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Impact of dye treatment as management strategy on available light may favour a highly invasive alien aquatic plant

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ABSTRACT

Attempts to control massive proliferations of invasive alien aquatic plants (IAAP) are often ineffective. A renewed interest for dye treatment is emerging aiming to control the submerged macrophyte *Myriophyllum heterophyllum* in France. We aimed to understand the effects of dye on this plant knowing about its adaptation to low light. In a 2 × 2 factorial design experiment, we assessed the effect of a dye mixture based on Brilliant Blue (E133) and Allura Red (E129) at high and low light intensities on light quantity and quality and how this might affect the plant's performance by measuring morphological and physiological traits. A multivariate analysis identified three groups – high light (HL) plants, plants in high light with dye (HLD) or low light (LL), and low light with dye (LLD) plants. HL plants performed well but showed stress signs, with a reduced main shoot length, a higher leaf dry matter content (LDMC) and a lower nitrogen content (N), not observed in plants grown in HLD or LL. HLD and LL plants exhibited a comparable total length growth rate to those in HL, but had lower LDMC and higher N contents. LLD plants performed poorly with the lowest growth and signs of physiological stress. Dye-induced changes in light quality only marginally affected the absorbance range of chlorophyll *b*, which apparently did not affect photosynthesis. Commercially available dyes currently used to control nuisance aquatic plants thus seem to have little or no effect on submerged macrophytes. The presence of dye may exacerbate negative effects of very low light intensities on the plant's growth. However, these very low intensities would only be reached during high-water levels or in winter, periods where the dye would rapidly be diluted. During summer, however, the application of dye may protect the plant from damaging light intensities and thus not be a good management strategy to control low light adapted invasive submerged macrophytes.

1. Introduction

Invasive alien aquatic plants (IAAP) have a proportionally higher appearance as preoccupying invasive species in the EU Invasive Alien Species Regulation 1143/2014 than terrestrial plants. This regulation requests for confinement and control of the listed species, while minimising collateral side effects on the ecosystems.

Massive proliferations of IAAP have many negative effects on biodiversity or ecosystem functions and services (Crane et al., 2022; Thiemer et al., 2021). Prevention, early detection and eradication at this stage are among the most effective management actions, but often difficult to achieve. Subsequently, an efficient management strategy comprising one or several control methods is required to reduce or eradicate established IAAP (Hussner et al., 2017). Very often, the demand for a control of heavy infestations comes from the recreational use of aquatic systems (Dutartre, 2004; Shackleton et al., 2019). Managers have to choose among a variety of control techniques to reduce plant biomass at least temporarily and to ensure the continuity of human activities. The most common used technique is mechanical harvesting (Hussner et al., 2017), known to be costly and unsustainable since it fosters dispersal through fragmentation and vegetative reproduction (Hussner and Krause, 2007; Madsen, 2000; Newman and Duenas, 2017). To avoid those disadvantages, managers have sought more affordable solutions such as the use of synthetic dyes to limit light availability for photosynthesis and eventually decrease plant biomass (Buglewicz and Hergenrader, 1977; Crane et al., 2022). First reports on the use of dyes to manage the proliferation of aquatic plants and algae date about 80 years (Eicher, 1947). The efficiency of dye treatments *in situ* have often been weak or difficult to disentangle from other variables such as temperature, available inorganic carbon, water flow, nutrient load or changes in

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natural light (Denys et al., 2014; Hintz, 2021; Madsen and Maberly, 1991). In addition, IAAP diverge much in their preference for different light intensities, with some even preferring shade conditions (Koleszár et al., 2022).

Submerged macrophytes are used to cope with changes in light quantity and quality because of movement and reflection at the water surface and increased attenuation of light with depth (Madsen and Maberly, 1991). Light availability can be reduced by the presence of suspended particles, phytoplankton, or other aquatic plants (Hintz, 2021). Consequently, different submerged macrophyte species exhibit morphological or physiological adaptations for low or high light, depending on their typical growth environment (Chen et al., 2016; Riis et al., 2012). Yet, a certain plasticity is also needed to account for the frequent and not always predictable changes in light quantity and quality. Such short-time acclimations will help the plants to compensate for unfavourable light environments (Barko and Smart, 1981; Guo et al., 2023; Hussner et al., 2011). For example, aquatic plants may respond to reduced light levels with stem elongation, a reduction in the number of side shoots and a greater root mass fraction (Barko and Smart, 1981). Plants may also acclimate to different light levels by physiological changes in the rate of photosynthesis, the ratio of chlorophyll *a* to *b* or the carotenoid content (Demmig-Adams and Adams, 1992).

Originating from the south-eastern part of the USA, *M. heterophyllum* is an evergreen macrophyte, invasive in the north-eastern part of the USA (Moody and Les, 2002), and also an invasive alien species in Europe since probably the 1940s (Anderson et al., 2015; Gross et al., 2020). The species seems currently to enhance its ecological niche (Gross et al., 2020), tolerating ranges of environment variables in France different from those previously observed in other European countries (Hussner and Krause, 2007; Pietsch and Jentsch, 1984) and in the USA (June-Wells et al., 2013; Thum and Lennon, 2010). This submerged macrophyte can form dense mats that may displace other species (Thum and Lennon, 2010), including species of high conservation value (e.g. *Luronium natans*) (Anderson et al., 2015). High economic costs result from clogged infrastructures, disturbance of aquatic recreational activities (Bailey and Calhoun, 2008) and decreased shoreline property values (Halstead et al., 2003). In recent years, a vast spread in North-eastern France has been observed, especially in small-gauge navigation channels and associated harbours, accounting currently for more than 900 km of affected waterways. In order to counter its development, there is a renewed interest in applying synthetic dyes to the water column to modify light availability and thus reduce the growth of this and other nuisance species. Yet, *M. heterophyllum* is known to be adapted to low light intensities (Hussner and Jahns, 2015).

Our objective was thus to understand the effects of dye application in waterbodies on *M. heterophyllum.* We set up our study in indoor microcosms to control environmental conditions, applying both high and low light levels with or without dye. We investigated dye effects both on light quantity and quality, and resulting morphological and physiological changes of the plant. We hypothesised that

- *M. heterophyllum* would display different morphological and physiological acclimations under different light conditions and changes in light spectra to the application of dye in the water column;
- Dye application under high light would not negatively affect this low-light adapted species.

2. Material and methods

2.1. Experimental set-up

We set up a factorial design experiment with two light levels (high/low) and with and without dye. The dye used was a mix of two food dyes, Brilliant Blue FCF (E133; CAS Number 3844-45) and Allura Red (E129; CAS Number 25956-17-6), commercialised in France as BactaPro®. The initial concentration for our experiment was at 2 mg l^{-1} E133 and 0.4 mg l^{-1} E129, calculated in order to mimic the reduction of light obtained at 1 m depth after dye spreading in a French ongoing field experiment. In particular, it has been being used since 2022 in the French inland yacht harbour of Saint-Jean-de-Losne (Burgundy) at concentrations of 0.2 mg l^{-1} E133 and 0.04 mg l^{-1} E129 in order to control the development of *M. heterophyllum*'s biomass. Shoots of *M. heterophyllum* were sampled in a small-gauge channel in North-eastern France (48°67' N, 5°82' E). They were brushed gently in order to remove debris and epiphytes. Apical shoots of 10 cm length were pre-cultured in a tank for 7 days at ~ 70 μmol photons m^{-2} s^{-1}. Sediment used was collected in a small gauge channel. For the experiment itself, we used three vases per condition (8 l, 19 cm Ø, 40 cm height) filled with tap water and planted four plants per vase each into a separate sediment container (PP, 52 mm Ø, 74 mm height, filled with 100 ml). Local tap water has an alkalinity of about 2–2.5 mM and a pH of about 7.57. Addition of the dye slightly increased the pH of the tap water to 7.65. As plants were cultivated individually in one pot with sediment and density of plants in the 8 l vases was not too high, this provided 12 replicates per condition and a total of 48 plants. At the beginning of the experiment, more than 60 plants were measured and weighed for fresh mass, then each 12 of them per condition were evenly distributed to the vases according to their length to avoid starting differences (1-way ANOVA, df = 4, F = 0.012, $P = 1$). Another set of 12 plants was used as starting control plants. They were freeze-dried individually to determine their dry mass and the DMC (dry matter content), the ratio between fresh and dry mass.

Light was provided by eight LED tubes (150 cm, ToLEDo T8 V3 4Ft 20 W 2000 lm 840), installed 105 cm above the table. The luminosity was adjusted with black gauze using a LI-COR spherical Quantum Sensor (LI-193) at ~ 100 (high light) and ~ 30 μmol photons m^{-2} s^{-1} (low light) at the bottom of the vases. The aim was to mimic an average luminosity in the field at a depth of 1 m with full sunlight and during cloudy days, respectively. The experiment was carried out for 14 days at the beginning of July 2023 in a culture room, with day/night light cycle of 16:8 h at 22±1 °C.

2.2. Plant morphological traits

At the end of the experiment, each plant was cleaned with a soft brush and the main stem and all side shoots were measured. We then dissected the plant in above- and belowground parts (the latter comprising roots and part of stem planted in sediment) in order to proceed to morphological traits measurements (Pérez-Harguindeguy et al., 2013). The 10-cm apical part of each main shoot was separated from the rest of the aboveground plant biomass and used for biochemical analyses. We determined the fresh weight for all plant parts and the dry weight after freeze-drying. Upper Leaves (UL) were separated from Upper Stem (US) in order to determine LMF (leaf mass fraction, based on the ratio of leaf and stem dry mass for this section) and LDMC (leaf dry matter content). We also determined the fraction of belowground biomass (BGF) as the ratio between above- and belowground parts. In order to consider the effect of branching, we calculated the ratio between the length of the main shoot to total shoot length including all side shoots (Length Ratio/LR).

Total shoot Relative Growth Rate (RGR) was calculated as $RGR_L = \frac{(\ln L_f - \ln L_0)}{t}$ where total shoot represents the main and lateral shoots of the plant together with branches, L_0 the initial and L_f the final total shoot length and t was the duration of the experiment in days. Dry Mass RGR was calculated in the same way: $RGR_DM = \frac{(\ln W_f - \ln W_0)}{t}$, W representing the total dry mass.

2.3. Plant chemistry

Lyophilised plant material was ground in a ball mill to fine powder. We used leaf biomass for the analysis of the plant chemistry. Carbon and nitrogen content were measured by elemental analysis (Thermoquest

NA2100 protein) from 2 mg samples weighed into tin capsules (5 × 9 mm). Phosphorus analysis was performed by spectrophotometry (Gallery Plus, Thermo Scientific) after alkaline mineralisation using the adapted standard NF EN ISO 6878 (April 2005). Stoichiometry was calculated as the molar ratios of C:N, C:P or N:P, respectively.

All spectrophotometric measures were performed in a Varian Cary 50 Bio UV/Visible spectrophotometer. All extractions were performed using 1 ml of solvent per sample in an Eppendorf Thermomixer (1200 rpm, 2 h with alternating 2 min shaking, 2 min quiet). After extraction, samples were centrifuged at 11,000 *g* (Hettich, Mikro 185) for 10 min. Chlorophyll *a* and *b* (chl *a*, chl *b*) and total carotenoids (car) were extracted with acetone and 0.1 M Tris-HCl, pH 7.8 at 80/20 [v/v] using about 0.5 mg leaf powder. Absorption was measured at 470, 664 and 647 nm. Pigment contents were calculated based on dry mass (dm) using the equations from Porra et al. (1989):

$$[\text{chl } a] = (12.25 * A_{664} - 2.55 * A_{647}) / \text{dm}$$

$$[\text{chl } b] = (20.31 * A_{647} - 4.91 * A_{664}) / \text{dm}$$

$$[\text{car}] = [(1000 * A_{470} - 3.27 * (12.25 * A_{664} - 2.55 * A_{647}) - 104 * (20.31 * A_{647} - 4.91 * A_{664})) / 229] / \text{dm}$$

We also calculated the mass ratio between chl *a* and chl *b* as chl *a*:*b*, and the ratio between chl (a + *b*) and car as indicators of light adaptation or stress reaction.

Anthocyanins (acy) were extracted from about 1 mg leaf powder using methanol, ultrapure water, and 37 % HCl at 80/20/1 [v/v/v]. Absorptions were read at 532 and 653 nm and concentrations calculated with the equation of Murray and Hackett (1991), using the molecular weight of cyanidin (an anthocyanin typically found in *Myriophyllum*) and its molar absorption coefficient:

$$[\text{acy}] = [(A_{532} - 0.24 * A_{653}) / 30] * 287.25 / \text{dm}$$

Total phenolic compounds (tpc) were extracted using 1 ml aqueous acetone 50/50 [v/v] for ~ 1 mg of leaf powder and then quantified according to the Folin-Ciocalteu method (Box, 1983). 300 µl of a saturated sodium carbonate solution (300 g l^{-1} Na_2CO_3) and 100 µl of Folin-Ciocalteu reagent (2 N; Merck) were successively added to tpc extracts dissolved in 2 ml ultrapure water. Absorption at 750 nm was read after 30 min, and concentrations calculated from a calibration curve using a tannic acid as standard.

2.4. Chlorophyll fluorescence measurements

After 14 days, the photosynthetic activity of upper leaves was measured using a Mini-PAM-II (Walz, Effeltrich, Germany). Fluorescence measurements (Fo, and Fm) were taken on one plant per vase, on a leaf harvested in the apical part at about 30 mm below the meristem. The maximal (Fm) and minimal fluorescence (Fo) were measured after 5-min dark-adaptation (Heidbüchel et al., 2019) to "open" all functional reaction centres. The optimal quantum ratio of PSII was calculated as follows: $\frac{Fv}{Fm} = \frac{Fm-F0}{Fm}$.

2.5. Dye action spectrum

At the end of the experiment, water samples were taken from the vases. At the same time, pigments (chlorophyll and carotenoids) were extracted from plant upper leaves in the vases that had received the different treatments, using the method described in Section 2.3. The absorbance spectra of water and pigment extracts were measured with a spectrophotometer (Varian Cary 50 Bio UV/Visible) between 400 and 700 nm, the spectrum useful for photosynthesis. The water and pigment spectra were normalised for comparison setting the highest absorbance peak of the dye mixture at 1, which was 630 nm, and setting at 1 the peak of chlorophylls in the red spectrum at 665 nm for the pigment extracts.

2.6. Statistical analysis

We discovered the presence of *Parapoynx stratiotata* larvae (Lepidoptera, Crambidae) on some of our plants and decided to exclude all vases concerned in the statistical analyses because even non-grazed plants may have undergone stress due to herbivory that could lead to changes in their morphology and physiology (see Fornoff and Gross, 2014 for possible changes). We thus conducted our analyses with n = 8 for HL and HLD, and n = 4 for LL and LLD treatments respectively, considering each individual plant as replicate as all plants had been grown in separate pots with sediment and in sufficiently large vases, excluding nutrient or space competition.

Principal Component Analyses (PCAs) were carried out separately on morphological and physiological parameters. A co-inertia analysis (followed by a Monte-Carlo test) was conducted on those results, to look for similarity of response patterns between these two categories of biological responses, together with ANOVAs or MANOVAs on the first axes to assess between-groups differences. Linear models were developed to assess the role of light intensity. Two-way ANOVAs were performed in order to compare the different treatments regarding morphological and physiological traits. Data were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Levene test) to meet the assumptions of ANOVA. Data was log transformed where appropriate. Otherwise, when necessary, Kruskal-Wallis tests were conducted. Significant differences between means were identified by the post hoc Tukey's HSD test for ANOVAs and Hotelling test for MANOVAs at the 0.05 significance level. Analyses were performed using the R software (version 4.3.2).

3. Results

3.1. Dye action spectrum

PPFD (Photosynthetic Photon Flux Density) values measured at canopy level in the vases ranged between 13.5 µmol photons m^{-2} s^{-1} for low light with dye (LLD), 25.9 for low light alone (LL), 44.2–47.7 for high light with dye (HLD) and 136.5–147.1 for high light alone (HL). The absorbance spectra of the coloured water from the vases used in the experiment and the absorbance spectra of the pigment extracts from the plants used in the experiment were normalised and superimposed (Fig. 1). In the blue and red regions of the visible spectrum respectively, there were slight peaks of the coloured water at 409 and 504 nm and a marked peak at 630 nm. Two peaks are consistent with the peaks of the two food pigments used in the dye, Allura Red [peak at 504 nm] and Brilliant Blue [630 nm]. In the pigment extracts, we observed four marked peaks, at 415, 432, 456 and 665 nm, corresponding to the absorbance regions of chlorophylls *a*, *b* and carotenoids. The absorbance peak of Allura Red is not overlapping with the main absorption of carotenoids and the blue region of chlorophyll absorbance. The

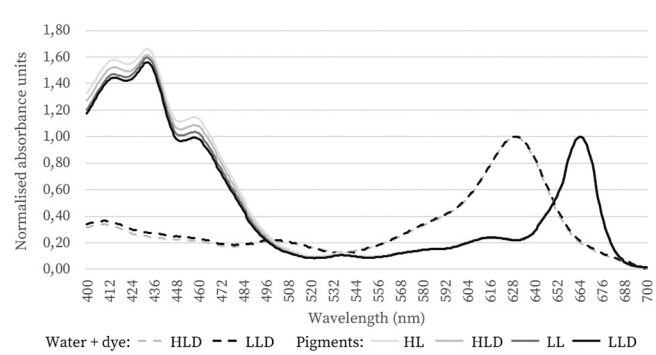

Fig. 1. Comparison of normalised absorbance spectra of dyed water and plant pigments for the different treatments. Peaks in the red spectrum (630 and 665 nm) were set at 1. Treatments: HL: High Light; HLD: High Light with Dye; LL: Low Light; LLD: Low Light with Dye.

absorbance peak of Brilliant Blue is marginally overlapping with the slope of the main absorbance peak of chlorophylls, covering the region where chlorophyll *b* has its maximum (647 nm). Yet, this main peak of Brilliant Blue is not affecting the maximum absorbance of chlorophyll *a* (Fig. 1).

3.2. Morphology

The results of PCA ordination based on morphological traits showed that samples separate along the first PCA axes. The first two axes of PCA ordination explained 67.4 % of total variance (Fig. 2). In particular, RGR, LDMC and LMF seem to drive the results for plants grown under high light alone, whereas the LLD condition seem to be negatively correlated to Belowground Mass Fraction (BGF) and positively to Length Ratio (LR). On the first PCA axis (45.8 % of explained variance), LL and HLD treatments do not differ from each other but from the 2 other treatments HL and LLD, which also differ from one another (Tukey's HSD, $P < 0.04$) (Fig. 2). On the second PCA axis (21.6 % of explained variance), only HLD treatment differs from LLD (Tukey's HSD, $P = 0.008$), whereas they could not be discriminated from any other. Treatments could neither be statistically separated from each other along the 3rd and 4th axes (24.2 % of explained variance). PCA inertia revealed that LMF, LR and both RGRs better explained the positioning along the first axis, whereas BGF and LDMC explained the results along the 2nd axis (Supplementary data, Table S1).

Linear models based on PPFD values showed an overall significant effect of light intensity on all traits measured. In particular, light intensity was positively correlated to all morphological variables, except length ratio, which displayed a negative correlation to light intensity (data not shown). At the highest light intensity, the plants performed best but developed shorter main stems and invested more in lateral shoots. We observed slight gradients between the 4 treatments for RGRs, DMC and BGF based on light availability: the higher the PPFD, the higher the values of these parameters; the opposite is true for Length ratio (Fig. 3). The length ratio showed significant differences (KW, $P < 0.001$) between treatments and was lower for the HL treatment compared to HLD (Welch's t-test, $P < 0.001$), LL (Welch's t-test, $P = 0.017$) and LLD (Welch's t-test, $P = 0.052$).

The LLD treatment showed a significantly lower RGR_L compared to other treatments (KW, $P = 0.03$). The result of the two-way ANOVA on RGR_DM also showed a significant effect of light, dye and the interaction of light and dye (Table 1) but again we observed only a significantly lower RGR_DM for plants grown under LLD than for the 3 other treatments. The results on LDMC highlighted a significant effect of the interaction of light and dye (Table 1), with a higher LDMC for the HL treatment compared to the HLD treatment (Tukey's HSD, $P = 0.02$). As for RGR_DM, the two-way ANOVA based on BGF indicated a significant effect of light, with a significant lower ratio for LLD than for HL alone (Tukey's HSD, $P = 0.02$). LMF was the only parameter to show a significant effect of dye according to the 2-way ANOVA, with a significantly lower proportion of leaves in plants grown in the vases with dye (Tukey's HSD, $P < 0.05$), only marginally significant for HLD compared to LL treatments (Tukey's HSD, $P < 0.09$).

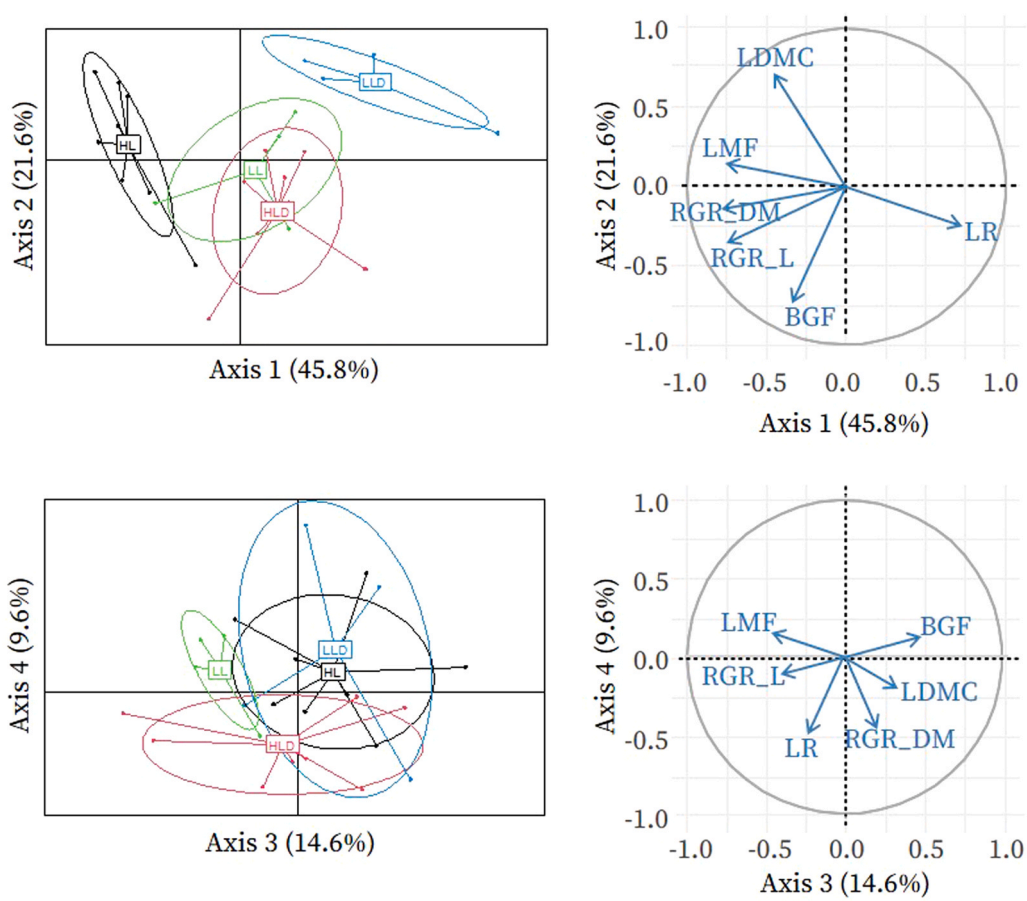

Fig. 2. PCA ordination of the morphological traits of *M. heterophyllum* grouped by treatment and corresponding correlation circles for the first 4 axes. Treatments: HL: High Light; HLD: High Light with Dye; LL: Low Light; LLD: Low Light with Dye. BGF – Below-Ground Mass Fraction; LDMC – Leaf Dry Matter Content; LMF – Leaf Mass Fraction; LR: Length ratio between main shoot and total shoot length; RGR_DM – Relative Growth Rate based on Dry Mass; RGR_L – Relative Growth Rate based on Length.

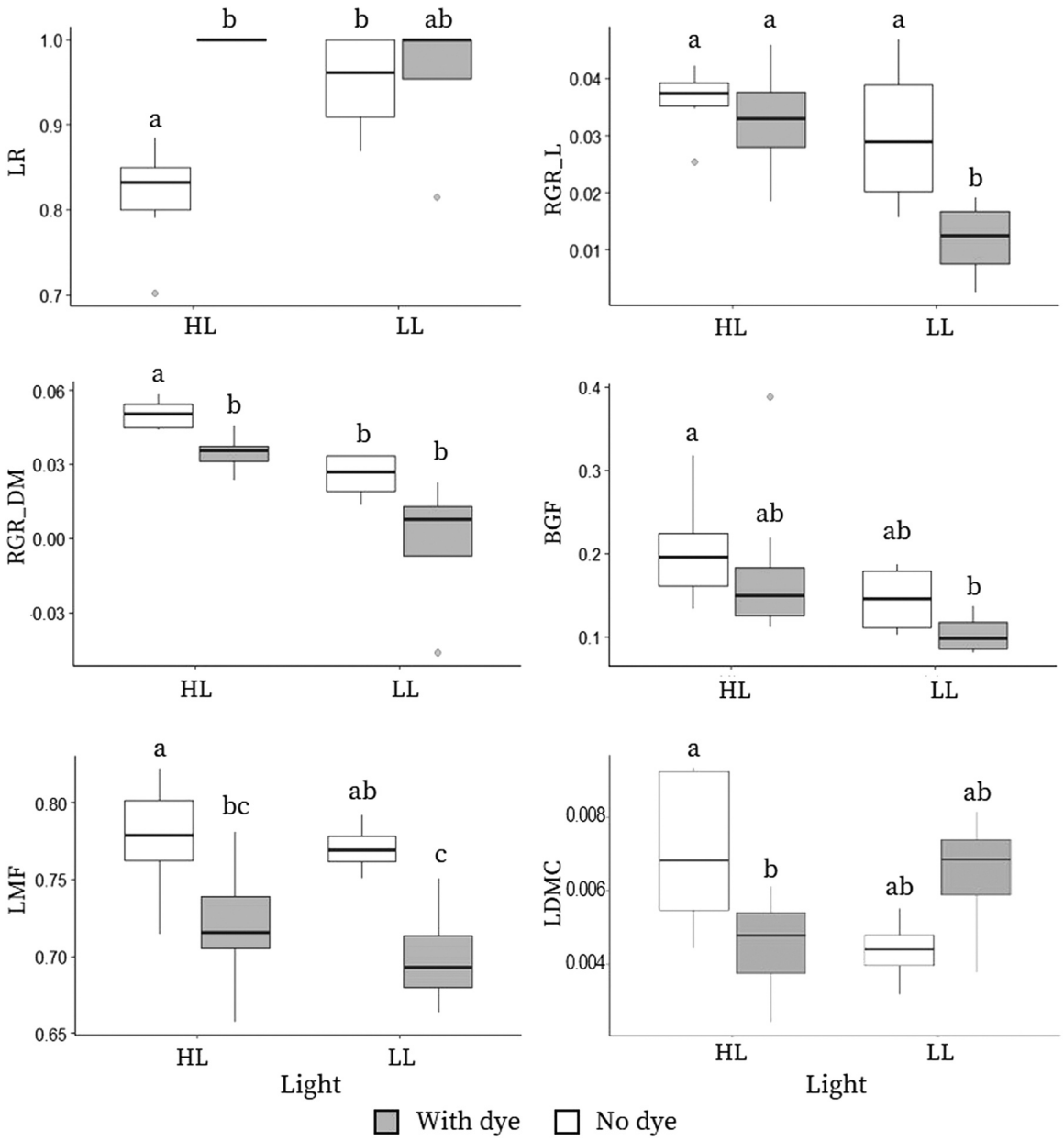

Fig. 3. Boxplots of Length Ratio (LR), RGR based on total shoot length (RGR_L) and on dry mass (RGR_DM), LDMC (Leaf Dry Matter Content), LMF (Leaf Mass Fraction) and Below-Ground mass Fraction (BGF) under the two light levels (High/Low) in absence or presence of dye. LR equals 1 for HLD (High Light with Dye) in the absence of side shoots. Coloured boxplots show the treatments with dye. Black letters indicate significant differences between treatments (Tukey's HSD or Welch's t-test, $P < 0.05$).

3.3. Physiology

The results of PCA based on physiological variables shows that the first two axes explained 61.2 % of total variability (Fig. 4). In particular, anthocyanins were positively correlated to HL and LLD. Pigment content, carbon and nitrogen seem to drive the results on LL and HLD treatments.

Within-group variability was too high to identify differences using only one axis. We thus explore the first 3 axes together with a MANOVA and determined that HL was statistically different from HLD (Hotelling, $P = 0.015$), LL ($P = 0.008$) and LLD ($P < 0.001$) and LLD differed also from the HLD treatment ($P < 0.001$). HLD and LL did not differ significantly ($P = 0.552$). Considering PCA inertia, pigments, both chlorophyll and carotenoids, and nitrogen explained mainly the positioning along the first axis, while phosphorus, carbon and anthocyanins better explained the results along the 2nd axis (Supplementary data, Table S2).

Linear models based on PPFD values showed a significant positive effect of light intensity on all morphological traits measured. Light intensity was negatively correlated to all physiological variables except tpc, P and the C:N molar ratio (data not shown).

3.3.1. Pigments

The lowest values for chlorophyll content were recorded for LLD and HL plants, with significant differences to the two other treatments (Tukey's HSD, $P < 0.035$) (Fig. 5). Carotenoid contents exhibit the same pattern with significant lower values for HL and LLD compared to HLD treatment (Tukey's HSD, $P = 0.001$ and $P = 0.011$ respectively) and to LL treatment (Tukey's HSD, $P < 0.001$ and $P = 0.003$ respectively) (Fig. 5). No significant effect neither by light nor by dye nor by the interaction of both was observed for the chlorophyll *a* to *b* ratio.

The content of total phenolic compounds displayed a great variability and we detected no significant difference between treatments.

Table 1
Results for morphological and physiological traits measured for plants grown under each treatment (HL = High Light, HLD = High Light with Dye, LL = Low Light, LLD = Low Light with Dye). **1a**: 2-way ANOVA was used when possible, showing the effects of 'light' (df = 1), 'dye' (df = 1) and their interaction ('light*dye', df = 1). **1b**: Kruskal-Wallis tests (df = 3) was used for variables where no transformation allowed to achieve normality and/or equality of variances, on morphological and physiological variables.

1a	Light		Dye		Light * dye	
	F	*P*	*F*	*P*	*F*	*P*
RGR_L	4.15	0.055	2.6	0.123	1.57	0.224
RGR_DM	19.77	< **0.001**	12.88	**0.002**	6.13	**0.022**
LDMC	0.33	0.57	2.46	0.133	10.55	**0.004**
LMF	0.86	0.366	21.01	**0.000**	0.18	0.676
BGF	8.18	**0.010**	2.65	0.120	0.32	0.580
chl *a*	1.98	0.175	5.52	**0.029**	45.72	**< 0.001**
chl *b*	1.81	0.194	3.8	0.065	34.15	**< 0.001**
chl (*a* + *b*)	1.95	0.178	5.37	**0.031**	43.77	**< 0.001**
chl (*a*:*b*)	0.00	0.986	0.03	0.859	2.28	0.147
car	0.85	0.367	1.85	0.189	34.74	**< 0.001**
chl (*a* + *b*)/car	2.56	0.125	10.32	**0.004**	16.69	**< 0.001**
tpc	1.93	0.180	3.14	0.092	0.15	0.703
acy	10.82	**0.004**	0.27	0.612	18.65	**< 0.001**
F*v*/F*m*	2.42	0.135	0.79	0.385	0.45	0.512
C	9.97	**0.005**	1.4	0.251	18.54	**< 0.001**
N	4.81	**0.040**	7.01	**0.016**	3.84	0.064
P	0.23	0.637	0.36	0.555	0.2	0.658
C:N	16.12	**<0.001**	9.26	**0.006**	1.21	0.285
C:P	0.08	0.779	0.97	0.336	1.47	0.240
N:P	2.37	0.139	3.61	0.072	1.87	0.187

1b	Treatment		
	Df	χ^2	*P*
LR	3	16.344	**< 0.001**

However, the variability in the anthocyanin content could be explained by significant effects of light and of the interaction of light and dye (Table 1). We observed a higher content in anthocyanin in plants grown under low light with dye (LLD) compared to HL (Tukey's HSD, $P = 0.033$), HLD (Tukey's HSD, $P < 0.001$) and LL (Tukey's HSD, $P = 0.02$) treatments. acy values were also significantly higher in HL compared to HLD treatment (Tukey's HSD, $P = 0.0039$) (Fig. 5). No significant difference was noted between the four treatments regarding the maximum photosynthesis yield (Fig. 5).

3.3.2. Stoichiometry

Plants in the HL treatment showed a significant lower nitrogen content compared to other treatments, with an effect of light and of dye ($P < 0.05$) (Table 1, Fig. 6). ANOVA results showed significant effects of light and of the interaction of light and dye for the carbon content (Table 1), with a significant lower amount of carbon in HL and LLD treatments compared to the HLD treatment (Tukey's HSD, $P = 0.01$ and $P < 0.001$ respectively) (Fig. 6). The LLD treatment was also significantly lower than the LL treatment (Tukey's HSD, $P = 0.047$). The C:N molar ratio was consequently higher for the HL treatment than for other treatments. No significant difference in phosphorus content was observed between treatments (Fig. 6).

The co-inertia analysis confirmed our observations and showed that both morphological and physiological variables responded in the same way (Monte-Carlo, $P < 0.05$).

4. Discussion

Despite variable or rather low effects of dye application as control measure of algal blooms or nuisance aquatic plant growth (Buglewicz and Hergenrader, 1977; Denys et al., 2014), this technique is again used in at least two sites in France to control the highly invasive alien submerged macrophyte *Myriophyllum heterophyllum*. Given the known very low light compensation point for this species and stress signs at high irradiance (Hussner, 2008; Hussner et al., 2010), we were interested to see how dye application would affect plant performance at both high and low light intensity. We used marketed products used in French waterways to see if they may have an impact on the plant's morphology and physiology. In general, our results showed three different ways of acclimation to the different light and dye conditions, as evidenced by the statistical results. Thus, these three groups will be discussed in the following: first, plants exposed to high light without dye (HL) that were growing well but exhibited some signs of stress. Second, plants in high light with dye (HLD) and those in low light without dye (LL), showing very similar performance. Finally, plants exposed to low light and dye (LLD) that performed poorly.

4.1. Impact of high light intensities on plant traits

With a lower Length Ratio (LR) and a higher growth rate based on dry mass (RGR_DM), the plants grown under high light seemed to have adapted their growth strategy to the high light intensity, investing more energy in branching and less in stem elongation to reach higher light conditions compared to all other treatments. Comparable changes have been observed in other invasive submerged macrophytes such as *M. spicatum*, *E. canadensis* or *E. nuttallii*, all showing a positive correlation between shade intensity and either branching or growth rate or both (Eller et al., 2015; Zefferman, 2015). Plants were also investing much in leaf biomass, as indicated by the high LMF of apical shoots. A rather high LMF has been observed for *M. heterophyllum* compared with other *Myriophyllum* species (Dülger and Hussner, 2017).

In the HL treatment, the lower chlorophyll content is probably a response to the high light intensity, as plants may not need to produce a high amount of photosynthetic pigments. Indeed, the content of chlorophyll in macrophytes is known to decrease with increasing irradiance for PPFD values above the minimal requirement for growth (Barko and Filbin, 1983). In that respect, our results are consistent with other studies that have shown that *M. heterophyllum* is a species very sensitive to irradiances higher than 100 μmol photons m^{-2} s^{-1}, where plants showed a marked decrease of pigments and some damages to photosynthetic apparatus (Hussner, 2008; Hussner et al., 2010). However, we neither observed significant higher chl *a*:*b* ratios, nor a higher carotenoid content, the latter potentially contributing to plant protection (Demmig-Adams, 1990; Lichtenthaler et al., 2013). Moreover, there was no reduction in maximum photosynthetic quantum yield (F*v*/F*m*), whereas sun plants are expected to display lower values than shade plants (Dale and Causton, 1992; Demmig-Adams, 1990; Lichtenthaler et al., 2013). The slightly higher anthocyanin levels in this treatment compared to HLD might indicate a response to the higher light intensities. Anthocyanins increase in general with high light exposure (Landi et al., 2015).

Nitrogen concentrations in plants grown in HL were lower than those in all other treatments, ranging from 1.7 % to 2.5 % of dry mass. These values are still above the critical concentration of 1.3 % found for sub-

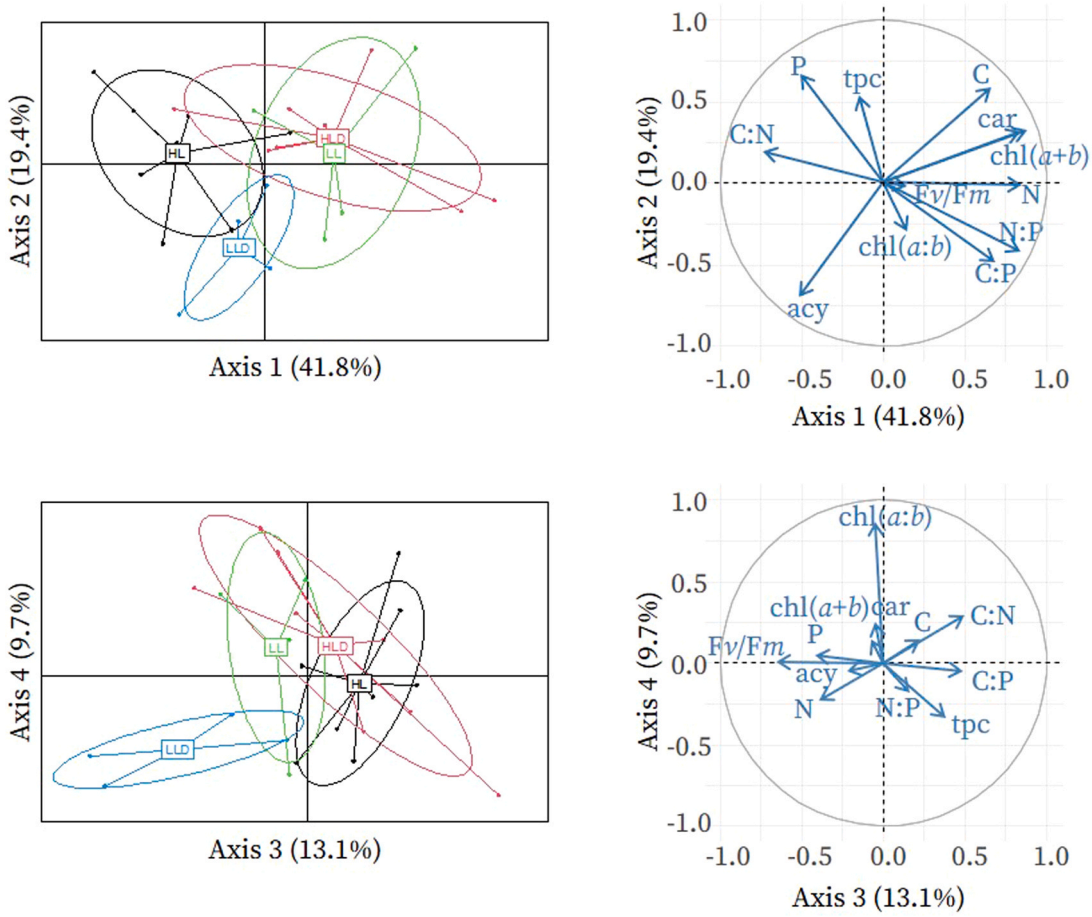

Fig. 4. PCA ordination of the physiological traits of *M. heterophyllum* grouped by treatment and corresponding correlation circles for the first 2 axes. Treatments: HL: High Light; HLD: High Light with Dye; LL: Low Light; LLD: Low Light with Dye. acy – anthocyanins; tpc – total phenolic compound; car – carotenoids. Stoichiometry is based on molar ratios of the respective elements.

merged macrophytes (Gerloff and Krombholz, 1966). Although the carbon content was also significantly lower, the reduction in N was larger, resulting in an overall significantly higher C:N molar ratio of 22.90 (± 2.2) compared to other treatments. This value is still well below reported critical upper C:N ratios identified for aquatic plants, which are 24 and 39 (based on mass ratios, equivalent to 28 and 45.5 as molar ratios as used in our study) for growth and yield rate, respectively (Demars and Edwards, 2007).

4.2. Plant traits at moderate light intensities

As evidenced by the statistical results for both morphological and physiological traits, plants showed in general comparable trait expression in the HLD and LL conditions. This might be because light intensities were moderate and sufficient for the plant to grow (Hussner and Jahns, 2015) without generating stress. In fact, light intensities for HLD (46 μmol photons m^{-2} s^{-1}) and LL (26 μmol photons m^{-2} s^{-1}) treatments were comparable with values we measured at 1 m depth in the field at sites with *M. heterophyllum* dominance, before and during dye application. We measured on average about 30–40 μmol photons m^{-2} s^{-1} at cloudy days at this depth (HG and EMG, unpublished data).

Plants in the HLD and LL treatments exhibited a similar length growth than those in HL, but had a lower LDMC, which might indicate a higher physiological activity as plants contain a higher relative content in water allowing for physiological processes (Nuttens et al., 2016).

Anthocyanins were lower in HLD and LL compared to other treatments, presumably indicating a lower level of light stress. Chlorophylls and carotenoids were also higher in both treatments and showing a higher chl(a + b)/car ratio, the latter indicating possibly less stressful conditions. A higher car/chl (a + b) ratio (inverse ratio) has been linked to stress caused by arsenic pollution in the congener *M. spicatum* (Gross et al., 2018).

Nutrient contents and the CNP stoichiometry found in our study were in line with usual values for aquatic plants, and showed no indication of N or P limitation or imbalance (Demars and Edwards, 2007).

4.3. Influence of low light intensities on plants traits

Plants in the LLD treatment had the lowest RGR based on total shoot length, and some plants even display a negative RGR based on dry mass. Plants had almost no side shoots but their main length shoot was not statistically shorter than in HL or LL treatments, suggesting that plants made thinner shoot to reach more favourable light conditions. In low light conditions, additional dye application reduced the photosynthetic photon flux density to 13.5 μmol photons m^{-2} s^{-1}. This value is below the threshold of ~ 20 μmol photons m^{-2} s^{-1} measured as the compensation point at 20 °C for *M. heterophyllum* (Hussner, 2008) and may explain the poor performance. A negative effect of low light on growth rate has also been noted in other *Myriophyllum* species, such as the IAAP *M. aquaticum* (Tan et al., 2019). Plants in the LLD treatment had also the lowest biomass allocation to belowground parts. This is coherent with a study on another IAAP, the emergent species *Ludwigia grandiflora*, showing a change in biomass allocation from below- to aboveground parts (Thouvenot et al., 2013). A low BGF would provoke a weaker anchorage in the sediment and less nutrient uptake. The handbook for plant functional traits by Pérez-Harguindeguy et al. (2013) also states that

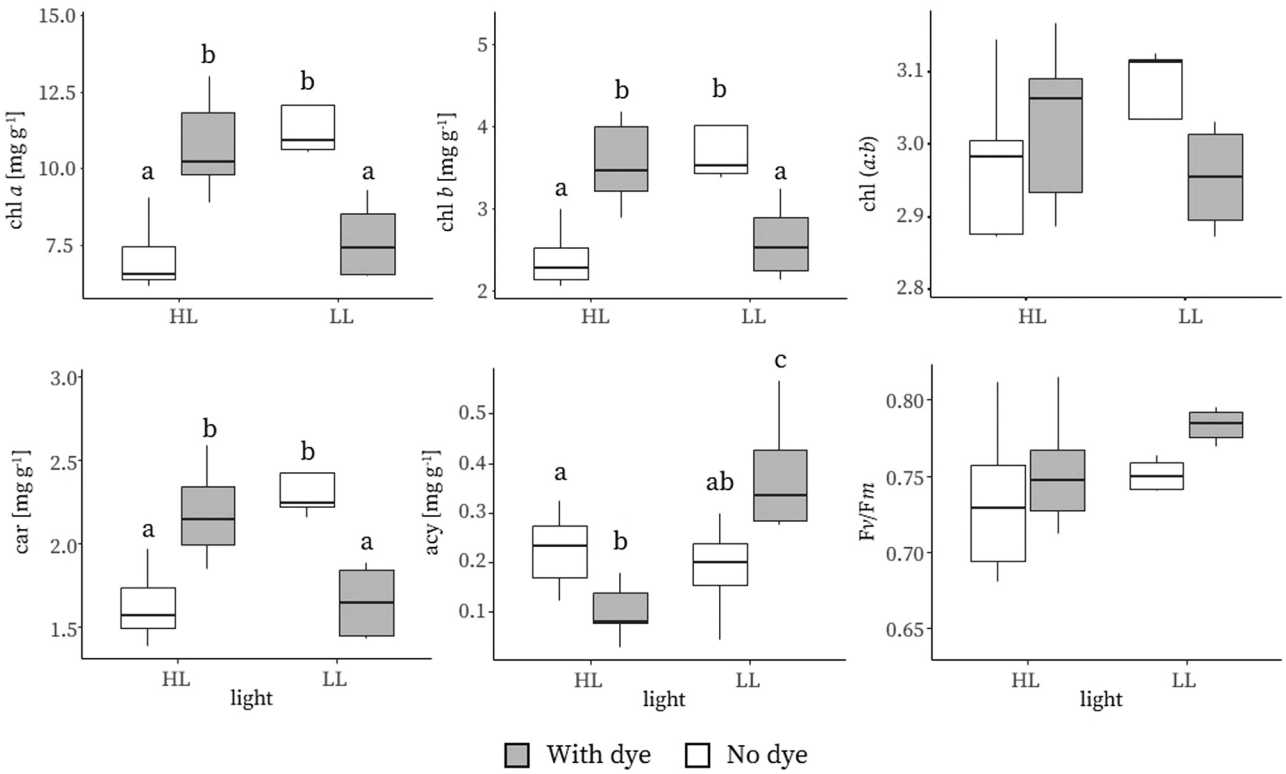

Fig. 5. Boxplots of physiological traits: Contents of chlorophyll, carotenoids and anthocyanins, chlorophyll *a* to *b* ratio (chl (*a*:*b*)) and Maximum Quantum Yield (F*v*/F*m*), in the four treatments. Coloured boxplots show the treatments with dye. Letters indicate significant differences between treatments (Tukey's HSD, $P < 0.05$). acy – anthocyanin; car – carotenoids.

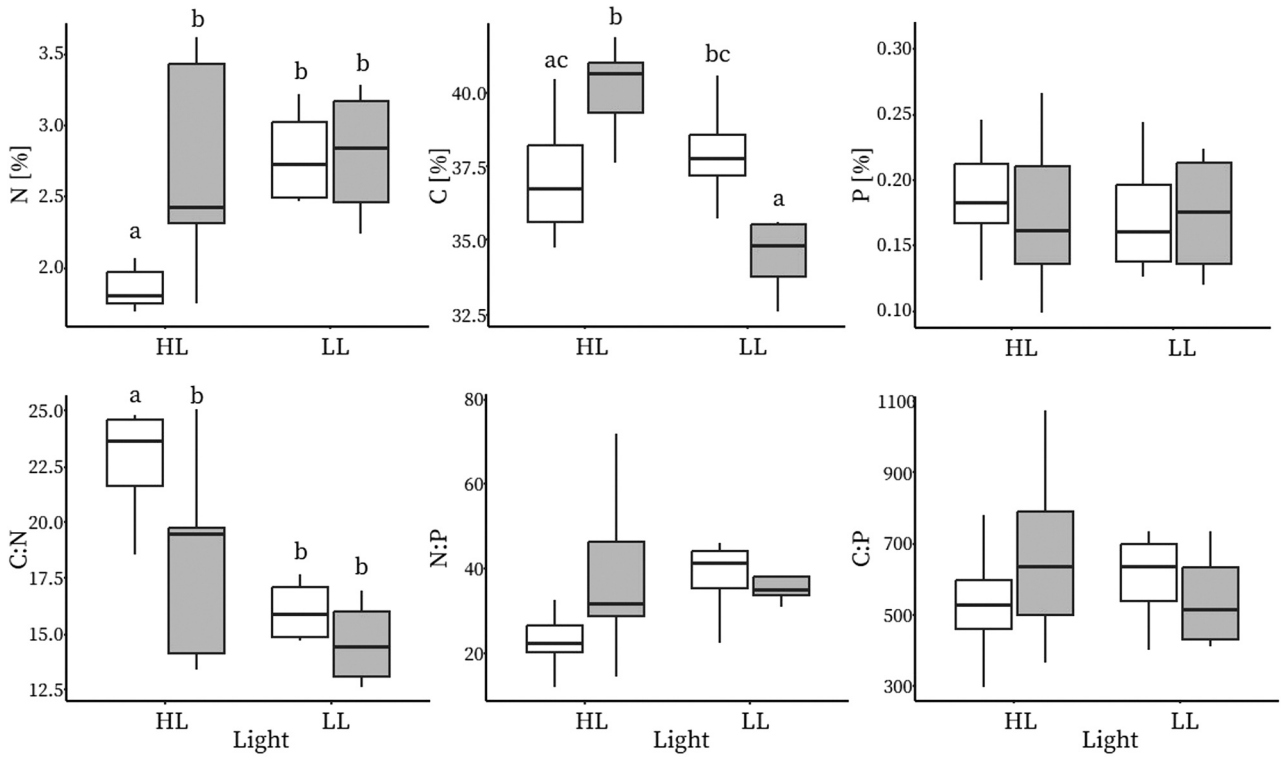

Fig. 6. Variability of carbon (C), nitrogen (N) and phosphorus (P) content provided as [%] based on dm, and stoichiometry expressed as molar ratios for each treatment. Letters shows significant differences between treatments (Tukey's HSD, $P < 0.04$).

seedlings showing plastic responses to low light, typically decrease their root mass fraction (RMF). LLD plants showed chlorophyll and carotenoid levels comparable to HL plants. Yet under the very low light conditions here, this rather means that the plants were incapable of increasing their photosynthetic pigments to enhance photosynthesis and thus growth. The fact that plants in the LLD condition also had the lowest C content confirms this interpretation, showing that carbon fixation was hampered. However, no effect on the nitrogen or phosphorus content was observed, probably because plants grew under non-limiting conditions for these macronutrients.

4.4. Dye application at different light intensities

Comparing the morphological traits across all treatments, we see that *M. heterophyllum* had adapted its growth strategy in favour of a higher main shoot to total shoot length (LR) at moderate and low light levels, and that high light levels enhanced branching. Plant performance at HLD and LL was often comparable to that at HL, in line with the low light adaptation known from this species (Hussner et al., 2010). The allocation to belowground biomass (BGF) tended to increase with light intensity. Based on our observations also in the field this might show that the plant tries to monopolise the available space with its roots, facilitating its establishment and dominance in invaded channels.

Our study revealed many interaction effects showing that the response of plants to dye treatment depends predominantly on the available light intensity, and little or not on light quality, partially confirming our first hypothesis.

The only trait that was solely affected by dye was the allocation to leaves in apical shoots (LMF), which was lower in the presence of dye at both light intensities. This is at first an unexpected result. The LMF is, among other traits such as the specific leaf area (leaf biomass divided by leaf area), in general a good predictor of the RGR of plants (Pérez-Harguindeguy et al., 2013). This is not the case for our plants. Maybe dye treatment enhanced the internode length or stem biomass. Still, the LMF remained high with around 0.7 also in the dye treatments, additionally plants have photosynthetic pigments in the stems, so these differences might not be that important for the plants.

Even if dyes based on mixing two or more colours have been developed and marketed over the years, blue dyes are nowadays most frequently used for algae and plant control for both aesthetic and cost reasons (Newman and Duenas, 2010). Hence, we showed that the absorbance spectrum of Brilliant Blue FCF only marginally overlapped with the absorbance region of chlorophylls in *M. heterophyllum*. In our experiment, the change in light quantity and quality had not affected the chl (*a*:*b*) ratio, that did not significantly differ between plants grown with and without dye. The chl *b* content did not differ between HLD and LL treatments. The dye alone had thus no significant effect on the chl *b* content in plants despite the partial overlap of the chl *b* spectrum with the dye spectrum around 640 nm, whereas any rise in chl *b* and consequently any lowering of the chl (*a*:*b*) ratio would have indicated an adaptation of the photosynthetic apparatus due to leaves receiving less blue and red light (Lichtenthaler et al., 2013, 1981).

Neither the maximum photosynthetic yield differed between treatments, evidenced by the F*v*/F*m* values obtained after dark adaptation. This is consistent with other microcosm studies showing that spectral changes caused e.g. by dyes might not hamper the growth of plants (Li et al., 2021). Further, possible short-term acclimation plasticity of a plant depends on its general adaptation to high or low light environments (Serôdio et al., 2017; Sharon et al., 2009)

A higher LDMC, as observed in the HL treatment, may be a sign of stress and the HLD treatment, with the concomitant reduction in light intensity, might even have benefited the plant. Such a situation has been pointed out in another study on dye treatment to manage the shoreline IAAP *Crassula helmsii* (Denys et al., 2014). The facilitation of growth of submerged species caused by light reduction has also been observed under other circumstances. Light reduction facilitated the growth of *C. demersum* in the presence of the free-floating *Lemna gibba* (Szabó et al., 2024). The application of dye in the water would for *M. heterophyllum* be a counter-effective management technique as it may act as a protection for the plant against too high irradiance. These findings confirm our second hypothesis.

Overall, our results argue in favour of a dye spreading when the light levels are already low in the ecosystem, but not during summer, when high light intensities dominate. Other studies proposed dye spreading before the plants start to grow, so that the plants are not yet high enough to capture the light they need to develop (Buglewicz and Hergenrader, 1977; Lynch Jr., 2006). If shade due to any colour change in light quality appear to work on the control of *E. canadensis* biomass for instance (Newman and Duenas, 2010), a recent mesocosm study showed that dye effects on invasive macrophyte species can be species-specific (Crane et al., 2022) and that this management strategy is not always the best choice. Our study underlines this point based on the findings of the strong interaction between available light intensity and the presence of dye on *M. heterophyllum*. Available light quantity influenced the performance of the plant, while changes in light quality by the applied dye mixture had little or no effect.

Beyond all management strategies, it should not be forgotten that dye application to aquatic ecosystems may affect native and non-invasive species, as well as other organism groups and thus global ecosystem functioning (Eicher, 1947). For the food dyes used in our study, mainly toxicological data for human consumption and ecotoxicological risks for aquatic systems from treated wastewater are available (Borzelleca et al., 1990; El-Wahab and Moram, 2013; Tiron et al., 2020), showing effects on aquatic organisms at concentrations much higher than those used in the current management approaches in France. However, few chronic or long-term ecotoxicological risk assessment for aquatic organisms have been carried out to date. Even less is known about the effects of dye degradation products or their association with sediments and suspended matter. Ozonation strongly increased the toxicity of brilliant blue E133 towards the key zooplankton species *Daphnia magna* (Tiron et al., 2020), showing the need for a more detailed ecotoxicological risk assessment for the aquatic environment. Maybe new dyes interfering more with the required light quality of nuisance plants can be developed, but they need to be both cost-efficient and non-toxic to the environment.

5. Conclusion

The high morphological and physiological plasticity of *M. heterophyllum* to different light intensities in the presence or absence of dye, as found in our study, caution against a general application of dye as management strategy for this species, especially as changes in light quality were not sufficient to change plant physiology. During summer, the application of dye in the ecosystem may protect the plant from damaging light intensities. The application of dye might be an option to attenuate already very low light intensities in order to weaken the plant. Currently ongoing field applications in France in open waterbodies such as canals and only during the growing season may not sufficiently lower the available light to reduce efficiently the performance of *M. heterophyllum*. The low light intensities obtained in our microcosm experiment in the LLD treatment correspond e.g. to winter conditions or to periods of heavy rainfall in Northern part of France, coinciding with high-water periods, during which the dye products in open waterbodies may rapidly be diluted. Consequently, a high-water turnover might reduce if not completely remove the potential effects of this type of management. In conclusion, this type of management should not be included when defining strategies to control *M. heterophyllum*.

CRediT authorship contribution statement

Mohamed A. Mahdjoub: Writing – review & editing, Investigation,

Formal analysis. **Hélène Groffier:** Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Elisabeth M. Gross:** Writing – review & editing, Validation, Supervision, Funding acquisition, Conceptualization. **Simon Devin:** Writing – review & editing, Validation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aquabot.2024.103809.

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