



Increased temperature reduces the positive effect of sulfide-detoxification mutualism on *Zostera noltii* nutrient uptake and growth

Jimmy de Fouw^{1,*}, Katrin Rehlmeier^{1,2}, Matthijs van der Geest^{3,4},
Alfons J. P. Smolders^{1,5}, Tjisse van der Heide^{2,6}

¹Department of Aquatic Ecology and Environmental Biology, Institute for Water and Wetland Research, Radboud University Nijmegen, Faculty of Science, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands

²Groningen Institute for Evolutionary Life Sciences (GELIFES), University of Groningen, PO Box 11103, 9700 CC Groningen, The Netherlands

³Wageningen Marine Research, Wageningen University & Research, PO Box 57, 1780 AB Den Helder, The Netherlands

⁴MARBEC, Université de Montpellier, CNRS, IRD, Ifremer, 34095 Montpellier, France

⁵B-WARE Research Centre, Radboud University, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

⁶Department of Coastal Systems, NIOZ Royal Netherlands Institute for Sea Research, PO Box 59, 1790 AB Den Burg (Texel), The Netherlands

ABSTRACT: Seagrass meadows form essential ecological components in coastal zones but are rapidly declining worldwide due to anthropogenic impacts, including eutrophication and climate change-related heat waves. An important consequence of increased eutrophication is organic matter input in the sediment, which, together with raised temperatures, stimulates the production of toxic sulfide. Although multiple recent studies have highlighted that seagrass can engage in a mutualistic relationship with lucinid bivalves alleviating sulfide toxicity in the rhizosphere, it remains unclear how this mutualism is affected by temperature and eutrophication. To unravel this relation, we investigated the response of the seagrass *Zostera noltii* to contrasting sediment organic matter conditions, temperatures and presence/absence of the lucinid clam *Loripes orbiculatus* in a full-factorial mesocosm experiment. Results demonstrate that temperature increased porewater sulfide and nutrient levels, particularly in treatments with high sediment organic matter content. Interestingly, *L. orbiculatus* reduced not only sulfide levels, but also porewater nutrients. The removal of sulfide in turn stimulated *Z. noltii* growth, despite lower nutrient availability. Finally, increased temperature suppressed the mutualistic benefit for *Z. noltii* as sulfide detoxification became hampered. We conclude that, as eutrophication remains an issue in the coastal zone and temperature extremes will likely become more common in the future, seagrass meadows where lucinids occur may need to increasingly rely on this mutualism. At the same time, however, this interaction will become more strained, with an inherent increasing risk of mutualism breakdown.

KEY WORDS: Environmental stress · Lucinidae · Marine mutualism · Seagrass · Sulfide · *Loripes orbiculatus*

1. INTRODUCTION

Seagrass meadows form essential ecological components of many coastal zones due to the high biodiversity they support and their extremely high pro-

ductivity. In addition, seagrass meadows are of great socio-economic importance, because they serve as carbon and nutrient sinks, storm buffers and fish habitat (e.g. Waycott et al. 2009, Unsworth et al. 2015). However, similar to other vital coastal ecosys-

*Corresponding author: j.defouw@science.ru.nl

© The authors 2022. Open Access under Creative Commons by Attribution Licence. Use, distribution and reproduction are unrestricted. Authors and original publication must be credited.

tems like coral reefs and tropical forest, seagrass beds are rapidly declining as a result of human impact, which is a major concern as their associated services are also lost (Waycott et al. 2009). Seagrass meadows are exposed to many anthropogenic pressures, such as climate change, coastal development and nutrient enrichment. Nutrient enrichment is an important driver of seagrass decline, especially in mesotrophic and eutrophic systems (Hughes et al. 2013, Östman et al. 2016). Excessive nutrient enrichment promotes growth of epiphytes, phytoplankton and macroalgae that outcompete seagrasses for light. In addition, decomposition of easily degradable dead algal matter may lead to increased ammonium and sulfide concentrations in the sediment and lower water layer, causing toxicity effects for seagrasses (e.g. van Katwijk et al. 1997, Brun et al. 2002, Burkholder et al. 2007). Sulfide toxicity in particular has been shown to be an important agent promoting large seagrass die-off events worldwide (e.g. Seddon et al. 2000, Holmer & Bondgaard 2001, Borum et al. 2005, de Fouw et al. 2016, Hall et al. 2016).

Apart from external nutrient loading, an internal seagrass feedback may also increase sediment nutrients and organic matter, stimulating sediment sulfide production. Seagrass meadows facilitate their own habitat and growth by increasing water clarity by trapping suspended particles and stabilizing sediments (Hansen & Reidenbach 2012). This positive facilitation, however, also results in a negative feedback as organic matter accumulates in the sediment (Hansen et al. 2000, Folmer et al. 2012), and sulfate-reducing bacteria living in anoxic sediments oxidize organic matter using sulfate instead of oxygen, producing sulfide as a waste product (Jørgensen 1982, Lamers et al. 2013). In addition to radial oxygen loss from seagrass roots to avoid sulphide intrusion and free sulphide-oxidising cable bacteria in the sediment (Hasler-Sheetal & Holmer 2015, Martin et al. 2019), members of the bivalve families Lucinidae, Thyasiridae and Solemya (Cavanaugh 1983, Dubilier et al. 2008) can play an important role by detoxifying the sulphides (e.g. Reynolds et al. 2007, van der Heide et al. 2012, de Fouw et al. 2016, Chin et al. 2020, van der Geest et al. 2020). Sulfide is used as an energy source by endosymbiotic chemoautotrophic bacteria living in the gills of lucinid bivalves (Cavanaugh 1983). The bacteria and the lucinid bivalves form a symbiotic relationship in which the clams transport sulfide and oxygen to their gills where the bacteria oxidize sulfide for synthesizing sugars that fuel the growth of both organisms (Cavanaugh 1983, van der Heide et al. 2012). In this

way, the seagrass and the lucinid–bacteria consortium engage in a facultative mutualism in which the seagrass provides the clams and their bacteria with sulfide and oxygen, while the seagrass profits from the removal of toxic sulfides (van der Heide et al. 2012, van der Geest et al. 2020, Chin et al. 2021).

As anaerobic degradation and related sulfide production are strongly temperature dependent, it is possible that the strength of the mutualism also depends on temperature. This idea is supported by a correlative meta-analysis showing that the occurrence of lucinids is positively related to temperature on a global scale (van der Heide et al. 2012). On the other hand, excessive temperature conditions can also trigger mutualism breakdown. This was highlighted by a recent study in a West African intertidal seagrass system. Here, seagrass degradation was initiated by high temperature associated with intense evaporation at low tide (desiccation stress). Desiccation disrupted the seagrass–lucinid mutualism, after which increased sediment sulfide levels amplified the seagrass die-off (de Fouw et al. 2016, 2018). Global climatic change is currently one of the major threats to coastal systems in general. Therefore, a better understanding of how the strength of the lucinid–seagrass mutualism is affected by the predicted temperature increase is important.

Sediments that are rich in organic matter, like those commonly found in more eutrophic systems, particularly in areas sheltered from currents and waves, will typically have high nutrient and sulfide levels. In such cases, detoxification by this facultative mutualism is most important to alleviate direct sulfide toxicity. Apart from its lethal phytotoxic effect at high concentrations, sulfide can also hamper nutrient uptake by plants when sublethal sulfide levels reach the root tissues and disrupt the cellular metabolism (see review by Lamers et al. 2013). As a consequence, mutualistic sulfide detoxification can also be important in nutrient-poor environments where nitrogen and phosphorus availability limit seagrass productivity (Touchette & Burkholder 2000, Burkholder et al. 2007). Here, sulfide may already inhibit seagrass growth at very low concentrations ($10 \mu\text{mol l}^{-1}$) (Calleja et al. 2007) by directly interfering with nitrogen and phosphorus uptake (Koch et al. 1990). Furthermore, such effects may be amplified in nutrient-poor carbonate-based sediments, as these are typically iron-poor and thus have little chemical sulfide binding capacity (Ruiz-Halpern et al. 2008, Holmer et al. 2009).

Although sulfide detoxification by lucinid clams may stimulate seagrass nutrient uptake, the seagrass–lucinid mutualism is still poorly understood regarding

nutrient dynamics. For instance, recent work revealed that the endosymbiotic gill-bacteria are also capable of nitrogen fixation (diazotrophy), which suggests that, apart from sulfide detoxification, the lucinid-bacteria consortium might also benefit seagrasses under nutrient-poor conditions by providing nitrogen (König et al. 2017, Petersen et al. 2017, Cardini et al. 2019). In contrast, other studies have shown that sediment porewater nutrients are decreased in the presence of lucinids, suggesting that lucinids could cause or amplify nutrient limitation for seagrasses (van der Heide et al. 2012, König et al. 2017, Chin et al. 2021). Clearly, the nutritional role of the facultative mutualistic interaction under different environmental conditions in seagrass systems is unclear.

To clarify the role of the lucinid-seagrass interaction under different anthropogenic stressors, a closer examination of the linkages among sediment porewater nutrient concentrations, nutrient uptake by seagrass and presence of lucinids is needed. Here, we shed light on the nutrient uptake by seagrass mediated by the facultative mutualism with the lucinid-bacteria consortium and the effect of a heat wave by increased water temperature. In a 12 wk mesocosm experiment using relatively nutrient-poor sediment, we investigated differences in biomass production of seagrass and porewater sulfide levels dependent on the presence of lucinids, increased water temperatures and addition of organic matter. Organic matter (dead algae) was added to stimulate both nutrient enrichment and sulfide production, resulting from decomposition by sulfate-reducing bacteria. Lucinids were expected to increase seagrass production following nutrient addition by lowering toxic sulfide concentrations. Furthermore, we hypothesized that organic matter addition combined with the presence of lucinids would result in the highest biomass production acting synergistically, since the plants would simultaneously profit from both nutrient increase and low sulfide levels. Moreover, we expected that the increased temperature combined with organic matter addition would result in higher sediment sulfide levels due to faster breakdown rates, and consequently to low seagrass production.

2. MATERIALS AND METHODS

2.1. Sample collection

In May 2019, the lucinid bivalves *Loripes orbiculatus*, apical shoots of the seagrass *Zostera noltii* and sediment were collected from an oligotrophic seagrass

meadow at Port Lazo (48.763° N, 2.989° W), Bretagne, France, and transported to the laboratory in Nijmegen, the Netherlands. Apical shoots and *L. orbiculatus* were collected from <15 cm sediment depth and separated from the sediment by carefully sieving in a 1 mm sieve. Sediment was collected by a shovel and sieved over a 1 mm sieve in the laboratory. Sediment porewater nutrient and sulfide concentrations were low in the upper sediment layer (0–10 cm) at this location, with 6.4, 24.0 and 5.3 $\mu\text{mol l}^{-1}$ phosphate, ammonium and sulfide, respectively (data from van der Heide et al. 2009), and an organic matter content of $2.18 \pm 0.06\%$ (mean \pm SD) (this study). Organisms were kept separately in open, aerated 100 l polyethylene containers with 15 cm of sediment and 20 cm of water for 5 wk to acclimatize. *Z. noltii* apical shoots were planted, and *L. orbiculatus* buried themselves naturally when placed on the sediment. The acclimation and experiment were performed in 2 separate, open 760 l water basins (height = 0.4 m) that allowed the manipulation of water temperature. During the experiment, including the acclimatization phase, water flow and oxygen saturation were maintained by air stones and aquarium water pumps. Artificial seawater was used (33–35 PSU Tropic Marin at 20°C). Constant salinity levels were maintained by addition of deionized water, replacing evaporated water. Salinity and water temperature were measured daily with a handheld multimeter (WTW, Multi 3420 multimeter), and 100% water changes were conducted every 2 wk to prevent algae growth. A handheld pH meter (Thermo Scientific, pH 450 meter kit) was used to determine surface water pH levels, which were maintained between 8.1 and 8.4 by CO₂ aeration. Light exposure had a duration of 16 h d⁻¹ and an intensity at the leaf surface in the water of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (according to van der Heide et al. 2012).

2.2. Experimental setup

The experiment consisted of a $2 \times 2 \times 2$ factorial design with 5 mesocosm replicates per treatment, all with *Z. noltii* distributed over 4 polyethylene tanks (100 l capacity each, included as a random factor). Depending on the treatment, each unit received *L. orbiculatus*, organic matter, ambient or increased temperature or a combination of 2 or all treatments, which resulted in 40 mesocosm units. The units consisted of 770 ml, 2-compartment PVC cylinders (height = 12 cm, diameter = 8.4 cm), which were closed at the bottom and open at the top. Compartments at the bottom (340 ml capacity, height = 6 cm) were sepa-

rated from the open upper compartments (420 ml capacity, height = 6 cm) by a perforated 3 mm PVC layer (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m692p043_supp.pdf). This layer was covered by a porous 0.1 mm membrane, allowing diffusion of fluids while preventing penetration of sediment particles (van der Heide et al. 2012, Chin et al. 2021). Lower compartments were filled with anaerobic seawater (34 PSU) and sealed with a rubber septum, allowing the injection of organic matter or seawater (see Fig. S1). The membrane allowed the injected content to gradually diffuse into the upper compartment. All upper compartments were filled with homogenized, sieved (1 mm mesh) sediment from the collection site and planted with seagrass. Prior to setting up the experiment, *Z. noltii* wet weight and *L. orbiculatus* length were determined. All mesocosm units received a total of 8 shoots of *Z. noltii* divided over 4 ramets with apical shoots (one 3-shoot, two 2-shoot and one 1-shoot ramets standardized by wet weight, 1.77 ± 0.11 g, mean \pm SD, ~ 1440 shoots m^{-2}). In each treatment involving *L. orbiculatus*, 7 individuals were standardized by length (8.79 ± 0.51 mm; mean \pm SD, 1263 ind. m^{-2}) and placed on the sediment surface of the mesocosms to allow them to bury in the sediment. Densities of *L. orbiculatus* and *Z. noltii* were within the ranges of densities observed in the field (van der Heide et al. 2012, van der Geest et al. 2020). The mesocosm units were equally randomly distributed over the 100 l polyethylene tanks ($n = 4$), with an equal number of units containing *L. orbiculatus* in both water basins. Positions of the units within a tank were randomized weekly to minimize possible differences in light levels and water flow around units.

Half of the units were kept at ambient temperatures (21°C) throughout the whole experiment. Units in the other half of the separated water basin were initially kept at the same temperature for 8 wk. After this adjustment period, temperatures were increased to simulate a heat wave that induced heat stress conditions. Initially, temperature was raised within 2 d to 27.5°C. Next, to prevent overshooting the temperature, water temperature was increased more gradually over the course of the following 4 wk to a maximum of approximately 30°C. Water temperature was monitored continuously at 15 min intervals in each tank with HOBO Pendant loggers (Onset) (Fig. S2).

After an acclimatization phase of 2 wk, units involving the injection treatment were injected with 2 ml of organic matter in the form of marine microalgae concentrate (Reed Mariculture, Shellfish Diet 1800) via the injection tube (rubber septum) in the bottom compartment (Fig. S1). This experimental

set-up mimics nutrient and sulfide increase in the sediment similarly to seagrass experiments in which slow-release fertilizer was used (Christianen et al. 2012, Govers et al. 2014, van der Geest et al. 2020), with the advantage that concentrations can be easily increased during the experiment. Organic matter injections were performed in Weeks 3, 8 and 11. Control units were injected with 2 ml of anaerobic seawater. Sulfide concentrations in sediment porewater were measured weekly (Fig. S3). Sediment porewater was drawn anaerobically from the upper compartment with a 5 cm Rhizon sampler (Rhizosphere Research Products) and directly transferred with an attached needle into vacuumized 12 ml glass flasks filled with 6 ml of sulfide anti-oxidation buffer. Sulfide concentrations were determined immediately with a silver/sulfide ion-selective electrode (HI4115; Hanna 244 Instruments).

At the end of the experiment, 5 cm Rhizon samplers were used to extract 24 ml of pore water from the main root zone (top 6 cm) of each upper compartment into 2 vacuumized 12 ml flasks. From these porewater samples, ammonium and phosphate concentrations were determined colorimetrically on an Auto-Analyzer 3 system (Bran & Luebbe or Skalar and Seal auto-analyzer), using salicylate and ammonium molybdate-based methods, respectively (Lamers et al. 1998, van der Heide et al. 2012). Following porewater collection, seagrass and lucinid clams were harvested. After determining the wet weight of seagrass above- and belowground biomass, samples were dried at 70°C for 3 d. Subsequently, biomass dry weight was determined. Total carbon and nitrogen concentrations in *Z. noltii* leaves were measured in dried tissues by a CNS analyzer (type NA1500; Carlo Erba Instruments). Total phosphorus was determined after digestion of dried *Z. noltii* leaves with nitric acid and analyzed using an inductively coupled plasma emission (ICP) spectrophotometer (ICP-OES iCAP 6000; Thermo Fisher Scientific).

2.3. Statistical analyses

Analyses were conducted with R statistical and programming environment (version 4.1.1, R Core Team 2021) to determine the effects of lucinid absence or presence, organic matter injection and ambient or increased water temperature. Each analysis consisted of a linear mixed effects model including seagrass biomass, porewater sulfide and nutrient concentrations and plant nutrient content as response variables, while lucinid absence or presence, organic matter injection

and ambient or increased water temperature served as independent variables. Possible differences due to non-identical conditions within the 4 water tanks were taken into account in all models by inclusion of the tanks as a random effect and were not significant for any of the models. In order to obtain normally distributed residuals to run linear modeling, data were log transformed when needed (see Table 1). Normality and homogeneity of variances were evaluated with graphical methods; quantile–quantile plots of residuals from generated models; and plots of model residuals versus fitted values. We tested if the interaction of the 3 independent variables had a significant effect at a significance level of $\alpha = 0.05$. p-values were computed by using Satterthwaite approximation for denominator degrees of freedom from the R package ‘lmerTest,’ and the function ‘rand’ was used to test the effect of the random effects (Kuznetsova et al. 2017).

3. RESULTS

3.1. Seagrass biomass

Live *Zostera noltii* belowground dry biomass was significantly higher in the presence of *Loripes orbiculatus*, while no effect of temperature or organic matter treatment was detected (Table 1). However, aboveground *Z. noltii* dry biomass was increased by the addition of organic matter and by the presence of *L. orbiculatus*, and was amplified when these 2 treatments were combined, resulting in a positive synergistic effect where *Z. noltii* aboveground biomass increased 1.5- and 2.5-fold in the presence of *L. orbiculatus* in plots without and with addition of organic matter, respectively. However, this effect was hampered by increased temperature, as the positive effect of *L. orbiculatus* presence was decreased by 1.9-fold, but only in plots where organic matter was added (Fig. 1a, Table 1). Consequently, we detected a significant 3-way interaction among temperature, organic matter addition and *L. orbiculatus* presence (Table 1).

Table 1. Results of ANOVA *F*-tests on type III linear mixed models of *Zostera noltii* biomass, sediment porewater chemistry and aboveground *Z. noltii* nutrient content, with treatment effects with or without organic matter (OM) addition, ambient or increased temperature (T) conditions and *Loripes orbiculatus* presence or absence (L). Water tank was considered a random factor. Data transformations are indicated. Significant ($\alpha = 0.05$) p-values are highlighted in **bold**

Response variable	Treatment	df	<i>F</i>	p
Belowground biomass	L	30.08	25.12	<0.0001
	OM	30.37	4.05	0.053
	T	1.96	1.86	0.308
	L × OM	30.08	1.34	0.257
	L × T	30.08	2.95	0.096
	OM × T	30.37	1.32	0.260
	L × OM × T	30.08	0.33	0.569
Aboveground biomass	L	30.13	26.61	<0.0001
	OM	30.61	29.69	<0.0001
	T	1.93	3.21	0.219
	L × OM	30.13	12.26	0.001
	L × T	30.13	10.29	0.003
	OM × T	30.61	11.58	0.002
	L × OM × T	30.13	4.24	0.048
Sulfide concentration (log10)	L	32.00	307.32	<0.0001
	OM	32.00	127.26	<0.0001
	T	32.00	27.60	<0.0001
	L × OM	32.00	0.96	0.344
	L × T	32.00	0.01	0.937
	OM × T	32.00	7.92	0.008
	L × OM × T	32.00	0.25	0.621
Porewater NH ₄ (ln)	L	32.00	443.63	<0.0001
	OM	32.00	305.73	<0.0001
	T	32.00	103.39	<0.0001
	L × OM	32.00	31.24	<0.0001
	L × T	32.00	2.32	0.137
	OM × T	32.00	15.12	<0.0001
	L × OM × T	32.00	12.83	0.001
Porewater PO ₄ (ln+1)	L	32.00	725.51	<0.0001
	OM	32.00	140.75	<0.0001
	T	32.00	5.07	0.031
	L × OM	32.00	3.28	0.080
	L × T	32.00	0.46	0.500
	OM × T	32.00	5.69	0.023
	L × OM × T	32.00	0.01	0.926
Total leaf nitrogen	L	30.17	86.13	<0.0001
	OM	30.77	50.18	<0.0001
	T	1.91	4.25	0.181
	L × OM	30.17	33.32	<0.0001
	L × T	30.17	10.88	0.002
	OM × T	30.77	18.60	<0.0001
	L × OM × T	30.17	13.00	0.001
Total leaf phosphorus	L	29.25	23.76	<0.0001
	OM	30.28	26.81	<0.0001
	T	1.85	10.34	0.094
	L × OM	29.25	23.76	<0.0001
	L × T	29.25	13.69	0.001
	OM × T	30.28	13.38	0.001
	L × OM × T	29.25	8.93	0.006

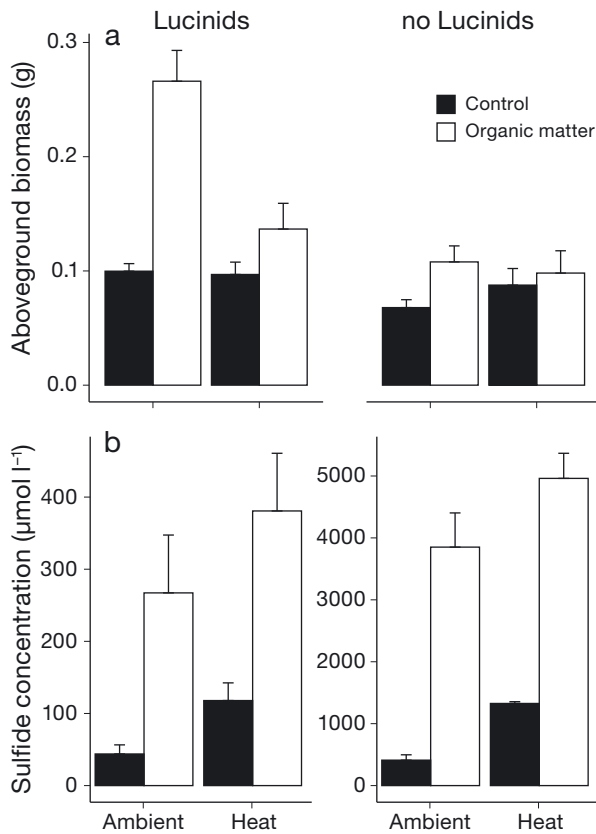


Fig. 1. *Zostera noltii* (a) aboveground dry biomass and (b) porewater sulfide concentration with and without organic matter addition and with and without the lucinid *Loripes orbiculatus* under ambient and increased temperature conditions after 12 wk. Bars represent mean \pm SE

3.2. Porewater sulfide and nutrients

Organic matter addition increased sediment porewater sulfide concentrations under ambient conditions 6 to 9 times with and without *L. orbiculatus*, respectively. This effect was amplified by 1.3 and 1.4 times at higher temperature, respectively, resulting in a positive antagonistic effect when these 2 treatments were combined (Table 1, Fig. 1b). The addition of *L. orbiculatus* dramatically decreased sulfide concentrations on average between treatments by 12 times, and it also led to decreased porewater ammonium and phosphate of similar magnitude, 17 and 22 times, respectively (Table 1, Fig. 2). Porewater ammonium was increased by both organic matter addition and by higher temperature (Fig. 2a). However, this temperature effect was diminished in organic matter treatments without *L. orbiculatus*, yielding a 3-way interaction among temperature, organic matter addition and *L. orbiculatus* presence (Table 1). Porewater phosphate was increased by organic matter ad-

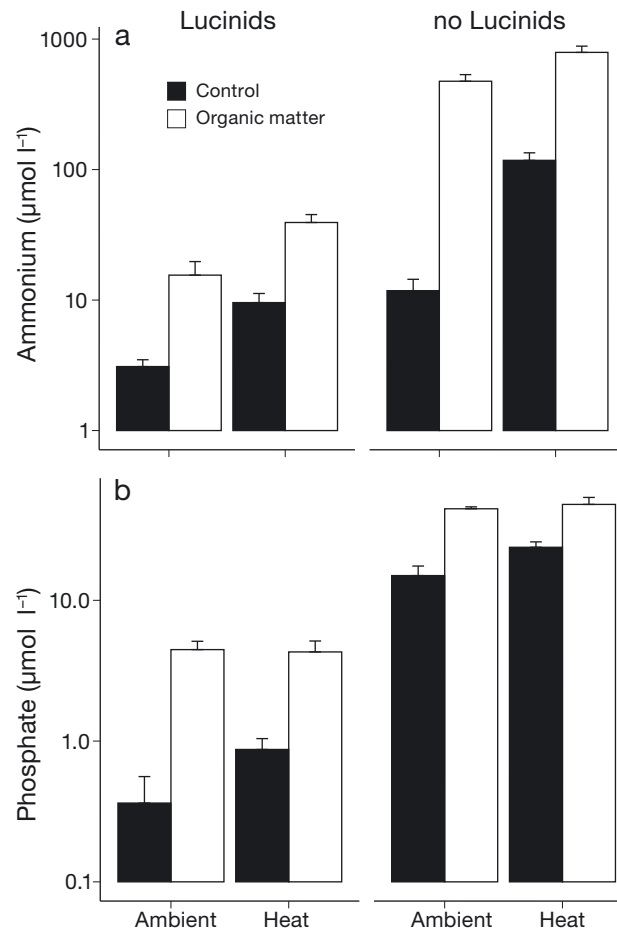


Fig. 2. Sediment porewater (a) ammonium and (b) phosphate with and without organic matter addition and with and without the lucinid *Loripes orbiculatus* under ambient and increased temperature conditions after 12 wk. Bars represent mean \pm SE

dition (Fig. 2b). Temperature also had a positive effect, but in general in the treatments without additional organic matter. The presence of *L. orbiculatus* decreased sediment porewater phosphate (Table 1).

3.3. Seagrass nutrient content

Total nitrogen content in aboveground *Z. noltii* was increased by *L. orbiculatus* presence (Fig. 3a) and addition of organic matter, and the latter was amplified in the presence of *L. orbiculatus* (Table 1). However, this positive effect of *L. orbiculatus* with organic matter addition was hampered by 64% when temperature was increased (Table S1). *L. orbiculatus* also had a strong positive effect on total leaf nitrogen in treatments without added organic matter. Temperature had a minor positive effect in treatments without

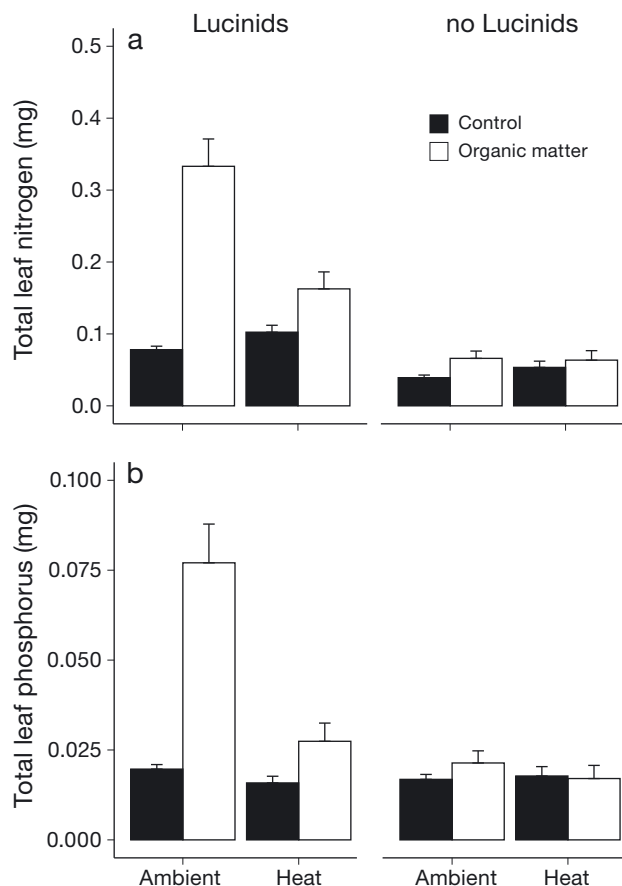


Fig. 3. (a) Total nitrogen and (b) total phosphorus in aboveground *Zostera noltii* with and without organic matter addition and with and without the lucinid *Loripes orbiculatus* under ambient and increased temperature conditions, after 12 wk. Bars represent mean \pm SE

L. orbiculatus. This resulted in all interactions becoming significant (Table 1). Similar to nitrogen, *L. orbiculatus* also had a positive effect on total phosphorus content in aboveground *Z. noltii* when organic matter was added, particularly under ambient temperature conditions, but this effect decreased by 89% when temperature increased (Fig. 3b; Table S1); here as well, all interactions were significant (Table 1). Average carbon content did not differ between treatments, and no major effects were detected (Table S1).

4. DISCUSSION

In this study, we aimed to clarify the role of the facultative lucinid–seagrass mutualism on growth and nutrient content by seagrass under enhanced organic loading and temperature conditions. Our mesocosm experiment shows that *Loripes orbiculatus* not only increased growth, but also stimulated nutrient con-

tent in *Zostera noltii*. However, we found that this effect on growth and nutrient content was hampered by high temperatures when organic matter was increased, despite the increase in porewater nutrients under these conditions. As expected, our organic matter addition and temperature increase treatment both stimulated porewater sulfide levels. In turn, sulfide was lowered by *L. orbiculatus*, facilitating higher nutrient uptake by seagrass resulting in higher biomass. Our results suggest that *Z. noltii* is capable of increasing nutrient uptake and growth due to the mutualistic interaction, but this effect was reduced when temperature was rapidly increased.

4.1. Temperature and sulfide stress effect

Z. noltii aboveground biomass increased in the presence of *L. orbiculatus* and reduced porewater sulfide concentrations with and without organic matter addition. This effect was hampered in the latter when temperature was suddenly increased, as the positive effect of *L. orbiculatus* presence was decreased (Fig. 1a). Thus, our results confirm that seagrass biomass is increased by the presence of endosymbiotic lucinid bivalves, due to their effect on toxic sulfide levels, which was also shown by earlier work (e.g. van der Heide et al. 2012, van der Geest et al. 2020). However, our results also show a temperature effect on seagrass biomass. Both bioirrigation and the endosymbiotic sulfide-oxidizing gill bacteria combined seem to be responsible for the reduction of porewater sulfide (see discussion in Chin et al. 2021). This sulfide reduction by *L. orbiculatus* appears to be negatively affected by temperature, as sulfide increased by 170% (44 to 118 $\mu\text{mol l}^{-1}$) and 43% (268 to 380 $\mu\text{mol l}^{-1}$) in treatments without and with organic matter addition, respectively, but this effect was not significant (Fig. 1b; Table S1). Therefore, we argue that the temperature-related increased sulfide concentrations alone cannot explain the reduced growth of *Z. noltii*. Although *Z. noltii* has a thermotolerance threshold over 38°C (Massa et al. 2009), results do indicate that *Z. noltii* is also directly negatively affected by the increased temperature in our experiment. Indeed, nutrient content was negatively affected by the cumulative effects of temperature and sulfide toxicity. Sulfide toxicity for *Z. noltii* can occur at a level above about 200 $\mu\text{mol l}^{-1}$ (van der Heide et al. 2012, Govers et al. 2014). Total nitrogen and phosphorus stored in seagrass leaves increased in the presence of *L. orbiculatus* under ambient conditions with and without organic matter addition (Fig. 3a). However, when tem-

perature increase was combined with organic matter addition, this *L. orbiculatus* effect decreased for both nitrogen and phosphorus. This result shows that nutrient uptake is reduced due to thermal stress and/or sulfide toxicity and can explain the reduced growth of *Z. noltii* with increasing temperature.

4.2. Plant nutrient origin

Although we cannot determine the direct origin of nitrogen in the seagrass, we suspect that sulfide detoxification by *L. orbiculatus* probably facilitates nutrient uptake, explaining the higher nutrient content (nitrogen and phosphorus) in the seagrass. Nutrient uptake by plants is hampered due to intrusion of phytotoxic sulfide into root tissue (see review by Lamers et al. 2013). In addition, bioirrigation may also cause the release of nutrients to the surface water, which possibly increases the flux of nutrients dissolved in the porewater for the seagrass roots, despite the overall decrease in sediment porewater nutrients in the presence of the lucinids (van der Heide et al. 2012, Chin et al. 2021). Cardini et al. (2019) suggested that *L. orbiculatus* may also provide a direct additional source of ammonium via nitrogen fixation by the endosymbiotic bacteria. Lucinid bivalves host several kinds of bacteria capable of diazotrophy and excrete ammonia directly in the sediment (Petersen et al. 2017, Cardini et al. 2019, Martin et al. 2020). Apart from bacteria hosted by the Lucinidae, there are also free-living diazotrophic bacteria in the sediment which can contribute to seagrass growth (Welsh 2000, Martin et al. 2020). It is not unlikely that sediment microbial assemblages may change under the presence of Lucinidae, which has been shown for other bivalve species (Ray et al. 2021), in turn positively affecting seagrass biomass. Our study is the first to show that nitrogen and phosphorus content increased in seagrass tissue in the presence of lucinids. However, we cannot quantify the contribution of nitrogen fixation on the ammonium availability for seagrass as suggested by Cardini et al. (2019) or the role of the detoxifying effect or bioirrigation by the Lucinid–bacteria consortium.

4.3. Trophic ecosystem importance

In eutrophic, sulfide-rich systems, mutualism may be more important for seagrass because of the direct sulfide-detoxifying role of the lucinids. In very oligotrophic systems, sulfide levels are generally expected

to be low. Because organic carbon in sediment is generally low in oligotrophic systems, the build-up of toxic porewater sulfide will also be low. Therefore, we argue that the direct effect of sulfide detoxification on seagrass growth is also generally low in oligotrophic systems. However, nitrogen availability is often a limiting factor for seagrass productivity, especially in oligotrophic systems (Agawin et al. 1996, Udy & Dennison 1997, Fourqurean & Zieman 2002). As seagrass growth is decreased by sulfide even at very low concentrations ($<10 \mu\text{mol l}^{-1}$) (Calleja et al. 2007, Lamers et al. 2013), nutrient uptake is likely also hampered, and mutualism may be important even in these low-sulfide, and low-nutrient environments.

4.4. General importance and implications for restoration

In our experiment, temperature increased sediment porewater sulfide, ammonium and phosphate concentrations simultaneously, due to increased anaerobic decomposition of organic matter in the lower compartments of the mesocosms. Under these conditions, *Z. noltii* biomass increased in the presence of *L. orbiculatus*, although the extent of possible benefits through nutrient uptake seems highly impaired by temperature stress. Therefore, in this study, temperature stress did not lead to a mutualistic breakdown; rather, it suppressed the mutualistic benefit for *Z. noltii*. Nevertheless, even under these conditions, *Z. noltii* biomass and nutrient content were still higher compared to the situation without *L. orbiculatus*, suggesting that the mutualistic relationship still holds and facilitates seagrass in stressful conditions. Similar results were shown in a field experiment where under manipulated enhanced sulfide stress conditions, the presence of *L. orbiculatus* was still beneficial for *Z. noltii* (van der Geest et al. 2020). In addition, in a tropical lucinid–seagrass interaction, the facilitation strength became even more important under a combination of reduced light and sulfide stress conditions (Chin et al. 2021). These observations also suggest that for seagrass meadows where the lucinid–seagrass facultative mutualism occurs, this association becomes more important with current global change. As climate change will lead to increased average temperatures as well as an increase in the number of marine heat waves, it seems likely that stress events will become a more common phenomenon (Smale et al. 2019, Laufkötter et al. 2020).

Our study highlights the importance of the facultative seagrass–lucinid mutualism for plant health. Re-

cently, studies have increasingly emphasized that many marine foundation species engage in facultative mutualisms, stressing the importance of considering these interactions when aiming to conserve habitats formed by these species (Renzi et al. 2019, van der Heide et al. 2021). In addition, within the context of restoration, the inclusion of these positive interactions may greatly increase restoration success. For example, work from US salt marsh experiments with cordgrass *Spartina alterniflora* shows that the inclusion of interspecific interactions such as with ribbed mussels *Geukensia demissa* may also greatly benefit restoration efforts (Angelini et al. 2016, Derksen-Hooijberg et al. 2018), and similar results were obtained in coral reefs with the addition of sponges (Biggs 2013). To our knowledge, there are currently no examples where lucinid bivalves were included in seagrass restoration programs. However, based on our findings and those of previous recent studies, we argue that co-transplantation of lucinids may enhance seagrass restoration success, particularly in sheltered areas with high nutrient loading (Gagnon et al. 2020). In addition to co-transplantation, it would be worthwhile exploring the possibility of cultivating lucinid bivalves which could be added to seagrass restoration projects without harming an existing seagrass meadow. We suggest that in seagrass restoration projects, managers should investigate whether such positive interactions naturally occur in the target location and under what conditions these interactions are vital for ecosystem functioning.

Data availability. Data are deposited in the Dryad Digital Repository available at <https://doi.org/10.17026/dans-x9d-ytww>

Acknowledgements. We thank Roy Peters, Fleur Boelens, Jolieke Siepman and Peter Cruijssen for technical assistance during fieldwork, collecting materials for the experiment and conducting the mesocosm experiment, and Paul van der Ven of the General Instrumentation facility at Radboud University Nijmegen for nutrient and elemental analysis. J.d.F. was supported by NWO Open Competition #ALWOP.203, and T.v.d.H. by NWO-Vidi Career #16588. M.v.d.G. was supported by a post-doctoral fellowship from the Mediterranean Centre for Environment and Biodiversity (LabEx-CeMEB) and received funding from the European Union's Horizon 2020 Research and Innovation Programme under grant agreement no. 869710.

LITERATURE CITED

- others (2016) A keystone mutualism underpins resilience of a coastal ecosystem to drought. *Nat Commun* 7:12473
- ✦ Biggs BC (2013) Harnessing natural recovery processes to improve restoration outcomes: an experimental assessment of sponge-mediated coral reef restoration. *PLOS ONE* 8:e64945
- ✦ Borum J, Pedersen O, Greve TM, Frankovich TA, Zieman JC, Fourqurean JW, Madden CJ (2005) The potential role of plant oxygen and sulphide dynamics in die-off events of the tropical seagrass, *Thalassia testudinum*. *J Ecol* 93:148–158
- ✦ Brun FG, Hernández I, Vergara JJ, Peralta G, Pérez-Lloréns JL (2002) Assessing the toxicity of ammonium pulses to the survival and growth of *Zostera noltii*. *Mar Ecol Prog Ser* 225:177–187
- ✦ Burkholder JM, Tomasko DA, Touchette BW (2007) Seagrasses and eutrophication. *J Exp Mar Biol Ecol* 350: 46–72
- ✦ Calleja ML, Marba N, Duarte CM (2007) The relationship between seagrass (*Posidonia oceanica*) decline and sulfide porewater concentration in carbonate sediments. *Estuar Coast Shelf Sci* 73:583–588
- ✦ Cardini U, Bartoli M, Lückner S, Mooshammer M and others (2019) Chemosymbiotic bivalves contribute to the nitrogen budget of seagrass ecosystems. *ISME J* 13:3131–3134
- ✦ Cavanaugh CM (1983) Symbiotic chemoautotrophic bacteria in marine invertebrates from sulphide-rich habitats. *Nature* 302:58–61
- ✦ Chin DW, de Fouw J, van der Heide T, Cahill BV and others (2021) Facilitation of a tropical seagrass by a chemosymbiotic bivalve increases with environmental stress. *J Ecol* 109:204–217
- ✦ Christianen MJA, Govers LL, Bouma TJ, Kiswara W, Roelofs JGM, Lamers LPM, van Katwijk MM (2012) Marine megaherbivore grazing may increase seagrass tolerance to high nutrient loads. *J Ecol* 100:546–560
- ✦ de Fouw J, Govers LL, van de Koppel J, van Belzen J and others (2016) Drought, mutualism breakdown and landscape-scale degradation of seagrass beds. *Curr Biol* 26: 1051–1056
- ✦ de Fouw J, van der Heide T, van Belzen J, Govers LL and others (2018) A facultative mutualistic feedback enhances the stability of tropical intertidal seagrass beds. *Sci Rep* 8: 12988
- ✦ Derksen-Hooijberg M, Angelini C, Lamers LPM, Borst ACW and others (2018) Mutualistic interactions amplify salt-marsh restoration success. *J Appl Ecol* 55:405–414
- ✦ Dubilier N, Bergin C, Lott C (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat Rev Microbiol* 6:725–740
- ✦ Folmer EO, van der Geest M, Jansen EJ, Olff H, Anderson TM, Piersma T, van Gils JA (2012) Seagrass–sediment feedback: an exploration using a non-recursive structural equation model. *Ecosystems* 15:1380–1393
- ✦ Fourqurean JW, Zieman JC (2002) Nutrient content of the seagrass *Thalassia testudinum* reveals regional patterns of relative availability of nitrogen and phosphorus in the Florida Keys USA. *Biogeochemistry* 61:229–245
- ✦ Gagnon K, Rinde E, Bengil EGT, Carugati L and others (2020) Facilitating foundation species: the potential for plant–bivalve interactions to improve habitat restoration success. *J Appl Ecol* 57:1161–1179
- ✦ Govers LL, de Brouwer JHF, Suykerbuyk W, Bouma TJ, Lamers LPM, Smolders AJP, van Katwijk MM (2014) Toxic effects of increased sediment nutrient and organic
- ✦ Agawin NSR, Duarte CM, Fortes MD (1996) Nutrient limitation of Philippine seagrasses (Cape Bolinao, NW Philippines): *in situ* experimental evidence. *Mar Ecol Prog Ser* 138:233–243
- ✦ Angelini C, Griffin JN, van de Koppel J, Lamers LPM and

- matter loading on the seagrass *Zostera noltii*. *Aquat Toxicol* 155:253–260
- ✦ Hall MO, Furman BT, Merello M, Durako MJ (2016) Recurrence of *Thalassia testudinum* seagrass die-off in Florida Bay: initial observations. *Mar Ecol Prog Ser* 560: 243–249
- ✦ Hansen JCR, Reidenbach MA (2012) Wave and tidally driven flows in eelgrass beds and their effect on sediment suspension. *Mar Ecol Prog Ser* 448:271–287
- ✦ Hansen JW, Udy JW, Perry CJ, Dennison WC, Lomstein BA (2000) Effect of the seagrass *Zostera capricorni* on sediment microbial processes. *Mar Ecol Prog Ser* 199:83–96
- ✦ Hasler-Sheetal H, Holmer M (2015) Sulfide intrusion and detoxification in the seagrass *Zostera marina*. *PLOS ONE* 10:e0129136
- ✦ Holmer M, Bondgaard EJ (2001) Photosynthetic and growth response of eelgrass to low oxygen and high sulfide concentrations during hypoxic events. *Aquat Bot* 70:29–38
- ✦ Holmer M, Pedersen O, Krause-Jensen D, Olesen B and others (2009) Sulfide intrusion in the tropical seagrasses *Thalassia testudinum* and *Syringodium filiforme*. *Estuar Coast Shelf Sci* 85:319–326
- ✦ Hughes BB, Eby R, Van Dyke E, Tinker MT, Marks CI, Johnson KS, Wasson K (2013) Recovery of a top predator mediates negative eutrophic effects on seagrass. *Proc Natl Acad Sci USA* 110:15313–15318
- ✦ Jørgensen BB (1982) Mineralization of organic matter in the sea bed—the role of sulphate reduction. *Nature* 296: 643–645
- ✦ Koch MS, Mendelssohn IA, McKee KL (1990) Mechanism for the hydrogen sulfide-induced growth limitation in wetland macrophytes. *Limnol Oceanogr* 35:399–408
- ✦ König S, Gros O, Heiden SE, Hinzke T and others (2017) Nitrogen fixation in a chemoautotrophic lucinid symbiosis. *Nat Microbiol* 2:16193
- ✦ Kuznetsova A, Brockhoff PB, Christensen RH (2017) lmerTest package: tests in linear mixed effects models. *J Stat Softw* 82:1–26
- ✦ Lamers LPM, Tomassen HBM, Roelofs JGM (1998) Sulfate-induced eutrophication and phytotoxicity in freshwater wetlands. *Environ Sci Technol* 32:199–205
- ✦ Lamers LPM, Govers LL, Janssen ICJM, Geurts JJM and others (2013) Sulfide as a soil phytotoxin—a review. *Front Plant Sci* 4:268
- ✦ Laufkötter C, Zscheischler J, Frölicher TL (2020) High-impact marine heatwaves attributable to human-induced global warming. *Science* 369:1621–1625
- ✦ Martin BC, Bougoure J, Ryan MH, Bennett WW and others (2019) Oxygen loss from seagrass roots coincides with colonisation of sulphide-oxidising cable bacteria and reduces sulphide stress. *ISME J* 13:707–719
- ✦ Martin BC, Middleton JA, Fraser MW, Marshall IPG, Scholz VV, Hausl B, Schmidt H (2020) Cutting out the middle clam: lucinid endosymbiotic bacteria are also associated with seagrass roots worldwide. *ISME J* 14:2901–2905
- ✦ Massa SI, Arnaud-Haond S, Pearson GA, Serrao EA (2009) Temperature tolerance and survival of intertidal populations of the seagrass *Zostera noltii* (Hornemann) in Southern Europe (Ria Formosa, Portugal). *Hydrobiologia* 619:195–201
- ✦ Östman Ö, Eklöf J, Eriksson BK, Olsson J, Moksnes PO, Bergström U (2016) Top-down control as important as nutrient enrichment for eutrophication effects in North Atlantic coastal ecosystems. *J Appl Ecol* 53:1138–1147
- ✦ Petersen JM, Kemper A, Gruber-Vodicka H, Cardini U and others (2017) Chemosynthetic symbionts of marine invertebrate animals are capable of nitrogen fixation. *Nat Microbiol* 2:16195
- R Core Team (2021) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna
- ✦ Ray NE, Hancock B, Brush MJ, Colden A and others (2021) A review of how we assess denitrification in oyster habitats and proposed guidelines for future studies. *Limnol Oceanogr Methods* 19:714–731
- ✦ Renzi JJ, He Q, Silliman BR (2019) Harnessing positive species interactions to enhance coastal wetland restoration. *Front Ecol Evol* 7:131
- ✦ Reynolds LK, Berg P, Zieman JC (2007) Lucinid clam influence on the biogeochemistry of the seagrass *Thalassia testudinum* sediments. *Estuaries Coasts* 30:482–490
- ✦ Ruiz-Halpern S, Macko SA, Fourqurean JW (2008) The effects of manipulation of sedimentary iron and organic matter on sediment biogeochemistry and seagrasses in a subtropical carbonate environment. *Biogeochemistry* 87: 113–126
- ✦ Seddon S, Connolly RM, Edyvane KS (2000) Large-scale seagrass dieback in northern Spencer Gulf, South Australia. *Aquat Bot* 66:297–310
- ✦ Smale DA, Wernberg T, Oliver ECJ, Thomsen M and others (2019) Marine heatwaves threaten global biodiversity and the provision of ecosystem services. *Nat Clim Change* 9: 306–312
- ✦ Touchette BW, Burkholder JM (2000) Review of nitrogen and phosphorus metabolism in seagrasses. *J Exp Mar Biol Ecol* 250:133–167
- ✦ Udy JW, Dennison WC (1997) Growth and physiological responses of three seagrass species to elevated sediment nutrients in Moreton Bay, Australia. *J Exp Mar Biol Ecol* 217:253–277
- ✦ Unsworth RKF, Collier CJ, Waycott M, McKenzie LJ, Cullen-Unsworth LC (2015) A framework for the resilience of seagrass ecosystems. *Mar Pollut Bull* 100:34–46
- ✦ van der Geest M, van der Heide T, Holmer M, de Wit R (2020) First field-based evidence that the seagrass–lucinid mutualism can mitigate sulfide stress in seagrasses. *Front Mar Sci* 7:11
- ✦ van der Heide T, Peeters ETHM, Hermus DCR, van Katwijk MM, Roelofs JGM, Smolders AJP (2009) Predicting habitat suitability in temperate seagrass ecosystems. *Limnol Oceanogr* 54:2018–2024
- ✦ van der Heide T, Govers LL, de Fouw J, Olf H and others (2012) A three-stage symbiosis forms the foundation of seagrass ecosystems. *Science* 336:1432–1434
- ✦ van der Heide T, Angelini C, de Fouw J, Eklöf JS (2021) Facultative mutualisms: a double-edged sword for foundation species in the face of anthropogenic global change. *Ecol Evol* 11:29–44
- ✦ van Katwijk MM, Vergeer LHT, Schmitz GHW, Roelofs JGM (1997) Ammonium toxicity in eelgrass *Zostera marina*. *Mar Ecol Prog Ser* 157:159–173
- ✦ Waycott M, Duarte CM, Carruthers TJB, Orth RJ and others (2009) Accelerating loss of seagrasses across the globe threatens coastal ecosystems. *Proc Natl Acad Sci USA* 106:12377–12381
- ✦ Welsh DT (2000) Nitrogen fixation in seagrass meadows: regulation, plant–bacteria interactions and significance to primary productivity. *Ecol Lett* 3:58–71