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Iron co-limitation of *Sargassum fluitans*

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ABSTRACT

In recent years, global distribution of holopelagic *Sargassum* spp. (sargassum) has extended from the subtropical Sargasso Sea and Gulf of Mexico into the tropical Atlantic. Climate and current patterns drive seasonal and year-to-year fluctuations of biomass in the ocean, but the underlying drivers of sargassum growth are poorly understood. Previous experimental studies showed that nitrogen (N) and phosphorus (P) can be limiting to sargassum. However, iron (Fe) also limits primary production in large parts of the ocean. We therefore (1) conducted a mesocosm experiment studying the effects of N+P and Fe addition on the growth rate and nutrient content of *Sargassum fluitans*, and (2) compiled literature on Fe tissue levels in sargassum throughout its distribution area. The Fe levels in collected experimental specimens (Mexican Caribbean) were like those previously reported near coastlines with low terrestrial nutrient runoff, and in the open ocean. The addition of Fe greatly boosted growth, averaging 0.13 doublings day^{-1}, 40 % faster than our controls, and maximum growth rate (doubling biomass in 5½d) was 46 % above previously reported maximal value. While oceanic Fe is relatively abundant in the tropical North Atlantic during rain episodes in the summers due to Saharan dust deposition, its availability is likely more limiting during other parts of the year, particularly in the western Caribbean. However, the true limiting potential of Fe depends on many factors. Our study suggests Fe co-limitation might occur widely and urges to include Fe availability in future sargassum forecasting models.

1. Introduction

Sargassum fluitans (Borgesen) Borgesen and *Sargassum natans* (Linnaeus) Gaillon (hereafter referred to as sargassum) are unique among macrophytes as they are holopelagic, living their entire life cycle in the open ocean (Brooks et al., 2018). These macroalgae used to be concentrated in the subtropical Sargasso Sea and Gulf of Mexico; until 2011, when large masses were reported for the first time in the northern tropical Atlantic, forming the 'Great Atlantic Sargassum Belt' (GASB) which stretches from equatorial Africa and Brazil up to the Gulf of Mexico (Wang et al., 2019). In the tropical Atlantic, and especially in the Caribbean, currents and winds cause the algae to wash ashore, where fast decomposition of the algal mass causes brown and murky waters, anoxia and production of toxic and malodourous hydrogen sulfide. These 'sargassum brown tides' occur Caribbean-wide and cause substantial ecological damage to seagrass meadows (van Tussenbroek et al., 2017), mangroves (Hernández et al., 2021), corals (Antonio-Martínez et al., 2020) as well socio-economic damage by making beaches

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unsuitable for tourists (Chávez et al., 2020). The GASB and concomitant coastal sargassum stranding show distinct spatial variation, seasonality and interannual variability, thought to be caused by changes in prevailing wind and current regimes as well as nutrient availability (Johns et al., 2020; Putman et al., 2018; Skliris et al., 2022; Wang et al., 2019). Recent studies have shed light on the role of currents, upwellings and winds in the origin of the recent sargassum blooms (Berline et al., 2020; Franks et al., 2016; Johns et al., 2020; Putman et al., 2020; Wang et al., 2019), but the fundamental question on which nutrients fuel the production of sargassum is still topic of discussion. The effects of nutrients on sargassum growth have not yet been fully explored, partly because sargassum could initially not be kept well under laboratory conditions (Magaña-Gallegos et al., 2023a). Earlier growth experiments demonstrated that nitrogen (N) and phosphorous (P) additions boosted sargassum growth in the Sargasso Sea (Lapointe, 1986, 1995). According to new evidence, increased N and P availability since 1980 may be fueling the Atlantic basin's sargassum blooms (Lapointe et al., 2021). However, the potential limiting of growth caused by iron (Fe) has not yet been addressed.

Iron is an essential micronutrient for almost all life. While it makes up 3.5 % of the earth's crust (Taylor and McLennan, 1985), its solubility in seawater in the presence of oxygen is extremely low. In the so-called high-nutrient low-chlorophyll regions, around 30 % of the ocean's phytoplankton primary production is limited by Fe. In these regions, dissolved inorganic nitrogen and phosphorus (DIN and DIP) are readily available at the surface, and phytoplankton blooms upon iron addition (Moore et al., 2013). In regions where light is not limiting, biological N_2 fixation, which has a high iron requirement, causes surface water depletion of either P or Fe (Wu et al., 2000). Sargassum in the Sargasso Sea has both photic (Carpenter and Cox, 1974; Phlips et al., 1986) and aphotic (Phlips and Zeman, 1990) N_2 fixation by cyanobacterial epibionts. These epibionts produce a substantial fraction of sargassum N requirements throughout the year in the Sargasso Sea, although incorporation into sargassum tissue has not been studied (Johnson et al., 2023). Phlips et al. (1986) estimated a population N requirement contribution ranging from 2 % to 32 %, whereas (Hanson, 1977) estimated it at 44 %. Recent work reaffirms the importance of N_2-fixation for sargassum. They (Johnson et al., 2023) found that N_2-fixation rates of epiphytic cyanobacteria on Gulf-Stream sargassum mats were variable but could rival planktonic rates, and could be a regionally important source of fixed nitrogen. Carpenter and Cox (1974) found no relation between dissolved nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) and N_2-fixation rates of epiphytic cyanobacteria and concluded that Fe was a likely limiting factor. Fe availability, in addition to increasing N by biological N_2 fixation, also increases access to P because Fe-rich alkaline phosphatase exoenzymes can access the oceanic DOP (dissolved organic phosphorus) pool, which is much larger than the DIP pool (Browning et al., 2017). Thus, Fe couples with oceanic N and P cycles.

In the Sargasso Sea, which historically served as the native habitat for sargassum before its recent expansion, Fe-limitation of primary production is frequent in certain regions (Menzel and Ryther, 1961; Rao and Yeats, 1984). Sargassum has lately extended into the tropical northern Atlantic region, which is very Fe-rich. However, Fe-limited areas exist in this region due to seasonal and interannual fluctuations in Fe input (Mills et al., 2004). The northwest African upwelling, equatorial upwelling, and open-ocean diffusive nutrient flux are oceanic (resupply) nutrient sources thought to be important for sargassum proliferation in the GASB (Johns et al., 2020; Wang et al., 2019). External nutrient sources implied in formation of the GASB are atmospheric deposition, mainly of Sahara dust, and riverine input, mainly from the Amazon and Orinoco in the west and the Congo in the east (Oviatt et al., 2019; see Table A.1 for an overview of some of the literature). Acting on an interannual scale, the Atlantic Meridional Mode and Atlantic Niño climate phenomena influence sea surface temperatures, winds and currents, patterns of rainfall and upwelling, and the migration of the Intertropical Convergence Zone (Marsh et al., 2021). The ITCZ is a low-pressure zone near the equator where trade winds converge. Its yearly migration causes sargassum aggregation, south-north transport, and seasonal changes in the transport of equatorial water into the Caribbean. The ITCZ controls upwelling patterns, and the heavy precipitation it causes increases flow from the Amazon and Orinoco rivers, as well as African dust deposition into the tropical Atlantic (Johns et al., 2020; Skliris et al., 2022).

The above-mentioned nutrient sources differ in the amounts of N, P and Fe they provide. Differences in biological requirements and remineralization rates result in discrepancies in stoichiometry among organisms and oceanic (resupplied) nutrient sources. Oceanic remineralization of P is twice as efficient as that of N (Letscher and Moore, 2015), and Fe has the largest downward flux because of scavenging - i.e. adsorption to particles (Rigby et al., 2020). An external source of Fe relative to P is thus always necessary. Rivers supply N, P and Fe, but are relatively more enriched in N (Lapointe et al., 2021). Atmospheric Saharan dust is the source of >80 % of dissolved Fe in North Atlantic surface waters (Conway and John, 2014). Close to West-Africa, dry deposition is important, but further away wet deposition is the main source of dust in the ocean, which varies seasonally (van der Does et al., 2021) and can be significantly influenced by individual dust plumes with highly variable extensions (Croot et al., 2004). North African dust export has increased by 46 % since pre-industrial times, and Fe from anthropogenic pollution is more soluble (Kok et al., 2023). Biomass burning can be a novel dissolved Fe source of relevance in the equatorial Atlantic (Hamilton et al., 2020; Lapointe et al., 2021). Atmospheric deposition is an important source of Fe, but it may also deliver P and N. According to Mills et al. (2004), the release of soluble P from Saharan dust is 3–8 times greater than that of Fe. This P derived from Sahara dust could potentially support up to 30 % of nitrogen (N_2) fixation in the North Atlantic, while Fe could support up to 100 %. The ITCZ shields the tropical south Atlantic from Fe-containing African atmospheric dust, thereby acting as an 'iron curtain' which divides the region in two distinct biogeochemical provinces (Capone, 2014; Schlosser et al., 2014), with the tropical south Atlantic experiencing Fe limitation, while the tropical north Atlantic and the ITCZ are enriched with Fe. Due to the huge temporal and regional changes in Fe availability and sargassum biomass in the GASB, Fe is expected to be growth limiting at times but not at others. This could be determined not just by local concentrations of N, P, and Fe, but also by luxury uptake, which is the accumulation of surplus N, P, and Fe for later use. Since the nutritional content of macroalgae tissue can accurately predict growth (Gagné et al., 1982; Lapointe, 1995), biological sargassum models should include this.

The aim of this research is to investigate if Fe modulates sargassum's growth response to N and P availability. For this an *ex-situ* experiment in which we introduced N+P, Fe, and N+P+Fe was carried out. The nutrient content of sargassum tissue was determined, and a comprehensive literature database was created to discover potential Fe limitation and luxury uptake. Furthermore, this study focuses on *S. fluitans* III, the predominant species and morphotype in the GASB (García-Sánchez et al., 2020). The findings of this study will help to better identify major drivers of sargassum dynamics, allowing for better growth projections and forecast models, which are required for identifying ways to mitigate the huge sargassum stranding in this region.

2. Materials and methods

2.1. Algae collection and experimental set up

Sargassum fluitans III was collected in May 2022 using a dip net from a pier in Puerto Morelos, Mexico (20 52'N, 86 52'W) and identified using previously described criteria (Magaña-Gallegos et al., 2023a; Parr, 1939). The sargassum was transported (< 20 min) to the experimental area at the Reef Systems Unit, Universidad Nacional Autónoma de México (UNAM) and cleaned in accordance with (Magaña-Gallegos et al., 2023a; Schell et al., 2015). The *ex-situ* experimental area included 16 independent recirculating outdoor cultivation tanks, each consisting of a 45 L sump from which water was pumped up into an 18 L bucket, to facilitate the movement of sargassum. The sumps were immersed in chilled seawater to maintain a constant water temperature (Magaña-Gallegos et al., 2023a; Magaña-Gallegos et al., 2023b). Seawater from the Puerto Morelos reef lagoon, with a salinity of 35 and pH of 8.2, was filtered through a sand filter prior to use (Model S360T2, Hayward Pool Products Inc., Elizabeth, NJ, USA) (Magaña-Gallegos et al., 2023b). After filtration, nutrient concentrations of the seawater were 0.9 ± 0.7 µmol l^{-1} NH_4^+, 0.4 ± 0.7 µmol l^{-1} NO_3^-, 0.06 ± 0.17 µmol l^{-1} PO_4^{3-}. Before the experiments began, different thalli were individually placed in each recirculating tank and acclimated to 28 °C for a whole day (Magaña-Gallegos et al., 2023b). Furthermore, when sargassum was collected, four thalli of *S. fluitans* III were collected and dried for elemental analysis. This sargassum corresponds to T0. Four thalli of the morphotypes I and VIII from *S. natans* were also collected for elemental analysis, with the end to add to the literature database we constructed.

2.2. *Experimental design*

After acclimation of the *S. fluitans* III thalli in the recirculating tanks, an apical section was taken from each thallus, while the rest of the thallus was discarded. Apical sections, between ~5.5 to ~6.5 g wet weight, were separated, weighed with an electronic balance (Scout Pro, SP 4001; Ohaus Corp, Pine Brook, NJ) after gently pressing between two paper towels, and individually added to the tanks. The 16 experimental units (recirculating tanks) were subjected to 4 different nutrient treatments with four replicates each during a five-day period. The treatments included the addition of N and P (as $NO_3^- + PO_4^{3-}$), Fe (Fe-EDTA), and N+P+Fe. The control received no additional nutrients. Reagent-grade $NaNO_3$, Na_2HPO_4, and EDTA ferric sodium stock solutions were used to add nutrients to each recirculating system according to the assigned treatments. The nutrients were added to each recirculating system on days one and four of the experiment at concentrations of 100 µmol NO_3^- l^{-1}, 10 µmol PO_4^{3-} l^{-1}, and 10 µmol Fe l^{-1}. These concentrations and application frequencies were determined in a pilot study. The experiment was repeated twice to increase the number of replicates to eight per treatment. After the experimental period, the wet weight of each thallus was determined, and the thalli were oven-dried at 40 °C and stored for further analysis. The growth rates were calculated using the initial (W_1) and final (W_2) wet weight measurements, and the relative growth rate (RGR) was calculated as doublings per day (Hanisak and Samuel, 1987; Magaña-Gallegos et al., 2023b):

$$RGR = \log_2 (W_2/W_1) / t$$

The difference in RGR between control groups of the two experimental series was not statistically significant (Student́s T-test, t value, df 5, = 0.49). Temperature was maintained at 28 ± 1 °C, the optimal temperature for the growth of *S. fluitans* III (Magaña-Gallegos et al., 2023b). The salinity levels were carefully monitored and maintained at a constant level of 35 by adding deionized water, whenever necessary. Light levels just above the sargassum ranged from 408 to 1068 µmol m^{-2} s^{-1} while five centimeters under the sargassum (underwater) ranged from 327 to 824 µmol m^{-2} s^{-1} (LI-1500, LI-COR, NE, USA). This was above saturation irradiance for sargassum (Hanisak and Samuel, 1987).

2.3. *Algae elemental content*

The stored samples collected at the beginning (T0) and end of the experiment were used to evaluate the individual elemental composition of all thalli at the facilities of Radboud University, Netherlands. Protocols as in Koks et al. (2022) were followed. Carbon and nitrogen concentration were measured on a CN elemental analyzer (Elementar Vario Micro Cube). Tissue concentrations of P, Fe, the micronutrients cobalt (Co), copper (Cu), manganese (Mn), molybdenum (Mo), nickel (Ni) and zinc (Zn) (Nowicka, 2022), and the toxic metal(loid)s chromium (Cr), arsenic (As), cadmium (Cd) and lead (Pb) were measured using an inductively coupled plasma optical emission spectrometer (ICP-OES, Thermo Fischer Scientific, Bremen, Germany) following microwave-assisted digestion.

2.4. *Literature review*

For the literature review, we searched for ‘Sargassum’ together with ‘iron’ or ‘Fe’ using Web of Science and Google Scholar, collected all records on holopelagic *Sargassum* spp. and extracted tissue Fe, P, C and N content values together with information on geographical location, date, habitat (shore or offshore), morphotype, sample state (wet or dry) and sample size. We only considered papers that reported Fe content of Sargassum tissue, as several papers that consider the effects of C, N and P content on growth of holopelagic *Sargassum* spp. already exist.

2.5. *Statistical analysis*

To test the effect of treatment on relative growth rate, a one-way ANOVA was used, followed by a Tukey HSD test. One outlier was deleted based on Grubb’s test. This outlier was a dying thallus. Elemental tissue concentrations are given on a weight basis, either ppm or % (for C and N), while ratios (C:N:P:Fe) are given on a molar basis. R version 4.3.2 was used for statistical analyses.

We explored if tissue stoichiometry of N, P and Fe was a predictor of growth rates. When comparing the relationship between stoichiometry and growth we should consider growth dilution. By compensating for the dilution of a nutrient by the growth of the specimen during the experiment, we can investigate if initial tissue nutrient content influenced the growth rate. By comparing the different nutrient addition treatments, we can evaluate if alleviation of a nutrient limitation can then cause availability of another nutrient (in tissue) to become growth limiting. Growth dilution was compensated for by multiplying nutrient content with the thallus final wet weight (W_2) divided by initial wet weight (W_2) measurements:

Adjusted nutrient content = (W_2/W_1) * nutrient content

Using analysis of covariance (ANCOVA) we examined the interactions between nutrient additions, tissue stoichiometry of N, P and Fe and growth rates (growth ~ NP treatment * Fe-EDTA treatment * covariate nutrient), using stoichiometry adjusted for growth dilution (initial tissue content). Note that in the adjusted tissue content dataset, nutrients that were added experimentally were not modified for specimens from those treatments, and neither was carbon as it was assumed to be non-limiting.

In Fig. A.1, relationships between micronutrients and toxic metal (loid)s and growth are plotted both adjusted and unadjusted. Only data from control treatments is included, to prevent interactions between added nutrients and measured elements.

We used As to validate growth dilution adjustment, as it is known to accumulate in sargassum over time (Gobert et al., 2022; Ortega-Flores et al., 2022). For this validation, we only looked at control groups to exclude effects of nutrient additions. The negative relationship between As and growth disappeared when compensating for growth dilution (seen in Fig. A.1). While the relationship between As content and growth was not statistically significant for both unadjusted and adjusted data ($F_{1,5} = 2.79$, $p = 0.16$, adjusted R-squared = 0.23) and ($F_{1,5} = 0.037$, $p = 0.85$, adjusted R-squared = −0.19), respectively). This lack of significance is attributed to the small sample size (n = 7).

3. Results

3.1. Growth rates

Nutrient addition significantly influenced the relative growth rate (RGR) of *S. fluitans* III (ANOVA $F_{3,27} = 5.6$, $p = 0.004$). Post-hoc comparisons (Tukey, $p < 0.05$) revealed that the N+P+Fe treatment outperformed the control ($p = 0.004$) and N+P ($p = 0.02$) treatments but not the Fe treatment ($p = 0.07$) in terms of RGR. Tukey's test revealed no significant difference between the N+P and Fe treatments or the control. Sargassum without any nutrient addition (controls) increased their wet weight on average by 34 % during the experiment, N+P treatment by 39 %, Fe treatment by 43 %, and N+P+Fe treatment by 62 %. The maximum mean growth rate expressed as doubling time was 5.5 days in the N+P+Fe treatment, compared to 7.3 days for the control.

3.2. Tissue nutrient and elemental content

The Fe treatment considerably increased tissue Fe concentration (Table 1), from ~41 ppm at T0 to 3300 ppm at the end of the experiment, and up to 2400 ppm in the N+P+Fe treatment. Furthermore, substantial increases were seen between T0 and control (111 ppm) and N+P (145 ppm) treatments. In N+P and N+P+Fe addition treatments, P contents increased with 322 % and 360 % respectively, while N content doubled. The Fe treatment saw a decrease in P and N contents. C content decreased in all treatments. While molar-based C:N ratios halved in the N+P and N+P+Fe treatments (Table 2), C:P ratio were 5 times lower in these groups. After Fe addition, Fe increased even more than P, shifting P:Fe ratios from 43 at T0 to 3 in N+P+Fe treatment and to 0.13 in the Fe only treatment. Concentrations of trace essential elements and toxic metal(loid)s are listed in Table A.2.

Table 1
Tissue nutrient contents (mean and upper and lower 95 % CI) in *Sargassum fluitans* III at the end of the experiments.

Group	Fe (ppm)	P (ppm)	C %	N %
T0	41 (35–47)	929 (744–1115)	27.1 (26.2–28.1)	0.8 (0.7–1.0)
Control	111 (56–166)	730 (612–849)	25.8 (25.1–26.4)	0.83 (0.66–1.01)
Fe	3332 (2333–4332)	642 (510–774)	25.3 (24.3–26.4)	0.64 (0.53–0.74)
N+P	145 (50–240)	3921 (3371–4472)	26.2 (25.4–27.0)	1.68 (1.45–1.91)
N+P+Fe	2407 (2012–2802)	4278 (3353–5204)	24.9 (23.6–26.2)	1.65 (1.44–1.86)

3.3. Correlations between stoichiometry and growth

In Fig. 2, growth rate is plotted against tissue C:N ratio (adjusted for growth dilution in control and Fe treatments, see Section 2.5). High C:N ratios correlate with slower growth. This is shown by an ANCOVA investigating the effect on growth of addition of iron, addition of N and P, tissue C:N ratio and the interactions between the three variables. Apart from the strong main effects of NP addition ($F_{1,23} = 7.64$, $p = 0.01$) and Fe addition ($F_{1,23} = 14.84$, $p < 0.001$), C:N ratio correlated strongly ($F_{1,23} = 8.06$, $p = 0.009$), which is also because N:P additions cause low C:N ratio. The interactions between NP addition and Fe addition ($F_{1,23} = 0.59$, $p = 0.45$), between NP addition and C:N ratio ($F_{1,23} = 2.46$, $p = 0.13$) and between Fe addition and C:N ratio ($F_{1,23} = 0.03$, $p = 0.87$) were not significantly correlated with growth. But the three-way interaction between NP addition, Fe addition and C:N ratio was ($F_{1,23} = 7.86$, $p = 0.01$) was significant. This shows that after the effects of additions have been accounted for, C:N ratio does influence growth rate. For C:P ratio and C:Fe ratio there were no interactions

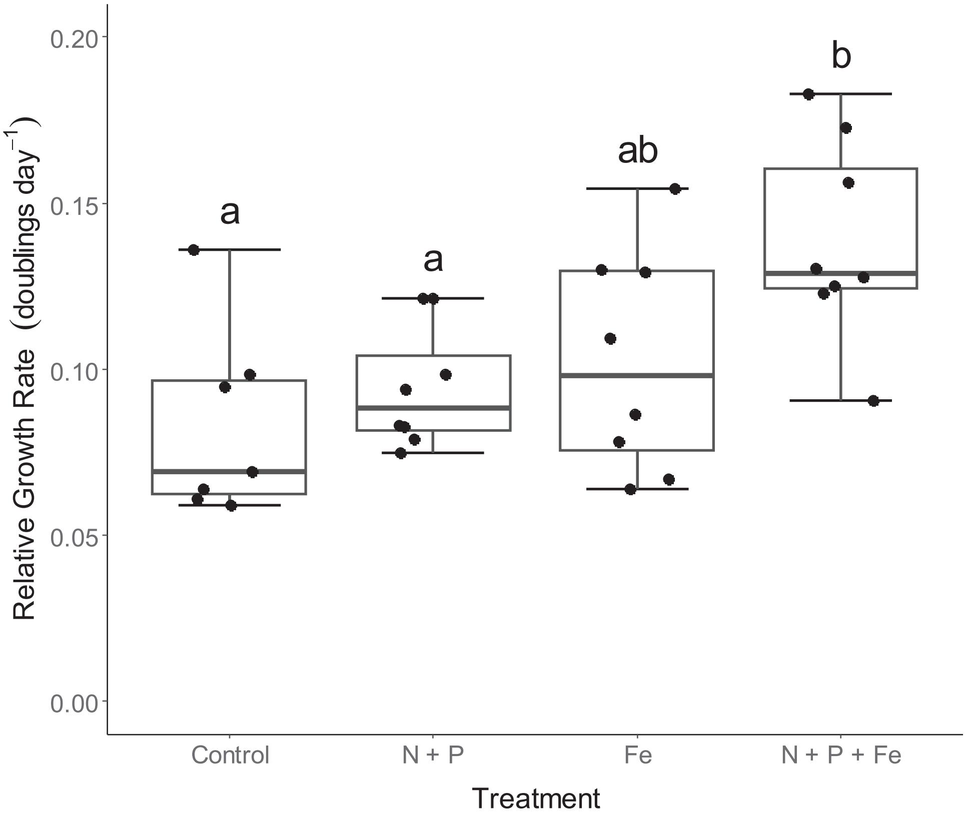

Fig. 1. Relative growth rate (RGR) of *Sargassum fluitans* III under different nutrient enrichment treatments. Boxplot shows quartiles, median, and significant differences. Letters indicate significantly different groups after application of a post-hoc Tukey test ($p < 0.05$).

Table 2
Tissue nutrient ratios (mean ± SD) in *Sargassum fluitans* III, at the end of the experiments.

	C:N	C:P	N:P	C:N:P	C:N:P:Fe	C:N:P: (Fe:P)
T0	38.4 ± 7.8	807 ± 253	21.0 ± 4.1	807:21:1	32051:882:43:1	807:21:1:0.0265
Control	37.7 ± 8.7	932 ± 145	25.3 ± 4.3	932:25:1	13932:420:16:1	932:25:1:0.0910
Fe	48.2 ± 10.5	1122 ± 491	22.8 ± 5.1	1122:23:1	393:8:0:1	1122:23:1:2.9531
N+P	18.7 ± 3.7	177 ± 33	9.5 ± 0.8	177:10:1	12707:682:72:1	177:10:1:0.0203
N+P+Fe	17.9 ± 2.1	160 ± 42	8.8 ± 1.4	160:9:1	503:29:3:1	160:9:1:0.3440

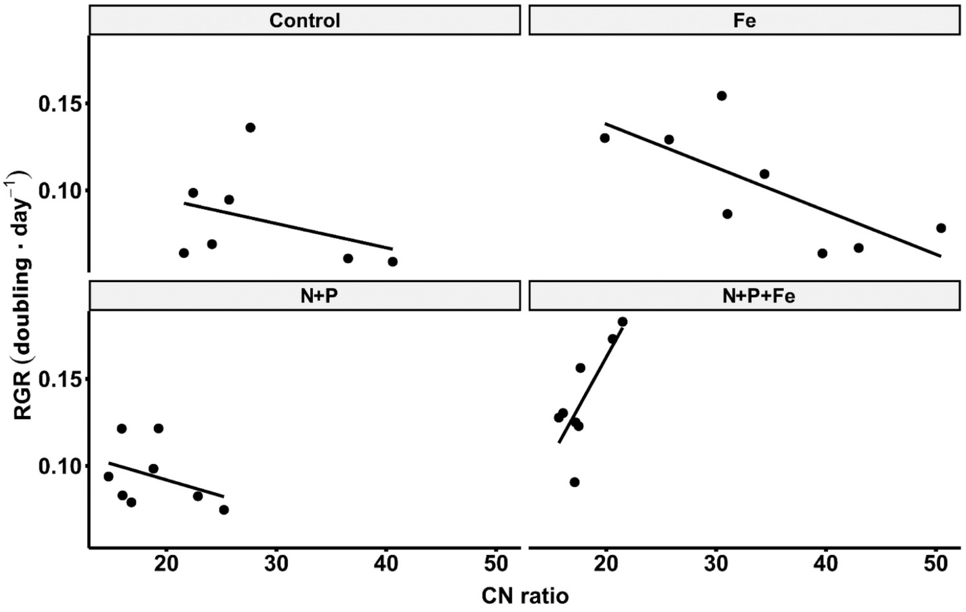

Fig. 2. Relationship between tissue C:N ratio and Relative Growth Rate (RGR) of *Sargassum fluitans* III under four nutrient regimes.

correlated with increased growth.

3.4. Literature review

Based on Fe, P, N and C stoichiometry of sargassum (Table A.3), we grouped entries in 3 groups, 'Tropical offshore Atlantic', 'Mexican coast', and 'other Caribbean coasts', based on distinct patterns in Fe content. The oceanic sargassum Fe had a median value of 34 ppm (mean 46, and range 5–300 ppm), while P had median 847 ppm (mean 993 and range 301–1646 ppm). No studies that reported Fe content from this region also reported N and C content. Sargassum collected along the Mexican coast (including the neritic zone) had a median Fe content of 22 ppm (mean 28 and range 2–177 ppm), while P had median 350 ppm (mean 500 and range 197–1398 ppm), and N had median 0.88 % (mean 0.99 and range 0.5–1.7 %). Shores elsewhere in the Caribbean had distinctly higher but variable Fe content, with a median Fe of 262 ppm (mean 533 and range 20–2473 ppm), with a median P of 700 ppm (mean 685 and range 110–1333 ppm), and median N of 0.92 % (mean 1.07 and range 0.65–1.72 %). *S. fluitans* from our study had higher mean Fe content (41 ppm) than the mean concentration reported for Mexican *S. fluitans* (22 ppm), as well as higher mean P (929 vs 355 ppm). No N content was reported for *S. fluitans* in elemental studies we found for this region, but our 0.85 % mean N in *S. fluitans* was a bit lower than for Sargassum spp. (0.99 %). Fe content was highest in *Sargassum fluitans* III, and lowest in *S. natans* VIII, with *S. natans* I presenting intermediate mean Fe concentration. For P content the order was *S. natans* I > *S. fluitans* III > *S. natans* VIII.

4. Discussion

Our experiment demonstrated that the growth response of *Sargassum fluitans* III to macronutrients N and P was driven by iron availability. The addition of N and P alone did not stimulate growth; however, when combined with Fe fertilization, unprecedented high growth rates (doubling biomass in 5½ days) were observed, suggesting that micronutrient (co-)limitation may be an important mechanism regulating sargassum blooms.

4.1. Sargassum growth and nutritional history

Plants in our N+P+Fe treatment exhibited an average growth rate of 0.13, with a maximum of 0.18 doublings day^{-1}, while control plants showed a growth rate of only 0.08 doublings day^{-1} which is within the range previously reported for *S. fluitans* (Table A.3). The highest growth rate of *S. fluitans* previously reported *in situ* was 0.12 doublings day^{-1} (with an average of 0.10), at Barbados (Corbin and Oxenford, 2023), while the highest growth rate reported in an *ex situ* nutrient addition experiment was 0.11 (Hanisak and Samuel, 1987), with apical growth tips grown in Provasoli's Enriched Seawater (Provasoli, 1968), a nutrient medium containing all essential nutrients. Interestingly, these were the studies where Fe was likely not limiting, as waters in Barbados are typically much richer than those in Mexico and Provasoli's enriched seawater contains micronutrients in addition to N and P. Our findings suggest that micronutrients like Fe play a crucial role in sargassum development and provide insight into tropical Atlantic sargassum blooms.

Our starting material (T0) had a C:N:P ratio of 807:21:1, which falls within the range observed for other *Sargassum* species (Atkinson and Smith, 1983), and brown algae in general (Lovelock et al., 2014). Lapointe (1995) found that *S. natans* (likely *S. natans* I) in oceanic Sargasso Sea water with mean C:N 50, C:P 877 and N:P 18 responded to both N and P addition, while neritic *S. natans* with C:N 28, C:P 347 and

N:P 10 responded weakly to N and not to P addition. Lapointe et al. (2014) found growth rates between 0.03 and 0.09 for neritic *S. natans* and *S. fluitans* with mean C:N 10 and C:P around 270, while oceanic specimens with C:N 18 and C:P around 800 had very low growth rates (0.005–0.02 for *S. natans*, *S. fluitans* not measured). We found that sargassum with comparatively high C:P and C:N ratios did not respond to addition of N and P without Fe addition, which can have played a role in previous experiments. It should be considered that these experiments and tissue content values are mostly from the subtropical Atlantic and not from the GASB. It is possible that sargassum growth is influenced by seasonal effects and regional conditions like temperature, e.g. (Magaña-Gallegos et al., 2023b).

Fe-limitation studies on macroalgae are scarce because Fe concentrations are usually not limiting in benthic habitats, with some exceptions such as certain areas along the Japanese coast. Matsunaga et al. (1994) found that in *Laminaria japonica* Areschoug, the critical and subsistence tissue levels for Fe were 14–21 ppm and 8 ppm, respectively. Nagai et al. (2014) found that Fe addition increased *in vitro* growth rates in *Sargassum horneri* (Turner) C.Agardh, *S. patens* C.Agardh, and *S. ringgoldianum* Harvey.

In benthic *Sargassum baccularia* (Mertens) C.Agardh critical levels (required for optimal growth) were 0.86 % for N and 0.09 % for P, while subsistence levels (below which growth ceases) were 0.6 % for N and 0.06 % for P (Schaffelke and Klumpp, 1998). Our starting material *S. fluitans* had values around these critical levels. Future work should investigate critical and subsistence levels of N, P and Fe in sargassum. In growth experiments it should be taken into account that initial tissue nutrient status may also affect response to nutrient additions, as Fong et al. (2003) showed that 4 species of tropical macroalgae had varied responses to nutrient pulses after a period of either nutrient sufficiency or depletion, with multiple species growing more during the 3-day experiment when they had low initial tissue nutrient content.

When interpreting stoichiometry, it should be considered that tissue nutrients may be only partially bioavailable, and that nutrient requirements differ in various ways. During our trials, sargassum increased total tissue Fe content 75-fold in 5 days, from 40 to 3000 ppm (Table 2). This is not all intracellular Fe, as sargassum has a high capacity for biosorption of metals to chelating compounds in cell walls (Gobert et al., 2022). Fe was present in our seawater and led to a significant difference between T0 and control tissue content (41 and 111 ppm), but most of this was likely surface-bound and not (directly) bioavailable.

Compared to N+P treatment, N+P+Fe addition resulted in slightly lower tissue CNP ratio (177:10:1 and 160:9:1, respectively) despite more growth which would dilute N and P in the tissues, indicating increased uptake of N and P.

4.2. Sargassum stoichiometry from literature

Our literature review on sargassum nutrient content across its basin-wide distribution showed clear patterns in Fe values. Equatorial/tropical Atlantic sargassum samples were low in Fe, but not as low as the samples from the Mexican coast, more distal to the Sahara dust source, and upwellings. Dassié et al. (2022) notice this pattern too. Sargassum sampled at the Mexican coast tends to be low in Fe compared to other coasts, possibly related to low Fe influx into the sea from the land, because of the absence of surface rivers due to the karstic nature of Yucatan Peninsula (Whelan et al., 2011). Along other coasts, Fe is very variable and often high due to terrestrial runoff. Ortega-Flores et al. (2022), correlated seasonal patterns in sargassum Fe content with dust deposition, with Fe content peaking in late summer.

P content of *S. fluitans* was even lower in Mexico (median 323 ppm) than reported from the P-limited Sargasso Sea (Gobert et al., 2022) (median 445 ppm). This was substantially lower than P in our starting material (929 ppm) which may indicate that P is usually limiting. Sargassum C and N content was not as often reported together with Fe as P was, and no data was available from oceanic waters. Lapointe et al. (2021) presented a database of sargassum C, N and P content, mainly focusing around the subtropical area, which shows that in the last decades tissue N has increased by 35 % while tissue P has decreased by 44 %. To be able to infer links between Fe and macronutrients from stoichiometry data, future studies should report C, N, P, Fe when reporting on sargassum tissue elemental content.

The three Sargassum morphotypes differ in their metal content with highest Fe concentrations in *S. fluitans* III and lowest concentrations in *S. natans* VIII (Fig. 3), which was also found by Cipolloni et al. (2022). This is likely due to differences in surface-to-volume ratio and chemical composition (e.g., phycocolloid content), allowing for more adsorption of Fe. Future research should determine if adsorbed Fe is an important source of intracellular Fe and if it can function as a storage mechanism for ‘luxury’ Fe, as it can in the brown algae *Ectocarpus siliculosus* (Dillwyn) Lyngbye (Miller et al., 2014) and *Macrocystis pyrifera* (Linnaeus) C. Agardh (Miller et al., 2016). If this mechanism exists, it could be that *S. natans* VIII’s lower surface-to-volume ratio (as compared to the other species/morphotypes) is a disadvantage under Fe-limited circumstances.

4.3. Nutrient limitation and co-limitation

Before the Earth became oxygenated, Fe was abundant in the ocean and it became incorporated into many of the metabolic pathways of the ancestors of modern algae (Morrissey and Bowler, 2012). It is involved in electron transport and functions as a cofactor for many enzymes like those involved in respiration, photosynthesis and nitrogen-fixation (Behnke and LaRoche, 2020; Hoffmann et al., 2007; Moore et al., 2009). Algae have developed a variety of different adaptations to current Fe-deficient conditions, especially in the pelagic environment where Fe is often limiting growth (Morrissey and Bowler, 2012; Schoffman et al., 2016). Fe can be a cofactor in enzymes which mitigate oxidative stress, which are important in photosynthesis, mitigating heavy metal pollution, and for several processes in aerobic respiration (Danouche et al., 2022). In some of these enzymes, Fe can be substituted by other metals, leading to interacting micronutrient quota. For example, Fe deficiency will cause increased oxidative damage due to electron leakage during photosynthesis, which decreases growth rate. Alleviation of Fe deficiency will reduce this damaging effect, but Mn also reduces the damage, and only when Fe and Mn are both replete can maximum growth rates be achieved (Schoffman et al., 2016). Iron also interacts with macronutrients. Interaction between Fe and nitrate N, involves the nitrate and nitrite reductase enzymes which have high Fe requirements. Nitrate reduction is the rate-limiting process in NO_3 uptake and assimilation (González-Galisteo et al., 2019). We gathered possibly relevant co-limitation pairs from the literature in Table A.5, which may be useful in interpreting (micronutrient) stoichiometry (Table A.2) and growth rate for the conditions where either N+P, Fe or all three are not limiting versus controls.

Liebig’s Law of the Minimum states that growth is dictated by the scarcest resource. It was originally applied to crop growth but has often been found to match observations in natural ecosystems. However, it does not describe certain types of co-limitation well. Saito et al. (2008) described the potential co-limitation pairs for phytoplankton and defined three types of co-limitation. Independent co-limitation (Type I) involves two biochemically independent nutrients, where both are equally limiting, and growth is only observed after adding both. In biochemical substitution co-limitation (Type II), two elements can substitute for the same biochemical role, either within the same macromolecule or by substituting one macromolecule for another. In biochemically dependent co-limitation (Type III), acquiring one nutrient depends on another. Gorban et al. (2010) described how for type I co-limitation, adaptation tends to equalize the degree of co-limitation by multiple resources (Law of the Minimum paradox), while type II and type III are ‘non-Liebig’ synergistic systems of factors, where adaptation

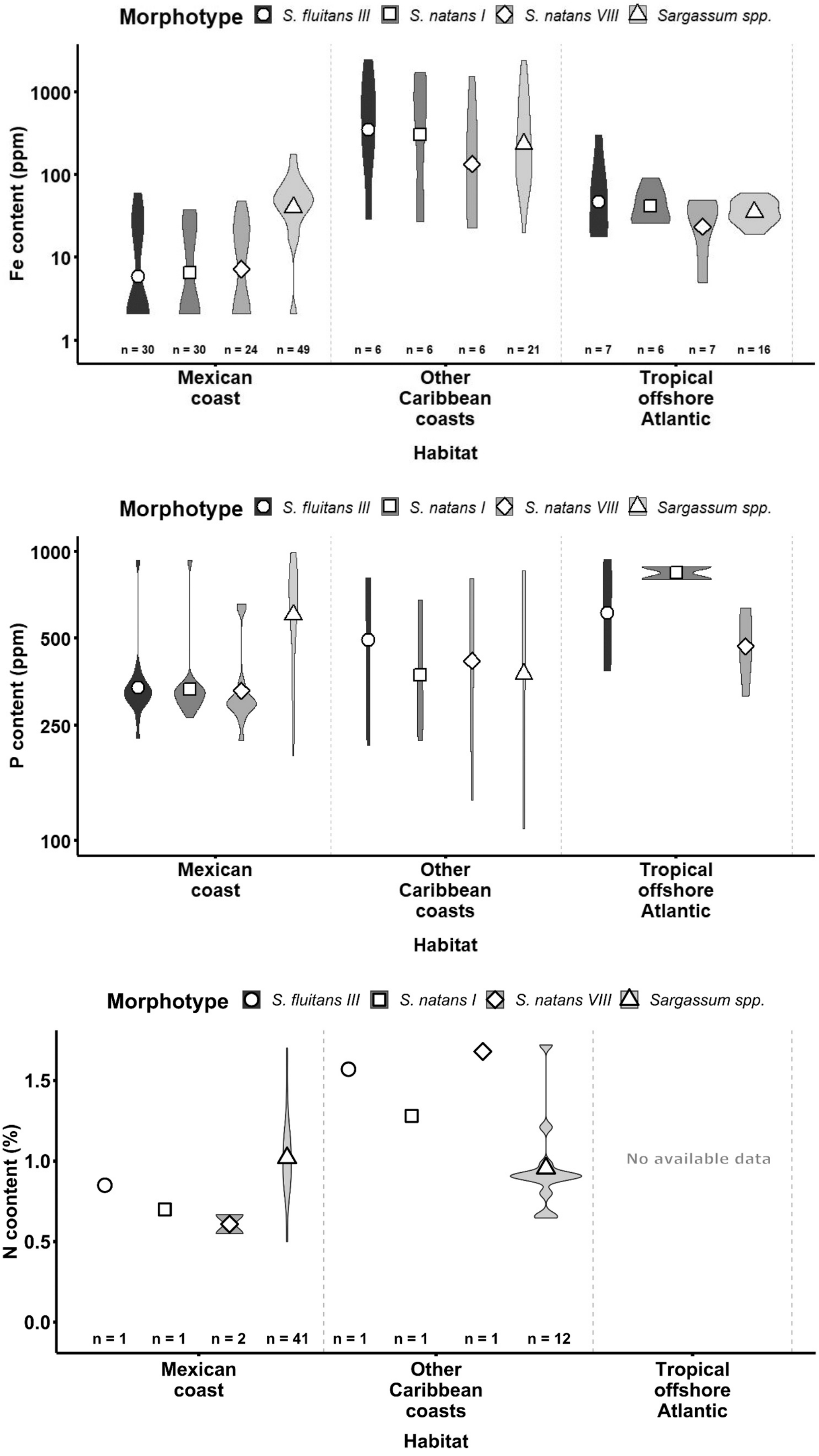

Fig. 3. Violin plots summarizing sargassum Fe, P and N content, grouped per zone and species. *Sargassum* spp. is a mix of species and morphotypes. Data from 20 publications for Fe, 9 for P, 7 for N, and own data from Mexico (this study, and 30 points from a cruise around Cozumel) and Bonaire (2 bays). Locations, morphotypes and repeated measurements differing at least 1 month are defined as separate entries.

will lead to a smaller number of important factors, thus more Liebig-type behavior (Law of the Minimum inverse paradox). Our results indicate a synergistic organization of the Fe-N/P co-limitation mechanism, where Fe is required to assimilate more N and P. The near-significant effect ($p=0.08$) of the Fe-only treatment when including pilots (Fig. A.2) might indicate that the specimens were initially Fe-limited and subsequently became co-limited.

The Fe co-limitation we present here should be viewed in the context of the high Fe requirements of both nitrogen fixation (Schoffman et al., 2016), shown to occur at high and variable rates in sargassum mats (Johnson et al., 2023) and of alkaline phosphatase, which allows use of DOP, shown to be increased in P-depleted sargassum (Lapointe, 1995).

4.4. Holobiont and aggregation scale effects

To translate results of *in vitro* nutrient additions to the field, the effects of aggregation into dense mats and interactions with its microbiome should be considered. An organism – here defined as a single sargassum specimen – including its associated (micro)biome forms an ecological unit known as the holobiont. Macroalgae epibionts can perform various tasks beneficial to the host including nutrient acquisition. The sargassum microbiome is diverse and variable (Theirlynck et al., 2023), profiting not only from the niche provided by sargassum structure but also from its substantial production of dissolved organic matter: around 10 % of production is exported as dissolved organic carbon (Powers et al., 2019).

Pelagic microalgae are known to benefit from bacterial excretion of siderophores, molecules that bind Fe and facilitate uptake (Amin et al., 2009). In floating *Sargassum horneri*, the dominant functions of the bacterial community were Fe and N transporters (Mei et al., 2019), and were expressed much more in a light-colored (low chlorophyl) patch with a thin mucus layer than in a dark-colored patch with a thick mucus layer.

Aggregation of sargassum into denser mats increases efficiency of internal cycling of nutrients. Concentrations of dissolved N and P have been found to be higher within mats (Aquino et al., 2022; Lapointe, 1995; Lapointe et al., 2021; Lapointe et al., 2014). Dissolved inorganic nitrogen (DIN) is produced by (microbial) remineralization of dissolved organic nitrogen (DON) produced by sargassum, and by (N_2-fixing) epibionts (Johnson et al., 2023). Part of DON, like urea, can also be taken up directly by sargassum (Han et al., 2018). The uptake of released adsorbed Fe (Miller et al., 2016; Miller et al., 2014) will also be more efficient in aggregations. Given that nitrate reduction requires Fe (Table A.5) and since available N in the ocean is a mix of nitrate, ammonium, and DON, with the latter two occurring substantially more in patches, Fe-limitation may occur at different tissue concentrations than in our study and depend on patch density. During sargassum aggregation under the ITCT, Fe should be less limiting because of wet deposition.

Sargassum mats are also more likely to retain Fe-rich dust particles. Benthic *Sargassum* species increased growth substantially when adsorbed particulate manner was present, presumably due to formation of a nutrient-rich boundary layer created by remineralization of nutrients from the particle by microbial epibionts (Schaffelke, 1999). Eichner et al. (2020) found that Fe from dust particles dissolved faster in the core of *Trichodesmium* colonies, presumably due to effects of bacterial siderophores. Low pH and low O_2 helps to dissolve Fe from dust particles, but in the colonies, these were not affected enough to cause this effect. *Trichodesmium* colonies are the closest analogue to sargassum mats in the open ocean, but sargassum mats have the potential to increase pH and O_2 effects. Potentially hypoxic conditions arise at night in sargassum mats during which Fe(III) is reduced and solubilized from dust particles or alginate or (other) ligands. Measurements of O_2 and pH in floating sargassum mats and the effect of dust in relation to these are worth investigating.

The increase in biomass and thus turnover caused by Fe, combined with high capacity for adsorption, adds to previous findings of an N_2-fixing epibiont community, use of DOP, and the need for Fe ligands could mean variations in Fe availability relate to rapid growth observed when sargassum aggregates in the GASB.

5. Conclusions

In conclusion, our research demonstrates that the micronutrient Fe, working in synergy with macronutrients N and/or P, substantially boosts the growth rate of *S. fluitans*. We observed a maximum growth rate that is 46 % higher than previously recorded, indicating a need to reassess earlier studies that focused solely on N and/or P limitation in sargassum. Present sargassum forecasting models primarily focus on drift patterns, neglecting variations attributable to biomass growth and decay. Recent models (Brooks et al., 2018; Jouanno et al., 2021; Marsh et al., 2021; Marsh et al., 2022) have begun incorporating biological factors, yet our findings suggest the necessity of updating these models to reflect revised maximum growth parameters. Our study proposes an adjustment of the maximum growth rate parameter to either 0.13 (our average) or 0.18 (our maximum), with a critical inclusion of Fe's role. The observed co-limitation in sargassum, featuring tissue Fe concentrations close to the global median in *Sargassum* species, highlights the importance of integrating Fe in models considering intracellular stoichiometry. This is particularly relevant given that micronutrients frequently limit growth in the ocean (McCain et al., 2021; Moore et al., 2013). Therefore, simultaneous reporting of N, P, and Fe tissue contents is vital for furthering research in this area.

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Leon Lamers: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Marieke van Katwijk:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Eden Magaña:** Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Brigitta van Tussenbroek:** Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition. **Marjolijn Christianen:** Writing – review & editing, Writing – original draft, Supervision. **Tjeerd Bouma:** Supervision, Funding acquisition. **Fons Smolders:** Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization. **Luuk Leemans:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.2024.103807.

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