), which in turn reflects the integration of the various processes involved in nutrient uptake and assimilation.

The leaf nitrogen content based on dm of *M. spicatum* ranged between 0.8 and 1.6 %, and declined at a high density of *C. globularis;* the effect remained based on AFDM. The N content was even lower than found before in situ (Hempel et al. 2009), but was still above the critical level of 0.75 % suggested by Gerloff (1975). The leaf phosphorus content was low but not affected by *C. globularis*. Values were in agreement with field data (Hempel et al. 2009; Gross 2009) but much lower than found in other studies (Gerloff 1975; Carpenter 1980; Schmitt and Adams 1981). The N/P molar ratio in *M. spicatum* was high and ranged between 33 and 110 in the presence of *C. globularis*, indicating P limitation (Loladze and Elser 2011). Plants on soils poor in phosphorus increase the toughness of leaves (Hidaka and Kitayama 2011), again providing evidence for a strong interaction of the various cell components as stated above. The C/N molar ratio even increased at high *C. globularis* densities, indicating a stronger effect on nitrogen than on carbon. Changes in the C/N ratio might imply decreased nitrogen availability (Ventura et al. 2008). It is possible that *M. spicatum* did not invest much in the light-harvesting machinery and also limited carbon fixation since leaf N is correlated to the photosynthetic activity (Blindow et al. 2003; Wang et al. 2008). Shading by *C. globularis* might have resulted in the decreased productivity of *M. spicatum*. This is supported by the weak but positive correlation between N and total chlorophyll, and a lower chl *a*/*b*-ratio in the presence of *C. globularis,* indicating acclimation to lower irradiance (Lichtenthaler 1987). However, leaf-mass fraction (Cornelissen et al. 2003) was unchanged between treatments, suggesting that *M. spicatum* was not capable of investing in a proportional increase in leaf tissue.

High root/shoot ratios are typically associated with infertile environments (Chapin 1980). The high root/shoot ratio found for *M. spicatum* compared to other studies (Knauer et al. 2008) indicates low nutrient availability. However, the ratio was not affected by *C. globularis* density, in contrast to findings for *P. pectinatus* competing with *Chara* (Hidding et al. 2010). We consider two possible explanations for the enhanced nutrient limitation in the presence of *C. globularis*: shading might have reduced sediment nutrient uptake because low light causes a decline in N and P uptake (Gross 2003; Wang et al. 2008), or shoot competition for nutrients may have contributed to the lower leaf nutrient contents (Wang et al. 2008).

Leaf nitrogen content and dry-matter content affect components of the carbon and/or nitrogen cycles at the leaf, whole-plant and ecosystem level (Garnier et al. 2004): ecosystems composed of slow-growing species with low leaf nitrogen but high dry-matter content producing low litter quality will show slow decomposition rates. High *C. globularis* densities resulted in a lower tissue quality indicated by low leaf nitrogen and dry-matter contents in *M. spicatum*, suggesting effects at the ecosystem level. Based on our results, we strongly suggest more studies taking into account variables such as plant ash and dry-matter content, specifically when focusing on stoichiometry and physiological activity. Abscised *M. spicatum* fragments had higher leaf carbon, nitrogen and phosphorus contents than main shoots. These autofragments were well equipped for dispersal, but *M. spicatum* could not benefit from this vegetative dispersal mechanism in the presence of *C. globularis*. Competition for space and nutrient limitation might have reduced autofragmentation (Wang et al. 2008).

In conclusion, a striking result of our study is that *C. globularis* benefits from nutrient-poor conditions and also strongly affects the performance of *M. spicatum* and possibly other tall growing angiosperms (Hidding et al. 2010). Our results may apply to regions where *M. spicatum* is an invasive species and re-oligotrophication is possible thus offering new possibilities for its biological control via *Chara* (Nichols 1991).

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