Experimental evidence for the decline of submerged vegetation in freshwater ecosystems by the invasive Chinese mitten crab (Eriocheir sinensis)

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Abstract

The Chinese mitten crab (*Eriocheir sinensis*) is a damaging invader which is designated as a species of Union Concern within the European Union. A negative impact of the crabs on macrophyte vegetation in lowland rivers is suspected but not yet proven or quantified. We have performed a mesocosm study that combines a density gradient of Chinese mitten crabs (0, 0.3, 1.0 and 2.5 ind. m^{-2}) with chemical stress (2350 µg EDTA L^{-1} + 258 µg glyphosate L^{-1}) or light limitation stress (-70% irradiance compared to control) on water plants (*Myriophyllum spicatum*). The results clearly demonstrate that the crabs are capable of removing plant shoots effectively which can lead to a complete elimination of the vegetation. Generally, the higher the crab density, the sooner the plants started to disappear and the sooner the vegetation was completely removed. Additional light and chemical stress accelerated this process: plant disappearance at a crab density of 0.3 ind. m^{-2} compared to 1.0 ind. m^{-2} in the control treatments. Video recording, plant strength and crab pinch strength measurements and stable isotope signatures of $\delta^{13}C$ and $\delta^{15}N$ in the Chinese mitten crabs and their possible food sources showed that directly eating the plants is causing only minor damage to the plants. Most damage comes from the movement of the crabs and crab-crab interactions during which they use their chelae to grasp the shoots. We conclude that a decline of vegetation as a consequence of Chinese mitten crab behaviour can be a realistic scenario in freshwater ecosystems and warrants close attention and monitoring. Being primary producers and ecosystems engineers, macrophytes are key species in these ecosystems, whose services are lost when they disappear and are difficult to restore.

Introduction

Invasive alien species (IAS) are major drivers for the loss of biodiversity and associated ecosystem services. The economic costs of these invasions, in Europe alone, are estimated to be 12 billion euro each year, with a strong likelihood to increase in the coming years (European Environment Agency (EEA) 2012). Not surprisingly, applied ecological research on IAS populations is considered as one of the most urgent nature conservation issues by the EEA. Member states are obliged to take measures to eradicate these species. The Chinese mitten crab (*Eriocheir sinensis* H. Milne-Edwards (Decapoda: Varunidae)) is one of the 49 species listed in EU Regulations (EU Regulation No 1143/2014). Since the first observation of the Chinese mitten crab in the German River Aller in 1912, this IAS has spread rapidly throughout many parts of Europe.

In vegetated lowland streams and rivers, aquatic plants provide many ecosystem services (e.g. O'Hare et al. 2018). They play a major role in the evaluation criteria of the European Water Framework Directive (WFD) and/or nature conservation programs (e.g. Natura 2000 goals). Since the summer of 2013, a nearly total absence of macrophytes is observed in a large part of the Grote Nete river in Belgium, where previously plant growth was abundant. Chinese mitten crabs were observed in the Nete catchment, but not exclusively in the section where the macrophytes were lost (VMM 2015; VMM 2017). These studies however, do not mention crab densities, which may have varied in different parts of the catchment. Chinese mitten crabs are omnivorous and opportunistic and feed on almost any organic food source they can find, including living macrophytes (Jin et al. 2001, 2003; Mao et al. 2016). Crayfish in general can threaten the development of submerged macrophytes (van der Wal et al. 2013). This may pose a threat to the resident flora and fauna and may lead to loss of biodiversity (Rogers 2000; Wójcik et al. 2015). Burrowing activities also often make river beds and banks more susceptible to erosion which can

induce resuspension of sediment, increase in turbidity and decrease in light availability (Bouma and Soes 2010; Jin et al. 2003; Wang et al. 2017). These effects increase the vulnerability of plants to uprooting, which may subsequently influence the release of nutrients and pollutants from the sediment (Wang et al. 2017). It has also been shown that macrophytes can be more susceptible to herbivory when they are already under stress (Hidding et al. 2016; Wang et al. 2017). The combination of increased environmental stress and an increased crab population in lowland rivers may thus pose a threat to aquatic flora (which could be the case in the aforementioned section of the Grote Nete river).

Increased concentrations of ethylenediaminetetraacetic acid (EDTA) and glyphosate were also often found throughout the Nete catchment, especially near the effluent of waste water treatment plants (VMM 2015; VMM 2017). EDTA is a chelating agent that forms very stable complexes with essential metal ions (e.g. Fe) and major cations such as Ca^{2+} and Mg^{2+}, which can result in limited uptake and ensuing deficiency of these elements in macrophytes (Hangarter and Stasinopoulos 1991). Glyphosate (N-(phosphonomethyl)glycine) is the active compound of various commercially available herbicides. It inhibits an enzyme in plants (5-Enylpyruvylshikimate-3-phosphate synthase, EPSPS) which is a key step in making aromatic amino acids, thereby preventing the synthesis of metabolites, including flavonoids, lignins and other phenolic compounds (Dill 2005). Insufficient phytotoxic evidence however, was found to point to the right cause of macrophyte loss in the Grote Nete river (VMM 2015).

The objective of this study is to investigate whether the activities of invasive Chinese mitten crabs (herbivory and cutting) can cause a decline of native mature aquatic vegetation. To achieve this objective, a mesocosm experiment was conducted in which the decline of vegetation patches was followed under different crab densities, and in combination with different types of abiotic stress factors on the vegetation. The abiotic stress factors (chemical stress and light limitation stress) were inspired by the case study described above (Grote Nete river), and the following hypotheses are put forward: (i) beyond a certain crab density threshold, macrophyte shoots are negatively affected by crab activity such as consuming or cutting plants; (ii) this can result in a decimation of the entire vegetation patch and a hampered regrowth from its root system; and (iii) abiotic stressors on plants (e.g. chemical and light limitation stress) can influence the level of damage caused by crab activity.

Material and Methods

Experimental setup

The experiment took place in 12 circular mesocosm tanks (2 m diameter). These tanks (ponds) are located in a greenhouse of the Mesodrome research facility at the University of Antwerp. The greenhouse is a semi-controlled environment in a sense that daylight (length and intensity) and temperature are natural, but other influences such as precipitation, wind etc. are controlled. The experiment was conducted between April and June 2017, which is during the growth period of the vegetation and during the anadromous migration of the juvenile crabs used in the experiment.

A layer of 5 cm coarse (0-2 mm) commercially bought river sand was added to each tank. Tap water was added to create a water depth of 0.5 m (sand bulk density = 1.97 g dry weight (DW) cm^{-}

^{3}; sand:water volume ratio = 0.1). Water quality parameters PO_4^{3-}, TDIN, pH, electric conductivity (EC) and dissolved oxygen were measured at the beginning of the experiment and subsequently monitored once per month (Table 1). A colorimetric segmented flow analyser was used for nutrient analysis (SAN++, Skalar, Breda, The Netherlands) and a WTW Multi 3430 SET F multimeter (Weilheim, Germany) for pH, EC and O_2 measurements. Water temperature was logged continuously with an iButton Hygrochron Temperature/Humidity Data Logger (Maxim Integrated Products, Sunnyvale, CA, USA). Light irradiance above the water surface was logged continuously with Hobo data loggers (Onset, S-LIA-M003, PAR Sensor). In the middle of each tank, a plastic tray was placed (33×41×10 cm). This tray was filled with the same sand as in the rest of the tank. The top was sealed with a mesh wire with a mesh size of 25 mm so that crabs could not fully dig into the sand to avoid uprooting, though their legs and chelae could still access the root system to a certain extent. In each tray 150 shoots of commercially bought *Myriophyllum spicatum* L. (Haloragaceae) were planted, with a combined weight of 90.0 ± 15.9 g fresh weight (FW) (7.5 ± 1.3 g DW). Furthermore, ceramic flower pots were added as a shelter for the crabs (one pot for each crab to avoid competition), and an air stone was installed to aerate the tanks. Finally, leaf litter consisting of 5 common tree species, *Quercus robur* L. (Fagaceae), *Q. rubra* L., *Fagus sylvatica* L. (Fagaceae), *Castanea sativa* Mill (Fagaceae) and *Populus*×*canadensis* Moench (Salicaceae), was added to provide an alternative food source for the crabs. Tree leaves were oven-dried for 72 h at 70 °C and then pre-conditioned for 1 week in pond water. The leaves were divided over 12 plastic trays (18×33×10 cm) and brought into the 12 tanks (650 g FW (163 g DW) per mesocosm). The trays were covered with a mesh wire (mesh size 25 mm) to keep the leaves in the trays and avoid floatation. Two holes of 5x5 cm allowed the crabs to easily access the tray. Plants

were allowed to acclimatise, root and grow in optimal conditions for 3 weeks before chemical and light limitation stress were imposed (Fig. 1).

Chemical stress was introduced by adding EDTA and glyphosate together to the first four tanks. EDTA was added as $Na_2H_2EDTA.2H_2O$ at a concentration of 2350 ± 58 µg L^{-1} measured five weeks after introduction (not filtered; Gas Chromatography, ISO standard 16588). Glyphosate was added as Roundup® which had a glyphosate concentration of 258 ± 27 µg L^{-1} measured five weeks after introduction (not filtered; Gas Chromatography, ISO standard 16588). Note that the added concentration of both chemicals at the beginning was higher (5000 µg EDTA L^{-1} and 500 µg glyphosate L^{-1}) since a certain fraction will degrade or can adsorb to surfaces (plastic of the containers, sand grains, organic matter etc.) and become inactive. The resulting measured concentrations are high, but are in the same order of magnitude of values found in the Grote Nete catchment, i.e. 2150 µg EDTA L^{-1} found in effluent of the wastewater treatment plant discharging into the Grote Nete river and up to 140 µg glyphosate L^{-1} found in waterways in Flanders (monitoring data Flemish Environment Agency, VMM)).

Light limitation stress was introduced as a ~70% light reduction to a second series of four tanks by covering the tanks with layers of a white, plastic shade cloth. The imposed light reduction was based on experiments by Barko and Smart (1981) and Zefferman (2004) in which the effect of different shading intensities on *M. spicatum* were tested. They demonstrated that a 70% light reduction resulted in similar shoot numbers and shoot lengths as in the control treatment (Barko and Smart 1981), and a limited reduced biomass and relative growth rate compared to the full light level (Zefferman 2004). Therefore, a 70% reduction can be considered to be stressful for the plants,

without impeding their growth. A small test prior to the experiment in which a comparable range of light reduction was used, showed similar results as the Barko and Smart (1981) and Zefferman (2004) experiments (unpublished data).

The remaining four tanks are the control treatments. One week after the addition of the light limitation and chemical stress, most of the shoots had reached the water surface and started to produce flowers which indicated they had become mature plants. At this moment, crabs were added to the tanks (Fig. 1). Crabs were preselected based on their condition (visual appearance of good health based on mobility, colour, and presence of all limbs) and similarity in size and weight (21.9 ± 2.1 g FW). No gender selection was made. For each stress treatment and control, 1 tank received no crabs (reference within each treatment), 1 tank received 1 crab, 1 tank received 3 crabs and 1 tank received 8 crabs. This corresponds to crab densities of 0 ind. m^{-2}, 0.3 ind. m^{-2}, 1.0 ind. m^{-2} and 2.5 ind. m^{-2}, respectively. Because related omnivorous Sesarmid crabs are known to supplement their diet with animal tissue such as fish carcasses, to provide a major part of their dietary nitrogen requirements, crabs were offered fish meat weekly (± 1 g anchovies per crab) to ensure a balanced diet and to avoid cannibalism (Thongtham and Kristensten 2005; Kristensen et al. 2010). Each two to five days, plant shoots that were cut by the crabs and floated in the tanks were retrieved, oven-dried at 70°C for at least 48 h and weighed. The experiment lasted for 25 days because at this time most of the vegetation in the stressed treatments with the highest crab density was gone.

After the experiment, the crabs were retrieved and weighed again, then stored frozen at -20 °C until further analysis. The remaining plant biomass in the trays was cut by hand just above the mesh wire, oven-dried at 70 °C for at least 48 h and weighed. The sum of the remaining plant

biomass and the floating plant biomass collected during the experiment gives the total plant biomass. All 12 trays were then together transferred to a tank with fresh tap water and in optimal conditions. The water quality was the same as at the start of the previous experiment (Table 1) and was not monitored further. Regrowth of the shoots was visually evaluated weekly during 7 weeks (short term period) after which the biomass was determined again by cutting all shoots just above the mesh wire, drying and weighing. The presence of flowers was noted. The trays were then placed back into the tank for a new, long term period of regrowth. After 1 year, all biomass was determined again by cutting all shoots just above the mesh wire, drying and weighing. The presence of flowers was again noted.

Plant nutrient concentrations

To compare the nutrient condition of the plants between the treatments, total phosphorus content of plants from all 12 tanks was determined at the end of the experiment according to Walinga et al. (1989): samples were digested with H_2SO_4, HOC_6H_4COOH and H_2O_2 and analysed on a colorimetric segmented flow analyser (SAN++, Skalar, Breda, The Netherlands). Total carbon and nitrogen content of plants from all 12 tanks was determined during the procedure for stable isotope analysis (see below).

Crab pinch test

To compare the force of the crabs with the resisting strength of the plants, crab pinch strength of 40 individuals of each sex and of different body mass was measured using a Charge Amplifier (Kistler Instrumente AG, Type 5995, Winterthur, Switserland). The crabs were preselected based on their condition (visual appearance of good health). Five pinches were measured for each crab,

using 1 chela at the time, and randomly alternating between chelae. Maximum pinch strength (out of 5) and average pinch strength (n = 5) are plotted against the crab biomass (g FW).

Plant shear test

Shearing tests were used to measure the stem resistance to fracture. For each treatment, the test was undertaken on 5 stems. Tests were conducted with a leaf-cutting device following Ang et al. (2008), mounted on a universal testing machine (Instron 5942, Canton, MA, USA). A single stainless-steel blade of a straight razor (Dovo, Solinge, Germany) was mounted on the moving head of the testing machine with an approach angle of 20°. The stem was positioned on 2 supports (with a 15 mm span), with the blade being equidistant from the 2 supports. The blade was moved downward at a constant speed of 10 mm s^{-1} shearing the stem into 2 parts. The maximum load applied to the leaf (N) was recorded with a frequency of 10 Hz and used to calculate the maximum force to shear the stem (N) and the shear strength (maximum force divided by the cross-sectional area, MPa). To take into account the high proportion of lacunae in stems of aquatic plants, the cross-sectional area used to calculate shear strength was the effective cross sectional area, calculated as the difference between total cross-sectional area and total lacuna area. This correction is used to quantify the effective cross-sectional area supporting forces in the shearing tests. To measure cross-sectional area of the stem sheared, thin cuts adjacent to the shearing plane were made. Images of the cuts were taken using a binocular and a digital camera and analysed with Leica Application Suite (v4.3, Leica Microsystems, Switzerland) to calculate total stem cross-sectional area (mm^2) and total lacuna area (mm^2).

Stable isotope analysis

To calculate the relative importance of the food sources, stable isotope signatures ($\delta^{13}C$ and $\delta^{15}N$) were measured for all different 'food web' components in the mesocosms; the crabs as consumers and *M. spicatum*, leaf litter and supplementary fish as food sources. Crabs were dissected to collect their gill tissue, which was subsequently freeze dried using a Heto PowerDry LL3000 (Thermo Scientific) and ground using a Retsch mixer mill (MM301). Only gill tissue was extracted for isotope analysis, due to its low turnover rate compared to other tissues (Lorrain et al. 2002). Dried *M. spicatum* shoots and leaf litter were ground with a Retsch ZM200 ultra-centrifugal mill. Powdered samples were weighed in silver cups and acidified with one drop of 5% hydrochloric acid, to remove any carbonates (Jacob et al. 2005), and oven-dried at 80 °C for 4 h after which the cups were folded and analysed. Sample weights were 5 mg for leaf litter and macrophytes, and 1 mg for crab tissue. Carbon and nitrogen contents were measured using a Flash EA 1112 Elemental Analyzer (Thermo Finnigan). The ^{13}C and ^{15}N stable isotope signatures were measured using a Delta V Advantage isotope ratio mass spectrometer (Thermo Finnigan) that was coupled, via a ConFlo III interface (Thermo Finnigan), to the Elemental Analyzer.

Video recording

Two crabs similar to ones used in the experiment, and a patch of 15 mature *M. spicatum* shoots were placed in a 100 L aquarium. Crab movements were observed with a camera (Sony CX550) during 3 consecutive 12 h day and 12h night cycles (using the infra-red mode of the camera). The aim was to record the interaction of the crabs with the plants in a qualitative way to observe whether crabs climb into the patch, if they use their chelae to grasp the shoots and whether they cut the shoots directly.

Statistical analyses

First, the impacts of the different treatments and crab densities on plant biomass is compared. An empirical model for the fraction of biomass remaining (FBR) as a function of time (t) is proposed:

$$Fraction\ of\ Biomass\ Remaining = \frac{1}{1+e^{-\delta[\tau-t]}} \qquad (1)$$

The FBR-model (Eq. 1) computes the fraction of biomass remaining after a time t of running the experiment. At time t = 0 s, the FBR equals one. When the experiment duration t approaches τ [units s], the FBR starts to decay at an exponential decay rate δ [units s^{-1}]. For t significantly larger than τ the FBR converges to zero. The larger is τ, the longer it takes for the system to start declining. Every dataset for FBR as a function of time from each mesocosm tank will have its own set of parameters τ and δ. The parameters are obtained by fitting the dataset to the model. By doing so the different stress impacts and crab densities can be compared using the corresponding parameters τ and δ. More detailed information, and every fit output is given in online supplementary material. All tests were performed in R 3.3.2 (R Development Core Team 2016).

Secondly, differences in crab fresh weight before and after the experiment and between stress treatments were tested. Mesocosm tanks with different crab densities but the same stress treatment were used as 4 replicas. Data were first checked for normality distribution via Shapiro-Wilk tests and visual inspection of Q-Q plots. Not normally distributed data were tested for significant differences among groups using Kruskal-Wallis tests and Dunn's post hoc tests. Normally distributed data were checked for equality of error variances using Levene's tests. Significant differences among groups were assessed using one-way ANOVAs with Tukey post-hoc tests for

equal variances or Welch tests and Games-Howell post-hoc tests for non-equal variances. Interaction could not be tested because of the experimental design, though this is not expected to happen and if it occurs it is included in the residual variation of the applied test. Additionally, differences in crab pinch strength between sexes was tested following the same statistical procedure as described above. Relationships between crab pinch strength and crab biomass were defined using Pearson correlation coefficients and tested for significance using two-tailed t-tests. All tests were performed in R 3.3.2 (R Development Core Team 2016).

Thirdly, mesocosm tanks with different stress treatments but with the same crab density were used as 3 replicates to test the effect of crab density on plant biomass. Mesocosm tanks with different crab densities but the same stress treatment were used as 4 replicas to test for differences in plant nutrient concentrations, plant shear force, and total plant biomass between different stress treatments. The same statistical procedure was used as described above for testing differences in crab characteristics.

Finally, stable isotope data was used to calculate the relative importance of the food sources in the crab's diet. The stable isotope mixing model 'Stable Isotope Analysis in R' (SIAR, Parnell and Jackson 2013) package (version 4.2) was used under 3.3.2 (R Development Core Team 2016) for this purpose. This Bayesian mixing model incorporates variation in the stable isotope ($\delta^{13}C$ and $\delta^{15}N$) signatures of the different food sources and the consumer and subsequently calculates density plots of credible intervals for the estimated dietary proportion of each food source (Parnell et al. 2010; Parnell and Jackson 2013). Furthermore, this mixing model allows the incorporation of food source C and N content, thereby enabling better resolution when analysing food sources with vastly different C and N concentrations (Phillips and Koch 2002), e.g. for omnivores that may

consume both nitrogen poor detritus and nitrogen rich animal material. Before incorporation in the model, the food source carbon and nitrogen stable isotope signatures were corrected for trophic fractionation by adding 0.8‰ and 2.6‰ respectively, for animals of which only muscle tissue was analysed (McCutchan et al. 2003).

Results

Impacts of the different stress treatments and crab densities on plant biomass

The results clearly demonstrate that the crabs were able to remove plant shoots effectively, leading to a complete elimination of the plant patch (Fig. 2). If no crabs were present (reference tanks), no shoots were cut. When crabs are present, many shoots were cut and the amount and timing was influenced by crab density and additional stress treatment. The fraction of biomass remaining as a function of time follows a sigmoid pattern (see Fig. S1-S3 in online supplementary material). For all experiments (light limitation- and chemical stress, and the control treatment), the threshold value (τ) exponentially decreased as a function of crab density with an exponential coefficient α_{light} and $\alpha_{chemical}$ and $\alpha_{control}$ respectively (see Fig. S5, S7, S9 in online supplementary material). So, the more crabs, the sooner the system will start declining: e.g., having 0.3 ind. m^{-2} in the control treatment (Fig. 2A), most biomass was still present by the end of the experiment (reduction of only 5%), whereas an increasing crab density in the control treatment led to a removal of all biomass by the end of the experiment (reduction of 100%), which occurred sooner at the highest crab density (at day 25 for 1.0 ind. m^{-2} and at day 12 for 2.5 ind. m^{-2}). No significant correlation between the rate of collapse (δ) and crab density was observed once the threshold was reached. When compared to the control experiment, there was a significant ($\alpha_{control}$ = [0.82 ± 0.11] $ind.^{-1}$; α_{light} = [2.30 ± 1.29] $ind.^{-1}$; $\alpha_{chemical}$ = [1.70 ± 0.55] $ind.^{-1}$) impact of light limitation stress and chemical

297 stress on the exponential decrease of (τ) as a function of crab density. In other words, when
298 compared to the control treatment, the start of vegetation decline will be sooner in case of light
299 limitation stress or chemical stress for the same crab density (Fig. 2B, C). No significant difference
300 was observed between the impact of light and chemical stress on macrophyte biomass
301 development.
302
303 *Crab characteristics*
304 Crab mortality during the experiment was low. Only 3 crabs were found dead in the first few days
305 of the experiment and were immediately replaced by a similar individual. The fresh weight of the
306 crabs at the end of the experiment had increased significantly from 21.9 ± 6.5 g to 33.6 ± 10.7 g
307 FW during the experiment of 25 days (Kruskal-Wallis test; $X^2_{df=1} = 29.2$; $p < 0.001$). Male crabs
308 had a significantly higher average and maximum pinch strength than females, proportional to their
309 body mass (Welch test, maximum strength; $F_{df=1,59} = 19.5$; $p < 0.001$, Average strength; $F_{df=1,61} =$
310 18.6; $p < 0.001$). Additionally, both pinch strengths showed a significant positive relationship with
311 body mass for both male and female crabs (Fig. 3).
312
313 *Plant characteristics*
314 Visually, the plants in each of the mesocosms appeared healthy at the end of the initial growth
315 period. They filled the entire water column, produced flowers and had no signs of necrosis or other
316 visual symptoms of aberrant growth. Physiologically, plants grown under light limitation stress
317 had significantly higher concentrations of N (29.8 ± 2.4 mg N g DW^{-1} (mean ± SD), one-way
318 ANOVA; n = 4; $F_{df=2,9} = 21.8$; $p < 0.01$) and P (2.4 ± 0.4 mg P g DW^{-1}, one-way ANOVA; n = 4;
319 $F_{df=2,9} = 15.2$; $p = 0.01$) than the plants from the control (15.7 ± 3.5 mg N g DW^{-1} and 1.1 ± 0.1 mg

320 P g DW^{-1}; n = 4) and chemical treatment (17.4 ± 5.2 mg N g DW^{-1} and 1.4 ± 0.3 mg P g DW^{-1}; n
321 = 4). Because carbon content did not vary significantly among the treatments, the C:N and C:P
322 ratios of plants grown under light limitation stress (C:N = 12.7 ± 0.2, C:P = 136.2 ± 9.3) were
323 consequently lower than in the control (C:N = 19.3 ± 1.5, C:P = 287.5 ± 27.3) and chemical
324 treatment (C:N = 16.6 ± 2.2, C:P = 210.2 ± 17.0), although these differences were not significant.
325 Plants grown under light limitation stress also had a significantly lower shear strength (0.17 ± 0.03
326 MPa) than the ones from the chemical treatment (0.26 ± 0.02 MPa, one-way ANOVA; $F_{df=2,12}$ =
327 4.3; p = 0.039). The maximum force to shear the stems of the control plants (0.66 ± 0.14 N) was
328 significantly higher than from plants from the light treatment (0.40 ± 0.1 N, Kruskal-Wallis test;
329 $X^2_{df=2}$ = 5.9; p = 0.049).
330
331 Total plant biomass (see methods) recovered from the reference tanks at the end of the end of the
332 experiment varied between 23.0 - 33.6 g DW, depending on the abiotic stressor: control > chemical
333 stress > light limitation stress (Fig. 4). Treatment type therefore did have a significant effect on
334 total plant biomass (one-way ANOVA; $F_{df=2,9}$ = 4.62; p = 0.042), with light limited plants having
335 a significantly lower biomass than plants from the control treatment (p = 0.038). Within each
336 treatment, total plant biomass was not significantly affected by crab density (one-way ANOVA;
337 $F_{df=3,8}$ = 1.46; p = 0.30). Nevertheless, large variations in total plant biomass between crab density
338 treatments exists, e.g. more than 50% less biomass if 2.5 ind. m^{-2} are present compared to the
339 reference situation (Fig. 4).
340
341 *Reconstructing crab diet*

342 Clear differences in $\delta^{13}C$ and $\delta^{15}N$ signatures could be observed between the crabs and the three
343 measured possible food sources (Fig. 5). In Fig. 5 the crabs are positioned at the upper left part of
344 the biplot, being relatively depleted in ^{13}C, but with high $\delta^{15}N$ signatures. Terrestrial leaves were
345 the most depleted in ^{15}N, followed by *M. spicatum* with slightly higher values and by supplemented
346 fish which was the most enriched in ^{15}N. Terrestrial leaves were the most depleted in ^{13}C in
347 comparison to *M. spicatum* and fish, which both had comparable $\delta^{13}C$ signatures.
348
349 Mixing model calculations showed that most of the crabs' diet consisted of terrestrial leaves and
350 fish meat, with a variable but smaller proportion consisting of *M. spicatum* (Table 2). The 5^{th}
351 percentile value of *M. spicatum* was always 0, indicating that the chance of *M. spicatum* not being
352 consumed was at least 5% (Table 2). The only treatments in which *M. spicatum* constitutes a
353 potential important food source (i.e. have a potential maximum contribution higher than 50%),
354 also had the highest uncertainty due to the low number of crabs being measured, inherent to the
355 crab density imposed. Differences in potential food sources between treatments and crab densities
356 were not consistent.
357
358 *Regrowth experiment*
359 On day 16 of the regrowth experiment, the first shoots reappeared in the patches which came
360 originally from the reference tanks (i.e. with no crabs). On day 47 (short term period), flowers
361 appeared again in the new-grown patches and all shoots were cut at that day. Biomass mainly grew
362 back in the patches that came originally from the reference tanks (Table 3), and one shoot grew
363 back in the tray that used to stay in the tank with the chemical treatment and with the highest crab
364 density. No plant regrowth was observed in any other trays (Table 3). After one year of regrowth

(long term period), the biomass of the patches that came originally from the reference tanks all had flowers and biomass was similar to the biomass in the previous year (Table 3, Fig. 4). All other trays only had a few shoots that grew back and not even all of them managed to produce flowers (Table 3).

Discussion

This study has shown that the Chinese mitten crabs can negatively impact freshwater flora. One crab (0.3 ind. m^{-2}), in combination with light limitation or chemical stress, resulted in complete eradication of the experimental vegetation patch in less than 25 days. Within the constraints of our experiment, this crab density could be considered as a threshold value above which vegetation is likely to be impacted by the crabs in presence of other stress factors. The value is similar to the density threshold of 0.25 – 0.5 ind. m^{-2} established by Jin et al. (2001) for macrophytes in the crabs' home territory in China. In all our experiments with 1.0 and 2.5 ind. m^{-2}, vegetation was severely diminished or completely gone within 25 days. However, since this is not a field study, the parameters presented here do not account for the many *in situ* variables which could mitigate or exacerbate the impact, including: the daily movements and seasonal migration of the crabs, presence or absence of other food sources, combination and intensity of additional biotic and abiotic plant (and crab) stress factors, and the ratio of plant coverage to crab density.

Locally and during certain periods of time (e.g. during migrations), it cannot be excluded that crab densities exceed the threshold value in the river resulting in the complete disappearance of aquatic vegetation. This is especially likely when the plants are already stressed by other factors (Hidding et al. 2016; Wang et al. 2017) and other food supplies are restricted. In a natural river, contrary to

our experimental conditions, several vegetation patches are present over a much larger area and crabs are free to move around. Such conditions probably gives patches time to recover rather than being continuously exposed to the same crab density (which was the case in our experiment). Exposure time is also dependent on vegetation coverage which may be higher in low-order streams (hence a higher threshold value for a given crab density) and lower in high-order streams (hence a lower threshold value for a similar crab density). In the case of our experiment, the above-mentioned threshold value of 0.3 ind. m^{-2} could be recalculated to the density of crabs per vegetated surface area, instead of per total surface area of the tank, which increases it to 7.4 ind. m^{-2} vegetation. This reasoning corroborates the conclusion of Wang et al. (2017) that plant resilience to disturbance from crabs is reduced in continuous presence of high crab densities. In order to estimate the risk for the vegetation it is advised to report crab density values in relation to the vegetation cover. Note that our experiment was executed with only 1 size class of crabs, and the threshold values observed will probably no longer hold when considering smaller (younger) or larger (older) crabs.

The regrowth experiment demonstrated that following removal by the crabs, plants were hindered in their ability to grow back in the short and long term, even after their optimal (a)biotic conditions were restored. This may point to a hysteresis effect in the critical crab density that removes vegetation and the critical crab density that allows recovery. The mesh wire prevented the crabs from burrowing into the sediment, but it is likely that their chelae and/or legs were able to reach and damage the root system, thereby inhibiting regrowth. Long-term experiments such as those presented herein are needed to simulate river restoration projects in which crab densities are reduced. A (semi) permanent reduction or even total loss of vegetation can have dramatic

411 consequences for the aquatic ecosystem since macrophytes are ecological engineers (Schoelynck
412 et al. 2012) that support several ecosystem functions in the river (Boerema et al. 2014; Carpenter
413 and Lodge (1986); O'Hare et al. (2018); Sand-Jensen et al. (1989). A sudden loss of vegetation
414 may induce sediment erosion, which can be intensified by the burrowing activities of the crabs and
415 which may further inhibit vegetation recovery in the long term.
416
417 Isotope analyses showed that only a minority of the plant biomass is actually assimilated and that
418 leaf litter was the main food source for the crabs. This corroborates the findings of Czerniejewski
419 et al. (2010) and Roswarne et al. (2016) who showed that macrophytes contribute approximately
420 10 - 16% of the crabs' diet, respectively (based on gut-content analyses), and with a mesocosm
421 experiment by Rudnick et al. (2005) showing that the main food source for crabs are tree leaves.
422 The crabs' cutting behaviour, and not consumption, was found to be the main cause of macrophyte
423 shoot removal, which corroborates previous studies on the destructive effects of (invasive) crayfish
424 and crabs (e.g. Jin et al. 2003; Lodge et al. 1994, van der Wal et al. 2013). Crab pinch strength
425 proved to be an order of magnitude higher than what is needed to cut the plant stems. Though
426 video recording did not observe crabs pinching through the stems and so removing macrophyte
427 fragments, crab movement through the vegetation and crab-crab interactions resulted in breaks and
428 snaps in macrophyte shoot tissue (Fig. 6). Therefore, we put forward the hypothesis that crabs
429 mostly do not pinch the shoots at full force, but rather just grasp them. Yet such action may be
430 enough to damage plant cells (trauma), causing the shoot to die locally (necrosis), which results in
431 plant fragments being repelled after which they start floating. The process of necrosis takes some
432 time, which may also explain the time lag between the introduction of the crabs and observation
433 of the first floating shoots. Variation in total macrophyte biomass was found among setups with

different crab densities under the same treatment. This variation can only be explained by a reduced plant growth in tanks where were present, because similar initial biomass was planted, abiotic circumstances were the same within the same stress treatment and crabs consumed only little biomass. Growing and branching potential of the macrophytes decreases with every trauma and repelled shoot. This results in a smaller shoot density, in lower biomass production and a higher risk for further crab damage.

The additional influence of abiotic stress had a significant effect on how fast vegetation will decline. In the presence of chemical stress, the start of vegetation loss appeared sooner in time and led to more severe losses after 25 days even at the lowest crab density, compared to the control treatment. Macrophytes exposed to chemical contaminants such as EDTA, glyphosate and its metabolite aminomethylphosphonic acid (AMPA) are expected to experience stress or injury which could accelerate necrosis in the shoots that were damaged by the crabs (Reddy et al. 2004). Note that the concentrations used in the experiment were at the upper end of what can be found in rivers in, for example, the Nete catchment but are realistic near the mixing zone of effluent discharge or during periods of drought and a reduced dilution effect of contaminants. Also note that we have used Roundup® to add glyphosate, but we did not analyse the adjuvants in the herbicide, which can also be stressful, nor are any interaction or additive toxicological effects between EDTA and glyphosate considered. Plants under the reduced light treatment reacted ambiguously. At the lowest crab density, plants were cut loose much sooner than in the control treatment, while this was much later at the two highest crab densities. The higher nutrient content (i.e. higher N and P content and lower C:N and C:P ratios) of plants grown under the light treatment would indicate that they are more nutritious and more likely to be consumed, if other factors

457 regarding plant palatability such as structural defences or secondary compounds are constant
458 between the treatments (Elser et al. 2000, Gross and Bakker 2012). Additionally, the lower shear
459 strength of these plants would make them easier to cut and more vulnerable to crab pinches than
460 in the other treatments. However, this effect was only clear in case of 0.3 ind. m^{-2} crab density
461 (notably where crab-crab interactions are not present). Additionally, we hypothesize that lower
462 light intensity may have itself had an ambiguous effect on the crabs' behaviour, as it mainly is a
463 nocturnal animal (Gilbey et al. 2008). Darker conditions might decrease the time that crabs hide
464 in the vegetation resulting in a lower plant trauma prevalence during crab-crab interactions, but it
465 might also have increased crab mobility which may result in higher plant trauma prevalence.
466 Specific behaviour experiments are needed to sort this out.
467
468 The impact of the Chinese mitten crab on aquatic ecosystems in Europe remains largely unknown.
469 However, our study suggests that the crab can potentially have a high impact on macrophyte
470 communities if crab densities are large compared to the standing macrophyte biomass. Actual
471 density data are not often published which is a shortcoming to estimate the risk for aquatic
472 vegetation. Yet absolute values of crab numbers caught show an increase across Europe in the last
473 decades, e.g. Baltic Sea region (Ojaveer et al. 2007), Spain (Garcia-de-Lomas et al. 2010), Poland
474 (Normant et al. 2000; Wójcik-Fudalewska and Normant-Saremba 2016), France, Belgium, The
475 Netherlands, Germany and UK (Herborg et al. 2003, 2005). Ecological niche modelling
476 demonstrated that most of Europe is vulnerable to invasion by Chinese mitten crabs and especially
477 rivers flowing into the Mediterranean Sea appear to be a highly suitable habitat (Herborg et al.
478 2007). With climate change, river water temperatures are projected to increase on average by 0.8–
479 1.6 °C by 2100 (some European river systems such as the Rhine, Danube and Rhone even up to

2.1 °C) (Van Vliet et al. 2013), which may favour the spread of the crabs (Herborg et al. 2007). At the same time, abiotic stress to macrophytes caused by climate change is also likely to increase in the near future (Short et al. 2016; Reitsema et al. 2018), which can make plants more vulnerable to crabs (Hidding et al. 2016). Many rivers may become devoid of macrophytes, which is currently the case for parts of the Grote Nete river in Belgium (VMM 2017). This may result in negative consequences for the aquatic system as a whole and makes it hard to reach the Water Framework Directive goals aiming at good water quality by 2027.

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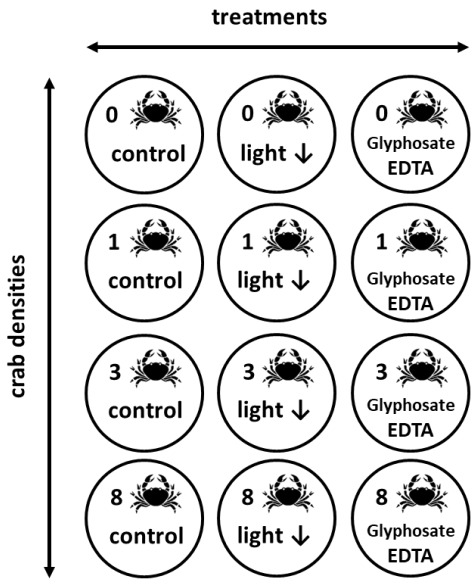

Fig. 1 Experimental setup. Twelve mesocosm tanks were installed combining 4 crab densities with 3 treatments.

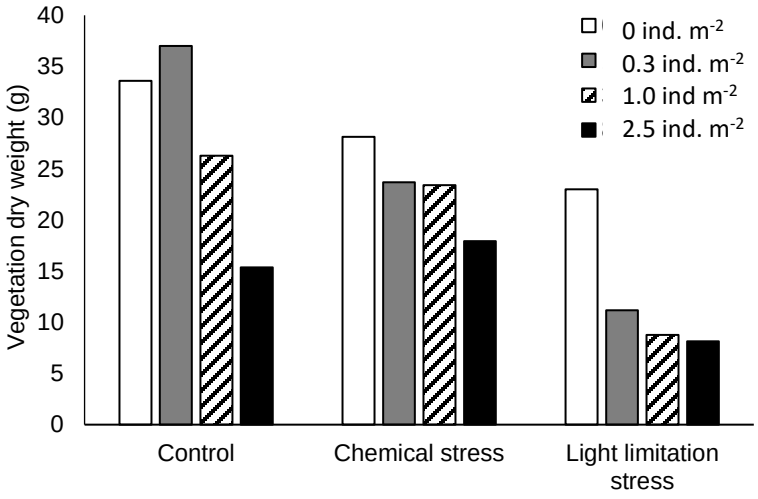

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Fig. 2 Total plant biomass in each of the treatments and per crab density. Total plant biomass is the sum of the floating plant biomass collected during the experiment and the remaining plant biomass at the end of the experiment.

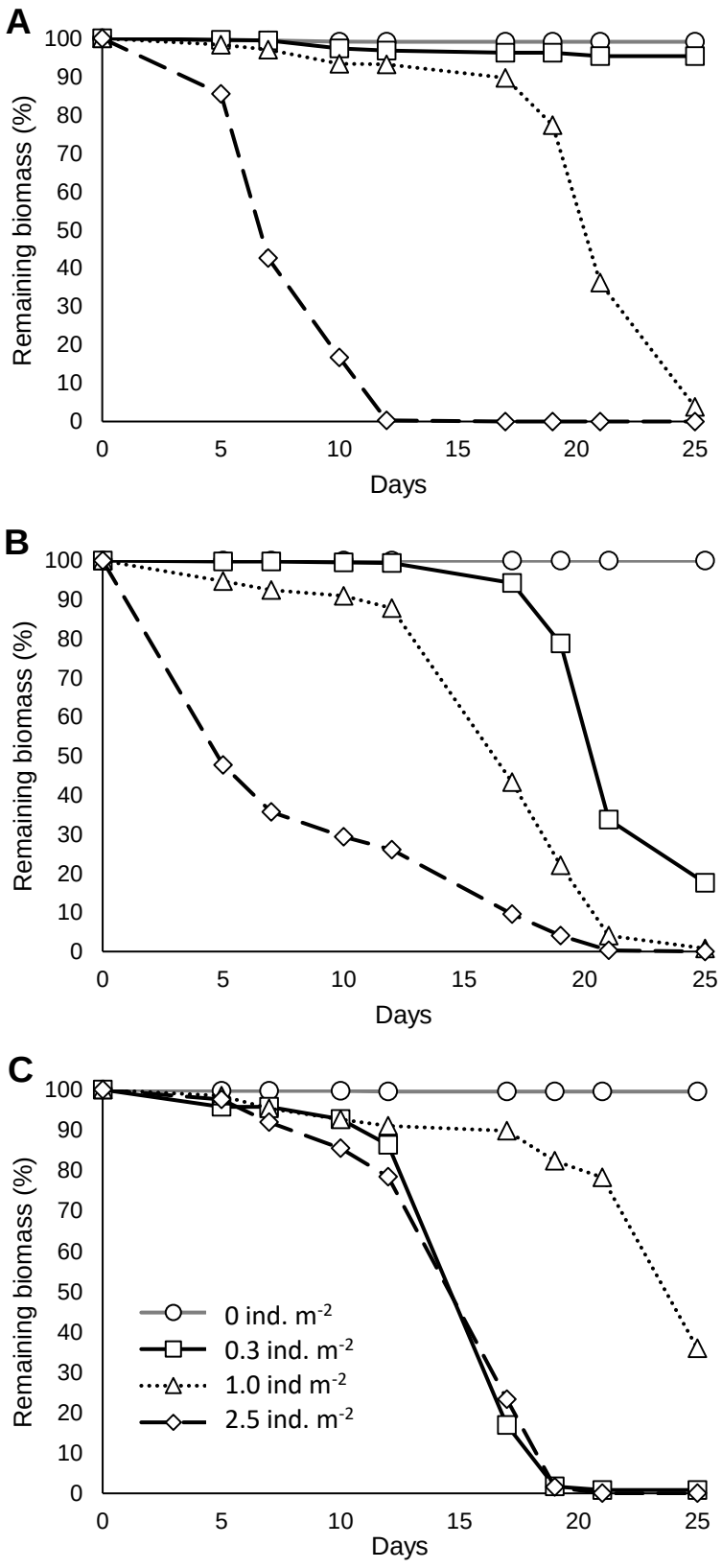

Fig. 3 Reduction of plant biomass in the control (A), chemical stress (B) and light reduction stress (C) ponds with different crab densities.

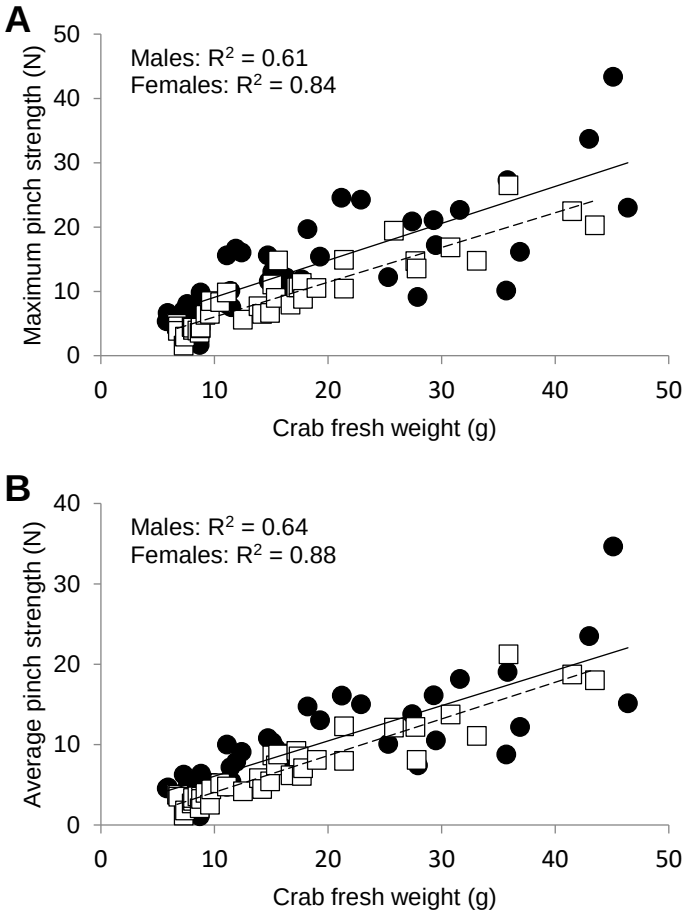

Fig. 4 Relationship between maximum (A) and average (B) crab pinch strength and crab fresh weight (g). Circles and solid trendlines represent male crabs (n = 40), while squares and dashed trendlines represent female crabs (n = 40). All relationships were significant at the $p < 0.01$ level.

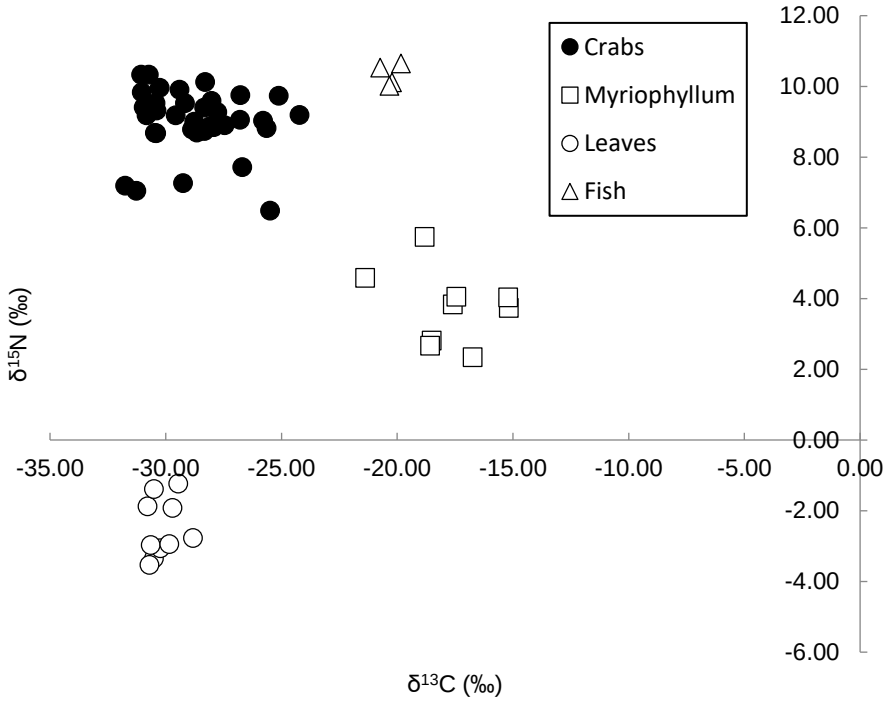

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Fig. 5 Stable isotope signatures of $\delta^{13}C$ and $\delta^{15}N$ of the Chinese mitten crabs and their possible food sources *M. spicatum*, terrestrial leaves and supplemented fish meat.

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Fig. 6 Images of video recording (A-C) and pictures (D-F) of the interaction of 2 Chinese Mitten Crab with a macrophyte patch. (A) A crab grasping the macrophyte with front chelae. (B) The crabs disturbing the macrophyte by clambering on top of it and substantially bending its stems. (C) A crab presumably eating the macrophyte. This goes on for a few minutes in this instance. (D - F) Breaks and snaps (trauma) in some of the macrophytes stems due to crab activities.

692 **Table 1** Abiotic conditions of the tanks per month and per treatment (control, chemical stress and
693 light limitation stress). Data are mean values ± standard deviation (n = 4) for PO_4^{3-}, total dissolved
694 nitrogen (TDIN), pH, electric conductivity (EC) and O_2. Temperature was logged continuously
695 and averaged for the whole month period (± standard deviation). Irradiance was logged
696 continuously and the median of all day-time values was calculated for the whole month period

	PO_4^{3-}	**TDIN**	**pH**	**EC**	**O_2**	**O_2**	**Temp**	**Irradiance**
Control	**(mg P L^{-1})**	**(mg N L^{-1})**	**(-)**	**(µS cm^{-1})**	**(%)**	**(mg L^{-1})**	**(°C)**	**(W m^{-2})**
April	0.02 ± 0.00	3.7 ± 0.0	8.6 ± 0.0	-	106 ± 1	10.3 ± 0.1	15.0 ± 1.0	163
May	<0.02	2.6 ± 0.1	8.9 ± 0.1	712 ± 27	126 ± 5	10.4 ± 0.3	23.3 ± 1.0	157
June	<0.02	0.2 ± 0.0	9.0 ± 0.3	664 ± 38	-	-	22.4 ± 1.0	230
Chemical								
April	0.02 ± 0.00	3.7 ± 0.0	8.6 ± 0.0	-	106 ± 0	10.3 ± 0.1	15.0 ± 1.0	163
May	<0.02	2.1 ± 0.1	9.0 ± 0.1	712 ± 27	131 ± 4	10.8 ± 0.3	22.8 ± 1.0	157
June	<0.02	0.0 ± 0.0	8.3 ±0.3	714 ± 24	-	-	21.6 ± 1.0	230
Light								
April	0.02 ± 0.00	3.7 ± 0.0	8.6 ± 0.0	-	108 ± 1	10.4 ± 0.1	15.5 ± 1.0	164
May	<0.02	2.7 ± 0.1	8.6 ± 0.0	752 ± 40	114 ± 1	9.4 ± 0.1	21.9 ± 1.0	56
June	<0.02	1.2 ± 0.2	8.4 ± 0.2	720 ± 37	-	-	20.9 ± 1.0	59

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Table 2 Potential contributions of the different possible food sources to the crabs' diets. Ranges represent 90% credible intervals (5–95 percentile ranges) with median contribution in parentheses, calculated using the SIAR mixing model. The analysis of the single crab in the control treatment had analytical errors and no data could be generated.

Treatment	**Crab densities (ind. m^{-2})**	***M. spicatum***	**Tree leaves**	**Fish**
Control	0.3	-	-	-
Control	1.0	0 - 0.45 (0.13)	0.35 - 0.81 (0.66)	0.13 - 0.29 (0.21)
Control	2.5	0 - 0.29 (0.05)	0.50 - 0.81 (0.73)	0.17 - 0.26 (0.21)
Light limitation	0.3	0 - 0.62 (0.27)	0.14 - 0.86 (0.52)	0.01 - 0.48 (0.16)
Light limitation	1.0	0 - 0.62 (0.31)	0.10 - 0.78 (0.45)	0.06 - 0.49 (0.22)
Light limitation	2.5	0 - 0.22 (0.05)	0.57 - 0.80 (0.73)	0.18 - 0.25 (0.22)
Chemical	0.3	0 - 0.64 (0.32)	0.06 - 0.78 (0.44)	0.01 - 0.54 (0.21)
Chemical	1.0	0 - 0.47 (0.16)	0.31 - 0.72 (0.63)	0.08 - 0.37 (0.19)
Chemical	2.5	0 - 0.29 (0.06)	0.53 - 0.86 (0.75)	0.13 - 0.24 (0.18)

Table 3 Regrowth of plant biomass after (a)biotic stresses were relieved. Data are plant dry mass (g DM) after a short and long term period. The symbol * indicate the presence of flowers at the time of evaluation.

Treatment	**Crab densities (ind. m^{-2})**	**Regrowth after 47 days (g DM)**	**Regrowth after 345 days (g DM)**
Control	0	6.9*	21.5*
Control	0.3	0	2.3*
Control	1.0	0	0.2
Control	2.5	0	0.4*
Light limitation	0	3.2*	14.6*
Light limitation	0.3	0	0.1
Light limitation	1.0	0	0.6
Light limitation	2.5	0	0
Chemical	0	6.7*	22.0*
Chemical	0.3	0	1.0*
Chemical	1.0	0	0.2
Chemical	2.5	0.1	4.2*