

Supporting Information

Biogeochemistry

Iron overload consequences for submerged plants stoichiometry, homeostasis and performance

Michał Rybak^{a*}, Kinga Drzewiecka^b, Magdalena Woźniak^b, Safa Öksüz^c, Michał Krueger^d,
Tadeusz Sobczyński^e, Izabela Ratajczak^b, Tomasz Joniak^a

^a Department of Water Protection, Institute of Environmental Biology, Faculty of Biology, Adam Mickiewicz University, Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland

^b Department of Chemistry, Faculty of Forestry and Wood Technology, Poznań University of Life Sciences, Wojska Polskiego 75, 60-625 Poznań, Poland

^c Department of Molecular Biology and Genetic, Faculty of Science and Literature, Afyon Kocatepe University, Gazligöl Yolu ANS Campus, 03200, Afyonkarahisar, Turkey

^d Faculty of Archaeology, Adam Mickiewicz University, Poznań, Uniwersytetu Poznańskiego 7, 61-614 Poznań, Poland

^e Department of Analytical Chemistry, Faculty of Chemistry, Adam Mickiewicz University, Uniwersytetu Poznańskiego 8, 61-614 Poznań, Poland

***Corresponding author:** e-mail: m.rybak@amu.edu.pl; phone: +48 61 829 5782

Materials and methods – supplementary information

Study species, culturing and experimental set-up

The model species was *M. spicatum*, a rooted, perennial submerged aquatic plant distributed worldwide. *M. spicatum* tolerates eutrophic conditions and is a very common species in both clear and turbid state waters (Song et al. 2017). Due to the habitat requirements, *M. spicatum* is highly vulnerable to restoration treatments, which makes it an excellent and universal research object. One hundred plants of *M. spicatum* used in the experiment were obtained from a specialist aquatic plant supplier and inserted into the experimental set immediately after delivery.

The substrates used in experiment were a natural lake sediment (the fraction 5-10 cm below the surface) collected from mesotrophic Strzeszyńskie Lake (N 52°27'39.84" E 16°49'29.16") and commercial fine-grained sand, rinsed several times with distilled water (1:1, v/v), both sieved through a 2 mm sieve and sterilised. The obtained substrate was characterised by pH=7.1 and 1.8 % organic matter content.

Ninety apical parts (10.0 ±0.5 cm length; without side-axis branches) were selected for the experiment. Three shoots were planted per pot (Ø 9 cm, height 10 cm), and inserted in substrate at 4 cm depth. Pots were filled with 6 cm of substrate covered by a 2 cm layer of sand to limit the nutrient flux between the substrate and the water column. Each pot was placed in a cylindrical glass microcosm (2.3 L) filled with 2.0 L of dechlorinated tap water, fulfilling all criteria for drinking water (Puche and Rodrigo 2015; Rojo et al. 2015). A set of 30 microcosms were placed in a cultivation room (temperature 22.5 °C, photosynthetically active radiation at the water surface 160.6 µmol m⁻² s⁻¹; light-dark regime 14:10 h). Cylinders were covered with perforated LDPE foil to reduce water evaporation and maintain gas exchange. Microcosms were left for one week to stabilize the conditions and for specimens' acclimation. The placement of microcosms was changed once every three days during the entire experiment to avoid a site effect.

Physicochemical analysis of water, sampling and plant functional traits

Physicochemical measurements were performed at the same time of the day as follows: 5 and 1 days before Apl (-5, -1, respectively), immediately before Apl, 4 hours and 1 day after Apl, and repeated every 2 days until the end of the experiment. The following parameters were measured: water and substrate pH, oxygen (CX-401, Elmetron) and electric conductivity

(EC; HI98129, Hanna Instr.), turbidity (TN-100, Eutech Instr.) and water colour (Nanocolor VIS, Macherey-Nagel). Substrate and plant sampling for chemical analyses and morphological traits measurements was performed at the end of the experiment. Specimens were cleaned of surface contamination firstly in tap water, then twice in deionized water and surface dried using paper tissues. Samples intended for pigment and biochemical analyses were shock-frozen in liquid nitrogen. Samples for functional traits and elemental composition were collaborative. Thus, firstly measurements of *M. spicatum* individuals were made. Afterwards, plants were dissected into root, stem and leaf fractions, dried in an electric dryer at 60 °C until constant weight, and then ground all together in a porcelain mortar to obtain a fine homogeneous powder. Elemental analysis of particular fractions was not performed due to low dry mass. The water samples were frozen until analyses and substrates samples were air-dried and ground in a porcelain mortar to obtain a homogeneous powder.

Morphological traits of each specimen were measured at the endpoint of the experiment as follows: main shoot length, root length, eventual side-axis length and their number, total fresh mass, root, stem and leaf mass fraction total dry mass. The relative growth rate (RGR) was calculated using the following formula: $RGR = \frac{\ln X_2 \times \ln X_1}{t_2 - t_1}$, where X_1 and X_2 are the mean length of stem at time t_1 (start of cultivation) and t_2 (experiment endpoint).

Elemental analyses

Water samples for dissolved inorganic phosphorus ($DIP = PO_4^{3-}$) and dissolved inorganic nitrogen ($DIN = NO_2^- + NO_3^- + NH_4^+$) were analysed after filtration through membrane filters (0.45 µm, Sartorius) using a flow injection analyser (FIA compact, MLE GmbH) according to ISO 15681-1:2005, ISO 13395:1996, ISO 11732:2005, respectively. Further calculations were performed based on DIP and DIN since only dissolved forms can be taken up by macrophytes and thus better reflect stoichiometric reliance than total forms (Reynolds and Davies 2007; Glibert et al. 2011).

The C and N contents in substrates and plant samples were determined using the Flash 2000 Series elemental analyser (Thermo Fisher Scientific). The concentrations of Fe, Ca, Mg, Zn, Cu and Co in water samples were analysed using the Spectra 280AA atomic absorption spectrometer (Agilent Technologies) with flame atomization. Chloride concentration was determined by silver nitrate titration with chromate indicator (ISO 9297:1989). The concentrations of P, Fe, Ca, Mg, Zn, Cu and Co in plants and substrates were analysed by

X-ray fluorescence spectrometry using Bruker Tracer III-SD handheld spectrometer. The distance between detector and sample was equal as the device was placed in laboratory, vertical position.

Pigment content and biochemical parameters of oxidative stress

Analysis of chlorophyll *a* (Chl-*a*), chlorophyll *b* (Chl-*b*) and carotenoids (Caro) was conducted using the spectrophotometric method (Nanocolor VIS, Macherey-Nagel) after cold extraction in 96% methanol following tissue homogenisation in liquid nitrogen (Lichtenthaler and Buschmann 2005).

The total phenolic content (TPC) was determined by the Folin-Ciocalteu method (Singleton et al. 1999) and total flavonoid content (TFC) by the aluminium chloride colorimetric method (Woisky and Salatino 1998) with a Cary 300 Bio UV-Visible scanning spectrophotometer (Varian). The results of TPC were expressed in mg of gallic acid equivalents per g of fresh tissue (mg GAeq g⁻¹ fresh weight, fw) and TFC in mg of quercetin equivalents per g of fresh tissue (mg Qeq g⁻¹ fw). The content of free salicylic acid (SA) was assayed according to the method of Yalpani et al⁵⁵, and analysed with a Waters Alliance 2695 Chromatograph coupled with a Waters 2475 Multi- λ Fluorescence Detector (Waters Corporation).

Statistical analysis

The differences between treatments with respect to the physicochemical and optical properties of water were assessed by within-subject analysis of variance with treatments as fixed factors and time as the repeated factor. Elements' concentration in water, substrates and plants, morphological traits of *M. spicatum*, pigment concentration and oxidative stress parameters were analysed by one-way analysis of variance. Tukey's HSD test was employed as a post-hoc analysis for significant effects. Required assumptions were assessed using Levene's, Mauchly's and Wilks' multivariate tests. Linear mixed-effects models (Bates et al. 2015) were used to test how element concentration in a plant is related to its concentrations in the environment (water and substrates). Created models were simplified by conducting backward elimination of water or substrates factors. The candidate models were then compared based on the Akaike information criterion (AIC), with the model characterised by the lowest AIC value considered as the best one. All models were validated by examining the distribution of residuals plotted against fitted values. Prior to the analyses, variables were log-transformed and are presented in

this scale. The elements below the detection limit were not included (N, P, Fe). Analyses were performed using the R software (ver. 3.5; R Development Core Team).

References

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Table S1 Effect of coagulant application on photosynthetic pigments and phenolic compound contents in *Myriophyllum spicatum* at the end of the experiment (mean value $\pm$ standard deviation) with one-way ANOVA results

Parameter, unit	Treatment				<i>F</i> df	p
	C	C1	C2	C3		
Chl-a, mg g ⁻¹ fw	5.9 $\pm$ 0.7	6.8 $\pm$ 1.3	8.0 $\pm$ 1.3	6.8 $\pm$ 0.7	3.2 3, 16	0.055
Chl-b, mg g ⁻¹ fw	4.6 $\pm$ 0.4	5.7 $\pm$ 1.3	6.6 $\pm$ 1.0	5.8 $\pm$ 0.4	3.9 3, 16	0.054
Caro, mg g ⁻¹ fw	0.52 $\pm$ 0.08	0.55 $\pm$ 0.19	0.60 $\pm$ 0.18	0.49 $\pm$ 0.22	0.2 3, 16	0.840
TPC, mg GAeq g ⁻¹ fw	31.3 $\pm$ 4.7	26.1 $\pm$ 3.2	23.1 $\pm$ 6.5	28.2 $\pm$ 6.7	1.1 3, 8	0.374
TFC, mg Qeq g ⁻¹ fw	11.0 $\pm$ 2.6	14.8 $\pm$ 4.2	8.5 $\pm$ 3.0	13.0 $\pm$ 2.7	2.1 3, 8	0.180
SA, μ g g ⁻¹ fw	1.65 $\pm$ 0.41	1.92 $\pm$ 0.64	1.79 $\pm$ 0.53	1.53 $\pm$ 0.86	0.1 3, 8	0.929

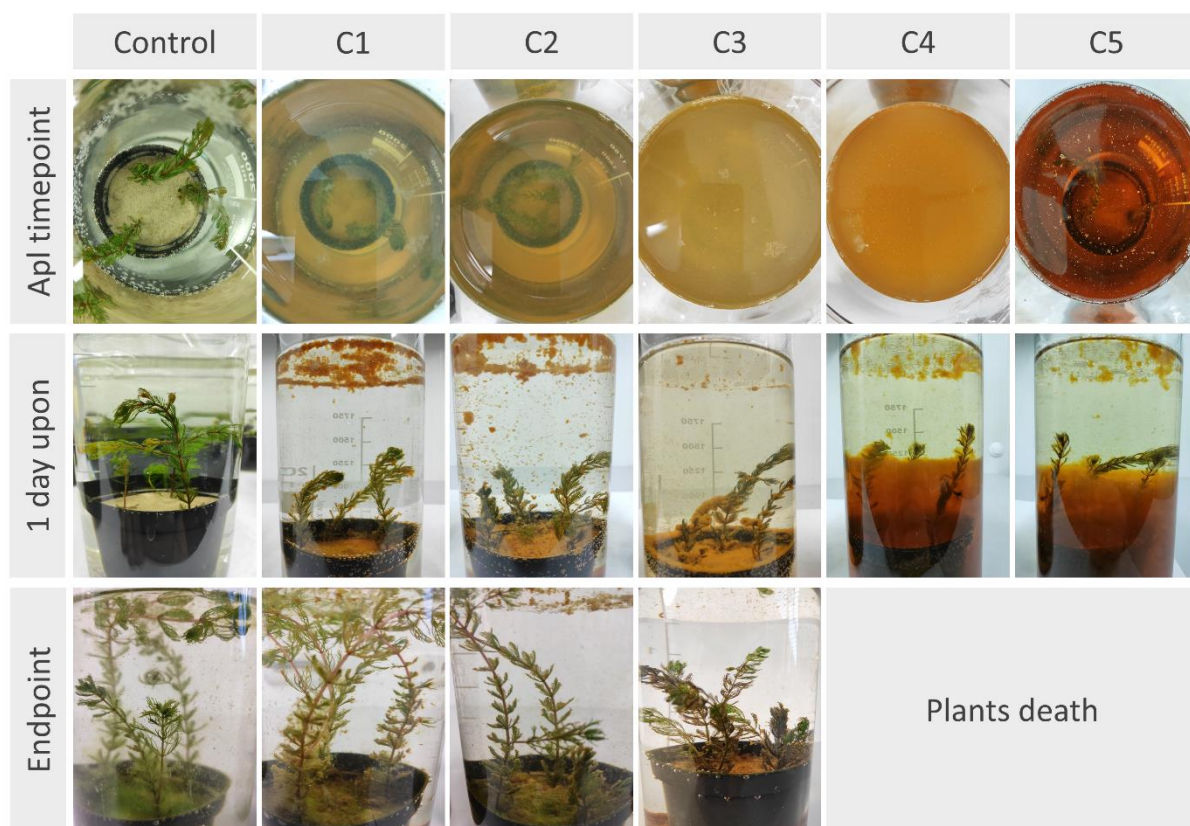


Fig. S1 Overhead view on microcosms immediately upon coagulant application (upper row); created flocks 1 day after application (middle row); *Myriophyllum spicatum* condition at the experiment endpoint (bottom row).

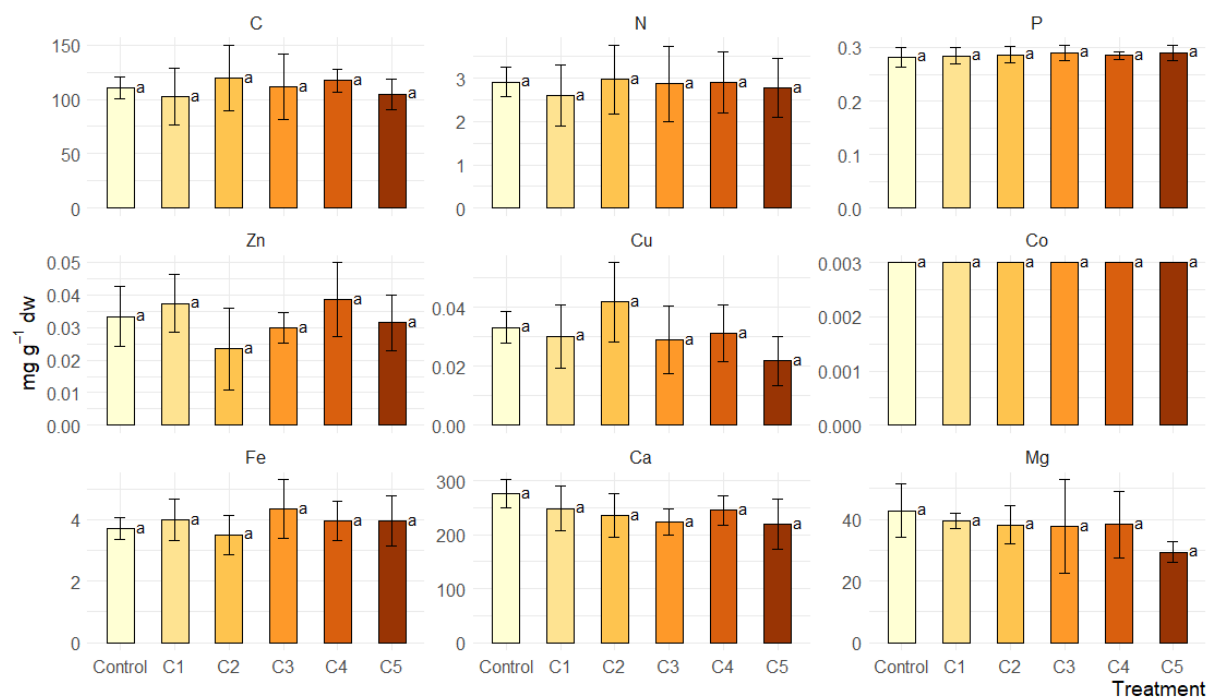


Fig. S2 Effect of coagulant application on element concentrations in substrates at the end of the experiment (mean value $\pm$ standard error); identical letters denote non-significant differences at $p < 0.05$.