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Toxic effects of increased sediment nutrient and organic matter loading on the seagrass *Zostera noltii*

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Abstract

As a result of anthropogenic disturbances and natural stressors, seagrass beds are often patchy and heterogeneous. The effects of high loads of nutrients and organic matter in patch development and expansion in heterogeneous seagrass beds have, however, poorly been studied. We experimentally assessed the *in situ* effects of sediment quality on seagrass (*Zostera noltii*) patch dynamics by studying patch (0.35 m diameter) development and expansion for 4 sediment treatments: control, nutrient addition (NPK), organic matter addition (OM) and a combination (NPK+OM). OM addition strongly increased porewater sulfide concentrations whereas NPK increased porewater ammonium, nitrate and phosphate concentrations. As high nitrate concentrations suppressed sulfide production in NPK+OM, this treatment was biogeochemically comparable to NPK. Sulfide and ammonium concentrations differed within treatments, but over a 77 days period, seagrass patch survival and expansion were impaired by all additions compared to the control treatment. Expansion decreased at porewater ammonium concentrations >2000 μmol L^{-1}. Mother patch biomass was not affected by high porewater ammonium concentrations as a result of its detoxification by higher seagrass densities. Sulfide

concentrations >1000 μmol L^{-1} were toxic to both patch expansion and mother patch. We conclude that patch survival and expansion are constrained at high loads of nutrients or organic matter as a result of porewater ammonium or sulfide toxicity.

1. Introduction

Seagrass beds are among the most productive and biodiverse ecosystems in the world (Hemminga and Duarte, 2000; Orth et al., 2006). Unfortunately, they are severely threatened by increasing human activities in coastal areas (Orth et al., 2006; Waycott et al., 2009). The most imminent problems for seagrass meadows are related to the large-scale eutrophication of coastal waters (Burkholder et al., 2007; Duarte, 2002; Orth et al., 2006; Waycott et al., 2009). Eutrophication results in light limitation for submerged species, as it promotes the growth of epiphytes and phytoplankton, but may also directly affect seagrass vitality through ammonium toxicity (Brun et al., 2002; Van der Heide et al., 2008; van Katwijk et al., 1997). High ammonium levels are known to be able to disrupt the uptake of essential cations such as potassium by higher plants, affect their pH regulation, and decrease overall growth rates (Britto and Kronzucker, 2002).

In addition, eutrophication related to land use change (including deforestation) often increases the organic matter input into coastal sediments, thereby not only increasing ammonium levels even further, but also fueling the production of sulfide (Burkholder et al., 2007; Van der Heide, 2009). Sulfide is a strongly phytotoxic compound, as it blocks the activity of cytochrome oxidase and other metal

containing enzymes (Lamers et al., 2013). It is formed in anaerobic sediments rich in organic matter, where sulfate is used as an alternative terminal electron acceptor during the microbial breakdown of organic matter (Gray and Elliott, 2009; Holmer and Nielsen, 1997). Sulfide toxicity, due to the build-up of sulfides in the sediment, may lead to massive seagrass die-offs (Borum et al., 2005; Carlson et al., 1994; Terrados et al., 1999).

The deterioration of habitat quality, including eutrophication and sulfide toxicity, has led to the complete disappearance of seagrass beds in some regions (Borum et al., 2005; Burkholder et al., 2007). In other areas, it has created heterogeneous, patchy seagrass landscapes (Duarte and Sand-Jensen, 1990; Frederiksen, 2004; Pasqualini et al., 1999) which reflect processes of recovery from disturbances (Brun et al., 2003a; Duarte and Sand-Jensen, 1990; Pickett and White, 1985). A patch, defined as "a surface area that differs from its surrounding in nature or appearance" (Turner et al., 2001), may result from both sexual and asexual colonization (Bostrom et al., 2006; Duarte and Sand-Jensen, 1990), and from habitat fragmentation of seagrass beds. Patches expand through shoot expansion, mainly through horizontal elongation of rhizomes at the patch edges (Duarte et al., 2006a; Marba and Duarte, 1998; Olesen and Sand-Jensen, 1994; Vermaat, 2009). Their shoot densities increase exponentially (self-accelerating) with increasing patch age and size (Bostrom et al., 2006; Sintes et al., 2005; Vermaat, 2009; Vidondo et al., 1997).

Most studies have tended to focus on large-scale processes (Bell et al., 1999) in seagrass beds, rather than on ubiquitous but small-scale dynamics. Studies

describing seagrass patch dynamics (Almela et al., 2008; Brun et al., 2003b; Campbell and Paling, 2003; Di Carlo and Kenworthy, 2008; Duarte and Sand-Jensen, 1990) focused on plant physiology rather than on the impact of environmental characteristics including local sediment biogeochemistry. Eutrophication and sulfide toxicity, two environmental stressors of seagrass meadows that have rapidly become common at a global scale, may not only contribute to the formation of patches, but can also be expected to affect patch expansion itself. As patchy seagrass meadows are expected to be more vulnerable to stressors than homogeneous fields due to their lower regrowth capacities and the higher impact of hydrodynamics (Bos and van Katwijk, 2007; Duarte and Sand-Jensen, 1990; Olesen and Sand-Jensen, 1994), it is important to know the effects of both important, potentially phytotoxic stressors on seagrass patches. To our knowledge, experimental manipulation of sediment quality to study the effects on seagrass patch expansion has never been carried out before. We therefore assessed the possible toxic effects of changes in biogeochemical sediment quality related to eutrophication (increased levels of nutrients and organic matter) on small-scale seagrass patches, using the fast growing *Zostera noltii* as a model species.

2. Materials and Methods

2.1 Study Area. The experiment was conducted in an intertidal *Zostera noltii* bed on the mudflats of Viane (51°39' N, 4°01' E), the Oosterschelde, in the southwestern part of the Netherlands. The Oosterschelde delta has a surface area of 351 km^2 and a tidal amplitude of 2.5-3 m (Troost et al., 2009). Mean surface water temperature

fluctuates between 0-22°C annually. Freshwater input into the former estuary has become highly limited after the implementation of major water works in the past, and salinity is generally around 30 (Nienhuis and Smaal, 1994).

Zostera noltii meadows cover around 75 ha of the mudflats in the Oosterschelde, growing mainly in sheltered areas near dikes. At the experimental site, *Zostera noltii* was growing within 30 meters of a dike, on a compact clay bank with a median grain size (D50) of 130 μm and % organic carbon of 0.72%. At the start of the experiment, the seagrass cover of the meadow was relatively homogeneous (2250 ± 124 shoots m^{-1}) and biogeochemical characteristics of the meadow were also determined (sulfide = 0.31 ± 0.22, NH_4 = 13.14 ± 2.09, NO_3 = 5.51 ± 1.44, PO_4 = 8.95 ± 0.84, μmol L^{-1}, n = 21). Experimental seagrass patches, as well as the natural bed, were located on the mudflat at an average height of 54 cm above Amsterdam Ordnance Datum (NAP), resulting in exposition to the atmosphere for on average 7 hours during each low tide, depending on weather conditions. This intertidal mudflat was flooded and drained with each tidal cycle (semidiurnal).

2.2 Experimental design. Circular *Zostera noltii* sods of 35 cm in diameter and 7 cm depth were carefully collected from a homogeneously covered meadow, and transplanted to a bare stretch of the mudflat between the dike and the seagrass bed nearby. This area is known to be a suitable seagrass habitat, as seagrass was present and vital at this site until it was removed during dike reinforcement works in the previous growing season. The sods were transplanted into circular spots of 105 cm diameter and 7 cm depth. Before placing the sods, the different sediment treatments (details next section) were applied on the bare sediment. The sods,

hereafter referred to as mother patches, were placed in the middle of the treated sediment spots and the remains were filled up with local sediment. Treatments were randomly assigned to the patches, using eight replicates (n=8) per treatment. The experiment was carried out for 77 days, from June till September 2009.

2.3 Sediment treatments: nutrient and organic matter addition. To study the separate and interacting effects of sediment eutrophication, we applied four different biogeochemical treatments: a control treatment (Control), an organic matter treatment (OM), a nutrient treatment (NPK) and a combined treatment of both nutrients and organic matter (NPK+OM). We fertilized NPK and NPK+OM plots with 1.11 kg m^{-2} Osmocote® slow release fertilizer (g:g:g ratio N:P:K 18:9:10) with a longevity of 8-9 months. The fertilizer was distributed evenly beneath the seagrass sods and the surrounding, bare sediment. Loading rates were around 57 mmol N m^{-2} day^{-1} and 13 mmol P m^{-2} day^{-1} (Christianen et al., 2012; Vanlent et al., 1995).

For the organic matter treatments (OM and NPK + OM), we added 2 g L^{-1} of organic matter (1 g starch + 1 g cellulose L^{-1} sediment), which equaled a loading rate of about 157 mmol C m^{-2} day^{-1} (for 47 days), under the seagrass sods, which was expected to stimulate sulfide production (Peralta et al., 2003; Ruiz-Halpern et al., 2008). All treatments were applied only once, at the start of the experiment. However, the NPK and NPK+OM treatments appeared to generate biogeochemically similar results, as high nitrate levels suppressed sulfide production. As a result, this unforeseen effect of combined NPK and OM addition did not result in an expected interaction treatment.

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2.4 Data collection in the field. Sediment samples were collected at the start of the experiment for chemical analyses to characterize the local habitat. Patch heights were measured using a RTK-DGPS (Real Time Kinematic Global Positioning System) (Van der Heide et al., 2010a), but did not differ significantly among treatments. During the experiment, sediment porewater samples were collected anaerobically at t= 0, 21, 49, 77 days, using 60 mL vacuumed syringes connected to ceramic soil moisture samplers (Eijkelkamp Agrisearch Equipment, Giesbeek, the Netherlands), which were placed in the top 7 cm of the sediment. For each experimental patch, two porewater samples were taken, one inside the mother patch and one just outside the patch, but within the sediment treatment area (potential expansion area). Samples were pooled for analysis. Of each anaerobic sample, 10 mL was used for sulfide analysis on the same day (see below), and 40 mL was frozen until further chemical analyses. After porewater extraction, shoot numbers within the transplanted patches and shoot numbers of the patch outgrowth (patch expansion) were monitored, by counting all shoots. After 77 days, the experiment was terminated and patch shoot density was determined. The patch expansion area (all seagrass biomass produced outside the 0.35 m diameter patch) was harvested for all patches and a small core (diameter 0.06 m) was taken inside the patch to determine patch biomass. Harvested samples were rinsed and split up into aboveground (leaves and sheath) and belowground (rhizomes and roots) biomass, and dried at 60°C until constant weight, and weighed (g DW).

2.5 Analysis of porewater and plant samples. Within 5 hours after sampling, total sulfide concentration in the porewater was measured in a mixture of 50% sample

and 50% Sulfide Anti-Oxidation Buffer (SAOB) (Lamers et al., 1998), using an ion-specific silver-sulfide electrode. Porewater ammonium and ortho-phosphate concentrations were measured colorimetrically (Skalar and Seal autoanalyzer), using ammonium-molybdate and salicylate (Lamers et al., 1998). Nitrate was determined by sulphanilamide, after reduction of nitrate to nitrite in a cadmium column (Wood et al., 1967).

To assess seagrass nutrient status, dry plant material was used to determine % C and % N of both leaves and rhizomes, using an elemental analyzer (Type NA 1500 Carlo Erba Thermo Fisher Science, USA), coupled online via an interface (Finnigan Conflo III) to a mass spectrometer (Thermo Finnigan Delta Plus, USA). Total phosphorus in seagrass tissue was measured on an inductively-coupled-plasma emission spectrophotometer (ICP) (Spectroflame, Spectro Inc.), after digestion of dry plant material with nitric acid and H_2O_2 (Smolders et al., 2006). Sediment grain size distribution was determined on dried and sieved (1 mm mesh size) samples by laser diffraction on a Malvern (Master 2000) particle size analyzer.

2.6 Statistical analysis. Normal distribution of the data was tested on the residuals, and negatively skewed data were transformed using the square root of the maximum of *x* minus *x* (Field, 2005) to meet assumptions of the ANOVAs (Table 1). Biomass and elemental composition were analyzed by 2-way ANOVAs to assess the effects of NPK and OM, with a 95% confidence interval We used R 3.01 to perform 2-way ANOVAs. Porewater nutrient and sulfide data were tested using 3-way repeated measure ANOVAs, to analyze the effects of time (t_0, t_{21}, t_{49}, t_{77}), NPK (+/-) and OM (+/-) (Table 2). We used the Greenhouse-Geisser correction when sphericity

assumptions were violated. PASW-SPSS 18.0 was used for repeated measures ANOVAs. Exponential regressions were tested with linear models after log transformation the variables on the Y-axis (biomass mother patch and expansion) and untransformed variables on the X-axis (sulfide and ammonium). For sulfide-biomass relationships, only values from the control and OM treatments were taken into account, and for the ammonium-biomass relationships, only results from the control, NPK and NPK+OM treatments. This separate testing of treatments was done to distinguish between the effects of high porewater sulfide or high ammonium, which could each result in a low biomass. Outliers were detected with Dixon's Q-test and removed from the data (Dean and Dixon, 1951).

3. Results

3.1 Porewater results. Both the NPK and OM treatment successfully altered biogeochemical sediment characteristics, as expected (Fig. 1). OM addition resulted in significantly elevated porewater sulfide levels (Fig. 1a) up to concentrations of 3000 μmol L^{-1} in the OM treatment (repeated measures ANOVA, $F_{(1.4)} = 6.05$, $P = 0.007$). In contrast, porewater sulfide levels did not increase in the NPK+OM treatment. High amounts of nitrate were released in both NPK treatments, and sulfide production was almost completely suppressed in the NPK + OM treatment ($F_{(1.3)} = 5.88$, $P = 0.007$), because nitrate is a thermodynamically more favorable electron acceptor than sulfate (Lucassen et al., 2004). The NPK+OM treatment therefore resulted in biogeochemical properties, which were similar to the NPK treatment. The NPK treatment significantly decreased porewater sulfide

concentrations relative to both the control and OM treatment (Fig. 1, Table 2, F(7,71), P = 0.023). Sulfide concentrations peaked at 3340 µmol L^{-1} on the 8th of August, but declined to 240 µmol L^{-1} by the 7th of September, suggesting that OM availability had been exhausted after the 8th of August (47 days). Furthermore, NPK addition resulted in significantly (Table 2) elevated porewater ammonium (F(1.8) = 5.87, P = 0.004), nitrate (F(1.5) = 4.678, P = 0.038) and phosphate concentrations (F(1.7) = 5.52, P = 0.026) (Fig. 1). Porewater ammonium levels in both nutrient treatments exceeded 4000 µmol L^{-1} (a more than 8-fold increase compared to the controls), and nitrate concentrations increased more than 50-fold compared to the controls up to >2000 µmol L^{-1}.

3.2 Biomass response. We observed seagrass expansion (seagrass growth outside the 0.35 m diameter patches) in 100% of the control treatments (n=8). In contrast, 37.5 % of the patches in the OM and the NPK+OM treatments and only 25 % of the transplanted patches in the NPK treatments expanded. Aboveground biomass of the mother patches decreased in all treatments compared to the initial biomass of 43 g DW m^{-2}, without significant effects of NPK (2-way ANOVA, F(1) = 2.53, P = 0.123) or OM (F(1) = 2.81, P = 0.110). Shoot densities (shoots m^{-2}) in the mother patches decreased by approximately 75% in all addition treatments, whereas they did not change in time in the control treatment (Table 2, Fig. 3). Belowground biomass of the mother patches increased in the control treatment, from an initial biomass of 31 g DW m^{-2} to a biomass of 40 g DW m^{-2} at the end of the experiment. In stark contrast, belowground biomass was reduced by almost 50 % compared to the initial biomass in all other treatments (Table 1, Fig. 2).

The aboveground biomass of the expansion did not differ among treatments (Table 1). In contrast, both NPK ($F(1) = 4.36$, $P = 0.046$) and OM ($F(1) = 4.22$, $P = 0.049$) addition negatively affected total patch aboveground biomass (Fig. 2, Table 1). Even though all patches received standardized treatments, the effects of NPK or OM addition on biomass parameters (mother patch and expansion biomass) showed high variability (Fig. 4), Some patches in the treatments (NPK, NPK+OM, OM) appeared to manifest less ammonium or sulfide stress (Fig. 4) as reflected by higher biomasses in these units. We therefore tested whether this heterogeneity could be explained by differences in the concentrations in sulfide accumulated, and indeed found a very strong correlation between biomass and porewater sulfide concentrations, ($R^2 = 0.69$, $P<0.001$ for mother patch and $R^2 = 0.70$, $P<0.001$ for patch expansion). Sulfide levels above 1000 µmol L^{-1} completely prevented expansion of patches (Fig. 4c) and resulted in their complete die-off (Fig. 4a). Some of the patches already suffered from lower sulfide concentrations. Low patch expansion biomass was also strongly related to high porewater ammonium concentrations (Fig. 4d, $R^2 = 0.61$, $P<0.001$), but we did not find effects on mother patch biomass (Fig 4b, $R^2 = 0.20$, $P>0.05$). We observed no patch expansion at ammonium concentrations >5000 µmol L^{-1}, but expansion was already strongly decreased by ammonium concentrations around 2000 µmol L^{-1} (Fig. 4d). Contrastingly, some mother patches were able to tolerate ammonium concentrations >7000 µmol L^{-1}.

3.3 Plant elemental composition. Leaf nitrogen (% N) content of the seagrass leaves of the expansion increased by NPK addition (2-way ANOVA, F(1) = 20.56, *P* <0.001, Table 3). Leaf phosphorous content, in contrast, was not significantly affected by any treatment. *Zostera noltii* did not seem to be nutrient limited in our experiment, as average leaf nitrogen (3.8 %) and phosphorous content (0.9 %) were well above the values for absolute nutrient limitation (Duarte, 1990). Total sulfur content of the leaves (% S) was 40 % lower in the NPK treatments (F(1) = 37.73, *P*<0.001,) than in the Control and OM only (OM) treatment (Table 3. 1. 1% versus 1.7%)

4. Discussion

Limitations to seagrass patch survival and development have typically been attributed to physical and biological stressors in literature (Sintes et al., 2005; Townsend and Fonseca, 1998). In this study, we show that also biogeochemical toxins, such as high nutrient and sulfide concentrations, can be highly detrimental to seagrass patch survival and expansion. As the high nitrate levels in the NPK treatment strongly decreased (natural) sulfide production in the sediment, interacting effects of sulfide and ammonium toxicity could not be investigated, and we will therefore discuss the effects of both stressors separately.

4.1 Ammonium toxicity. This is the first study that actually shows a causal relationship for porewater ammonium and toxicity effects in seagrass. Previous

studies mainly focused on the effects of high nitrate or ammonium concentrations in the surface water layer (Burkholder et al., 1992; Christianen et al., 2011; Van der Heide et al., 2008; van Katwijk et al., 1997). In a correlative study (Short, 1983b) already suggested that porewater nitrogen concentrations of 10 – 100 mmol m^{-2} were associated with decreasing eelgrass shoot densities. Ammonium concentrations >25 μM in the water column are known to be potentially lethal to *Zostera* spp. (Brun et al., 2002; Van der Heide et al., 2008; van Katwijk et al., 1997). The porewater ammonium concentrations we found to completely restrict patch expansion, >2000 - 4000 μmol L^{-1}, are more than a hundred times higher, although negative effects on biomass were already visible at 1000 μmol L^{-1}. We could therefore consider *Zostera noltii* a fairly persistent species for soil ammonium toxicity.

The high threshold values we found compared to surface water ammonium experiments indicates that *Zostera noltii* is much more resistant to sediment eutrophication than to water column eutrophication (Peralta et al., 2003). This is to be expected, as porewater nutrient concentrations are known to be at least ten times higher than ambient water column concentrations (Burkholder et al., 2007; Touchette and Burkholder, 2000). This difference could also be related to a higher affinity of leaves for ammonium as compared to roots (Thursby and Harlin, 1982). Also, sediment pH is usually lower than water column pH as a result of higher CO_2 concentrations (pH 7.5 vs. 8.2 on average), which might result in lower ammonium uptake rates in the roots (Toetz, 1973). Furthermore, most water column eutrophication experiments were conducted with *Zostera marina*, and *Z. noltii* may

be more resistant to eutrophication of the water column than *Z. marina*, and vice versa, *Z. marina* may be less resistant to high porewater ammonium levels.

The negative effects of biogeochemical stressors seemed to be less pronounced for the mother patch than for patch expansion (Fig. 4). This indicates that the lower shoot density and biomass in the expansion area makes the plants more vulnerable to toxicity, because toxicity cannot be alleviated by joint detoxification or by growth dilution (Van der Heide et al., 2010b). Both processes are strongly affected by population density, and result in denser vegetation being better able to cope with toxins and showing higher growth rates (Van der Heide et al., 2010b). This implies that sparse and patchy vegetation can be expected to be more vulnerable to ammonium toxicity than densely vegetated seagrass beds. At the same time, however, sparse patches may collect lower amounts of detritus leading to lower porewater ammonium and sulfide concentrations. Although even small patches of *Zostera noltii* seem to be persistent to high porewater ammonium, they were not able to expand or grow during these conditions in our experiment.

Another possible effect of direct nutrient toxicity may be an imbalanced carbon-nitrogen ratio due to increased carbon demand (Touchette et al., 2003). We did find a slightly significant effect of NPK addition on C:N ratios (11:1 vs. 12:1) in our experiment, which could imply that this treatment may have resulted in a carbon-nitrogen imbalance in the plants.

Our treatments resulted in very high porewater ammonium concentrations (up to 7000 µM), which is probably the result of the sediment properties (clay) at our experimental site. However, such high porewater ammonium concentrations (1000 up to 7000 µM) have been observed before in eutrophic coastal sediments (Fourqurean et al., 1992; Murray et al., 1978; Pages et al., 2012). And the high porewater ammonium concentrations in our experiment enabled us to investigate the effects of this wide range of ammonium concentrations, which has been observed in natural coastal sediments.

4.2 Sulfide toxicity. High sulfide concentrations of the porewater appeared to be another major constraint for seagrass patch expansion. Sulfide is known to be very toxic to seagrasses (Calleja et al., 2007; Koch and Erskine, 2001; Lamers et al., 2013; Marba et al., 2006; Terrados et al., 1999) and Van der Heide et al. (2012) showed a negative effect on *Zostera noltii* biomass production at porewater sulfide levels <200 µmol L^{-1}. We found similar threshold values in our field experiment and also found that patch expansion was entirely blocked by sulfide concentrations >1000 µmol L^{-1}. At low sulfide levels, seagrass is usually able to cope with sulfide by oxidizing it in the root zone (Holmer and Bondgaard, 2001; Marba et al., 2006; Pedersen et al., 1998). However, if either sulfide levels become too high, as in our experiment, or when seagrass photosynthesis is constrained or biomass too low, sulfide intrudes into the plants (Garcia et al., 2013; Holmer and Kendrick, 2013). It then interferes with the cytochromes in the electron transport chain, resulting in a negative energy balance, which eventually causes seagrass mortality (Erskine and Koch, 2000; Holmer and Bondgaard, 2001).

Our results showed that lower sulfide stress is reflected in the sulfur content of *Zostera noltii* leaves and that sulfide intrusion and sulfur storage already takes place at sulfide concentrations >200 μM (control). It is however remarkable that sulfur storage in the leaves of *Zostera noltii* did not differ between control (ambient sulfide levels) and OM treatments (elevated sulfide levels). This may be due to decreased vitality of the roots in the high-sulfide treatment (OM), which could severely decrease the uptake of sulfur by the roots.

4.3 Effects on patch development. Patch growth is a self-accelerating process, but it is usually slower than the potential growth rates, as physical and biological stressors interfere (Sintes et al., 2005; Townsend and Fonseca, 1998). Our study adds biogeochemical stressors to this process of patch development. We observed patch expansion in the control patches, but when we added biogeochemical stressors, patch growth was severely limited by ammonium or sulfide toxicity.

4.4 Ecological implications. Eutrophication is one of the major causes of seagrass decline worldwide (Duarte, 2002; Orth et al., 2006; Waycott et al., 2009). As it is known to promote high concentrations of ammonium and sulfide in the porewater (Hauxwell et al., 2001; Short, 1983a; Smolders and Roelofs, 1995), both factors represent important biogeochemical stressors and are expected to influence seagrass expansion processes at a global scale. Although the occurrence of patches is a natural phenomenon in seagrass meadows (Duarte et al., 2006a), the level of

patchiness is strongly increasing as a result of various human activities (Bostrom et al., 2006).

Patches are very dynamic and normally ensure recovery of seagrass meadows (Duarte et al., 2006b). As we seek to preserve these constantly changing systems (preservation paradox; (Pickett and White, 1985)), it is vital to gain a better understanding of the effects of stressors on system dynamics. The present study showed, for the first time, that patch survival and expansion can be severely influenced by common biogeochemical stressors related to eutrophication, i.e. increased ammonium and sulfide levels, which not only led to thinning of the patches but also precluded patch expansion. Density-dependent alleviation of ammonium and sulfide toxicity will decrease due to the thinning, and subsequently accelerate the decline.

Acknowledgements

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Table 1. Results of 2-way ANOVAs (NPK * OM) for all shown variables of *Zostera noltii* after 77 days. *$0.01 \leq p \leq 0.05$. **$0.001 \leq p \leq 0.01$. ***$p \leq 0.001$. ns = not significant.

Parameter	NPK					OM					NPK*OM					Error	transformation
	df	MS	F	P		df	MS	F	P		df	MS	F	P			
Total patch biomass (g DW m^{-2})	1	2298	2.14	0.155	ns	1	2782	2.59	0.119	ns	1	7808	7.27	0.012	*	32.8	Sqrt(max(x))-x
Total patch aboveground biomass (g DW m^{-2})	1	859	4.36	0.046	*	1	832	4.22	0.049	*	1	1399	7.10	0.013	*	14.0	Sqrt(max(x))-x
Total patch belowground biomass (g DW m^{-2})	1	347	0.86	0.36	ns	1	571	1.42	0.243	ns	1	2597	6.45	0.017	*	20.1	Sqrt(max(x))-x
Mother patch total biomass (g DW m^{-2})	1	1152	1.26	0.271	ns	1	1569	1.71	0.202	ns	1	6182	6.73	0.015	*	30.3	Sqrt(max(x))-x
Mother patch aboveground biomass (g DW m^{-2})	1	392	2.53	0.123	ns	1	436	2.81	0.11	ns	1	992	6.39	0.017	*	12.4	Sqrt(max(x))-x
Mother patch belowground biomass (g DW m^{-2})	1	196	0.54	0.467	ns	1	259	0.71	0.406	ns	1	2012	5.53	0.026	*	19.1	Sqrt(max(x))-x
Patch expansion total biomass (g DW m^{-2})	1	393	2.53	0.123	ns	1	436	2.81	0.105	ns	1	991	6.39	0.017	*	12.5	Sqrt(max(x))-x
Patch expansion aboveground biomass (g DW m^{-2})	1	196	0.54	0.569	ns	1	259	0.71	0.406	ns	1	2012	5.53	0.026	*	19.1	Sqrt(max(x))-x
Patch expansion belowground biomass (g DW m^{-2})	1	1152	1.26	0.272	ns	1	1569	1.71	0.202	ns	1	6182	6.74	0.0149	*	30.29	Sqrt(max(x))-x
Total N (%)	1	1.40	20.56	<0.001	***	1	0.06	0.805	0.387	ns	1	0.00	0.21	0.888	ns	0.27	
Total P (%)	1	0.09	2.97	0.116	ns	1	0.00	0.05	0.821	ns	1	0.01	0.31	0.591	ns	0.18	
Total C (%)	1	13.3	9.69	0.009	**	1	0.79	0.58	0.463	ns	1	4.21	3.01	0.106	ns	0.53	

	df		F	p		df		F	p		df		F	p		
Total S (%)	1	1.12	37.73	<0.001	***	1	0.02	0.738	0.410	ns	1	0.01	0.27	0.613	ns	0.17
C:N (molar ratio)	1	4.87	8.15	0.015	*	1	0.92	1.55	0.237	ns	1	0.38	0.63	0.443	ns	0.77
N:P (molar ratio)	1	52.8	12.78	0.005	**	1	0.38	0.09	0.767	ns	1	1.28	0.31	0.590	ns	2.03

Table 2. Results of the 3-way (time*NPK*OM) repeated measures ANOVAs (t_0, t_{21}, t_{49}, t_{77}) for shoot densities (m^{-2}) and all relevant porewater nutrients. F values and significance levels are shown for all the main effects and interactions * $0.01 \leq P \leq 0.05$. ** $0.001 \leq P \leq 0.01$. *** $P \leq 0.001$. ns = not significant. df stands for adjusted degrees of freedom as a result of the Greenhouse-Geisser correction for violated sphericity..

	Time				Time*NPK				Time*OM				Time*NPK*OM			
	df	F	P		df	F	P		df	F	P		df	F	P	
Shoots mother patch (shoots m^{-2})	1.4	5.623	0.009	**	1.1	1.88	0.226	ns	1.2	1.666	0.252	ns	1.1	7.429	0.034	*
Shoots patch expansion (shoots m^{-2})	1.1	5.884	0.041	*	1.1	2.098	0.189	ns	1.2	1.735	0.227	ns	1.1	0.733	0.435	ns
Sulfide (μmol L^{-1})	1.2	9.100	0.019	*	1.2	7.709	0.023	*	1.4	6.047	0.007	**	1.3	5.882	0.007	**
Ammonium (μmol L^{-1})	1.8	7.683	0.007	**	1.8	5.870	0.004	**	1.3	0.956	0.390	ns	1.6	0.662	0.501	ns
Nitrate (μmol L^{-1})	1.5	5.070	0.034	*	1.5	4.678	0.038	*	1.3	1.326	0.295	ns	1.6	1.215	0.322	ns
Phosphate (μmol L^{-1})	2.1	13.569	0.000	***	1.7	5.418	0.026	*	1.2	1.754	0.225	ns	3.0	0.701	0.562	ns

Table 3. Elemental composition of the leaves. Molar C:N:P ratios and percentages (g^{-1}) of

leaf tissue nutrients.

Treatment	C:N:P	% C	% N	% P	% S
Control	121:10:01	39.3	3.83	0.89	1.77
NPK	161:15:01	40.3	4.40a	0.65	1.11
NPK+OM	140:14:01	41.3	4.56	0.75	1.11
OM	114:10:01	38.0	3.94	0.87	1.66

Figure legends

Figure 1. Porewater concentrations of sulfide (a), phosphate (b), ammonium (c) and nitrate (d) during the experiment. Error bars represent SEM (n=8).

Figure 2. Aboveground (a) and belowground biomass (b) of patch expansion (dashed bars) and mother patch (straight line); dashed lines indicate initial aboveground and belowground biomasses. Error bars represent SEM of total aboveground and belowground biomass, n = 8.

Figure 3 Shoot densities of the mother patch (a) and in the expansion area (b) in time; error bars represent SEM, n = 8.

Figure 4. Scatterplots of mother patch biomass and weighted average porewater sulfide concentrations (a) and ammonium concentrations (b), and expansion biomass and weighted average porewater sulfide concentrations (c) and ammonium concentrations (d) with exponential regression lines and related R^2 values.
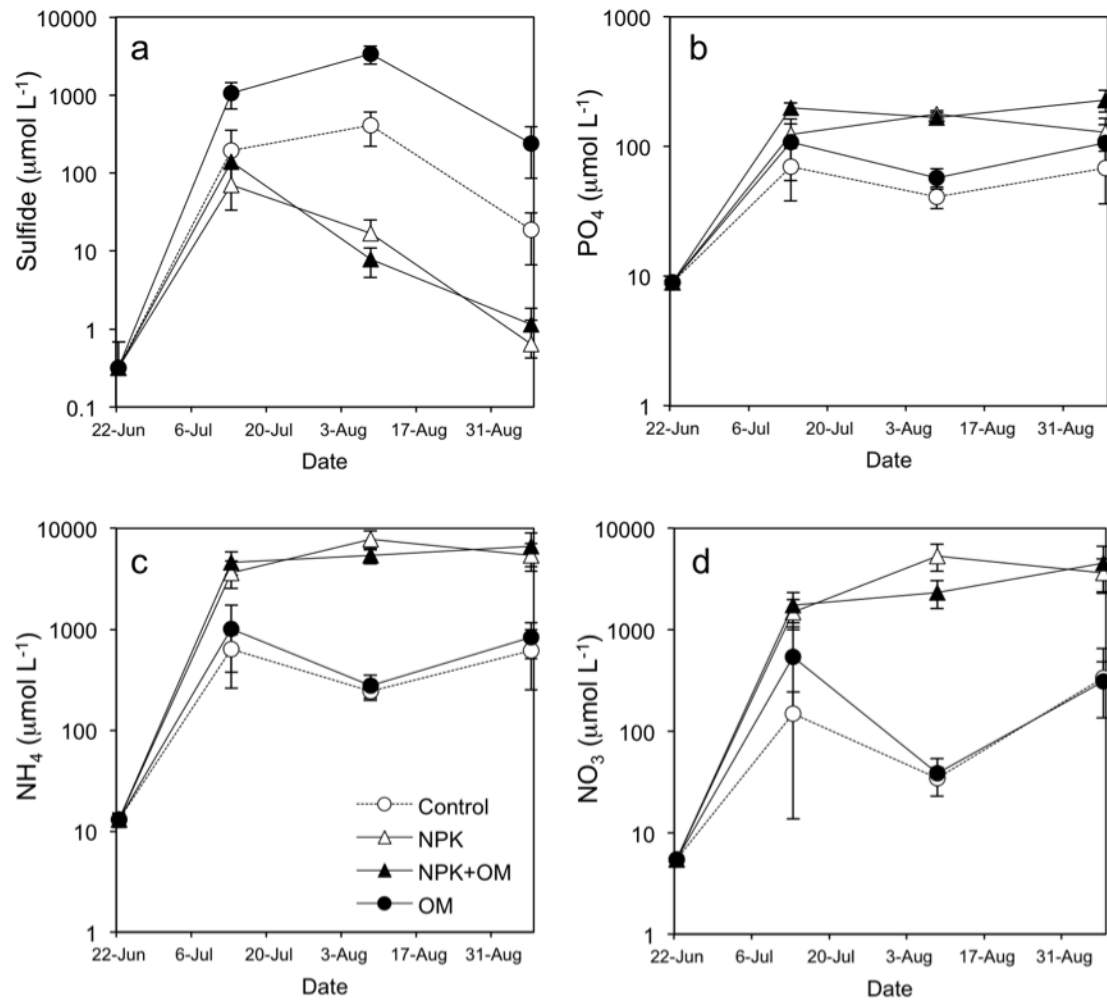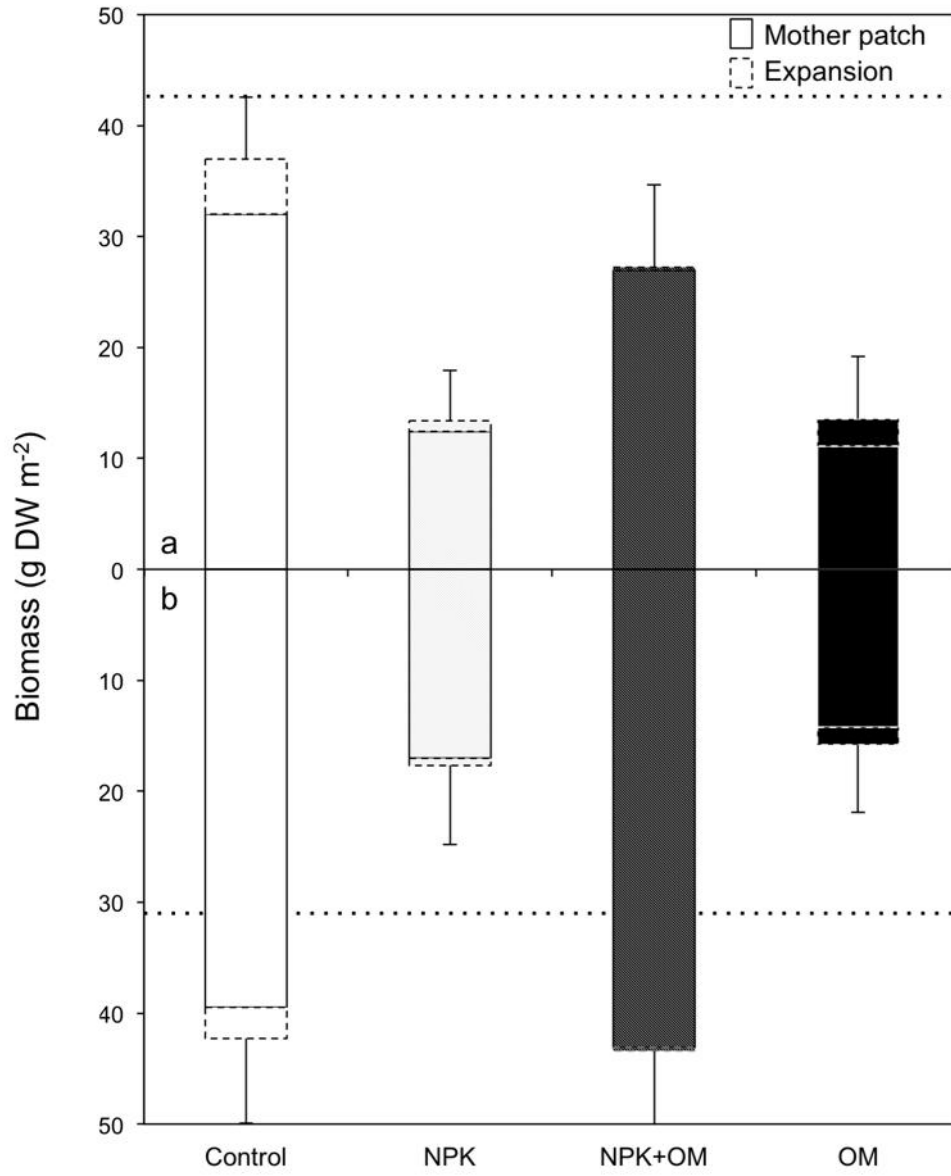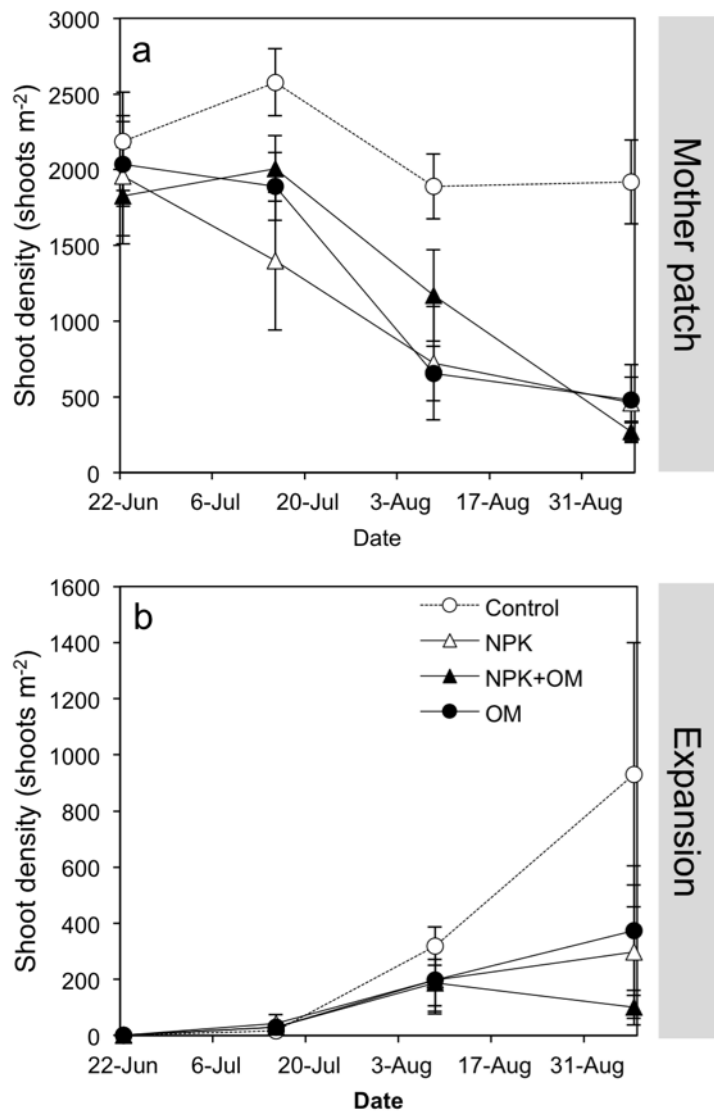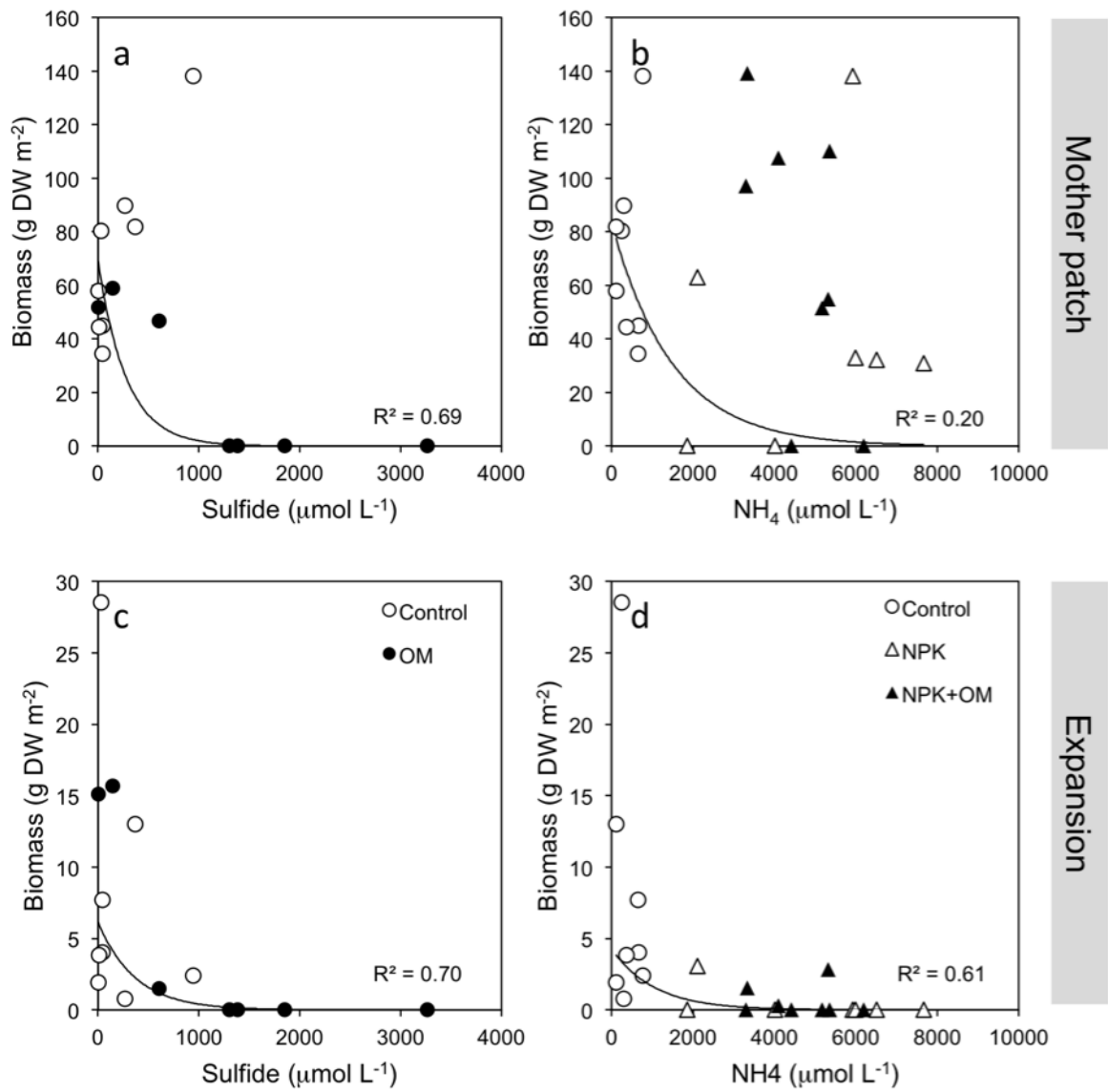
Figure 1

Figure 2

Figure 3

Figure 4