THE EFFECTS OF BIOGEOCHEMICAL STRESSORS ON SEAGRASS ECOSYSTEMS

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geboren op 16 september 1985
te ’s-Hertogenbosch
"What we know is a drop, what we do not know is an ocean"

ISAAC NEWTON
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Seagrasses are aquatic angiosperms, the only flowering plants that are well adapted to live and reproduce in saline waters. Although they look somewhat similar to terrestrial grasses (Figure 1.1), they are not related to this group of plants, but evolved from freshwater macrophytes about 100 million years ago (den Hartog 1970, Les et al. 1997). The 12 genera of seagrass species belong to four different families: Zosteraceae, Posidoniaceae, Cymodoceae, and Hydrocharitaceae, forming an ecological rather than a taxonomical group (den Hartog and Kuo 2006). Like terrestrial grass species, seagrasses can form extensive meadows in shallow coastal waters all over the world (Green and Short 2003). The depth limit of seagrasses is determined by the water clarity, as they need light for photosynthesis, and the seagrass species Posidonia oceanica can be found up to 50 m depth in the clearest parts of the Mediterranean (Duarte 1991). In contrast, seagrasses in murky waters may grow to very shallow depths of only 1 m. Next to subtidal species, which grow constantly submerged, there are also seagrasses that occur in intertidal areas, where mainly desiccation stress and hydrodynamics determine their upper depth limits (Leuschner et al. 1998, Bjork et al. 1999).
The natural value of seagrass beds is often unknown or unrecognized compared to the attractive and colorful neighboring coral reefs, but the importance of seagrass ecosystems is comparable to that of coral reefs, mangroves and salt marshes in terms of ecosystem services and ecological richness. More importantly, these coastal ecosystems are often interconnected by means of migrating animals, nutrient fluxes, and organic carbon (Nagelkerken 2000, Cowen et al. 2006, Gillis et al. 2014). Seagrass ecosystems rank among the most productive and most valuable ecosystems on earth, in terms of value ($ per hectare (Costanza et al. 1997). This value can be attributed to the many ecosystem services that seagrass beds can provide: high rates of production and nutrient cycling (Duarte and Chiscano 1999), carbon sequestration (Fourqurean et al. 2012), coastal protection by attenuation of waves and currents and by stabilizing the sediment (Christianen et al. 2013), nursery habitat for commercial fish species (Nagelkerken 2000), and habitat and food for many endangered species (Valentine and Heck 1999, Christianen et al. 2012).

**Seagrass beds under threat**

Regrettably, seagrass beds have been declining rapidly over the past decades: with about 7% per year since 1990 – a rate of decline comparable to that of coral reefs and tropical rainforests (Orth et al. 2006, Waycott et al. 2009). The main reason for this decline is the increase of human activities in coastal areas (Short and Wyllie-Echeverria 1996). Nowadays, billions of people live in coastal areas all over the world (Cohen et al. 1997, Small and Nicholls 2003), and in a few decades, probably 50% of the entire human population will be living within 150 km from the shore (Cohen 2003). This development has lead to a steep increase of human activities in coastal areas such as dredging, aquaculture, sewage discharge, industrial activities, and deforestation (Cohen 2003, Mora 2008), which severely threaten coastal ecosystems, including seagrass beds.

These activities have resulted in eutrophication, high trace metal levels, habitat degradation, and increased water column sediment loads, which negatively affect seagrass ecosystems. In addition, also climate change may put pressure on global seagrass ecosystem functioning (Orth et al. 2006, Waycott et al. 2009). In theory, climate change may lead to circumstances favorable to seagrasses, as they evolved about 100 million years ago in times of higher CO2 concentrations, temperatures and sea levels. However, current global change is much more rapid than ancient change rates and it may thus be hard for seagrasses to adapt to present changing environmental conditions (Orth et al. 2006), especially since an additional array of other stressors synergistically threaten seagrass ecosystems. The most imminent threat to seagrass ecosystems is eutrophication (Burkholder et al. 2007). Coastal nutrient loads have strongly increased, due to agricultural run-off and untreated sewage discharge in bays, lagoons and estuaries (Short and Wyllie-Echeverria 1996, Burkholder et al. 2007). These high nutrient levels lead to increased turbidity and lower light-availability for seagrasses, as these favor fast growing plankton and macroalgae species at the expense of seagrasses (Figure 1.2) (Hauxwell et al. 2003, Kemp et al. 2005). Additionally, eutrophication may also directly affect seagrasses by promoting ammonium and sulfide toxicity (van Katwijk et al. 1997, Koch and Erskine 2001, Van der Heide et al. 2008).

Disappearance of seagrass beds also implies the loss of important ecosystem services that seagrasses provide, such as coastal protection (Christianen et al. 2013), fisheries (Gillanders 2006), and carbon sequestration (Fourqurean et al. 2012). Moreover, seagrass beds are strongly connected to other coastal ecosystems so degradation or disappearance of seagrasses may also affect ecosystem functioning and ecosystem services (e.g. fisheries) of nearby coral reefs, mangroves, and salt marshes. Therefore, stressors threatening seagrass beds and thus other coastal ecosystems should be recognized and halted to prevent further loss of coastal key-ecosystems and related ecosystem services. Fortunately, there is an increasing number of marine protected areas (MPAs) that include seagrass beds (Orth et al. 2006), and there have been worldwide initiatives on the restoration of seagrass meadows (Van Katwijk et al. submitted).

**Seagrass dynamics and restoration**

Seagrass beds form naturally dynamic landscapes which are maintained through asexual clonal expansion, sexual recruitment and the turnover of shoots (Duarte et al. 2006). These processes act over various spatial and time scales and unbalances in in these dynamic processes may result in changes on a meadow scale, such as patchy and heterogeneous landscapes, which may be more vulnerable to stressors than healthy seagrass beds (Chapter 2). Unbalanced seagrass dynamics may even result in catastrophic declines, due to altered disturbance-recovery dynamics (Chapter 4), which we discussed above (1.1) (Duarte et al. 2006). As seagrass beds have been disappearing on a global scale, many attempts have been made to restore seagrass beds to their original value. However, due to positive feedbacks in seagrass beds, seagrass restoration has been proven difficult (Van der Heide et al. 2007, van Katwijk et al. submitted). In the Netherlands, we have also been involved in large-scale mechanical seagrass transplants in the southwestern delta (Oosterschelde) (Box 1.1). In this thesis we answer some questions concerning...
these transplants: we investigate how important starch reserves are for the winter survival of transplanted seagrasses (Chapter 3) and we look further into the competition between *Zostera noltii* and the bioturbating lugworm *Arenicola marina* (Chapter 9) which has become more intense since lugworm numbers have been increasing in the Oosterschelde (Suykerbuyk et al. 2012).

**Box 1.1 Seagrass restoration: a case study from the Oosterschelde**

In the Dutch Oosterschelde Delta, Dike reinforcements have endangered the red-list seagrass species *Zostera noltii*, which was growing on the intertidal mudflats in the vicinity of the dikes. Therefore, large sods of *Zostera noltii* have been transplanted to several suitable locations within the Oosterschelde (Figure 1.3) (Giesen et al. 2012). These transplantations have been carried out in 2007, 2008, 2010, 2011, and 2012, and in total, about 3000 m² of dwarf eelgrass has been transplanted. As large-scale seagrass restoration is generally more successful than small-scale restoration (Van Katwijk et al. submitted), seagrass sods of 1.5x0.75 m were moved mechanically (Figure 1.4) and placed on a thick shell-layer to prevent lugworm bioturbation (Suykerbuyk et al. 2012).

**Feedbacks and seagrass ecosystem functioning**

Seagrasses are considered as ecosystem engineers, which are species that “directly or indirectly modulate the availability of resources to other species, by causing physical state changes in biotic or abiotic materials. In doing so, they modify, maintain and recreate habitats” (Jones et al. 1994). Thus, seagrasses are able to manipulate their abiotic and biotic environment, which enables them to improve their own growing conditions with increasing density (Van der Heide et al. 2007, Van der Heide 2009). They can alleviate hydrodynamic stress by attenuating waves and currents (Fonseca and Cahalan 1992, Peterson et al. 2004), stabilize the sediment and entrap sediments and organic matter (Gacia et al. 2002, Carr et al. 2010). These processes reduce sediment resuspension and therefore improve the water clarity and subsequent light availability to seagrasses. Another important feedback mechanism in seagrass beds is density-dependent toxicity (Van der Heide et al. 2010), where e.g. ammonium toxicity (in the water layer) and sulfide toxicity (in the sediment) may be reduced with increasing seagrass densities as result of joint-detoxification, either by joint uptake of toxic compounds (reduced nitrogen) or by joint-prevention of intrusion (sulfide) by aeration of the sediment (Chapter 4).

A probable outcome of the presence of strong positive feedbacks in seagrass ecosystems is the existence of nonlinear dynamics or bistability (Scheffer et al. 2001, Scheffer and Carpenter 2003), which implies that systems may suddenly collapse when disturbances drive the system past a certain critical threshold (Scheffer et al. 2001, Folke et al. 2004). Such an unexpected collapse has also
occurred in the Wadden Sea where extensive subtidal *Zostera marina* beds, suddenly and completely disappeared in the 1930, and have not recovered ever since (den Hartog and Polderman 1975, Giesen and van Katwijk 1990, Van der Heide et al. 2007).

**Biogeochemical processes and nutrient cycling**

Seagrasses live at the interface of the sediment and the water column. They may protrude into the water column from centimeters (Halophila spp.) up to meters (7m – *Zostera caulescens*) (Koch et al. 2007), and they may root in the sediment to up to a meter depth (*Posidonia oceanica*) (Mateo et al. 1997). Seagrasses not only produce organic matter, but also entrap organic matter, nutrients and fine sediment (Gacia and Duarte 2001, Gacia et al. 2002, Bos et al. 2007). This promotes very high rates of mineralization and nutrient cycling in seagrass beds, especially compared to unvegetated sediments (Marba et al. 2006). Seagrass beds may therefore be considered as biogeochemical hotspots (McClain et al. 2003, Layman et al. 2013). Decomposition of organic matter in seagrass sediments (both trapped allochthonous OM and senescent plant material) is an important source of nitrogen and phosphorus, which are the principal nutrients for seagrass growth (Pedersen and Borum 1992, Holmer et al. 2001). When organic matter input is enhanced by, for instance, eutrophication, increased decomposition rates may lead to such high nutrient availability which may yield either 1) direct toxic effects (Chapter 2) or 2) promote epiphyte blooms on the seagrasses (Burkholder et al. 2007). In contrast to a surplus of nutrients, seagrasses in oligotrophic areas may also be limited by either nitrogen or phosphorus availability (Bulthuis et al. 1992, Udy and Dennison 1997), which are the principal nutrients for seagrass growth (Touchette and Burkholder 2000). Nutrient limitation of seagrasses depends generally on the type of sediment (grain size and origin) and the distance to a nutrient source (Erfemeijer and Middelburg 1993, Fourqueuran and Zieman 2002, van Katwijk et al. 2011). In literature, seagrasses growing on carbonate sediments are considered to be P-limited, while seagrasses growing on terrigenous sediments may be more N-limited (Erfemeijer and Middelburg 1993, Udy and Dennison 1997). As an adaptation to nutrient limitation, seagrasses are able to take up nutrient by both leaves and roots (Short 1987, Burkholder et al. 2007).

**Sulfur cycling**

Decomposition of organic matter mainly occurs anaerobically, as oxygen is rapidly depleted by the high microbial activity in the uppermost sediment layer (Holmer and Nielsen 1997). An alternative, but in seawater abundant, electron acceptor in the mineralization processes is sulfate (~28 mmol L⁻¹) (Schultz and Zabel 2006, Canfield and Farquhar 2009). Therefore, most organic matter is decomposed by sulfate reducing bacteria (Kristensen et al. 2000, Holmer et al. 2001). Sulfate reduction by sulfate reducing bacteria (Equation 1.1) takes place in the highly reduced organic sediments of seagrass beds, where organic matter is used as electron donor, and sulfate as electron acceptor (Jorgensen 1982). Dissolved free sulfides (H₂S, HS⁻, S²⁻) are produced in the process (Lamers et al. 2013).

\[
\text{SO}_4^{2-} + 2 \text{CH}_2\text{O} \rightarrow \text{H}_2\text{S} + 2 \text{HCO}_3^- \quad \text{Equation 1.1}
\]

Sulfide is a potent phytotoxin, which affects plant physiology and energy balance through interference with cytochromes in the electron transfer chain (Bagarinao 1992, Erskine and Koch 2000). In addition, seagrass ecosystem functioning may be affected, as large-scale vegetation diebacks as a result of long-term exposure to high sulfide levels have been observed (Carlson et al. 1994, Koch and Erskine 2001, Borum et al. 2005). Seagrasses can naturally protect themselves against sulfide intrusion by radial oxygen loss in the rhizosphere (Pedersen et al. 1998), which can locally oxidize sulfides or prevent reduction of sulfates (Lamers et al. 2013). In addition, seagrasses can also form an iron hydroxide-rich zone in the rhizosphere, which may be used to trap sulfides (Deborde et al. 2008). However, when very high sulfide concentrations accumulate in the sediment or in periods of low radial oxygen loss, such as during the night, toxic sulfide may intrude the plants and cause seagrass mortality (Marba et al. 2006). External inputs of organic matter in seagrass beds by anthropogenic eutrophication may therefore not only affect seagrasses by reduced light conditions, but also by increased sulfide stress (Marba et al. 2006). Sulfide levels in the sediment may also be reduced by microbes living on the aerobic/anaerobic interface. Chemolithoautotrophic bacteria such as aerobic *Beggiatooa* or anaerobic *Thiobacillus* convert toxic sulfides to sulfur in an energy yielding process (Jorgensen 2006, Gosh and Dam 2009). Some marine animals, which live in environments rich in inorganic reduced sulfur compound (sulfides), harbor symbiotic sulfide-oxidizing bacteria in their tissues, which provide them with a source of energy (Seckbach 2004). Examples are the giant tube worm *Riftia* that is common near sulfide-rich hydrothermal vents in the deep sea, and bivalves of the Lucinidae family (*Cavanaugh 1983, Taylor and Glover 2006*). In this thesis, we uncover an omnipresent sulfide-relieving mechanism in global seagrass beds involving Lucinid bivalves owing to a three-stage mutualism (chapter 5).

**Trace metals and petrochemicals**

Next to biogeochemical stressors, which result from eutrophication, chemical stressors such as trace metals and petrochemicals may affect seagrass health and ecosystem functioning (Ralph et al. 2007). Trace metals are naturally present in the marine environment as a result of weathering and erosion of rocks (Batley 1987, Prange and Dennison 2000), and many trace metals also function as micronutrients for seagrasses (Fe, Cu, Ni, Zn). However, trace metal pollution in coastal areas,
originating from sewage, mining, industries, agriculture, and atmospheric deposition (Irvine and Birch 1998, Guzman and Garcia 2002), has lead to elevated trace metal concentrations in waters and sediments and introduced high levels of detrimental heavy metals (Cd, Pb, Hg) into the coastal environment (Costa et al. 2012). Trace metal pollution can be harmful to seagrasses, by negatively affecting metabolic activities and photosynthetic rates (Conroy et al. 1991, Ralph and Burchett 1998, Macfarlane and Burchett 2001), but more importantly, they may also accumulate in the seagrass dominated foodweb, with toxic effects to higher trophic levels (Prange and Dennison 2000). Seagrasses can therefore be used as first-level indicators for trace metal pollution in coastal ecosystems (Chapter 8).

Contamination of marine systems by petrochemicals is often concurring with trace metal pollution (Thorhaug et al. 1986). Petrochemical pollution originates mainly from oil industries (leakage, spills and refineries) and harbors (Lewis and Pryor 2013). Intertidal seagrasses may be threatened by smothering, which results in reduced growth rates and mortality, whereas leaf densities and flowering are affected in subtidal seagrasses (Dean et al. 1998, Peirano et al. 2005, Ralph et al. 2007). There is however a lack of data to support long-term effects of trace metal and petrochemical pollution on seagrasses and seagrass communities.

**Species interactions in seagrass beds**

Seagrass beds support a very diverse community of flora and fauna (Figure 1.5), which depend on seagrasses for food, shelter and nursery habitat (Valentine and Heck 1999, Nagelkerken 2000, Williams and Heck 2001). A large group of herbivores directly depends on seagrass beds as their most important food source; this includes mesograzers (amphipods, isopods, gastropods), large herbivores such as grazing geese and swans, and endangered mega herbivores such as dugongs and green turtles (Valentine and Heck 1999, Goecker et al. 2005, Heck and Valentine 2006). It has been suggested that the latter used to play a pivotal role in shaping tropical seagrass ecosystems, but as these mega herbivores have severely declined in numbers due to overexploitation, top-down effects have become less significant than before (Jackson 2001).

Furthermore, many other species living in seagrass beds do not directly rely on seagrasses, but are an important part of the food web (Bostrom et al. 2006). Apex predators, such as shark, rays, and sea otters may even protect seagrasses from the detrimental effects of eutrophication by consuming crabs that prey on seagrass epiphyte herbivores (Hughes et al. 2013). And shorebirds, such as the red knot, may affect the intertidal faunal community living in seagrass beds, by consuming large quantities of burrowing mollusks (van Gils et al. 2012). Next to epifauna, endobenthic fauna also plays an important part in the seagrass community. Lucinid bivalves, which live in-between the seagrass roots, even affect the sediment biogeochemistry (Chapter 5, Chapter 7) and are involved in a mutualistic relationship with seagrasses worldwide. Endobenthic fauna may also display contrasting characteristics compared to seagrasses; burrowing lugworms destabilize the sediment, while seagrasses stabilize the sediment (Chapter 8). These antagonistic characteristics may lead to an interesting competition between seagrasses and lugworm, of which they outcome may be context dependent. The interaction between bioturbation lugworms and seagrasses is further studied in Chapter 6.

Lastly, seagrass beds also function as important nursery areas for many coral reef fish and shellfish species, which include a number of commercially and recreationally important species (Nagelkerken 2000, Heck et al. 2003, Delatorre Castro and Ronnback 2004).

**Main objectives of this thesis**

As described above, the main objectives of this thesis were to assess the effects of biogeochemical stressors, mainly sulfide, nutrients and trace metals on seagrass ecosystems. We thus studied seagrass dynamics and species interactions involving biogeochemical stressors. The questions and hypotheses addressed in this thesis are summarized in Table 1.1. In addition, all species studied in this thesis are introduced in box 1.2.
**Box 1.2 Species in this Thesis**

**Zostera noltii** or dwarf eelgrass is the principal species in this thesis. This tiny *Zostera* species occurs along the coast of the northeastern Atlantic, from the Baltic Sea to the Banc d’Arguin in Mauritania (Green and Short 2003). This species can grow in the intertidal area up to the shallow (1-2 m) subtidal. Leaves from *Zostera noltii* can grow up to 20 cm in length and to 0.5-1.5 mm in width. *Zostera noltii* is a perennial species and can reproduce both clonally and with seeds (Moore and Short 2007). *Zostera noltii* areas have been declining in the last decades in several areas in Western Europe (Martin et al. 2010, Suykerbuyk et al. 2012).

**Thalassia testudinum** or turtle grass is a widely distributed seagrass species, which can be found in shallow coastal zones in tropical and subtropical areas of the Western Atlantic (van Tussenbroek et al. 2006). Beds of *Thalassia testudinum* can be found in estuaries and coastal lagoons where it often occurs in the vicinity of mangroves and coral reefs. In most places, a strong interaction between these three tropical ecosystems is formed by both biotic and abiotic components (Nagelkerken 2010). The lush *Thalassia* beds in the Caribbean and along the southern coast of North America also serve as important nursery habitats for many coral reef fishes (Nagelkerken 2000), and as foraging habitat for the endangered green turtle (Williams and Heck 2001).

**Syringodium filiforme** or manatee grass is a tropical seagrass species, which can be found in coastal waters from the Gulf of Mexico to the Caribbean Sea (den Hartog and Kuo 2006). It usually occurs in waters between 1-3 meters depth, but can also occur in much deeper waters. *Syringodium filiforme* is an important food source for grazer such as parrotfish, manatees, sea urchins, and surgeonfish (Zieman et al. 1989).

**Arenicola marina** or lugworm is a large marine worm, which belongs to the phylum of Annelida. The lugworm can be found in intertidal areas all over North-Western Europe and can be recognized by the typical casts on top of the sediment. The lugworm is a burrowing, bioturbating animal, a deposit feeder, which reworks the sediment. Historically, *Arenicola marina* is found to co-occur with the seagrass species *Zostera noltii* (Jacobs et al. 1983), but has recently been found to hamper the recolonization of seagrasses in certain areas (Suykerbuyk et al. 2012).

**Loripes lucinalis** is a lucinid bivalve, which hosts endosymbiotic sulfide oxidizing bacteria in its gills. Bivalves from the genus of Lucinidae are known to be omnipresent is seagrass beds all over the world, where they form a mutualistic relationship with seagrasses (Chapter 7). *Loripes lucinalis* occurs from the French and British coast in the north, all the way to in Mauritania in the south, and the Black Sea in the East.
Chapter 1  Introduction

QUESTION

CHAPTER 2  What are the biogeochemical constraints for seagrass patch survival and expansion?

CHAPTER 3  What are the bottlenecks for the winter survival of Zostera noltii in the Oosterschelde?

CHAPTER 4  What are the effects of local environmental conditions and feedbacks on the resilience of seagrasses?

CHAPTER 5  What is the overall status of trace metals in seagrass beds worldwide and in the Caribbean in particular?

CHAPTER 6  What is the nutrient status of the seagrass beds on Curaçao and Bonaire?

CHAPTER 7  How do seagrasses worldwide cope with sulfide stress in organic sediments?

CHAPTER 8  What is the biogeochemical effect of lugworm activity on seagrasses?

CHAPTER 9  What happens with the three-stage seagrass-lucinid-gill-bacteria symbiosis in seagrass beds under desiccation stress?

HYPOTHESIS

High porewater sulfide concentrations may limit seagrass patch expansion and survival.

Low autumn starch reserves and low winter shoot densities may limit winter survival of Zostera noltii.

We expect lower seagrass recovery rates in low-density beds, especially with high sulfide concentrations, as feedbacks such as joint detoxification will be less efficient in lower seagrass densities.

There has not yet been a complete literature review on trace metals in seagrass beds, we therefore provide a benchmark.

Eutrophication may threaten the important nursery habitats on Curaçao and Bonaire.

A possible mutualistic interaction between seagrasses, lucinid bivalves and their symbiotic gill-bacteria may reduce the build-up of toxic sulfides.

We hypothesize that soil aeration by lugworm bioirrigation and soil reworking may relieve sulfide stress for seagrasses.

A possible mutualism-breakdown could accelerate habitat degradation.

Table 1.1 Overview of questions and hypotheses in this thesis
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.
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. Patch expansion 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.

MATERIALS AND METHODS

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 km² 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, NH₄=13.14±2.09, NO₂=5.51±1.44, PO₄=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 exposure 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).
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.

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.

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NPK</th>
<th>OM</th>
<th>F</th>
<th>P</th>
<th>Error</th>
<th>Transformation</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total patch biomass (g DW m⁻²)</td>
<td>1</td>
<td>2.28</td>
<td>2.14 0.155</td>
<td>MS</td>
<td>1</td>
<td>2.28 2.14 0.155</td>
<td>MS</td>
</tr>
<tr>
<td>Total patch aboveground biomass (g DW m⁻²)</td>
<td>1</td>
<td>1.35</td>
<td>0.86 0.36</td>
<td>MS</td>
<td>1</td>
<td>1.35 0.86 0.36</td>
<td>MS</td>
</tr>
<tr>
<td>Total patch belowground biomass (g DW m⁻²)</td>
<td>1</td>
<td>1.13</td>
<td>1.26 0.271</td>
<td>MS</td>
<td>1</td>
<td>1.13 1.26 0.271</td>
<td>MS</td>
</tr>
<tr>
<td>Total patch expansion biomass (g DW m⁻²)</td>
<td>1</td>
<td>1.39</td>
<td>2.53 0.123</td>
<td>MS</td>
<td>1</td>
<td>1.39 2.53 0.123</td>
<td>MS</td>
</tr>
<tr>
<td>Total patch expansion aboveground biomass (g DW m⁻²)</td>
<td>1</td>
<td>1.20</td>
<td>0.54 0.467</td>
<td>MS</td>
<td>1</td>
<td>1.20 0.54 0.467</td>
<td>MS</td>
</tr>
<tr>
<td>Total patch expansion belowground biomass (g DW m⁻²)</td>
<td>1</td>
<td>0.68</td>
<td>0.69 0.562</td>
<td>MS</td>
<td>1</td>
<td>0.68 0.69 0.562</td>
<td>MS</td>
</tr>
<tr>
<td>Total N (%)</td>
<td>1</td>
<td>1.40</td>
<td>2.08 0.850</td>
<td>MS</td>
<td>1</td>
<td>1.40 2.08 0.850</td>
<td>MS</td>
</tr>
<tr>
<td>Total P (%)</td>
<td>1</td>
<td>1.17</td>
<td>1.32 0.166</td>
<td>MS</td>
<td>1</td>
<td>1.17 1.32 0.166</td>
<td>MS</td>
</tr>
<tr>
<td>Total C (%)</td>
<td>1</td>
<td>1.19</td>
<td>1.25 0.116</td>
<td>MS</td>
<td>1</td>
<td>1.19 1.25 0.116</td>
<td>MS</td>
</tr>
<tr>
<td>Total S (%)</td>
<td>1</td>
<td>1.12</td>
<td>1.29 0.099</td>
<td>MS</td>
<td>1</td>
<td>1.12 1.29 0.099</td>
<td>MS</td>
</tr>
<tr>
<td>C:N (molar ratio)</td>
<td>1</td>
<td>0.09</td>
<td>0.29 0.059</td>
<td>MS</td>
<td>1</td>
<td>0.09 0.29 0.059</td>
<td>MS</td>
</tr>
<tr>
<td>N:P (molar ratio)</td>
<td>1</td>
<td>0.74</td>
<td>0.77 0.050</td>
<td>MS</td>
<td>1</td>
<td>0.74 0.77 0.050</td>
<td>MS</td>
</tr>
</tbody>
</table>
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).

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 Confo 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 H2O2 (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.

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 2.1). 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 (t0, t21, t49, t77), NPK (+/-) and OM (+/-) (Table 2.2). We used the Greenhouse-Geisser correction when sphericity...
The 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).

RESULTS

Porewater results

![Figure 2.1 Porewater concentrations of sulfide (a), phosphate (b), ammonium (c) and nitrate (d) during the experiment. Error bars represent SEM (n=8).](image)

Both the NPK and OM treatment successfully altered biogeochemical sediment characteristics, as expected (Figure 2.1). OM addition resulted in significantly elevated porewater sulfide levels (Figure 2.1a) 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 (Figure 2.1, Table 2.2, $F_{7.71}, P=0.023$). 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 2.2) elevated porewater ammonium ($F_{1.8}=5.87$, $P=0.004$), nitrate ($F_{1.5}=4.678$, $P=0.038$) and phosphate concentrations ($F_{1.7}=5.52$, $P=0.026$) (Figure 2.1). 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.

![Figure 2.2 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.](image)

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 2.2, Figure 2.3). 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 2.1, Figure 2.2).

The aboveground biomass of the expansion did not differ among treatments (Table 2.1). 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 (Figure 2.2, Table 2.1). 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 (Figure 2.4). Some patches in the treatments (NPK, NPK+OM, OM) appeared to manifest less ammonium or sulfide stress (Figure 2.4) 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 expansion. Sulfide levels above 1000 \(\mu\)mol L\(^{-1}\) completely prevented expansion of patches (Figure 2.4c) and resulted in their complete die-off (Figure 2.4a). Some of the patches already suffered from lower sulfide concentrations. Low patch expansion biomass was also strongly related to high porewater ammonium concentrations (Figure 2.4d, \(R^2=0.61, P<0.001\)), but we did not find effects on mother patch biomass (Figure 2.4b, \(R^2=0.20, P=0.05\)). We observed no patch expansion at ammonium concentrations >5000 \(\mu\)mol L\(^{-1}\), but expansion was already strongly decreased by ammonium concentrations around 2000 \(\mu\)mol L\(^{-1}\) (Figure 2.4d). Contrastingly, some mother patches were able to tolerate ammonium concentrations >7000 \(\mu\)mol L\(^{-1}\).

**Figure 2.3** Shoot densities of the mother patch (a) and in the expansion area (b) in time; error bars represent SEM, \(n=8\).

**Figure 2.4** 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.

**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 2.3). 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 2.3. 1.1% versus 1.7%).
Table 2.3 Elemental composition of the leaves. Molar C:N:P ratios and percentages [g\(^{-1}\)] of leaf tissue nutrients.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C:N:P</th>
<th>%C</th>
<th>%N</th>
<th>%P</th>
<th>%S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>121:10:01</td>
<td>39.3</td>
<td>3.83</td>
<td>0.89</td>
<td>1.77</td>
</tr>
<tr>
<td>NPK</td>
<td>161:15:01</td>
<td>40.3</td>
<td>4.40</td>
<td>0.65</td>
<td>1.11</td>
</tr>
<tr>
<td>NPK+OM</td>
<td>140:14:01</td>
<td>41.3</td>
<td>4.56</td>
<td>0.75</td>
<td>1.11</td>
</tr>
<tr>
<td>OM</td>
<td>114:10:01</td>
<td>38.0</td>
<td>3.94</td>
<td>0.87</td>
<td>1.66</td>
</tr>
</tbody>
</table>

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 μmol L\(^{-1}\) 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). 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 μmol L\(^{-1}\) 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 (Figure 2.4). 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 (Fourquean 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.

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 μmol L\(^{-1}\) (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.

**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.

**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**

We like to acknowledge Marloes Hendriks, Madelon van de Kerk, Nel Govers, Camiel Doorenweerd, Jeroen Veeken, Eric Gerritsen, Bruce Schoelitz, Lucien Hanssen, Steef Hanssen and Jochem Vink, who were indispensable in the field and the laboratory. We are also grateful to Martin Versteeg, for his help in the field, to Jelle Eygensteyn, for his help with chemical analyses, and to Jim de Fouw for statistical advise. We also thank two anonymous reviewers for their contribution to the improvement of the manuscript. Rijkswaterstaat Zeeland and Projectbureau Zeeweringen funded this research.
Key-ecosystems such as seagrass beds are disappearing on a global scale. In order to counter-act local loss of seagrass beds, seagrass restoration projects have been carried out with varying degrees of success. As seagrass biomass peaks in summer, most restoration projects are monitored during this period, while wintering processes are largely ignored. We here attempted to elucidate some important bottlenecks for wintering survival in temperate areas of the intertidal model species Zostera noltii, using a three-year transplant-monitoring dataset and a field experiment. We found that next year’s transplant success could not be predicted based on the preceding year’s growth success, emphasizing the winter as a crucial period for survival of seagrass transplants. In addition, transplant success was neither determined by abiotic site characteristics. Low autumn rhizome starch concentrations in unsuccessful transplants, compared to successful transplants and natural beds, hinted at the importance of starch for winter survival. Hence, we tested the importance of starch, accumulated in autumn, versus the importance of the presence of sparse aboveground photosynthetic winter biomass for winter survival of seagrass transplants in a field experiment. We clipped the overwintering-leaves of three natural beds that naturally varied in their autumn rhizome starch concentrations. Decreased leaf densities in winter did not affect seagrass biomass in June, nor did this treatment affect rhizome starch concentration in June. Autumn rhizome starch reserves did however provide a good indication of next year’s growth success, confirming the importance of starch reserves for winter survival. We thus conclude that autumn rhizome starch can be a good predictor of next year’s growth success, whereas the preceding growing season shoot density and the presence of leaves during the winter were bad indicators of next year’s growth success.
INTRODUCTION

Seagrass beds are important coastal ecosystems, as they are highly productive, support high biodiversity, and can play a role in coastal protection by reducing wave energy and through sediment stabilization (Christianen et al., 2013; Duarte, 2002; Fourqurean et al., 2012; Hendriks et al., 2008). However, seagrass beds are currently disappearing on a global scale, mainly due to anthropogenic disturbances (Orth et al., 2006; Waycott et al., 2009). Therefore, many restoration projects have been carried out, with varying degrees of success (Fonseca et al., 2001), which cannot always be attributed to prevailing abiotic conditions (Orth et al., 2009; Suykerbuyk et al., submitted; Van Katwijk et al., 2009). As seagrass restoration projects are often costly, it is important to select suitable sites and to predict or identify bottlenecks that may affect these restoration attempts (Short et al., 2000; Short et al., 2002).

We expect winter processes to be important bottlenecks for transplant winter survival, based on the observation that transplant survival in winter is limited. Temperate and subtropical seagrasses typically display a seasonal biomass peak of high biomass during the growing season (summer), and a strongly decreased biomass during the winter (Duarte, 1989). Seagrass restoration sites are therefore typically monitored during the growing season, whereas wintering processes, which may also affect restoration success, are only occasionally studied (e.g. Marion and Orth, 2012; Vermaat and Verhagen, 1996). However, to date there are no quantitative analyses on the generality of this phenomenon or on what factors affect winter survival. So our overall objective is to identify parameters that have an indicative value for the winter survival of transplants.

Important processes that affect wintering seagrasses in temperate areas are the low temperatures and low light levels, which greatly decrease seagrass productivity. As photosynthesis is reduced, the carbon balance of seagrasses, which is determined by the carbon gain (photosynthesis) and carbon demand (respiration and growth), can become negative (Alcoverro et al., 2001; Alcoverro et al., 1999). During this stressful period, seagrasses become dependent on their carbohydrate reserves (Alcoverro et al., 1999; Brun et al., 2008; Zimmerman et al., 1995), which are thus important to seagrass winter survival.

For seagrasses, the most important non-soluble carbohydrate for long-term storage is starch (Pirc, 1989). Starch is mainly stored in the rhizomes, and produced in periods with a positive carbon balance (Olive et al., 2007; Zimmerman and Alberte, 1996). Wintering seagrasses are thus dependent on the production of starch reserves in the preceding growing season. The amount of starch needed for winter survival depends on both internal and external factors; respiration can be affected by temperature and light, but also by biomass and photosynthetic activity (Alcoverro et al., 1999; Fourqurean and Zieman, 1991; Marsh et al., 1986; Vermaat and Verhagen, 1996). In addition, environmental stressors such as ammonium toxicity and sediment anoxia can increase carbon demand (Brun et al., 2008). Growth is another important process that contributes to carbon demand (Alcoverro et al., 1999). Although growth is marginal during the winter months (0.015 cm² cm⁻² day⁻¹ versus loss rates of -0.110 cm² cm⁻² day⁻¹ in Zostera noltii) (Vermaat and Verhagen, 1996), carbon demand for growth in early spring can deplete carbohydrate reserves.

In this study, we aimed to further elucidate mechanisms of seagrass winter survival with regard to seagrass transplants and to look for indicators of winter survival, as we noticed that many restoration efforts fail over winter, whereas other sites have been successful, without having a clear indication of distinctive abiotic differences among sites. Our goals were therefore: to test if and how next year’s growth success depends on 1) shoot densities in the preceding growth season, 2) the presence of leaves in winter, and 3) the level of the starch reserves. To answer these questions, we combined monitoring data from large-scale transplants (Figure 3.1) with a winter leaf removal field experiment, using Zostera noltii as a model species.

MATERIALS AND METHODS

Location

We studied the winter survival of the natural Zostera noltii beds and transplanted Zostera noltii beds in the Oosterschelde Delta, The Netherlands (Figure 3.1a, 51°39’ N, 4°01’ E). The Oosterschelde has a surface area of 351 km², a tidal amplitude of 2.5-3 m (Troost et al., 2009) and freshwater input is highly limited,
resulting in a salinity of 30 PSU (Nienhuis and Smaal, 1994). Intertidal Zostera noltii meadows cover around 75 ha of the mudflats in the Oosterschelde and are located on relatively stable sediments such as clay banks, saltmarsh remnants and natural shell layers (Suykerbuyk et al., 2012). Intertidal Zostera noltii in the Oosterschelde, which is sometimes referred to as Zostera noltei, are generally not nutrient limited (Govers et al., 2014a) and light availability (when submerged) is around 3-4.5 m secchi disc visibility in summer (Wetsteyn and Kromkamp, 1994).

**Monitoring transplants and natural seagrass beds**

As a part of a large-scale seagrass transplantation project, aiming to mitigate seagrass damage caused by dike reinforcements, seagrass sods were mechanically transplanted to 10x10 m or 15x15 m plots on several mudflats in the Oosterschelde (for details, see Suykerbuyk et al. (2012)) (Figure 3.1). Plots were monitored in spring and summer from spring 2009 – autumn 2011; we counted shoot densities and determined seagrass surface area plot⁻¹ with a Real Time Kinematic differential GPS (RTK-dGPS, Trimble, USA). Samples for starch analysis were taken from a number of natural seagrass beds in the Oosterschelde. In addition to distinguishing between successful and unsuccessful transplant sites, we compared shoot densities plot⁻¹ in the preceding June (year⁻¹). If shoot numbers were <15% of the shoot numbers in the preceding September (year⁻¹), we marked those plots as unsuccessful. For transplanted plots in the year of transplanting, we compared shoot densities plot⁻¹ in June and September. If shoot numbers had decreased in this period (shoot numbers September<June), we marked those plots as unsuccessful. We classified a transplant site as successful if there were more successful than unsuccessful plots and vice versa. A single transplant site could thus be classified as successful in one year and unsuccessful in the next.

To distinguish between successful and unsuccessful transplant sites, we compared shoot densities plot⁻¹ in September with shoot densities plot⁻¹ in the preceding September (year⁻¹). If shoot numbers were <15% of the shoot numbers in the preceding year, plots were marked as unsuccessful. For transplanted plots in the year of transplanting, we compared shoot densities plot⁻¹ in June and September. If shoot numbers had decreased in this period (shoot numbers September<June), we marked those plots as unsuccessful. We classified a transplant site as successful if there were more successful than unsuccessful plots and vice versa. A single transplant site could thus be classified as successful in one year and unsuccessful in the next.

**Leaf removal experiment set-up**

Winter survival of Zostera noltii occurs by a short rhizome with a single shoot, which encloses an active meristem (Vermaat & Verhagen 1996) (Figure 3.2). To test the effect of leaf presence and autumn starch levels on seagrass winter survival, we selected three locations based on their differential starch concentration of rhizomes in November 2012. The average starch concentrations were 57.3 mg g⁻¹ DW in Dortsman Noord (DMN – high starch, Figure 3.1b N2), 38.8 mg g⁻¹ DW in Oostdijk (OD – medium starch, Figure 3.1b N3), and 18.8 mg g⁻¹ DW in KATS (Kats – low starch, Figure 3.1b N4). Next to starch concentration, the length and biomass of the rhizome fragments (in November) also varied between sites at the start of the experiment: rhizome fragments were longer and heavier at OD (medium) and Kats (low) (14±0.44 mm, 3.7±0.07 mg DW and 14±0.40 mm, 4.7±0.08 mg DW respectively) than at DMN (high) (11±0.23 mm, 3.2±0.01 mg DW). A more detailed description of abiotic site characteristics is given in Table 3.2.

On each site, we selected five paired plots of 50x50 cm in November 2012, which were marked with bamboo poles and monitored every month in the period of November 2012 to June 2013. We monitored leaf photosynthetic performance (PAM), as stress indicator (Beer et al., 2001; Brun et al., 2008), shoot density m⁻² and rhizome starch in each of these plots. In one of each paired plot, all visible aboveground biomass was removed monthly from November - January, which resulted in the leaf treatments + leaf and – leaf (n=5). Rhizomes were cut around the plot borders after each visit to prevent allocation of reserves into the plot. The experiment was terminated in June and the inner 30x30 cm square was harvested.

**Processing leaf removal experiment samples**

For the leaf PAM measurements, we collected fresh leaf material (~100 leaves) once a month on all three experimental sites in the period of November 2012 to May 2013. The material was randomly collected just next to the plots. Fresh leaf material was stored in a moist and dark cooler for transport to the lab where we measured photosynthetic performance (leaf fluorescence) on the day of collection. Photosynthetic performance was generally high (>0.7 Fv Fm⁻¹; for details, see results section) indicating that transport did not cause stress to the plants (Beer et al., 2001; Ralph and Burchett, 1998). Leaf fluorescence was measured with a Junior PAM (Walz Co, Effeltrich, Germany) on 20 rinsed and randomly selected leaves from each location (n=20). Plants were allowed to adapt to the dark for at least 1h before the saturating pulse was given (Beer et al., 1998; Christianen et al., 2011). Photosynthetic yield (maximum photochemical quantum yield of photosystem II) was calculated based on maximum photochemical efficiency of PSII (Fm-Fo)/Fm). Fm is the maximum phyto-fluorescence after dark-adaptation and Fo is the minimum fluorescence of the chlorophyll. Photosynthetic yield was
### Table 3.1

<table>
<thead>
<tr>
<th>Site</th>
<th>Grain size</th>
<th>Sediment C</th>
<th>Leaf %N</th>
<th>Leaf %C</th>
<th>NH$_4$</th>
<th>NO$_3$</th>
<th>PO$_4$</th>
<th>PO$_4$</th>
<th>H$_2$S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural populations</td>
<td>115a</td>
<td>4.60</td>
<td>0.44</td>
<td>0.06</td>
<td>3.33</td>
<td>0.06</td>
<td>36.1</td>
<td>0.45</td>
<td>5.11</td>
</tr>
<tr>
<td>Successful transplants</td>
<td>133a</td>
<td>7.90</td>
<td>0.24</td>
<td>0.02</td>
<td>3.28</td>
<td>0.08</td>
<td>36.6</td>
<td>1.28</td>
<td>8.82</td>
</tr>
<tr>
<td>Unsuccessful transplants</td>
<td>122a</td>
<td>4.70</td>
<td>0.26</td>
<td>0.03</td>
<td>3.32</td>
<td>0.04</td>
<td>36.3</td>
<td>0.98</td>
<td>9.26</td>
</tr>
</tbody>
</table>

### Table 3.2

<table>
<thead>
<tr>
<th>Location</th>
<th>Code</th>
<th>Starch</th>
<th>Grain size</th>
<th>Sediment C</th>
<th>Leaf %N</th>
<th>Leaf %C</th>
<th>NH$_4$</th>
<th>NO$_3$</th>
<th>PO$_4$</th>
<th>H$_2$S</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMN</td>
<td>N2</td>
<td>257.3</td>
<td>high</td>
<td>1030.49</td>
<td>3.11</td>
<td>19.97</td>
<td>0.85</td>
<td>19.67</td>
<td>11.56</td>
<td>exposed</td>
</tr>
<tr>
<td>OD</td>
<td>N3</td>
<td>338.8</td>
<td>medium</td>
<td>780.44</td>
<td>2.82</td>
<td>2.75</td>
<td>0.00</td>
<td>10.55</td>
<td>23.48</td>
<td>sheltered</td>
</tr>
<tr>
<td>Kats</td>
<td>N4</td>
<td>418.8</td>
<td>low</td>
<td>117 NA</td>
<td>3.02</td>
<td>6.75</td>
<td>0.70</td>
<td>7.65</td>
<td>0.00</td>
<td>intermediate</td>
</tr>
</tbody>
</table>

Simultaneous with the PAM measurements, we counted shoot numbers in the plots and sampled aboveground/belowground tissue in the vicinity of the plots (5 m). These biomass samples were also transported to the lab, rinsed and split into rhizome and shoots. Harvested samples were treated the same way after termination of the experiment.

Figure 3.3

Rhizome starch concentration in spring and autumn 2009-2011 of natural populations, successful and unsuccessful transplants. The data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the transplants was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing. Normal distribution of the data was tested with the Shapiro Wilk test, and data were log-transformed prior to testing.
RESULTS

**Transplants and natural beds**

We found no correlation between the shoot densities in summer and the shoot densities in the following spring in the transplant plots ($R^2=0.02$, Figure S3.1). These results indicate that other processes than the preceding year’s growth success play a role in predicting growth success in June. Subsequently, we compared rhizome starch concentration of transplanted seagrass patches with seagrass from the natural seagrass beds in the Oosterschelde (Figure 3.3b). Starch concentration varied seasonally, with the highest starch concentration in autumn and lowest starch concentration in spring. Starch concentration of all the unsuccessful transplant sites was always significantly lower than rhizome starch concentration from the successful transplants and the natural beds (Linear mixed model, $F_{2.160}=23.63$, $P<0.001$), which not differed mutually (Figure 3.3b). This difference was greater at the end of the growing season (autumn) than at the start, which suggests that seagrasses of both natural beds and successful transplants had more reserves to spend during the winter months than the unsuccessful transplants. In contrast, we did not find any significant differences in measured abiotic characteristics between successful and unsuccessful transplant sites (Table 3.1).

**Leaf removal experiment**

Seagrass photosynthetic performance (maximum quantum yield) decreased dramatically in the winter (RPM ANOVA, $F_{3.6}=282.25$, $P<0.001$), with the lowest quantum yield in February 2013. In March (2013), photosynthetic performance strongly increased, back to growing season levels (Figure 3.4a). Photosynthetic performance did not vary between locations ($F_2=0.54$, $P=0.562$, $n=20$). In contrast, shoot density did vary between locations ($F_2=21.23$, $P=0.001$, $n=5$), and the lowest shoot densities were observed in KATS (low), with 2-3 times lower shoot densities than DMN (high) and OD (medium) (Figure 3.4b). Shoot density did not differ significantly between the latter two locations (Figure 3.4b). Shoot density also decreased during winter ($F_1=7.496$, $P=0.019$), but at least some aboveground biomass was present at all locations during the entire winter (<2000 shoots m$^{-2}$ compared to >10,000 shoots m$^{-2}$ in summer). Shoot density strongly increased after the winter at OD (medium) and DMN (high), but further decreased at KATS (low). The latter may reflect a lack of starch to enable regrowth. Rhizome starch concentration (Figure 3.4c) dropped during winter on all locations ($F_2=11.054$, $P=0.001$, $n=5$), and although DMN (high) and OD (medium) started off with different rhizome starch concentration in November 2012, there was no difference between the two locations during winter.

Rhizome starch however remained significantly lower at Kats (low) ($F_2=43.77$, $P=0.001$) than at the other two locations during the entire winter. Starch concentration increased again from May (2013) onwards. Although photosynthetic performance (Figure 3.4a) and shoot densities started to increase again around March (2013), in DMN (high) and OD (medium) (Figure 3.4b), rhizome starch concentration dropped from November (2012) onwards to the end of April (2013), and only started to increase again from May (2013) onwards.

![Figure 3.4](image-url) (a) PAM yield, (b) shoots m$^{-2}$, and (c) rhizome carbohydrate concentration of *Zostera noltii* on three different locations. Displayed values are means and error bars represent standard errors (SE).

![Figure 3.5](image-url) Effects of the leaf removal treatment (+leaf and -leaf) on (a) seagrass biomass, and (b) rhizome carbohydrate concentrations in June, at the end of the experiment on three locations: DMN (high), KATS (low), and OD (medium). Displayed values are means and error bars represent standard errors (SE).
Leaf removal treatments resulted in lower shoot densities at DMN (high) (Linear mixed model, F1,53=7.547, P=0.008) and OD (F1,53=23.58, P<0.001), where shoot densities dropped by 700-1500 shoots m⁻² in the –leaf (leaf removal) treatments. The leaf removal treatment however, did not result in a significant decrease in shoot density at Kats (low) (F1,53=0.607, P=0.439), where standing biomass was very low to begin with (Figure 3.4b). Although we successfully reduced shoot densities, we did not observe any effect of this treatment on seagrass biomass at the end of our experiment in June 2013, on any of the locations (Linear mixed model, F1,53=0.861, P=0.362) (Figure 3.5a). Seagrass biomass differed significantly between all three locations (ANOVA, F1,27=31.68, P<0.001), with biomass OD (medium) > DMN (high) > Kats (low) and Kats having a total biomass m⁻² that was 10x lower than at OD (medium) (Figure 3.5a). Rhizome starch concentration did however not differ anymore between locations in June 2013 (ANOVA, F2,26=3.207, P=0.057), despite the differences in autumn. Leaf removal treatment did not affect rhizome starch concentration in June 2013 (Linear mixed model, F1,25=1.81, P=0.191) (Figure 3.5b).

\[ y = 67.69x - 723.43 \]  

Equation 3.1

Rhizome starch concentration in November 2012 was positively related to shoot density in June 2013 (R²=0.51, P=0.006) (Figure 3.6), showing the importance of a good start-off before winter for the success in next growing season. The relationship between November rhizome starch and shoot densities in the next June could be described according to the following equation (Figure 3.6).

In this equation (Equation 3.1), \( x \) describes rhizome starch levels in November (in mg g⁻¹ DW) and \( y \) describes shoot densities (m⁻²) the following June.

\[ y = 67.69x - 723.43 \]

Equation 3.1

**Figure 3.6** The relationship between rhizome carbohydrate concentration in autumn and next year’s shoot density in June.

**Discussion**

Seagrass restoration projects have had varying degrees of success, which sometimes remained unexplained by abiotic site characteristics, due to the high variability in some abiotic parameters and the lack of continuous measurements (Orth et al., 2009; Van Katwijk et al., 2009). We found that the winter period may be an important bottleneck for temperate seagrass transplants, but indicators to forecast transplant performance after winter were lacking. Autumn rhizome starch however, turned out to be an important indicator of growth success (shoot densities) in the following growing season.

**Starch reserves and carbon balance**

Rhizome starch reserves are important to wintering seagrasses, which experience decreased light availability, and our study shows that rhizome starch concentrations can be indicative of next year’s growth success of transplants. Starch reserves reflect the carbon balance of wintering seagrasses. Decreasing starch concentrations indicate a negative carbon balance, while increasing starch concentrations reflect a positive carbon balance, such as we observed from May onwards.

Wintering in small, unbranched rhizome fragments (Vermaat and Verhagen, 1996) limits the total starch storage capacity (g rhizome⁻¹), but also reduces respiration costs of the belowground parts. In addition, biomass partitioning between aboveground and belowground parts is critical for the winter carbon balance, as belowground tissue is very important for starch storage and for anchorage. However, this tissue is also heterotrophic, relying on photosynthates from the shoots (Olive et al., 2007). High belowground to aboveground biomass ratios, such as we observed in Kats (low), may generate high respiration rates (Fourqurean and Zieman, 1991). As a result, such populations may even need higher carbohydrate reserves to survive the winter.

Based on our findings, we could predict a minimum starch level needed for winter survival of *Zostera noltii*. By solving equation 3.1 (\( y = 0 \)), a minimum of 10.69 mg g⁻¹ starch is needed to yield more than 0 shoots m⁻² in the next spring. However this number is just an indication as this result is based on observations during only one winter and starch demand may vary among winters due to varying wintering conditions. Furthermore, minimum starch levels for wintering will also vary among species (Silva et al., 2013), as carbon demand depends on plant respiration rates and species specific morphology (Ralph et al., 2007; Silva et al., 2013).

As autumn starch concentration is indicative of growth success in the next season, starch storage in the preceding growing season is very important to monitor in restoration projects. Starch storage at the end of the growing season depends on processes that determine carbon gain (light, temperature, shoot densities) (Ruiz...
and Romero, 2003), but also the internal carbon demand (Alcoverro et al., 2001). The internal carbon demand is affected by respiration and growth, which are in turn affected by factors such as the weather, sediment conditions, sediment dynamics, and hydrodynamics (Alcoverro et al., 2001; Burke et al., 1996; Herzka and Dunton, 1997). Starch storage at the end of the growing season may be positively affected by selecting suitable sites for transplants, which allows for sufficient photosynthetic gains and relatively low demand due to adverse growth conditions such as sufficient light and nutrient availability and beneficial sediment conditions (Ruiz and Romero, 2003). Finally, autumn starch concentration may not be the only important determinant of seagrass winter survival. Other processes that may influence winter survival are for instance winter storms and resulting wave-driven sediment dynamics or erosion by ice scouring (Vermaat and Verhagen, 1996). The short roots of Zostera noltii, may give limited anchorage to persist these kind of physical disturbances (Cabaco and Santos, 2007; Han et al., 2012). Thus, populations with relative high starch reserves may remain sensitive to winter loss if the restoration site is too exposed or has too mobile sediment.

Cost-benefit analysis: winter leaves, a burden or an asset?

We found that photosynthetic performance of Zostera noltii leaves strongly decreased during the winter months, which indicates that plants are stressed (Brun et al., 2008; Howarth and Durako, 2012) by winter conditions (possibly low light levels and low temperatures), which did not vary among sites. In addition, Vermaat and Verhagen (1996) found highly decreased photosynthetic rates in January compared to July, but also stated that leaf photosynthesis is still important to support maintenance during winter. Next to maintenance, ongoing photosynthesis may be an important mechanism to protect belowground tissue and stored carbohydrates against anoxia, as produced oxygen may help to aerate the rhizosphere and prevent prolonged periods of anaerobic respiration (Alcoverro et al., 1999). Seagrasses are not only more vulnerable to intrusion of toxic sulfides during anaerobiosis (Lamers et al., 2013), but metabolites such as ethanol, amino acids, and organic acids may also drain carbohydrate reserves (Hemminga, 1998; Smith et al., 1984). In addition, maintaining a shoot during winter may also be beneficial when light levels and photosynthetic performance increase again in early spring. Instead of increasing carbon demand to grow new leaves, the old shoot can directly be used for photosynthesis.

Despite the above-described benefits of maintaining a leaf-bearing shoot during winter, we did not find any negative effect of our leaf removal treatment on seagrass spring biomass. We therefore conclude that the presence or absence of leaves in winter does not affect next year’s growth success for the individual wintering shoots.

CONCLUSIONS AND PERSPECTIVES

Winter survival of temperate seagrasses is largely determined by plant carbohydrate reserves, and autumn starch concentrations can be used as indicators for the success of seagrass transplants in the next growing season. This knowledge may be very useful, since abiotic site characteristics seem to give a very limited indication of transplant success and winter survival. Additionally, high starch reserves also enable an earlier growth-start when the carbon balance is still negative, as was observed at our study sites. This early growth by branching may rapidly increase shoot numbers and thus photosynthetic rates and the resulting storage of starch. Due to this positive feedback, seagrasses on early growth sites may probably store more starch throughout the growing season and will have a better chance of survival in the next winter.

Indicators, such as autumn starch reserves may not only be used to predict transplant success, but also to determine the timing of transplanting, as to make sure that transplanted plants have enough reserves for recovery and regrowth (Zimmerman et al., 1995). From these seagrass wintering mechanisms, lessons may be learnt with respect to other processes that may drain carbohydrate reserves during stressful periods. Examples of such stressors are limited light availability due to eutrophication or dredging (Brun et al., 2008; Erftemeijer and Lewis, 2006; Ralph et al., 2007; Touchette and Burkholder, 2000), and the process of transplanting/moving seagrasses (Sheridan et al., 1998). Starch reserves may also be used to predict chances of survival in highly turbid waters (Burke et al., 1996). Events that may drain carbohydrate reserves, e.g. dredging and transplanting (Ruiz and Romero, 2003), could thus be more conveniently timed when the carbon balance of the involved seagrasses is taken into account. We would therefore recommend timing of such disturbances at the start of the growing season (late spring), when the carbon balance is already positive and seagrasses don’t have to rely completely on their reserves. Additional high growth rates in this period may also help seagrasses to recover fast from disturbances such as dredging and transplanting. Hence, a better understanding of the carbon balance of wintering seagrasses may contribute to better protection and restoration of seagrasses.
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Figure S3.1 Correlation between shoot densities in summer and shoot densities in the following spring of the transplants in the first 36 months after transplanting.
Chapter 4

FEEDBACKS AND LOCAL ENVIRONMENTAL SETTINGS AFFECT PERSISTENCE AND RECOVERY DYNAMICS OF A COASTAL ECOSYSTEM

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Human activities in coastal areas have altered background conditions and introduced new disturbance regimes, leading to the global degradation of coastal ecosystems. It is important to assess ecosystem resilience to disturbances to predict and thereby prevent disappearance of these ecosystems. To better understand resilience, it is essential to gain understanding of how local background conditions and feedbacks may interact with disturbances. We therefore studied two aspects of resilience: persistence to and recovery from stressors and small-scale disturbances. We questioned how 1) local sediment conditions, 2) enhanced stress through application of a standardized stressor (OM-addition), and 3) density-dependent feedbacks, determined local persistence (i.e., growth) and disturbance recovery (i.e., regrowth), using intertidal seagrass beds (Zostera noltii) as model systems. The study was carried out at three field sites across Europe, encompassing a gradient of sediment conditions, where we applied a small-scale disturbance (i.e., creating a gap). Persistence and recovery greatly differed between locations and revealed some clear general patterns. Recovery was locally decreased by high sulfide concentrations resulting from OM-addition. Persistence was much less affected by OM-addition, because dense seagrass stands alleviated sulfide toxicity (joint-detoxification). The interaction between the stress treatment (OM-addition) and local sediment conditions determined local porewater sulfide concentrations and subsequent effects on local persistence and recovery. As a result, joint-detoxification was conditional, namely less important in coarse-grained than in fine-grained sediments. Our results illustrate the complexity of assessing ecosystem resilience resulting from the intricate interplay between interacting local conditions, feedbacks and disturbances affecting persistence and recovery dynamics.
INTRODUCTION

Many valuable coastal ecosystems such as coral reefs, seagrass meadows, mangroves and salt marshes are globally declining as a result of human-induced stressors and disturbances such as eutrophication (Burkholder et al. 2007), increased trace metal levels (Eisler 1981), impoverished trophic relationships (Estes et al. 2011), and climate change (Hughes 1994, Alongi 2002, Orth et al. 2006, Waycott et al. 2009, Deegan et al. 2012). To counteract such losses, attempts have been made to restore these valuable habitats, but restoration of coastal ecosystems has proven to be notoriously difficult (Rinkevich 1995, van Katwijk and Hermus 2000, Zedler 2000, Van der Heide et al. 2007, van Katwijk et al. submitted). Therefore, managers and conservationists have been trying to predict and thereby prevent ecosystem collapses both by looking for early warning indicators and by trying to estimate the resilience of ecosystems to further stresses and disturbances (Elliott et al. 2007).

Quantifying ecosystem resilience has however proven to be challenging, which is also reflected by the ongoing debate on the definition of resilience (Peterson et al. 1998, Folke et al. 2004, Elliott et al. 2007). Resilience has been described as the capacity to deal with disturbances either as ‘a measure of persistence of systems and of their ability to absorb change and disturbance and still maintain the same relationships between populations or state variables’ (Holling 1973) or as ‘the time needed to return to the equilibrium state’ (Pimm 1984). So in a broad sense, resilience refers to both the capacity to persist stresses and to recovery from disturbance, implying that measuring resilience should focus on quantifying both continued growth following a stress and regrowth following disturbances. Quantifying both aspects may however be complicated by interacting effects between stress and disturbances.

Understanding interacting effects between stress and disturbances on resilience is highly important, as human activities have in many cases caused both enhanced disturbance regimes that may lead to ecosystem degradation (Orth et al., 2006; Waycott et al., 2009), and enhanced background stress levels (Nystrom and Folke, 2001). Little is known about the effects of background stress levels on the capacity to recover after disturbances in coastal ecosystems, but it is very likely that an increase in anthropogenic background stressors affects ecosystem resilience to disturbances. (Hughes et al. 2003). Even in the absence of anthropogenic stressors, local differences in natural environmental conditions such as sediment characteristics or hydrodynamic regimes, are likely to represent different stress levels that may also affect the resilience of coastal ecosystems to disturbances.

Foundation species, or ecosystem engineers, are key-components of most valuable ecosystems. Ecosystem engineers such as seagrasses, corals, mangroves and salt marsh plants alter their abiotic and biotic environment (Jones et al. 1994) resulting from positive feedback mechanisms (Van der Heide et al. 2011). A well known example of a positive feedback in vegetated coastal ecosystems, is the attenuation of waves and currents by seagrasses and salt marsh vegetation (Bouma et al. 2005). This results in stabilization of coastal sediments (Bos et al., 2007; Christianen et al., 2013) and the trapping small particles from the water column (Granata et al. 2001, Hendriks et al. 2010) which generally leads to an improved water clarity and thereby enhanced growth for seagrasses (Van der Heide et al. 2011). Similar positive feedback mechanisms exist for other coastal ecosystems (Allen 2000, Huisman et al. 2009, Wild et al. 2011, Alteri et al. 2013, Gillis et al. 2014), and typically require a minimum population density and/or coverage (Bouma et al. 2009). Therefore, it may be expected that stressors and disturbances have a bigger effect on ecosystem resilience when breaking self-facilitating positive feedbacks, e.g. by reducing density.

We thus questioned how 1) local environmental stress levels, 2) enhanced stress levels by application of a standardized stressor (organic matter addition to induce sulfide stress), and 3) density-dependent feedbacks, determined ecosystem resilience in terms of local persistence (i.e. growth) and disturbance recovery (i.e. regrowth), using intertidal seagrass beds (Zostera noltii) as model systems. Seagrasses are relevant model species as seagrass beds have been experiencing an accelerating loss due to anthropogenic stressors and disturbances (Waycott et al. 2009). Furthermore, Zostera noltii can easily been found on sites with contrasting local background stress levels (both natural and human-induced) and differing local densities, which may be expected to alter positive feedback strength. Moreover, additional stress can easily be applied in a standardized form, by organic matter addition (Govers et al.).

MATERIALS AND METHODS

Site description

The experiment was conducted in intertidal Zostera noltii beds in three countries in Western Europe: France, The Netherlands, and Germany (Figure 4.1). For the experiment, we selected a high-density and low-density bed on each location. The French site was located on the mudflats of Saint-Jacut-de-la-mer (hereafter referred to as St Jacut), on the north coast of Brittany (48°36’14 N, 2°11’41 W). This site was characterized by the highest sediment organic matter (OM) levels of all sites, and a very fine grain size (Table 4.1), due to aquaculture and limited circulation in the bay of Arguenon (Varcourt and Bonnot-Courtois 1992). The Dutch site was located on the mudflats of Viane, in the Oosterschelde Delta (51°61’88 N, 4°01’77 E), and was characterized by both intermediate sediment organic matter levels and grain size (Table 4.1). Finally, the German site was situated on the mudflats of Rantum, on the barrier island Sylt (hereafter referred to as Sylt).
in the northern Wadden Sea (54°47 30 N, 8°17 43 E), and was characterized by coarse-grained sediment and low %OM (Table 4.1). Sediment Organic matter (%OM) was significantly higher in the high-density beds compared to the low-density beds on all locations (Linear mixed model, \(F_{1,39}=12.1, P=0.001\)) (Table 4.1). In contrast, median grain size (D50) was significantly lower in the high-density beds than in the low-density beds (\(F_{1,44}=5.6, P=0.022\)). Exposure time of the intertidal *Zostera noltii* beds during low tide was between 5-6 hours for all three sites. The experiment was conducted from late June to the end of August in the summer of 2011 and lasted for 52 days.

**Experimental set-up**

On all three sites, we selected a high- and a low-density bed (Table 4.1), in the vicinity (max 100 m distance) of each other, to keep environmental characteristics as similar as possible. High-density and low-density sites were significantly different in terms of biomass on all locations (linear mixed model, \(F_{1,32}=29.3, P<0.001\)). In each bed, we selected 12 plots in a homogenously vegetated area, where we created circular 35 cm diameter gaps, in which we removed all seagrass biomass at t0. The removed biomass was collected and taken to the lab for further analysis. The gap was at the center of a larger 105 cm diameter circle (the experimental plot) along which we cut the rhizomes to a depth of 10 cm with a knife.

![Map of Western Europe with the locations of the field sites: St Jacut in Brittany, France, Viane in the Oosterschelde, The Netherlands, and the island of Sylt, Germany.](image)

**Figure 4.1** Map of Western Europe with the locations of the field sites: St Jacut in Brittany, France, Viane in the Oosterschelde, The Netherlands, and the island of Sylt, Germany.

We randomly applied one of two stress-treatments to the plots (n=6): a control treatment (Control) or an organic matter addition treatment (+OM). For the OM-treatment, we produced OM-sticks consisting of labile organic matter (1:4 starch and sucrose) and water. 45 Sticks containing 20 g OM each were regularly distributed in the sediment (every 15x15 cm, to a depth of ~7 cm) in the large 105 cm circle. The labile OM was only reactive when it slowly dissolved from the sticks; we thus created a slow-release carbon source. To compensate for the physical disturbance of placing the sticks in the sediment, we applied a similar disturbance to the control plots using a knife. Next, we monitored the plots after 30 days and after 52 days.

Before the start of the experiment, we randomly selected 8 sites in each meadow (in both the high- and low-density beds), where we collected sediment samples and porewater samples. Porewater samples were collected anaerobically in the top 6 cm of the sediment with vacuumed 60 ml syringes, connected to Rhizon soil moisture samplers (Eijkelkamp Agrisearch Equipment, Giesbeek, the Netherlands). At t30 and t52 days, we collected porewater samples in the experimental plots. Two samples were collected in each gap (hereafter referred to as gap) and subsequently pooled and two samples outside the gap, but inside the experimental plot (hereafter referred to as meadow), were collected in the same way. Seagrass biomass was sampled a t0 inside the gap as all biomass was removed from the gap. At t30, we sampled biomass outside the gap (in the meadow, within the experimental plot) with two small 6 cm diameter cores per plot, and at 52 we harvested all regrown biomass from the gap and again sampled two 6 cm diameter cores outside the gap.

**Sample analysis**

40 ml of each porewater sample was frozen after collection and transported to the lab for further analysis. The remainder of the samples was immediately used to measure total sulfide concentration in the porewater, 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. Nitrate was determined by sulphanilamide, after reduction of nitrate to nitrite in a Cadmium column.

Seagrass biomass samples were rinsed and sorted into aboveground and belowground biomass and shoot numbers were counted. Subsequently, sorted seagrass was freeze-dried and weighed. Seagrass leaf nitrogen and carbon concentrations were determined on grinded freeze-dried plant material, using an elemental analyzer (Type NA 1500 Carlo Erba Thermo Fisher Science, USA), coupled online via an interface (Finnigan Conflo III, USA) to a mass spectrometer.

**Table 4.1** Sediment and seagrass characteristics of the low and high-density bed at each location.

<table>
<thead>
<tr>
<th>Location</th>
<th>Density</th>
<th>Sediment %OM</th>
<th>Sediment D50 (μm)</th>
<th>Biomass t52 (g DW m⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>St Jacut (FR)</td>
<td>low</td>
<td>2.93</td>
<td>132</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>3.77</td>
<td>119</td>
<td>266</td>
</tr>
<tr>
<td>Viane (NL)</td>
<td>low</td>
<td>1.26</td>
<td>198</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>2.83</td>
<td>171</td>
<td>108</td>
</tr>
<tr>
<td>Sylt (DE)</td>
<td>low</td>
<td>0.46</td>
<td>325</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>0.64</td>
<td>334</td>
<td>139</td>
</tr>
</tbody>
</table>
(Thermo Finnigan Delta Plus, USA). Total phosphorus in seagrass tissue was measured on an inductively coupled plasma emission spectrophotometer (ICP) (Spectroflame, Spectro Inc., USA), after digestion of freeze-dried plant material with nitric acid and H$_2$O$_2$.

Sediment grain size distribution was determined on dried and sieved (1 mm mesh size) sediment samples by laser diffraction on a Malvern (Master 2000, UK) particle size analyzer. Sediment organic matter (%) was determined by weight loss on ignition (LOI) at 550°C.

Statistical analysis
We used two-way ANOVAs for data with two factors (density * treatment) and linear mixed models to test for the effects of joint detoxification (density * treatment * gap/meadow) with plot as a random factor (error term in aov). We used a stepwise multiple linear regression with a backward selection to test which measured biotic and abiotic parameters highly affected seagrass recovery and persistence biomass. For the backward stepwise multiple linear regression we first included all possible explaining parameters for recovery biomass (biomass meadow, sulfide gaps, sulfide meadow, NH$_4$ gap, NH$_4$ meadow, PO$_4$ gap, PO$_4$ meadow). Sulfide, NH$_4$ and PO$_4$ were included as the weighted average per plot (separately for gap and meadow). The final adjusted models were the most reduced models, which were not significantly worse (P<0.05) than the full model that included all factors, and model selection was based on the Akaike information criterion (AIC). Normality of the models was tested on the residuals, and data were log-transformed prior to analysis to meet the model assumptions of normally distributed data. Final models resulting from the stepwise multiple linear regressions were also tested for independence with the Durbin-Watson test and colinearity was checked using the variance inflation factor (vif) (Field et al. 2012). We used R 2.15 for all statistical analysis.

RESULTS
Comparison of persistence (growth) and recovery (regrowth)

Although we found no significant effect of density on recovery biomass in St Jacut (2-way ANOVA, F$_{1,20}=1.8$, P=0.196), we observed a significant decrease in recovery as a result of the OM- addition (F$_{1,20}=4.6$, P=0.045) (Figure 4.2a). Then, on the other two sites (Viane and Sylt, Figure 4.2b & c), we did not find a significant effects of OM-addition on recovery, but we did find a significant lower recovery biomass on the low-density sites (Viane: F$_{1,20}=24.6$, P<0.001, Sylt: F$_{1,20}=22.3$, P=0.001). Both St Jacut sites, the high-density site of Viane, and the low-density site of Sylt had comparable recovery biomass (2-4 g DW m$^{-2}$). However, we found almost 5x higher recovery biomass on the high-density site of Sylt than on the above sites (Figure 4.2c).

We observed a different trend in the persistence (growth) biomass, which was the increase in seagrass biomass of the meadow between t52 and t0 (Figure 4.2d). Persistence was highest by far on the high-density site of St Jacut (Figure 4.2d), namely an increase of ~150 g DW m$^{-2}$ in 52 days, while we observed about 50% less growth (persistence) on St Jacut low-density and Viane high-density sites (Figure 4.2d-f). The lowest persistence was observed on the Viane low-density site (<10 g DW m$^{-2}$ in 52 days). In both St Jacut and Viane, the low-density sites were significantly less persistent than the high-density sites (F$_{1,20}=11.2$, P=0.003, F$_{1,20}=70.0$, P<0.001 for St Jacut and Viane respectively) (Figure 4.2b & c). Additionally, persistence in Viane considerably decreased as a result of the OM-addition (F$_{1,20}=5.5$, P=0.030), while persistence was not significantly affected by OM-addition on the other two sites, even though some plots decreased in biomass during the experiment, which resulted in negative persistence (or growth, Figure 4.2d, low-density +OM).

![Figure 4.2](image-url)
Location and density effects on sulfide stress treatments

OM-addition successfully led to significantly elevated porewater sulfide concentrations in the gaps on all three locations (2-way ANOVAs, St Jacut $F_{1,20}=27.2$, $P<0.001$, Viane $F_{1,20}=8.4$, $P=0.009$, Sylt $F_{1,20}=8.5$, $P=0.008$) (Figure 4.3a-c). In St Jacut, the OM-treatment resulted in sulfide levels higher than 1000 μmol L$^{-1}$. However, we also detected rather high sulfide concentrations in the control treatments (200-300 μmol L$^{-1}$) in St Jacut. OM-addition resulted in enhanced sulfide concentrations of 270 and 1000 μmol L$^{-1}$ in the low- and high-density respectively. Finally, The lowest sulfide concentrations were measured in Sylt, where sulfide concentrations reached up to 350 μmol L$^{-1}$ in the OM-treatments, but always kept below 50 μmol L$^{-1}$ for the controls. We did not find an effect of density on gap sulfide concentrations.

Addition of OM also significantly increased porewater sulfide concentrations outside the gap, in the meadow (Figure 4.3d-f) on all locations but Viane (NL) (St Jacut $F_{1,20}=27.2$, $P<0.001$, Viane $F_{1,20}=8.4$, $P=0.009$, Sylt $F_{1,20}=8.5$, $P=0.008$) (Figure 4.3a-c). In St Jacut, OM-addition resulted in sulfide levels higher than 1000 μmol L$^{-1}$. However, we also detected rather high sulfide concentrations in the control treatments (200-300 μmol L$^{-1}$) of St Jacut. In Viane, OM-addition resulted in sulfide concentrations of 270 and 1000 μmol L$^{-1}$ in the low- and high-density respectively. Finally, The lowest sulfide concentrations were measured in Sylt, where sulfide concentrations reached up to 350 μmol L$^{-1}$ in the OM-treatments, but always kept below 50 μmol L$^{-1}$ for the controls. We did not find an effect of density on gap sulfide concentrations.

As a proxy for joint detoxification, we calculated the difference in porewater sulfide concentrations between gaps and the surrounding meadow, assuming that all local conditions are more or less equal, except for vegetation density (Figure 4.4). We found a significant joint detoxification effect in St jacut (Figure 4.4a, Linear Mixed Model, $F_{1,10}=4.7$, $P=0.037$), where joint detoxification was as high as ~1000 μmol L$^{-1}$ (50% reduction) in the high density OM-treatment. In Viane, we only found a significant joint-detoxification effect in the OM-treatment (Figure 4.4b, $F_{1,10}=8.9$, $P=0.005$) as higher seagrass densities outside the gaps reduced sulfide concentrations by 150-500 μmol L$^{-1}$ (>90% reduction) to non-lethal levels (<50 μmol L$^{-1}$). Additionally, we also observed joint-detoxification of about 30-130 μmol L$^{-1}$ in Sylt, but surprisingly, only in the low-density beds (Figure 4.4c, $F_{1,10}=5.5$, $P=0.024$), as the difference in sulfide between gap and meadow was higher on the low-density beds than in the high-density beds.

Identifying main drivers of seagrass persistence and recovery

Multiple linear regression analyses revealed which measured factors explained most of the variation in persistence and recovery biomass for all three locations (Table 4.2). In St Jacut, 35% of the variation in recovery biomass could be explained by sulfide ($P=0.004$) and PO$_4$ (not significant) concentrations in the gap. The same two factors, as well as the meadow biomass outside the gap explained 71% of
the variation in recovery in Viane (P=0.003, P=0.054, and P<0.001 for gap PO₄, gap sulfide and meadow biomass respectively). In Sylt, 38% of the variation was explained by gap PO₄ concentrations (P=0.335) and meadow biomass (P=0.035). For persistence (i.e., growth), 33% of the variation in St Jacut was explained by sulfide concentrations in the meadow, sulfide in the gap, and PO₄ in the meadow (P=0.023, P=0.064, P=0.062 for sulfide gap, sulfide meadow and PO₄ meadow respectively). In Viane, porewater sulfide concentrations in the meadow alone explained 42% of the variation in persistence (P<0.001). Also in Sylt, porewater sulfide concentrations in the meadow were important and together with porewater PO₄ concentrations in the meadow explained 41% of the variation in persistence (growth) in Sylt.

Table 4.2 Results from the stepwise backwards multiple regression analysis of continuous biotic and abiotic variables on persistence and recovery biomass. Biomass is in g DW m⁻² and PO₄ and sulfide concentrations are in μmol L⁻¹.

<table>
<thead>
<tr>
<th>Location</th>
<th>Persistence (growth biomass)</th>
<th>Adjusted model</th>
<th>ΔR²</th>
<th>B</th>
<th>SE B</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>St Jacut</td>
<td></td>
<td>Adjusted model</td>
<td>0.33</td>
<td>6.64</td>
<td>0.73</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Sulfide gap</td>
<td></td>
<td>0.29</td>
<td>0.12</td>
<td>0.09</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>Sulfide meadow</td>
<td></td>
<td>-0.18</td>
<td>0.09</td>
<td>0.45</td>
<td>0.062</td>
</tr>
<tr>
<td></td>
<td>PO₄ meadow</td>
<td></td>
<td>-0.90</td>
<td>0.45</td>
<td>0.67</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Viane</td>
<td></td>
<td>Adjusted model</td>
<td>0.42</td>
<td>13.51</td>
<td>2.51</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Sulfide meadow</td>
<td></td>
<td>-2.67</td>
<td>0.67</td>
<td>0.840</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sylt</td>
<td></td>
<td>Adjusted model</td>
<td>0.41</td>
<td>0.26</td>
<td>1.27</td>
<td>0.840</td>
</tr>
<tr>
<td></td>
<td>Sulfide meadow</td>
<td></td>
<td>0.29</td>
<td>0.11</td>
<td>0.015</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>PO₄ meadow</td>
<td></td>
<td>1.67</td>
<td>0.70</td>
<td>0.70</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Summarizing, variation in recovery was mostly explained by porewater sulfide concentrations in the gap (St Jacut, Viane) and meadow biomass (Viane, Sylt), whereas persistence variation was mostly explained by porewater sulfide concentrations in the meadow (Viane, Sylt) and PO₄ concentrations in the meadow (Sylt).

Discussion

Human activities have enhanced background stress levels and disturbance regimes leading to the worldwide degradation of ecosystems (Alongi 2002, Hughes et al. 2003, Dudgeon et al. 2006, Waycott et al. 2009, Deegan et al. 2012). Little is known about the interaction between background stress levels and disturbances, although it is very likely that high background stress levels affect ecosystem resilience to disturbances (Nystrom and Folke, 2001). Furthermore, stressors and disturbances may have disproportionate effects on ecosystem resilience when breaking self-facilitating positive feedbacks that typify many foundation species. We therefore studied how local environmental conditions and potential positive feedbacks affect resilience of a vegetated coastal ecosystem (seagrass bed). We found distinct differences in local persistence and recovery among our three contrasting study sites as a result of interacting local background conditions and the applied stressor (OM): high sulfide levels negatively affected both persistence and recovery, although the level of this stressor was largely determined by local (sediment) conditions. In addition, sulfide concentrations were reduced outside the experimentally created gaps (meadow) compared to inside the gaps, which may be attributed to joint detoxification by higher seagrass densities outside the gap. This mechanism seemed particularly relevant to seagrass beds with sediment conditions that may promote high sulfide concentrations, or possess high seagrass densities (i.e. St Jacut). Hence, we found that persistence and recovery of a vegetated coastal ecosystem after disturbance were determined by interacting local background settings, the applied stressor, and a positive feedback.

Persistence and recovery

Ecosystems frequently experience both natural and human-induced disturbances, resulting in a heterogeneous landscape reflecting recovery processes (Pickett and White 1985, Brun et al. 2003). Gaps in vegetated coastal habitats, caused by disturbances such as burial, anchoring and dredging may quickly recover by clonal rhizomal growth of plants at the edges of the gaps (Rasheed 1999, Kenworthy et al. 2002, Ewanchuk and Bertness 2004, Macreadie et al. 2014). Recovery of the gaps may therefore depend on the surrounding shoot density or biomass, such as observed in our experiment (Figure 4.2, Table 4.2). Persistence and recovery differed greatly between the studied seagrass beds (Figure 4.2), which may be a
result of different background conditions affecting persistence (i.e. growth) and interacting with recovery processes. We observed local differences in background sediment conditions (median grain size and soil organic matter content), which interacted with the applied stressor (OM-addition). These conditions may be the result of a biotic feedback loop, that is, higher seagrass densities trap more fine sediment and organic particles than lower densities (Hendriks et al. 2008). Alternatively, high sediment organic matter content may result from an external input of organic matter such as eutrophication or aquaculture (Cancemi et al. 2003, Burkholder et al. 2007). This leads to higher sediment organic matter loads, which promotes decomposition by sulfate reducing bacteria, a process which results in the production of toxic sulfides (Jørgensen 1984). Moreover, fine-grained sediments (Table 4.1, St Jacut, Viane) stimulate anaerobic conditions, which are ideal for sulfate reduction (Jørgensen 1982) and are characterized by reduced exchange rates between the porewater and surface water, promoting the build-up of toxic sulfides. Adding up to complexity, high seagrass densities may enhance joint detoxification of high sulfide concentrations as indicated by our study, see below. The observed differences between sites illustrate the importance of local stressors/background conditions when dealing with disturbances, as stressors may affect or even interact with disturbance events. Similar responses may be observed in other ecosystems, such as in coral reefs, where susceptibility of coral reefs to disturbances may also be site specific, and depending on background stress levels (Brown 1997).

Furthermore, when considering the response of a system after a disturbance and including persistence (i.e. local growth) and recovery (i.e. regrowth), we can distinguish between four different system responses (Figure 4.5c). Interestingly, three possible outcomes are represented by the contrasting responses of our experimental sites (Figure 4.5d). These possible outcomes, or ‘strategies’ are plotted in a scatterplot (Figure 4.5a) where the strength of the process is plotted on the 1:1 diagonal, with the most resilient sites in the upper right corner, whereas the dominance of the process (persistence vs. recovery) is plotted on the other diagonal. We can use this plot to scale-up from our small-scale disturbances to landscape-scale effects. A highly persistent and quickly recovering habitat could be considered resilient as such a system will be able to (quickly) return to its equilibrium (the pre-disturbed state) after a disturbance. This conceptual model may also be applicable to other ecosystems such as salt marshes and coral reefs. For instance, a highly resilient coral reef will not only be able to persist prevailing background stressors, but will also be able to recover quickly from disturbances such as a tropical storm (Figure 4.5a) (Gunderson 2000, Folke et al. 2004). Contrastingly, cordgrass in salt marshes may be able to persist in large patches, whereas recovery/recruitment in drought-induced die-off areas may be limited (Angelini and Stillman 2012).

Sulfide is a potent soil phytotoxin (Lamers et al. 2013), known to generate toxic effects in freshwater macrophytes (Geurts et al. 2009), seagrasses (Koch et al. 2007, Holmer et al. 2011, Van der Heide et al. 2012), salt marshes (Koch et al. 1990, Lee 1999), and mangroves (McKee et al. 1988). Sulfide may negatively affect plant performance, and build-up of high sediment sulfide levels has even resulted in vegetation diebacks (Carlson et al. 1994, Terrados et al. 1999). High vegetation densities may counteract intrusion of toxic sulfide by loss of oxygen in the rhizosphere (Pedersen et al. 1998, Lee 1999), but low vegetation biomass may not be able to prevent toxic effects of sulfide. In line with this, we observed reduced porewater sulfide concentrations in the meadow (high ambient...
seagrass densities) compared to inside the created gaps (low density), which may be attributed to joint stress alleviation by high densities. Contrastingly to our expectations, we did not observe reduced sulfide concentrations on the high-density sites compared to the low-density sites as a result of entrapment of organic matter and fine sediments in dense vegetation. These conditions promote rather than reduce sulfide production. We did however observe joint-detoxification effects when locally comparing the densely vegetated meadow to the gaps. These observations imply that there is a positive feedback between the concentration of a toxicant and the population density or biomass; higher population biomass may lessen toxic effects, which could in turn stimulate growth (Van der Heide et al. 2010). Density-dependent toxicity has been described for a number of different systems and organisms (e.g. freshwater algae, Pickhardt et al. 2002), yeast, Greig and Travisano (2008), salt marsh plants, Altieri et al. (2013), including seagrasses (Van der Heide et al. 2008, Van der Heide et al. 2010, Govers et al.), and may also be applicable to other ecosystems. Population density may affect toxicity either by joint-detoxification, a process in which the toxicant is actively broken down by the exposed organism, or by growth-dilution. Growth-dilution is a mechanism involving non-degradable toxicants (such as trace metals) where the toxicant is stored in lower (non-lethal) concentrations, spread over a higher density/biomass (Van der Heide et al. 2010). However, we showed that joint-detoxification is conditional, and depending on the level of the toxicant (Figure 4.4c), which in turn results from and interacts with local background conditions (sediment characteristics).

CONCLUSIONS AND IMPLICATIONS

We are the first to show that local environmental settings, such as sediment conditions, and feedbacks may (interactively) affect persistence and recovery of a coastal ecosystem (seagrass bed). We found distinct differences between sites in persistence and recovery after small-scale disturbances (creation of a gap) (Figure 4.3), which we could relate to background conditions interacting with an applied stressor (OM addition to induce sulfide stress) and joint-detoxification of sulfides by high vegetation biomass. Our results indicate that it is important to identify possible feedbacks in ecosystems that affect resilience and to map local conditions to assess possible interactions between stressors and disturbances. We thus found that prevailing background conditions (sediment characteristics) may disturb system recovery from local disturbances (i.e. St Jacut, Viane). Similar mechanisms may be present in other vegetated ecosystems, where recovery of vegetation may also be hampered by high local sediment sulfide concentrations by a lack of stress amelioration by high densities (joint-detoxification) (Altieri et al. 2013). Non-rooting ecosystems such as corals may be typified by other interactions between background stressors and disturbances. For instance, coral reefs with high fishing pressure may be more vulnerable to eutrophication pulses due to a lack of herbivorous fish consuming resulting macroalgal blooms (Hoey and Bellwood 2011). This again illustrates the complexity of assessing ecosystem resilience for conservation and management, and we stress the importance of including feedbacks and interactions between stressors and disturbances in the assessment of ecosystem resilience.

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Seagrasses as indicators for coastal trace metal pollution: a global meta-analysis serving as a benchmark, and a Caribbean case study

Laura L Govers, Leon PM Lamers, Tjeerd J Bouma, Jelle Eygensteyn, Jan HF de Brouwer, A Jan Hendriks, Chantal M Huijbers, Marieke M van Katwijk (2014)

Seagrass beds are highly productive coastal ecosystems providing a large array of ecosystem services including fisheries and carbon sequestration. As seagrasses are known to be highly sensitive to anthropogenic forcing, we evaluated the use of trace metal concentrations in seagrasses as bioindicators for trace metal pollution of coastal regions at both global and local scale. We carried out a meta-analysis based on literature data to provide a global benchmark list for trace metal accumulation in seagrasses, which was lacking in literature. We subsequently carried out a case study at the Caribbean islands of Curaçao and Bonaire to test for local-scale differences in trace metal concentrations in seagrasses, and internal metal allocation. The benchmark and local study show that trace metal concentrations in seagrass leaves, regardless of the species, can vary over a 100 to 1000-fold range, and are related to the level of anthropogenic pressure, making seagrasses highly valuable indicators.
INTRODUCTION

Billions of people live in coastal areas all over the world and it is expected that, in a couple of decades, even more than 50% of the expanding human population will be living within 150 km from the shore (Cohen, 2003; Cohen et al., 1997; Small and Nicholls, 2003). This leads to a steep increase in anthropogenic activities in coastal areas such as dredging, aquaculture, industrial activities and pollution, sewage discharge, and deforestation (Cohen, 2003; Mora, 2008). These activities severely threaten coastal ecosystems including coral reefs (Mumby et al., 2006; Mumby et al., 2007), mangroves (Valiela et al., 2001) and seagrass beds (Waycott et al., 2009), which not only provide a large suite of ecosystem services (Costanza et al., 1997), but are also strongly interconnected by fluxes of nutrients and dissolved organic matter, and by animal migration (Cowen et al., 2006; Nagelkerken, 2000).

To get insight into the extent and the spatial variation of anthropogenic pressure on coastal ecosystems and to locate sources of pollution, there is a strong need for good indicators. Bioindicators, including a variety of organisms such as clams, plants, copepods and microorganisms, can be used to identify anthropogenic disturbances and preferentially provide early warning signals for pollution or degradation (Linton and Warner, 2003). As they accumulate pollutants, these organisms also reflect low intensity, but chronic impacts, in contrast to physical or chemical parameters which often only present a snapshot of environmental conditions (Linton and Warner, 2003). Additionally, bioindicators can provide information on multiple spatial scales, as most ecosystems are heterogeneous, and are able to differentiate between natural variation and anthropogenic disturbance (Markert et al., 1999; Martínez-Crego et al., 2008).

Seagrasses are known to be good bioindicators (Lee et al., 2004; Orth et al., 2006) as they are widespread and sensitive to environmental changes (Bhattacharya et al., 2003; Ferrat et al., 2003; Udy and Dennison, 1997; Walker and McComb, 1992), and are able to integrate ecological conditions and processes over various timescales from weeks to years (Gonzalez-Correa et al., 2005; Madden et al., 2009; Meehan and West, 2000). Seagrass bioindicators have predominantly been used in the Mediterranean and in Florida, where several complex indices have been developed based on seagrass characteristics ranging from the individual physiological level to the community level (Bennett et al., 2011; Lopez y Royo et al., 2011; Montefalcone, 2009; Moreno et al., 2001; Romero et al., 2007). However, most of the proposed indicator values are specific for areas and species, and can therefore not be used at a global scale.

Seagrasses have been shown to be indicative of trace metal pollution (Lafabrie et al., 2007; Lafabrie et al., 2008; Sundelin and Eriksson, 2001), and as this type of pollution is becoming a major threat to coastal ecosystems in rapidly developing countries (Li et al., 2007), there is a strong need for reliable trace metal bioindicators. To use seagrasses as such, they need to be highly responsive in order to be able to detect differences at both local and global scale. As they are primary producers providing stock food to a large variety of coastal herbivores, seagrasses can also be expected to be indicative of trace metal concentrations at higher trophic levels. Their concentrations may therefore also be used to detect possible threats to ecosystem services such as fisheries. However, as of today, literature does not provide a complete reference overview of trace metal levels in seagrasses on a global scale. We therefore conducted a meta-analysis to compile this benchmark of global trace metal concentrations in seagrass leaves.

In addition, we studied potential trace metal pollution in seagrasses on a local scale to test whether it was possible to detect local-scale differences in trace metal concentrations in seagrasses. We focused on two Caribbean islands: Curacao and Bonaire. The variation of anthropogenic pressure at an island scale makes these islands very suitable for this study. Moreover, the Caribbean represents a typical tropical area in which coastal ecosystems, including seagrass beds, are suffering from anthropogenic impacts such as coastal development, tourism and growing industries and harbors (Phillips, 1992), oil drilling and accompanying spills, trace metal pollution (Thorhaug et al., 1985; Vera, 1992), and eutrophication (Burkholder et al., 2007; Short and Wyllie-Echeverria, 1996). However, the effects of pollution on seagrass meadows have been poorly studied in this area.

Figure 5.1 Overview of all seagrass locations included in the meta-analysis (Table 5.4) on trace metals in seagrass leaves, and the distribution of the tropical seagrass genus Thalassia (adapted from Green & Short 2003).
Materials and Methods

Global meta-analysis of trace metal levels in seagrass

We compiled a benchmark database (Table S5.1) for trace metal levels (Co, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Zn) in seagrass leaves (μg g⁻¹) using Web of Science (ISI; search: seagrass AND metals), data from grey literature, and additional unpublished data of the authors on Zostera noltii (Mauritania and The Netherlands), Amphibolis antarctica (Australia) and Halodule uninervis (Indonesia). Belowground and whole plant data were excluded. Data were derived from either tables or figures in the selected papers, and each unique location as stated in the paper was used. The meta-analysis included data from 47 different studies on seagrass beds all over the world (Figure 5.1, Table S5.1). Replicate data points, including replicates from different seasons, were averaged per location, and data points were divided into polluted and unpolluted sites based on the description of the sites in the studies. Data included in our benchmark database spanned a 40-year period, from the 1970s up to 2011.

Table 5.1. General characteristics of the bays sampled. Bays varied in size and exposure to the open sea (related to the width of the bay mouth). The observed potential stressors are listed for each bay. The total number of sampling points refers to the number of replicates in each bay. Abbreviations of seagrass species: Tt=Thalassia testudinum, Sf=Syringodium filiforme, Hw=Halodule wrightii and Rm=Ruppia maritima. Only tissue of Thalassia testudinum was used for the trace metal analyses.

<table>
<thead>
<tr>
<th>Bay</th>
<th>Island</th>
<th>Surface (km²)</th>
<th>Width bay mouth (m)</th>
<th>Seagrass species</th>
<th>Local disturbance</th>
<th>Total # sampling points</th>
<th># Sampling points seagrass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boka Ascension</td>
<td>Curaçao</td>
<td>0.05</td>
<td>200</td>
<td>Tt, Sf, Hw</td>
<td>Pristine, turtle grazing</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Outer bay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inner bay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lac Bay</td>
<td>Bonaire</td>
<td>7.5</td>
<td>1600</td>
<td>Tt, Sf, Hw, Rm</td>
<td>Pristine, light recreation</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Piscadera Bay</td>
<td>Curaçao</td>
<td>0.75</td>
<td>90</td>
<td>Tt, Sf</td>
<td>Sewage discharge, boating</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Santa Anna Bay</td>
<td>Curaçao</td>
<td>4</td>
<td>230</td>
<td>none</td>
<td>Heavy industry; oil refinery</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Sint Joris Bay</td>
<td>Curaçao</td>
<td>2.5</td>
<td>240</td>
<td>Tt</td>
<td>Pristine, some waste dumping</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Spanish Water Bay</td>
<td>Curaçao</td>
<td>3</td>
<td>90</td>
<td>Tt</td>
<td>Domestic sewage, boating</td>
<td>14</td>
<td>14</td>
</tr>
</tbody>
</table>

Local study

Samples were collected in January 2010 on the islands of Curaçao (12°04’ N, 68°51’ W) and Bonaire (12°15’ N, 68°28’ W) (Figure 5.2). We sampled six different inland bays, which included 50% of all the islands’ bays with seagrass and 90% of the total seagrass area, varying in their levels of anthropogenic disturbance (Table 5.1). The bays are dominated by mangrove (Rhizophora mangle) communities along the shores, and by subtidal seagrass beds of turtle grass (Thalassia testudinum) and manatee grass (Syringodium filiforme). On the island of Curaçao, we sampled in Piscadera Bay, Spanish Water Bay, Boka Ascension, Santa Anna Bay and Sint Joris Bay, and on Bonaire, Lac Bay (Figure 5.2). Bays varied in size and morphology (Table 5.1), but they all experienced minimal wave stress and had a very limited tidal range of 30 cm (De Haan and Zaneveld, 1959). Sediment grain size of the bays ranged from coarse carbonate sediments on exposed sites (mean D50=600 μm) to finer grained, sandy sediments in the more sheltered areas (mean D50=240 μm) (Kuenen and Debrot, 1995). Boka Ascension was divided into inner and outer bay, as a large 10 cm deep shoal strongly limited water exchange between both parts.

Seagrass and porewater sampling

Although we sampled all seagrass species present in the bays, we mainly focused on Thalassia testudinum as this species and the very closely related Thalassia hemprichii are widely distributed among the tropics (Figure 5.1) (Green and Short, 2003; van Tussenbroek et al., 2006). Moreover, species of the genus Thalassia grow in shallow waters directly adjacent to coastal areas, are easy to recognize and are late successional species, which are able to accumulate trace metals (Fourquarean and Zieman, 2002).
Sampling sites were 20 m from the shore at most, and could be reached directly or by boat. Piscadera Bay is the most anthropogenically-impacted bay where seagrass is still present, although it only grows in the shallowest parts of the murky waters. In contrast, Lac Bay represents the most pristine seagrass system, as it is a well-protected nature reserve. For each bay, samples were collected in gradients from the pollution source to the bay inlet (Table 5.1). At sites including seagrass, both porewater and seagrass samples were taken in the seagrass bed; at sites lacking seagrass, porewater samples were taken from the bare sediment.

On each sampling site, two porewater samples were collected within 1 m to include the natural biogeochemical heterogeneity of the sediment, and pooled. Samples were collected anaerobically, using 60 mL vacuumed syringes connected to ceramic soil moisture samplers (pore size 0.15 µm; Eijkelkamp Agrisearch Equipment, Giesbeek, the Netherlands) placed in the top 7 cm of the soil (Govers et al., 2014). Surface water samples were collected similarly in the upper 5 cm of the water column to filter samples prior to laboratory analyses. After measuring their salinity and pH values, samples were frozen on the day of sampling and stored until further analytical analysis. On each sampling site, a pooled sample of at least 10 seagrass shoots with their belowground biomass was manually collected for each species at water depths between 0.5 and 2.5 m, while snorkeling.

Sample analysis
Seagrass samples were split up into roots, rhizomes, sheaths and leaves, and all epiphytes were carefully removed using a scalpel. Subsequently, the samples were dried at 60°C for 48 hours, weighed (g dry wt) and ground. Prior to elemental analyses, samples were digested in 5 mL pressure tubes, using 10 mg sample, 200 µL H_2O_2 and 700 µL HNO_3 in an autoclave for 30 min. at 121°C (ML autoclave, Tuttnauer, the Netherlands) and diluted with 9.1 water to 10 mL. Parallel analyses confirmed that the results of this method did not differ from those of another commonly used digestion method (Smolders et al., 2006) using 50 mg sample, 1 mL H_2O_2 and 2 mL HNO_3 in a digestion microwave (Ethos D, Milestone, Italy) diluted to 25 mL. Total concentrations of trace metals and other elements (Al, As, Ca, Cd, Co, Cr, Fe, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Si, Sr, Zn) in seagrass tissue (leaves and rhizomes) were measured by inductively coupled plasma emission spectrometry (IRIS Intrepid II, Thermo Electron Corporation, Franklin, MA, USA). Standard references (IPE-858, IPE-137; WEPAL, the Netherlands) were included in the analysis, and the average deviation amounted to 3.1%. Trace elements and trace metals in filtered, 3 times diluted porewater and surface water samples were measured using inductively coupled plasma emission spectrometry as described above.
Figure 5.3 An overview of trace metal (Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Zn) concentrations in seagrass leaves from literature. The number of unique sample locations is indicated by n, displayed values are mean values as described in literature. The dotted line indicates the median value of all literature values. Grey triangles show mean trace metal concentrations measured in this study for 5 different bays (for number of replicates, see Table 5.1). Hg was not measured in our samples. Note that the logarithmic scales of the x-axes vary for the different metals.
Statistical analysis
Displayed values are means±standard error (SE). The number of replicates for each bay is displayed in Table 5.1. To compare the conditions among bays, we used a one-way ANOVA, with a homogeneity test prior to the analysis. If equal variances could not be assumed, data were log-transformed. We used a Tukey post-hoc test when the assumptions for the ANOVA were met, and a Games-Howell post-hoc test otherwise. Significant differences (P<0.05) are indicated by different letters. When comparing two different means (distance to residential areas in Spanish Water Bay), we used an independent t-test (see appendix). Correlations were tested using Pearson’s (parametric), or Spearman’s (non-parametric) correlation coefficient. All statistical tests were performed in IBM SPSS Statistics 19.0 and R 2.13.

RESULTS
Global benchmark study
To get insight into the trace metal concentration ranges at a global scale, as well as to put our own results into perspective, we plotted our data together with the median values of leaf metal values found in literature (Figure 5.3) for locations all over the world (Figure 5.1). Globally, trace metal concentrations in seagrass leaves ranged from <0.03 μg g⁻¹ dry wt for Cd to >4000 μg g⁻¹ dry wt for Fe (Figure 5.3). For cadmium (Cd), cobalt (Co), copper (Cu) and lead (Pb), most of the levels measured in seagrass leaves of Curaçao and Bonaire were well below median benchmark values. For iron (Fe), nickel (Ni) and zinc (Zn), in contrast, values were mostly above median values of our global benchmark database, and chromium (Cr) values were well above median values (Figure 5.3).

In our meta-analysis, we found significantly higher mean trace metal concentrations in seagrass leaves of polluted sites than in unpolluted sites for Cu, Hg, Ni, Pb, and Zn (Welch’s t-test, P<0.05 Table S5.2). Leaf trace metal concentrations of polluted sites were on average 2 times higher (81 vs. 45 μg g⁻¹ dry wt for Zn, 15 vs. 8 μg g⁻¹ dry wt for Ni, 15 vs. 9 μg g⁻¹ dry wt for Cu), 3 times higher (15 vs. 5 μg g⁻¹ dry wt for Pb) or 4 times higher (0.13 vs. 0.03 μg g⁻¹ dry wt for Hg) on polluted sites compared to unpolluted sites. This implies that seagrass leaves can indeed be used as first-level bioindicators for trace metal pollution.

Internal distribution of trace metals
To investigate the internal allocation of metals to the different plant parts, we measured trace metal concentrations in both leaves and rhizomes of Thalassia testudinum. All essential metals (Cu, Fe, Ni, Zn, Cr) displayed significant, positive correlations between leaves and rhizomes (linear regression, R²>0.15, P<0.001; Figure 5.4). For the non-essential metals, however, we only found such relationship for Cd (linear regression, R²=0.17, P<0.001; Figure 5.4). We found the strongest relationships (high R² values) for the essential metals that were present in the highest concentrations in the plants: Fe, Zn and Cu. Although data for most metals in the graph were near the 1:1 line (Figure 5.4), some metals (e.g. Fe) were mainly accumulated in belowground parts, while others (e.g. Zn) were more concentrated in leaves.

Local trace metal concentrations in porewaters and leaves
In general, the polluted Piscadera Bay showed the highest leaf metal concentrations, while the protected Lac had the lowest levels for almost all measured metals (Table 5.2). In strong contrast, concentrations of trace metals in porewater and surface water samples (Table S5.3) did not show significant differences among the separate bays. In addition, we did not find any significant correlations between porewater and leaf concentrations of trace metals.

DISCUSSION
There is a strong need for bioindicators that can be used to assess the actual status and health of coastal ecosystems globally in relation to anthropogenic pressure, and to metal pollution in particular (Linton and Warner, 2003; van Katwijk et al., 2011). We therefore compiled the first global literature overview for seagrass metal levels, which is important as a benchmark for seagrass research related to metal pollution. In addition, we studied the use of seagrasses as bioindicators for trace metal pollution in the poorly studied Caribbean seagrass beds of two islands where various levels and sources of anthropogenic stressors were present. Based on our benchmark results, we could show that leaf concentrations of especially Cr, Fe, Ni and Zn were high at our case study sites.
Table 5.2 Trace metal concentrations in seagrass leaves (in mg g⁻¹, mean values per bay, for number of replicates see Table 5.1) of Thalassia testudinum in all studied bays. Significant differences (P<0.05) are indicated by different letters. Ranking is the mean pollution ranking of all 8 metals.

<table>
<thead>
<tr>
<th>Bay</th>
<th>Cd</th>
<th>Co</th>
<th>Cr</th>
<th>Cu</th>
<th>Fe</th>
<th>Hg</th>
<th>Ni</th>
<th>Pb</th>
<th>Zn</th>
<th>Ranking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boka Ascension Bay</td>
<td>0.93a</td>
<td>3.10ab</td>
<td>32.01ab</td>
<td>10.27b</td>
<td>3725.63c</td>
<td>NA</td>
<td>9.49b</td>
<td>5.93b</td>
<td>74.97b</td>
<td>2</td>
</tr>
<tr>
<td>Lac Bay</td>
<td>0.44a</td>
<td>0.08a</td>
<td>29.50a</td>
<td>1.36a</td>
<td>115.48b</td>
<td>NA</td>
<td>3.41a</td>
<td>2.37b</td>
<td>184.67a</td>
<td>5</td>
</tr>
<tr>
<td>Piscadera Bay</td>
<td>0.79a</td>
<td>3.57ab</td>
<td>30.34a</td>
<td>13.83ab</td>
<td>1044.48a</td>
<td>NA</td>
<td>18.13c</td>
<td>2.83ab</td>
<td>295.52a</td>
<td>1</td>
</tr>
<tr>
<td>Sint Joris Bay</td>
<td>0.61a</td>
<td>3.02b</td>
<td>23.98ab</td>
<td>17.21ab</td>
<td>1003.03a</td>
<td>NA</td>
<td>9.89b</td>
<td>0.97b</td>
<td>248.58a</td>
<td>3</td>
</tr>
<tr>
<td>Spanish Water Bay</td>
<td>0.69a</td>
<td>0.85ab</td>
<td>15.57a</td>
<td>31.02b</td>
<td>523.94a</td>
<td>NA</td>
<td>8.81b</td>
<td>2.71b</td>
<td>168.53a</td>
<td>4</td>
</tr>
</tbody>
</table>

**Global-scale benchmark**

We have here compiled a review of, to our best knowledge, all available literature on trace metal concentrations in seagrass leaves. Seagrasses are known metal accumulators, and in our literature study, we found a wide, 100 – 1000-fold, range in concentrations for all individual metals (Figure 5.3), which underscores the suitability of seagrasses as sensitive bioindicators for the detection of trace metal pollution.

Literature data were, however, not equally divided among regions, and especially seagrass species occurring in the Mediterranean (e.g. Posidonia oceanica) have been very well studied. Some studies may show a bias for metal-polluted areas (Table S5.1, Table S5.2). Furthermore, for some metals such as Co and Hg, few data were available in the literature (Figure 5.2). Data on trace metal pollution in tropical areas, where pollution and degradation of coastal ecosystems is increasing at an alarming rate, is mostly lacking. This data gap could rapidly be filled by using ‘easy to pick’ seagrasses in the areas that have not been covered yet, for example plants of the widespread Thalassia genus (Figure 5.1).

Our global analysis of trace metal concentrations of polluted vs. unpolluted sites (Table S5.2) clearly shows that leaf trace metal values are significantly elevated (2-4 x higher) on polluted sites compared to unpolluted sites. This analysis proves that seagrass leaves can be used as first-level indicators for trace metal pollution of coastal areas.

Seagrass trace metal values are also known to vary seasonally, with lower trace metal values in the growing season than in the dormant season (Li and Huang, 2012; Schlacher-Hoenlinger and Schlacher, 1998). As most studies are conducted during the growing season (Table S5.1), our benchmark study may underestimate rather than overrate seagrass trace metal levels. Furthermore, seasonal differences were moderated in our data- set by averaging sample points from different seasons. It would however be very interesting to further investigate the importance of seasonality for the use of seagrasses as bioindicators for trace metal pollution.

**Local study**

We assessed trace metal concentrations in seagrasses as an indicator for their level of pollution in coastal areas and, more importantly, their biological availability. The bioavailability of metals and their mobility in the sediment is determined by their chemical speciation (Morillo et al., 2004), but seagrasses are also able to take up trace metals from the water column (Batley, 1987; Bond et al., 1985). Similar to nutrient measurements in abiotic compartments, measurements of trace metals in the water only provide a snapshot of trace metal loads as they are subjected to large variations in concentrations and fluxes (Ralph et al., 2006), while seagrass trace metal concentrations display a longer-term trace metal accumulation and related stress (Lafabrie et al., 2007; Lafabrie et al., 2008; Sundelin and Eriksson, 2001).

Seagrass trace metal concentrations from the sampled bays indicated that Piscadera Bay, with a sewage outlet, was the most heavily polluted, whereas the conservation area Lac showed the lowest trace metal concentrations of all sampled bays, which is in accordance with our expectations. In contrast, porewater and surface water data did not provide this information, and were not correlated to plant concentrations, as expected.

The comparison of our own data to our worldwide benchmark list shows that levels of Cr appeared to be exceptionally high in all our study sites. Surprisingly, Cr levels appeared to be also high in the relatively undisturbed Lac, which suggests an external source of this metal. This is even more striking, because it is considered one of the least bioavailable metals in marine sediments (Morillo et al., 2004), which may imply that the Cr loads in our sampled bays are even higher than we might expect based on our results.

Although the concentrations of trace metals varied among sample points and between bays, it appeared to be very difficult to locate a point source for trace metals (Table S5.3 and Table S5.4) (Pergent-Martini and Pergent, 2000). Literature (Guzman and Garcia, 2002; Irvine and Birch, 1998) suggests that trace metal pollution in marine environments may originate from several processes and non-point-sources: runoff, flooding, mining, sewage, erosion, overuse of agrichemicals, industrial waste, atmospheric deposition, ports and refineries. We speculate that trace metals in the seagrass beds of Curacao originate from sewage (Piscadera Bay, Spanish Water Bay), ports and boating, but may also be of terrestrial origin, as high loads of terrigenous sediments are expected to have invaded the water due to deforestation and the construction of terrestrial drainage areas (Kuenen and Debrot, 1995). Furthermore, Curacao is home to a large oil refinery and two of its bays (Caracas Bay and Bullen Bay, not sampled) have been subjected to frequent oil spills and accompanying pollution in the past (Nagelkerken and Debrot, 1995). This oil pollution may also have reached other, nearby bays.
Internal distribution

We compared metal concentrations in the different plant parts and found significant correlations between leaf and rhizome metal concentrations of the essential elements (Figure 5.3). As plants need these elements either directly as micronutrients (Cu, Fe, Ni, Zn) or as an essential element (Co) for the nitrogenase enzymes fueling $N_2$-fixation by associated microorganisms (Welsh, 2000), they possess mechanisms for active allocation for most of these metals (Adriano, 2001; Marschner, 1995). As the concentrations represent redistribution in addition to uptake, we cannot draw conclusions about specific uptake ratios for leaves and rhizomes. In addition, all metals, including the non-essential elements showed fairly similar metal concentrations for both the leaves and rhizomes (close to the 1:1 line), which underlines the use of seagrass leaf material as indicator for trace metals.

Seagrasses as indicators for bioaccumulation

Although we mainly focused on the use of trace metal concentrations in seagrasses as indicator for pollution, the accumulation of metals may also affect seagrass health. Many trace metals are naturally abundant in seagrass beds (Batley, 1987; Prange and Dennison, 2000), but high concentrations can become toxic to seagrass and also be indicative of toxicity to other coastal organisms (Macinnis-Ng and Ralph, 2002; Prange and Dennison, 2000; Ralph and Burchett, 1998). Even sublethal levels of trace metals may have large effects on seagrass dominated ecosystems, as they are persistent and may accumulate in the food web, with toxic effects at higher trophic levels (Ikem and Egiebor, 2005; Schüürmann and Markert, 1998). In addition, trace metal accumulation in plants is often associated with changes in photosynthetic rates (Conroy et al., 1991; Macfarlane and Burchett, 2001; Prange and Dennison, 2000) and inhibited metabolic activity (Ralph and Burchett, 1998). This may lead to decreased growth rates or even result in plant die-off (Clijsters and Van Assche, 1985).

Trace metal accumulation in seagrasses can be used as a first level measurement to assess the contamination of the specific marine environment (Prange and Dennison, 2000). Moreover, as accumulation magnifies in the food web for some trace elements, high trace metal levels in primary producers such as seagrasses may indicate serious trace metal pollution in the whole food web.

Conclusions and recommendations

Summarizing, we here present a global list for trace metal concentrations in seagrass leaves, which can be used in further studies to relate local trace metal pollution to a benchmark list. Moreover, we showed that seagrasses in general, and Thalassia spp. in particular, can be used as an easy to sample and widespread bioindicator species for trace metal pollution of especially Cu, Hg, Ni, Pb, and Zn. As little information is available on the effects of trace metal pollution on seagrass physiology and on the bioaccumulative effects of trace metals in seagrass-based foodwebs, we strongly recommend further research on these topics.

Acknowledgements

We would like to thank M. Grol, for her logistic support. We would also like to acknowledge the employees of Carmabi, Curacao. STINAPA Bonaire extended the permit for the samples for Lac, and we would like to thank E. Beukenboom, R. de Leon and F. van Slobbe for making this arrangement. We would also like to thank R. Peters for sample processing and R. van Hintum for his help with some of the spatial analyses. We finally thank two anonymous reviewers for their valuable comments on an earlier version of the manuscript.
Table S5.1 Studies used for the global meta-analysis of trace metal concentrations in seagrasses. Data were derived from 47 different studies. NA means not available.

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### Chapter 5: Pollution

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Table S5.2 Leaf trace metal concentrations (μg g⁻¹, mean values) of unpolluted and polluted (as defined in papers) sites in the global meta-analysis. Statistical differences between mean leaf trace metal concentrations of unpolluted and polluted sites are indicated by: ns=not significant, *=P<0.05, **=P<0.01 and ***=P<0.001. NA: not available due to very limited data for Co on polluted sites.

Table S5.3 Trace metal concentrations (μg L⁻¹, mean values per bay) of porewater and surface water. Statistical differences are indicated by different letters. nd: not detectable; concentrations below detection limit (<0.1 μg L⁻¹).

Table S5.4 P-values for distance analyses of trace metal contents of Thalassia testudinum leaves and distances to the bay mouth. *=P<0.05; NA: not available due to the low number of data.

Table S5.5 P-values of point source independent t-tests in Spanish Water Bay. We tested for significant differences in seagrass leaf trace metal content of Thalassia testudinum near coastal residences (<200 m) and further away from coastal residences (>350 m). *=P<0.05.

Table S5.6 P-values of Pearson’s correlation tests. We tested the correlations of distance to the sewage pipe in Piscadera bay and trace metal contents of seagrass leaves. We found a significant positive correlation between distance from the sewage pipe and leaf Ni content (R²=0.54, P=0.048), which indicated that the source of Ni is probably located outside of the bay, or Ni availability was lower near the sewage pipe. *=P<0.05.
Marine beds are globally declining due to human activities in coastal areas. We here aimed to identify threats from eutrophication to the valuable seagrass beds of Curaçao and Bonaire in the Caribbean, which function as nursery habitats for commercial fish species. We documented surface- and porewater nutrient concentrations, and seagrass nutrient concentrations in 6 bays varying in nutrient loads. Water measurements only provided a momentary snapshot, due to timing, tidal stage, etc., but Thalassia testudinum nutrient concentrations indicated long-term nutrient loads. Nutrient levels in most bays did not raise any concern, but high leaf %P values of Thalassia in Piscadera Bay (~0.31%) and Spanish Water Bay (~0.21%) showed that seagrasses may be threatened by eutrophication, due to emergency overflow of waste water and coastal housing. We thus showed that seagrasses may be threatened and measures should be taken to prevent loss of these important nursery areas due to eutrophication.
INTRODUCTION

Seagrass beds are key coastal ecosystems, which support high biodiversity and provide important ecosystem services such as carbon sequestration, fisheries, and coastal protection (Christianen et al., 2013; Fourqueuran et al., 2012; Heck et al., 2003). However, seagrass meadows are rapidly declining all over the world, due to increasing anthropogenic activities in coastal areas (Waycott et al., 2009). Human pressures on coastal areas in the Caribbean are also increasing, and factors like booming tourism, growing industries, oil drillings and spills, trace metal pollution and eutrophication threaten coastal ecosystems (Phillips, 1992; Short and Wyllie-Echeverria, 1996; Thorhaug et al., 1985).

An important stressor that has not been well studied in the Caribbean is eutrophication (but see Gast et al. (1999)). Eutrophication can potentially lead to degradation or complete disappearance of seagrass beds due to epiphyte overgrowth and/ or light limitation (Burkholder et al., 2007; Kuenen and Debrot, 1995). We therefore aimed to identify and to quantify the effects of eutrophication on the seagrass beds of the Caribbean islands of Curacao and Bonaire. On these islands, the seagrass beds form essential nursery habitats for many commercially important fish species (Huijbers et al., 2013). However, seagrasses are present in bays with varying degrees of anthropogenic impacts (Debrot and Sybesma, 2000). Hence, we studied 1) the nutrient status of seagrasses in 6 bays on Curacao and Bonaire, 2) the indicator value of seagrass leaf nutrient concentrations, and 3) possible threats of eutrophication in Curacao and Bonaire bays.

MATERIALS AND METHODS

Samples were collected in January 2010 on Curacao (12°04’ N, 68°51’ W) and Bonaire (12°15’ N, 68°28’ W), Netherlands Antilles. We sampled six different non-estuarine inland bays (50% of all bays with seagrass, which includes >90% of the total seagrass area) varying in anthropogenic disturbance levels (Table 6.1). The estuarine inland bays (50% of all bays with seagrass, which includes >90% of the total seagrass area) varying in anthropogenic disturbance levels (Table 6.1). The distances between sampling points and key-point sources (residential areas, sewage pipes) were measured using the ruler tool in Google Earth™.

At each sampling site, a minimum of 10 shoots with belowground biomass was manually sampled while snorkeling and pooled at depths between 0.5 and 2.5 m. All seagrass species present at a sampling site were collected. At each sampling site, two porewater samples were collected anaerobically, using 60 ml vacuum syringes connected to ceramic soil moisture samplers (Eijkelkamp Agrisearch Equipment, Giesbeek, the Netherlands) placed in the top 7 cm of the soil. These duplicate porewater samples were subsequently pooled per sampling point. Surface water samples were collected similarly in the upper 5 cm of the water column. Samples were frozen on the day of sampling, for further analysis.

Seagrass samples were split up into roots, rhizomes, sheaths, and leaves, and all epiphytes were carefully removed using a scalpel. Subsequently, the samples were dried at 60°C for 48 hours, weighed (g DW) and ground. %C and %N of both leaves and rhizomes were determined with an elemental analyzer (Type NA 1500 Carlo Erba, Thermo Fisher Scientific Inc., USA), coupled online via an interface (Finnigan Conflo III) to a mass-spectrometer (Thermo Finnigan DeltaPlus, USA). Total concentration of phosphorous in seagrass tissue was measured with inductively-coupled-plasma emission spectrometry (IRIS Intrepid II, Thermo Electron Corporation, Franklin, MA, USA), after digestion with nitric acid, following Smolders et al. (2006) and as previously used for seagrass habitats by Govers et al. (2014a).

Porewater and surface water ammonium and ortho-phosphate concentrations were measured colorimetrically (Bran & Luebbe Autoanalyzer III, Seal Analytical, UK), using ammonium-molybdate and salicylate (Lamers et al., 1998). Nitrate was determined by sulphanilamide, after reduction of nitrate to nitrite in a Cadmium column and as previously used for tropical seagrass habitats by Christianen et al. (2012).

We additionally conducted a literature study to compare our Thalassia leaf nutrient concentrations to literature values for eutrophic and pristine Thalassia meadows (Table 6.4). References included in this table were found by using ISI Web of Science with the key words Thalassia AND nutrient*, Thalassia testudinum AND nutrient*, Thalassia hemprichii AND nutrient* or the same key words with either nitrogen or phosphorus instead of nutrient*.

Displayed values are means±standard error (SE), the number of replicates for each bay is indicated in Table 6.1. To compare conditions between bays, we used a one-way ANOVA with a Tukey HSD post-hoc test. Normality was tested prior
to analysis with a Shapiro Wilk test and non-normal data were log-transformed prior to testing. To compare two different means (residential areas, literature values), we used an independent T-test. Correlations were tested with Pearson’s correlation coefficient. Statistical tests were performed in IBM SPSS Statistics 19.0 and R 2.15.

**Table 6.1** Characteristics of the sampled bays on the islands of Curaçao and Bonaire with the number of sampling points per bay. Seagrass species abbreviations: Tt=Thalassia testudinum, Sf=Syringodium filiforme, Hw=Haloëdile wrightii, and Rm=Ruppia maritima.

<table>
<thead>
<tr>
<th>Bay</th>
<th>Island</th>
<th>Surface (km²)</th>
<th>Width bay mouth (m)</th>
<th>Seagrass species</th>
<th>Local disturbance</th>
<th>Total # sampling points</th>
<th># Sampling points seagrass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boka Ascension Bay</td>
<td>Curaçao</td>
<td>0.05</td>
<td>200</td>
<td>Tt, Sf, Hw</td>
<td>Plastic pollution, turtle grazing</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Lac Bay</td>
<td>Bonaire</td>
<td>7.5</td>
<td>1600</td>
<td>Tt, Sf, Hw, Rm</td>
<td>Protected, light recreation</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Piscadera Bay</td>
<td>Curaçao</td>
<td>0.75</td>
<td>90</td>
<td>Tt, Sf</td>
<td>Sewage discharge, boating</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Santa Anna Bay</td>
<td>Curaçao</td>
<td>4</td>
<td>230</td>
<td>none</td>
<td>Heavy industry; oil refinery</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Sint Joris Bay</td>
<td>Curaçao</td>
<td>2.5</td>
<td>240</td>
<td>Tt</td>
<td>Some waste dumping</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Spanish Water Bay</td>
<td>Curaçao</td>
<td>3</td>
<td>90</td>
<td>Tt, Sf, Hw, Rm</td>
<td>Domestic sewage, boating</td>
<td>14</td>
<td>14</td>
</tr>
</tbody>
</table>

**RESULTS**

Abiotic measurements such as surface water and porewater nutrient concentrations appeared to give a very limited indication of prevailing nutrient loads as we found no significant differences among bays (Table 6.2). However, surface water measurements indicated high nitrogen and phosphorus loads in St Anna Bay (13.64 and 1.83 μmol L⁻¹ respectively) and Boka Ascension Bay (9.88 and 2.07 μmol L⁻¹ respectively).

In contrast, tissue nutrient concentrations in *Thalassia testudinum* tissue reflected nutrient loads accumulated over a longer period (Table 6.3). Leaf %N concentrations were highest in Piscadera Bay (2.16±0.22 %N) and lowest in Sint-Joris Bay and Lac Bay (1.66±0.09 %N and 1.78±0.09 %N respectively). Leaf %P was also highest in Piscadera Bay, (0.25±0.03 %P), and lowest in Lac Bay (0.17±0.00 %P). We therefore identified Piscadera Bay as the bay with the highest nutrient loads, whereas Lac Bay and Sint Joris Bay had generally lower nutrient loads.

Mean leaf nutrient concentrations in Spanish Water Bay were not high compared to the other bays, possibly because of high mixing rates. However, when we looked at the effects of local eutrophication by coastal residential areas, we found a significance increase of >10% in *Thalassia* leaf %P in the vicinity (0-200 m) of residential areas.

**Table 6.2** Porewater and surface water total nitrogen (NH₄⁺ NO₃) and PO₄ concentrations in μmol L⁻¹. Displayed data are means (# replicates in Table 6.1), and significant differences (ANOVA) are indicated by letters (a, b, etc.). However, as all results did not differ significantly, everything is indicated by the letter a.

<table>
<thead>
<tr>
<th>Bay</th>
<th>Total N</th>
<th>PO₄</th>
<th>Total N</th>
<th>PO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boka Ascension Bay</td>
<td>18.30a</td>
<td>4.72a</td>
<td>9.88</td>
<td>2.07a</td>
</tr>
<tr>
<td>Lac Bay</td>
<td>25.11a</td>
<td>2.16a</td>
<td>4.35</td>
<td>0.62a</td>
</tr>
<tr>
<td>Piscadera Bay</td>
<td>8.05a</td>
<td>1.49a</td>
<td>6.95</td>
<td>0.83a</td>
</tr>
<tr>
<td>Santa Anna Bay</td>
<td>26.40a</td>
<td>2.54a</td>
<td>13.64</td>
<td>1.83a</td>
</tr>
<tr>
<td>Sint-Joris Bay</td>
<td>6.69a</td>
<td>2.80a</td>
<td>2.30</td>
<td>0.83a</td>
</tr>
<tr>
<td>Spanish Water Bay</td>
<td>6.73a</td>
<td>2.07a</td>
<td>5.63a</td>
<td>0.91a</td>
</tr>
</tbody>
</table>

**Table 6.3** Mean leaf nutrient concentrations of *Thalassia testudinum* in all sampled bays. Ratios are mol ratios. Significant differences between bays (ANOVA) are indicated by letters (a, b, etc.), as found by post-hoc comparisons. The same letters indicate non-significant differences between groups; different letters indicate significant differences between groups.

<table>
<thead>
<tr>
<th>Bay</th>
<th>%C</th>
<th>%N</th>
<th>%P</th>
<th>C:N</th>
<th>N:P</th>
<th>C:P</th>
<th>C:N:P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boka Ascension Bay</td>
<td>30.84a</td>
<td>2.02a</td>
<td>0.18a</td>
<td>18a</td>
<td>25a</td>
<td>451ab</td>
<td>451:18a</td>
</tr>
<tr>
<td>Lac Bay</td>
<td>34.05a</td>
<td>1.78a</td>
<td>0.17a</td>
<td>21a</td>
<td>24a</td>
<td>524bc</td>
<td>524:21a</td>
</tr>
<tr>
<td>Piscadera Bay</td>
<td>34.88a</td>
<td>2.16a</td>
<td>0.25a</td>
<td>19a</td>
<td>19a</td>
<td>366bc</td>
<td>366:19a</td>
</tr>
<tr>
<td>Sint-Joris Bay</td>
<td>33.27a</td>
<td>1.66a</td>
<td>0.18a</td>
<td>24a</td>
<td>20a</td>
<td>476bc</td>
<td>476:24a</td>
</tr>
<tr>
<td>Spanish Water Bay</td>
<td>33.93a</td>
<td>1.96a</td>
<td>0.19a</td>
<td>20a</td>
<td>23a</td>
<td>461b</td>
<td>461:20a</td>
</tr>
</tbody>
</table>
m) of residential areas (T-test, P=0.009), compared to seagrass stands >350 m of residential areas (Figure 6.1b). In addition, leaf %N also appeared to increase slightly (~10%) near residential areas, but in contrast to %P; this increase was not significant (P=0.166) (Figure 6.1a). Increased nutrient availability near residential areas was also observed in the high macroalgal densities (Halimeda sp., see also (Kuenen and Debrot, 1995; Slijkerman et al., 2011)) in between Thalassia testudinum stands. In addition to the results of Spanish Water Bay, Thalassia testudinum tissue nutrient concentrations seemed to indicate increased nutrient loads near the emergency overflow pipe (<500 m) of Piscadera Bay, as Thalassia leaf %N (2.6%) and %P (0.31%) were strongly elevated (indicated by grey triangles) compared to leaf nutrient concentrations near the bay mouth (1.9 %N and 0.21 %P) (Figure 6.2) and compared to literature data for pristine Thalassia beds (Table 6.4). In contrast, Syringodium filiforme did not display such an increase in leaf nutrient concentrations (i.e., 1.2 %N and 0.18 %P nearest to the emergency overflow pipe and 1.4 %N and 0.19 %P nearest to the bay mouth). No seagrass was sampled within a distance of 460 m from the emergency overflow pipe; as no seagrass was present this close to the outlet. In Santa Anna Bay, no seagrass was found, so this bay could not be included in the seagrass nutrient analysis.

Based on our literature study (Table 6.4), we found that Thalassia leaf nutrient concentrations are generally between 1.68-3.02 %N and 0.14-0.7 %P for systems that were marked as eutrophic (or polluted), and between 0.91-2.4 %N and 0.073-0.18 %P for pristine Thalassia beds. Although overlapping, both Thalassia leaf %N and leaf %P values differed significantly between eutrophic and pristine sites (T-tests, P<0.001, P<0.007 for %N and %P respectively).

**DISCUSSION**

Seagrass nutrient concentrations reflected nutrient loads over a longer period, which confirms previous work (Ferdie and Fourqurean, 2004; Udy and Dennison, 1997; van Katwijk et al., 2011). In contrast, abiotic parameters (surface and porewater nutrient concentrations) gave a very limited indication of prevailing nutrients loads, which agrees with earlier observations that such measurements only provide a momentary snapshot (Short and McRoy, 1984), affected by timing, tidal stage, terrestrial runoff, freshwater input, and rapid uptake of nutrients by plankton, seagrasses and macroalgae (van Katwijk et al., 2011). However, mean surface water total nitrogen (>4 μmol L⁻¹) and phosphate (>0.6 μmol L⁻¹) levels from most bays were well above the values indicated as eutrophic reef waters by Gast et al. (1999) and other coastal Caribbean lagoons (Carruthers et al., 2005; Olsen and Valiela, 2010; Peterson et al., 2012). Our values thus seem to indicate that nutrient levels can be above the threshold values for eutrophication for corals (Gast et al., 1999), and also hint at possible negative effects of high nutrient levels for seagrasses. Especially St Anna Bay (or Harbor Bay), where no seagrass can be found, displayed high surface water nutrient levels, possibly, seagrasses have disappeared due to eutrophication in this heavily polluted bay (Gast et al., 1999). Yet, to gain a more complete picture of long-term nutrient loads in these bays, we mainly focused on the indicator value of seagrass tissue nutrient concentrations in this study.

We found that Thalassia leaf nutrient concentrations for pristine seagrass beds are generally between 0.91-2.4 %N and 0.05-0.18 %P, whereas leaf nutrient concentrations found in nutrient enriched systems are between 1.87-3.02 %N and 0.14-0.70 %P (Table 6.4). When comparing these literature values with our data, Lac Bay, St Joris Bay, and Boka Ascension Bay fall within the limits of oligotrophic/pristine systems for both nitrogen and phosphorus. In addition, Thalassia testudinum leaf nitrogen concentrations also indicate limited nitrogen availability (<2.4 %N) in the other bays. However, relatively low nitrogen concentrations may also be the result of the terrigenous sediments in these bays, which may promote nitrogen limitation in seagrasses, due to higher phosphorus availability in terrigenous than in carbonate sediments (Erftemeijer and Middelburg, 1993; Touchette and Burkholder, 2000). Or possibly, sea turtle grazing, such as in Boka Ascension Bay, may promote nutrient export, thereby lowering bay nutrient loads and protecting seagrasses from eutrophication effects (Christianen et al., 2012). Total Phosphorus concentrations of both Piscadera Bay and Lac Bay may indicate excess phosphorus availability, based on comparison with literature data, which confirms the presence of point sources (coastal residentialities and emergency overflow pipe) of eutrophication. Thus, our data, supported by our literature overview, show that Thalassia leaf nutrient concentrations (especially P) are commonly elevated in the vicinity of a nutrient source and can thus be used as bioindicator for nutrient pollution.
## Table 6.4

<table>
<thead>
<tr>
<th>Study</th>
<th>Species</th>
<th>Location</th>
<th>Classification</th>
<th>Specifics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thalassia testudinum</td>
<td>Florida, USA</td>
<td>Eutrophic</td>
<td>Near bird colony island</td>
<td>Fourqurean et al. (1992)</td>
</tr>
<tr>
<td>2</td>
<td>Thalassia testudinum</td>
<td>Bahamas</td>
<td>Eutrophic</td>
<td>Offshore 12 n</td>
<td>Fourqurean et al. (1992)</td>
</tr>
<tr>
<td>3</td>
<td>Thalassia testudinum</td>
<td>Florida, USA</td>
<td>Eutrophic</td>
<td>Near bird colony island</td>
<td>Fourqurean et al. (1992)</td>
</tr>
<tr>
<td>4</td>
<td>Thalassia hemprichii</td>
<td>China</td>
<td>Eutrophic</td>
<td>Low intertidal</td>
<td>Zhang et al. (2014)</td>
</tr>
<tr>
<td>5</td>
<td>Thalassia testudinum</td>
<td>Barbados</td>
<td>Pristine</td>
<td>NA</td>
<td>Patriquin (1972)</td>
</tr>
<tr>
<td>6</td>
<td>Thalassia testudinum</td>
<td>Mexico</td>
<td>Pristine</td>
<td>Reef, oligotrophic</td>
<td>Gallegos et al. (1993)</td>
</tr>
<tr>
<td>7</td>
<td>Thalassia testudinum</td>
<td>Puerto Rico</td>
<td>Pristine</td>
<td>Surrounded by agricultural/urban land, but decoupled from watershed</td>
<td>Olsen and Valiela (2010)</td>
</tr>
<tr>
<td>8</td>
<td>Thalassia testudinum</td>
<td>Bahamas</td>
<td>Pristine</td>
<td>Tidal channel, mature leaves with epiphytes</td>
<td>Capone et al. (1979)</td>
</tr>
<tr>
<td>9</td>
<td>Thalassia testudinum</td>
<td>Jamaica</td>
<td>Pristine</td>
<td>No nitrogen rich upwelling</td>
<td>Peterson et al. (2012)</td>
</tr>
<tr>
<td>10</td>
<td>Thalassia testudinum</td>
<td>Florida, USA</td>
<td>Pristine</td>
<td>Unpolluted bay</td>
<td>Campbell et al. (2012)</td>
</tr>
<tr>
<td>11</td>
<td>Thalassia testudinum</td>
<td>Bahamas</td>
<td>Pristine</td>
<td>Near a city</td>
<td>Gallegos et al. (1993)</td>
</tr>
<tr>
<td>12</td>
<td>Thalassia testudinum</td>
<td>Mexico</td>
<td>Pristine</td>
<td>&lt;120 m from bird colony island</td>
<td>Fourqurean et al. (2001)</td>
</tr>
<tr>
<td>13</td>
<td>Thalassia testudinum</td>
<td>Mexico</td>
<td>Pristine</td>
<td>Reef, oligotrophic</td>
<td>Fourqurean et al. (1993)</td>
</tr>
<tr>
<td>14</td>
<td>Thalassia hemprichii</td>
<td>Indonesia</td>
<td>Pristine</td>
<td>Close to river input</td>
<td>van Katwijk et al. (2011)</td>
</tr>
<tr>
<td>15</td>
<td>Thalassia hemprichii</td>
<td>Indonesia</td>
<td>Pristine</td>
<td>Far away from river input</td>
<td>van Katwijk et al. (2011)</td>
</tr>
<tr>
<td>16</td>
<td>Thalassia hemprichii</td>
<td>Indonesia</td>
<td>Pristine</td>
<td>No nitrogen rich upwelling</td>
<td>Stapel and Hemminga (1997)</td>
</tr>
<tr>
<td>17</td>
<td>Thalassia hemprichii</td>
<td>Indonesia</td>
<td>Pristine</td>
<td>Barang Lompo, 3rd leaf</td>
<td>Stapel and Hemminga (1997)</td>
</tr>
<tr>
<td>18</td>
<td>Thalassia hemprichii</td>
<td>Indonesia</td>
<td>Pristine</td>
<td>Gusung Tallang, 3rd leaf</td>
<td>Stapel and Hemminga (1997)</td>
</tr>
</tbody>
</table>

We found a difference in nutrient accumulation in the leaves between a late and early successional seagrass species in the vicinity of a source of eutrophication (emergency overflow pipe, Piscadera Bay). In contrast to Thalassia testudinum, the fast growing Syringodium filiforme did not accumulate nutrients in the eutrophic bay, but seem to have used the extra nutrients for growth (pers. observations, Figure S6.1). Leaf nutrient concentrations of the late successional Thalassia testudinum were however >25% higher (0.31 %P, 2.6 %N) in vicinity of the overflow pipe than in plants near the bay mouth (0.21 %P, 1.9 %N). Christianen et al. (2011) found similar differences in leaf nutrient levels between an early successional and a late successional species, which might be explained by differences in growth strategy. An early successional species, such as Syringodium filiforme generally shows higher production rates (Barber and Behrens, 1985) and shoot turn-over rates (2.0 yr⁻¹; Gallegos et al. (1994)), and is able take up nutrients faster than late successional species (Duarte, 1991; Rollon et al., 1998). In contrast, the late successional species Thalassia testudinum is a slow growing species with low shoot turn-over rates (0.6 yr⁻¹; Gallegos et al. (1993)), which accumulates nutrients in the leaves (Carruthers et al., 2005; McGlathery et al., 1994). Thalassia testudinum proved also to be a good indicator of point sources of eutrophication in Spanish Water Bay, as leaf %P was elevated in the vicinity of residential areas up to concentrations >0.18% which may be
indicative of eutrophication according to literature values (Table 6.4). However, additional to leaf nutrient concentrations (%N, %P), nitrogen isotopic ratios would have provided even more detailed information on anthropogenic nutrient input in the studied bays (Mutchler et al., 2007; Schubert et al., 2013).

CONCLUSIONS

We conclude that that leaf nutrient values of *Thalassia testudinum* may be used as bioindicator values for point sources of eutrophication. Additionally, we identified potential threats of eutrophication to seagrasses on Curaçao in Spanish water Bay (residential areas) and Piscadera Bay (emergency overflow discharge); nutrient levels in the other sampled bays did however not raise any concern yet. The seagrasses of Piscadera Bay have already retreated to the shallowest areas (<1m, pers. observations) of the murky waters and are under threat of complete disappearance with a further increase of nutrient loads. Moreover, *Thalassia testudinum* in Spanish Water Bay has been declining for some time (Kuenen and Debrot, 1995), and we are the first to suggest (leaf %P values) that this may be due to excess anthropogenic nutrient input by coastal residencies. This bay, with the largest seagrass area of Curaçao (Debrot et al., 1998; Kuenen and Debrot, 1995), highly contributes to coral reef fish populations by functioning as a nursery habitat (Huijbers et al., 2013). Possible disappearance of seagrasses due to eutrophication may therefore have serious consequences for the ecological and economical values of the coastal ecosystems of Curaçao. We have shown that some bays at Curaçao are subjected to excess nutrient inputs, which may already have contributed to seagrass loss and linked ecosystem services. We therefore hope that measures will be taken to prevent further loss of valuable seagrass beds due to eutrophication at Curaçao.

ACKNOWLEDGEMENTS

We would like to acknowledge Monique Grol, Chantal Huijbers for logistic support, Elsmarie Beukenboom, Ramon de Leon and Frank van Slobbe from STINAPA Bonaire for the sampling permit for Bonaire, and we would also like to thank Jelle Eygensteyn and Paul van de Ven for assistance with the lab analyses.
Seagrasses evolved from terrestrial plants into marine foundation species around 100 million years ago. Their ecological success, however, remains a mystery as natural organic matter accumulation within the beds should result in toxic sediment sulfide levels. Using a meta-analysis, a field study and a laboratory experiment, we reveal how an ancient three-stage symbiosis between seagrass, lucinid bivalves and their sulfide-oxidizing gill-bacteria reduces sulfide stress for seagrasses. We found that the bivalve-sulfide-oxidizer symbiosis reduced sulfide levels and enhanced seagrass production as measured by biomass. In turn, the bivalves and their endosymbionts profit from organic matter accumulation and radial oxygen release from the seagrass roots. These findings elucidate the long-term success of seagrasses in warm waters, and offer new prospects for seagrass ecosystem conservation.
Seagrass meadows are important ecological and thus economic components of coastal zones worldwide (Larkum et al. 2006, Waycott et al. 2009). In many areas, coral reefs and seagrass meadows are tightly linked habitats that form the basis for marine biodiversity (Nagelkerken 2009). Seagrasses serve as keystone habitat for migrating coral reef species, thousands of other animals including waterbirds, fish, dugongs, manatees and turtles, are important carbon and nutrient sinks, and are important to fisheries and coastline protection (Larkum et al. 2006, Nagelkerken 2009, Waycott et al. 2009). Dense seagrass meadows attenuate currents and waves and trap pelagic and benthic organic matter in the sediment (Larkum et al. 2006, van der Heide et al. 2007, van der Heide et al. 2011). Owing to a lack of oxygen in many coastal marine sediments, an important fraction of organic matter is decomposed by bacteria that use the abundant sulfate in seawater as an electron acceptor instead of oxygen, and produce toxic sulfide as a metabolic end product (Jorgensen 1982). Although seagrasses transport oxygen into their roots and the surrounding rhizosphere (radial oxygen release) (Larkum et al. 2006, Calleja et al. 2007), sulfide production outpaces oxygen release under warmer conditions, resulting in sulfide accumulation and seagrass mortality (Larkum et al. 2006, Calleja et al. 2007, Koch et al. 2007). Seagrass beds tend to accumulate organic matter and so it is expected that seagrass beds would build up toxic sulfides and hence have a limited productivity and diversity (Larkum et al. 2006). But this is not the observed case and the underlying reason for the long-term persistence of seagrass ecosystems is an enigma (Figure 7.1a).

We tested the hypothesis that a three-stage symbiosis between seagrasses, associated burrowing lucinid bivalves and their symbiotic gill-bacteria contribute to reducing the cyclic build-up of sulfide (Figure 7.1b-d). Paleo-records suggest that the Lucinidae and their endosymbiotic relation date back to the Silurian (Liljedahl 1991, Distel 1998, Taylor and Glover 2000), but that they increasingly diversified since the evolutionary emergence of seagrasses in the late Cretaceous (Stanley 1977, Larkum et al. 2006, Taylor et al. 2011). Seagrass communities later became widespread in the Eocene and Lucinid remains frequently occur in association with their deposits since (Taylor et al. 2011, Vermeij 2011). Lucinid and their gill-inhabiting bacteria have a symbiosis in which the bivalves transport sulfide and oxygen to their gills (Figure 7.1d) where the bacteria oxidize sulfide for synthesizing sugars that fuel growth of both organisms (Cavanaugh 1983, Johnson et al. 1994, Anderson 1995, Reynolds et al. 2007, Childress and Girguis 2011). We hypothesized that seagrass meadows may provide an optimal habitat for these bivalves and their symbionts by indirectly stimulating sulfide production by high organic matter input, and by providing oxygen through radial oxygen release from the roots. In turn, lucinids remove sulfide, which could relieve any stress caused to seagrass growth by sulfide accumulation as organic matter is degraded (Figure 7.1a & b).

Indirect support for our hypothesis was provided by a worldwide meta-analysis of 84 studies describing the fauna of seagrass beds in 83 sites covering the entire climatic distribution of seagrasses, combined with a 110-point field survey that we conducted at Banc d'Arguin, Mauretania (Schanz et al. 2002). The meta-analysis reveals a relationship that covers 11 out of 12 seagrass genera (and Ruppia spp.) and at least 18 genera of Lucinidae (Figure 7.1 & Table S7.1). Only meadows of Phyllospadix spp., a seagrass genus that grows on bare rock, do not associate with Lucinidae. The association spans six out of seven continents, with bivalve densities ranging from 10 to over 1000 individuals per m². The bivalves were present in 97% of the tropical seagrass sites, 90% of the subtropical meadows and 56% of the temperate seagrass beds surveyed, indicating that the association may be dependent on temperature-related sulfide production (Koch et al. 2007). Furthermore, results from our field study showed a positive correlation between seagrasses and lucinids that explained 42% of their respective variation (Pearson’s r=0.65; Figure 7.2).

![Figure 7.1 Presence (green; dark points are quantitative, light points are qualitative) and absence (red) of lucinids in seagrass ecosystems based on our meta-analysis. The bivalves were present in 97% (93% of the quantitative sites) of all tropical seagrass beds, 90% (83% of the quantitative sites) of the subtropical beds and 56% (50% of the quantitative sites) of the temperate seagrass meadows. The seagrass-lucinid association spans six out of seven continents, at least 18 genera of Lucinidae (Figure 7.1 & Table S7.1). Only meadows of Phyllospadix spp., a seagrass genus that grows on bare rock, did not contain Lucinidae. The analyzed ecosystems generally contained high (~100 ind. m⁻²) to extremely high densities (>1000 ind. m⁻²) of lucinids (Table S7.1).](image-url)
top sections of 40 two-compartment columns (Figure S7.3), which were placed in a large seawater basin. The lower compartment of each column contained anaerobic seawater and an injection tube through which sulfide was added twice a week in half of the columns. The injected sulfide was allowed to diffuse into the top section through a porous membrane.

The presence of Loripes, and to a lesser extent of Zostera decreased sediment sulfide levels. After five weeks, pore water sulfide concentrations in the top sections of the sediment controls reached about 400 µmol L$^{-1}$, while the semi-weekly addition of sulfide caused levels to increase to nearly 2700 µmol L$^{-1}$ (Figure 7.2a). The presence of Zostera decreased sulfide levels to around 200 µmol L$^{-1}$ in the controls and 2200 µmol L$^{-1}$ in the sulfide addition treatments. In contrast, sulfide levels remained low when Loripes was present (~15 µmol L$^{-1}$), even in the sulfide addition treatments. As expected, the oxygen detection depth was reduced when sulfide was added, but increased when only Loripes, but not Zostera was present, due to sulfide-oxidation and intake of surface water (Figure 7.2b). Zostera alone did not significantly affect sediment oxygen conditions. Strikingly, the joint presence of Zostera and Loripes enhanced oxygen detection depth beyond that of their separate effects.

Our experiment showed that Zostera production is facilitated by Loripes; both in the control and in the sulfide addition treatments. In the treatments without Loripes, sulfide addition reduced Zostera shoot biomass to 50% of the controls (Figure 7.3a). Reduced shoot biomass was accompanied by decreased root biomass (Figure 7.3b) and impaired phosphate uptake (Schanz et al. 2002). In contrast, the addition of Loripes increased Zostera shoot biomass 1.9-fold and root weight 1.5-fold seen in the sulfide addition treatments. In the treatments without additional sulfide, the presence of Loripes increased both shoot and root weight by 1.4-fold and 1.3-fold respectively.

Loripes condition, expressed as the flesh/shell dry weight ratio, was positively affected by sulfide addition (Figure 7.3c). Furthermore, the addition of Zostera did not affect Loripes in the units where no sulfide was added, but improved the bivalve's condition in the sulfide treatments. As hypothesized, the positive effect of Zostera on Loripes seems to result from radial oxygen release from the seagrass roots (Figure S7.1b). Although sulfide was almost completely removed in all Loripes treatments (Figure 7.2a), the bivalve was less able to profit from the addition of sulfide in the absence of Zostera (Figure 7.3c). This indicates that at least in the Loripes units without seagrass, sulfide was not completely oxidized by the symbiotic bacteria because of oxygen limitation.

Overall, our results confirm our hypothesis that a three-stage symbiosis between seagrass, lucinids and sulfide-oxidizing bacteria reduces sulfide stress in seagrass meadows. Even though radial oxygen release by Zostera noltii and of seagrasses in general is limited (Caffrey and Kemp 1991, Sand-Jensen et al. 2005), Loripes in our experiment clearly benefited from the increased oxygen input in the sediment. In the field, the positive effects of seagrasses on lucinids are not confined to sediment oxygenation alone, but also by indirectly stimulating sulfide production and releasing dissolved organic molecules (Larkum et al. 2006, Reynolds et al. 2007). The positive effects
of Loripes on Zostera in our experiment could not be explained by differences in nutrient availability (Schanz et al. 2002). Plants were not nutrient limited, but both Zostera and Loripes significantly lowered dissolved ammonium and phosphorus in the sediment pore water, whereas sulfide addition increased nutrient availability (Figure S7.4). We found that in our experiment, the negative effects of sulfide addition on Zostera biomass could not fully be prevented by Loripes addition (Figure 7.3a), despite the removal of almost all sulfide by Loripes after three days. As the observed experimental effects could not be attributed to differences in nutrient availability, this is most likely caused by the pulsed nature of our sulfide supply. This may have led to short periods of exposure of Zostera to toxic sulfide levels.

Coastal ecosystems, and seagrass meadows in particular, are currently declining at an alarming and increasing rate worldwide, leading to loss of biodiversity (Waycott et al. 2009). Extensive restoration efforts have had little success so far (<30%), despite their extremely high costs ($±100,000 per ha) (Fonseca et al. 2001). Similar to the function of mycorrhizae, pollinators or seed dispersers in terrestrial systems (van der Heijden et al. 1998, Bascompte and Jordano 2007, Bastolla et al. 2009), our findings indicate that restoration efforts should not only focus on environmental stressors like eutrophication, sediment run-off or high salinity as a cause of decline, but should also consider internal ecological interactions such as the presence and vigor of symbiotic or mutualistic relations. Breakdown of symbiotic interactions can affect ecosystem functioning, with bleaching events in coral reefs as a clear example (Carpenter et al. 2008). Similar to the well-known symbiosis between corals and their unicellular algal endosymbionts (Baker et al. 2009), our findings indicate that restoration efforts should not only focus on environmental stressors like eutrophication, sediment run-off or high salinity as a cause of decline, but should also consider internal ecological interactions such as the presence and vigor of symbiotic or mutualistic relations. Breakdown of symbiotic interactions can affect ecosystem functioning, with bleaching events in coral reefs as a clear example (Carpenter et al. 2008). Similar to the well-known symbiosis between corals and their unicellular algal endosymbionts (Baker et al. 2009), we conclude that symbioses, rather than one defining species forms the foundation of seagrass ecosystems.

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Materials and Methods

Meta-analysis

To test the seagrass-lucinid association, we performed an extensive, worldwide meta-analysis that covered the entire climatic distribution of seagrasses. Criteria for including a study were: (1) seagrasses were present at the site, and (2) when Lucinidae were present, they were found inside the seagrass bed. In total, we analyzed 84 studies that sampled the fauna of seagrass beds in a total of 83 areas (temperature range=1 to 33°C, mean=22°C). Overall, 36 sites were from tropical areas, 31 from subtropical and 16 from temperate areas; quantitative data were available for 46 out of 83 sites. Apart from the geographical location of each site, and the seagrass and lucinid families found, we also report the annual seawater temperature range. These were obtained from freely available satellite imagery of the long-term monthly means (1971 – 2000) of the sea surface temperature (NOAA/OAR/ESRL/PSD 2011).

Field study

We conducted a field survey at Banc d’Arguin (Mauritania) to test the strength of the relation between seagrass biomass and lucinid density. Banc d’Arguin consists of about 500 km² of intertidal flat dominated by mixed meadows of Zostera nolitii, Halodule wrightii and Cymodocea nodosa that are inhabited by the lucinid bivalve Loripes lacteus (Wolff et al. 1993). In total, we sampled 110 stations across seven intertidal flats. Loripes was sampled up to a depth of 20 cm using a cylindrical 15 cm diameter PVC core sampler and seagrass was sampled with a 7 cm diameter corer. Each sample was sieved over a 1 mm mesh sieve. Next, Loripes was counted and seagrass biomass was determined after drying for 24 h at 70°C. Prior to linear regression analysis, Loripes counts and seagrass dry weight from the cores were transformed with the Box-Cox procedure to achieve normality and homoscedasticity (Box and Cox 1964).

Laboratory experiment

Organisms and sediment for the experiment were collected in Arcachon Bay (southwest France) and transported at 15°C to the laboratory, where both species were separately acclimatized for three weeks in 100 L polyethylene tanks. Zostera units contained 15 cm of sediment and 20 cm of surface water; Loripes tanks contained 30 cm of sediment and 5 cm of surface water. We used artificial seawater (33-35 PSU Tropic Marin at 20°C) throughout the acclimatization period and during the experiment; pH was kept at 8.1 to 8.3 by CO2 aeration. Light period was 16 h day⁻¹; intensity at the leaf surface was 300 μmol m⁻² s⁻¹, similar...
to growing season conditions in the field (Isaksen and Finster 1996). During this three-week period, we did not observe any bivalve mortality, and seagrasses exhibited healthy vegetative growth.

**Experimental setup**

The lower 6 cm tall sections of 40 two-compartment PVC columns (diameter 8.4 cm) were filled with anaerobic seawater (Figure S7.3). These 330 mL sections contained an injection tube and were separated from their upper compartments through a porous 0.1 mm membrane. Sediment was passed through a 1 mm sieve and transferred to the upper 12 cm tall sections (surface area: 0.0055 m²). Depending on the treatment, each unit then received either 1) *Loripes*, 2) *Zostera*, 3) both *Zostera* and *Loripes*, or 4) no further treatment. Nine *Loripes* specimens were added to each *Loripes* treatment (~1600 ind. m⁻²; mean shell length ~9 mm) and 5 seagrass ramets with 2 or 3 shoots (12 shoots in total) were planted in each unit containing *Zostera* (~2200 sh. m⁻²; ~0.12 g shoot, ~0.06 g rhizome and ~0.03 g DW root biomass per column). Each ramet contained one apical shoot to allow vegetative growth. Pilot experiments showed that this approach ensured consistent colonization of the units within the two-week adjustment period, with no detectable mortality of the plants. Densities of both species were well within reported ranges of densities in the field (up to 23000 sh. m⁻² for *Zostera* and 3700 ind. m⁻² for *Loripes*) (Vermaat and Verhagen 1996, Johnson et al. 2002, van der Geest et al. 2011).

A full factorial experiment was designed with eight treatments and five replicates per treatment. The columns were randomly placed in a 40 cm high 250 L polyethylene basin where water flow and oxygen saturation (measured with a 556 Multi Parameter Sampler, Yellow Springs Instruments) were maintained by two aquarium water pumps, and pH was kept constant (8.1-8.3) by CO₂ aeration. After setup, the units were allowed to adjust for two weeks. During this period, sulfide levels in the treatments containing *Loripes* stabilized at ~7 μmol L⁻¹, while sulfide in treatments without *Loripes* increased to ~233 μmol L⁻¹. Following the adjustment period, the experiment was performed for five weeks. Sulfide levels in the lower compartments of the sulfide addition treatments were increased twice a week by 3.3 ml injections of 100 mmol L⁻¹ Na₂S solution with pH adjusted to sediment conditions (pH 7.5) with HCl, while control treatments were injected with anaerobic water. Before each injection, we used 5 cm Rhizon samplers to extract 3 ml of pore water from the main root zone (top 6 cm) of each upper compartment into vacuumized 30 mL flasks containing 3 ml Sulfide Anti-Oxidation Buffer (SAOB). After each sampling, columns were re-randomized in the basin to minimize possible differences in light levels and water flow velocities between units. Sulfide concentrations were determined immediately with an ion selective silver/sulfide electrode (Thermo Scientific (USA), Orion 9416 BN; reference electrode: Orion 900200). Oxygen detection depth was measured after five weeks with an oxygen-sensitive microelectrode (Microscale Measurements, 1 mm tip). Ammonium, nitrate and total dissolved phosphorus in the sediment pore water were also measured after five weeks. We used 5 cm Rhizon samplers to extract 10 ml of pore water from the main root zone (top 6 cm) of each upper compartment into vacuumized 30 mL flasks. Ammonium and nitrate concentrations were determined colorimetrically. Ammonium was measured with salicylate (Lamers et al. 1998) and nitrate was determined by sulfanilamide after reduction of nitrate to nitrite in a cadmium column (Wood et al. 1967). Dissolved phosphorus was measured on an Inductively Coupled Plasma emission spectrophotometer (ICP; Spectroflame, Spectro). Total nitrogen concentration in *Zostera* leaves was measured in freeze-dried tissues by a CNS analyzer (type NA1500; Carlo Erba Instruments, Milan, Italy) (Lamers et al. 1998). Total phosphorus was measured by ICP after digestion with nitric acid (Lamers et al. 1998). *Zostera* shoot, root and rhizome biomass and *Loripes* flesh were measured as dry weight after 24 h of freeze-drying. *Loripes* shell weight was measured after drying for 24 h at 70°C. *Loripes* condition was expressed as flesh/shell dry weight ratio, which is a commonly used size-and-age independent measure of fitness in bivalves (Lucas and Beninger 1985). Sulfur contents in the *Loripes* tissues were measured on ICP, following nitric acid digestion.

**Statistical analyses**

Data were tested for normality prior to analysis. Sulfide data were analyzed with Repeated-Measures three-factor ANOVA. All other variables were analyzed by two- or three-factor ANOVA. All relevant and/or significant effects and interactions are mentioned in the figure legends or supporting text. A complete overview of the statistical output for Figures 7.2, 7.3 and S7.4 is provided in Table S7.2.

**Supporting text**

Both *Zostera* and *Loripes* significantly lowered dissolved ammonium and phosphorus in the sediment pore water, while sulfide addition increased their availability (Figure S7.4). Nitrate concentrations were 0.8±0.9 μmol L⁻¹ on average with no significant differences between treatments. Mean leaf nitrogen and phosphorus content were 1.78±0.26 and 0.15±0.02% dry weight respectively, which is around reported median values from the field for both (1.8 and 0.2 %DW respectively) (Duarte 1990). None of the treatments had any significant effect on leaf nitrogen. Leaf phosphorus content was unaffected by *Loripes*, but decreased significantly in the sulfide addition and sulfide addition with *Loripes* treatments (from 0.17±0.01 to 0.13±0.01 %DW; ANOVA: F₁,₅₀=29.0, P<0.001). Apparently, high sulfide levels impaired phosphorus uptake by *Zostera* in the sulfide addition treatment, leading to decreased leaf phosphorus content,
despite high dissolved phosphorus availability in the pore water (Figure S7.4). Our pulsed sulfide addition also seemed to impair phosphorus uptake in the sulfide addition with *Loripes* treatment, which, by interacting with the reduced dissolved phosphorus pool may have limited growth of *Zostera* under our conditions (Figure 7.3).

Sulfide addition resulted in a significant increase in the relative (ANOVA: $F_{1,16}=13.8, P=0.002$) and absolute sulfur content (ANOVA: $F_{1,16}=24.1, P<0.001$) in the flesh of the bivalves. Relative sulfur content was 2.0±0.2% (g:g) in the control treatments and 3.0±0.9% in the sulfide addition treatments. The total amount of sulfur stored in *Loripes* tissues per unit was 1.3±0.2 mg in the control treatments and 3.0±1.1 mg in the sulfide addition treatments. These results suggest that the increased sulfide availability led to increased storage of sulfur in the tissues of the bivalves, for instance as sulfur granules in the gills (Anderson 1995). We found no significant effects of *Zostera* on *Loripes* sulfur content.

**SUPPORTING FIGURES**

**Figure S7.1** (a) Seagrasses generally create a negative feedback on their own growth through organic matter accumulation, which stimulates production of toxic sulfide by heterotrophic sulfate-reducing bacteria. (b) We propose in this study that the presence of lucinid bivalves and their sulfide-oxidizing gill-symbionts breaks the negative feedback, resulting in a network of positive interactions. (c) The bivalves are found in high abundances in the root zones of seagrass meadows in warmer, mild temperate to tropical regions where sulfide production rates are high. (d) They occur in the anoxic zone of the sediment and use their highly extensile foot to create tubes for sulfide mining, export of waste products and import of oxygen and CO$_2$ from the sediment pore water and surface water (Anderson 1995, Reynolds et al. 2007). Both sulfide and oxygen are transported to the gills where chemoautotrophic bacteria oxidize sulfide for synthesizing sugars that fuel growth of both the bacteria and the bivalve (Johnson et al. 1994, Anderson 1995, Reynolds et al. 2007, Childress and Girguis 2011).

**Figure S7.2** Positive correlation (Pearson’s $r=0.65$) between seagrass biomass and *Loripes* density on Banc d’Arguin. *Loripes* counts and seagrass dry weight from the cores were transformed using the Box-Cox procedure prior to plotting and the regression analysis (see Materials & Methods).
Chapter 7  Biogeochemical species interactions

Figure S7.4  Pore water ammonium and dissolved phosphorus contents after five weeks; error bars represent SEM (n=5). Ammonium (a) was lowered significantly by Zostera (ANOVA: F1,32=59.7, P<0.001) and Loripes (F1,32=505.9, P<0.001), while sulfide addition caused an increase (F1,32=35.2, P<0.001). We found significant interactions between all treatments (Z*L: F1,32=57.1, P<0.001; Z*S: F1,32=73.3, P<0.001; L*S: F1,32=39.3, P<0.001; Z*L*S: F1,32=68.5, P<0.001). The treatment effects on dissolved phosphorus (b) were similar to ammonium, with significant effects of Zostera (F1,32=58.2, P<0.001), Loripes (F1,32=562.1, P<0.001) and sulfide addition (F1,32=19.6, P<0.001). We found significant interactions of Zostera and Loripes (F1,32=55.1, P<0.001), and Loripes and sulfide addition (F1,32=28.2, P<0.001).

Figure S7.3  Schematic drawing of the setup of an experimental unit. The dimensions of the top section were chosen to fit the organisms and to resemble field conditions. The lower section was kept large enough to allow rapid mixing and upward diffusion. Sulfide was injected twice a week in the sulfide addition treatments and allowed to diffuse from the lower compartment into the upper section through a 0.1 mm porous membrane.

Supporting tables

Table S7.1  Lucinid bivalve densities found in seagrass beds. These data provide a basic indication of the association between seagrasses and lucinids worldwide. Temp. depicts the mean annual temperature range based on the sea surface temperature (°C); Clim. indicates type of climate (tropical, subtropical or temperate); Lucinid density (spatial average): +=1-10; ++=11-100; +++=101-1000; ++++=>1000 ind/m² p=present (no abundance data); u=uncertain; 0=absent.

<table>
<thead>
<tr>
<th>Area (source)</th>
<th>Temp.</th>
<th>Clim.</th>
<th>Seagrass genus</th>
<th>Lucinid genus</th>
<th>Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chesapeake Bay (Orth 1973)</td>
<td>1 – 23</td>
<td>Temp.</td>
<td>Zostera</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Apalachee Bay, Florida (Lewis and Stoner 1981)</td>
<td>18 – 29</td>
<td>Subtr.</td>
<td>Syringodium, Thalassia</td>
<td>Codakia</td>
<td>+</td>
</tr>
<tr>
<td>Biscayne Bay, Florida (Moore et al. 1968)</td>
<td>24 – 30</td>
<td>Subtr.</td>
<td>Halodule, Syringodium, Thalassia</td>
<td>Anodontia, Codakia, Lucina</td>
<td>++/++++</td>
</tr>
<tr>
<td>Florida Bay, Florida (Reynolds et al. 2007)</td>
<td>24 – 30</td>
<td>Subtr.</td>
<td>Halodule, Syringodium, Thalassia</td>
<td>Anodontia, Codakia, Lucinesca</td>
<td>++/++++</td>
</tr>
<tr>
<td>Indian River lag., Florida (Mikkelsen et al. 1995)</td>
<td>23 – 29</td>
<td>Subtr.</td>
<td>Thalassia</td>
<td>Lucina</td>
<td>p</td>
</tr>
<tr>
<td>Pensacola Bay, Florida (Stoner et al. 1983)</td>
<td>18 – 29</td>
<td>Subtr.</td>
<td>Halodule</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Redfish Bay, Texas (Center for Coastal Studies 1996)</td>
<td>19 – 29</td>
<td>Subtr.</td>
<td>Halodule, Thalassia</td>
<td>Anodontia, Lucina, Phacooides</td>
<td>p</td>
</tr>
<tr>
<td>Gulf of California, Mexico (Torra Cosio and Bourillón 2000)</td>
<td>19 – 30</td>
<td>Subtr.</td>
<td>Zostera, Halodule, Ruppia</td>
<td>Codakia, Divalinga</td>
<td>p</td>
</tr>
<tr>
<td>Bahia de Chetumal, Mexico (Quesada et al. 2004)</td>
<td>27 – 29</td>
<td>Trop.</td>
<td>Syringodium, Thalassia</td>
<td>Codakia, Lucina</td>
<td>p</td>
</tr>
<tr>
<td>Turneffe Islands, Belize, Mexico (Hauser et al. 2007)</td>
<td>27 – 29</td>
<td>Trop.</td>
<td>Thalassia</td>
<td>Codakia, Parvilucina</td>
<td>p</td>
</tr>
<tr>
<td>Bahama’s (Brissac 2009)</td>
<td>24 – 29</td>
<td>Trop.</td>
<td>Thalassia</td>
<td>Codakia</td>
<td>p</td>
</tr>
<tr>
<td>Area (source)</td>
<td>Temp.</td>
<td>Clim.</td>
<td>Seagrass genus</td>
<td>Lucinid genus</td>
<td>Density</td>
</tr>
<tr>
<td>--------------</td>
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<td>---------------</td>
<td>---------</td>
</tr>
<tr>
<td>St Croix, Virgin Islands (Ferguson and Miller 2007)</td>
<td>26 – 29</td>
<td>Trop.</td>
<td>Halodule, Syringodium, Thalassia</td>
<td>Codakia, Divalinga, Lucina, Parvilucina</td>
<td>p</td>
</tr>
<tr>
<td>Guadeloupe (Gros et al. 2003)</td>
<td>26 – 29</td>
<td>Trop.</td>
<td>Thalassia</td>
<td>Anodontia, Codakia</td>
<td>p</td>
</tr>
<tr>
<td>Martinique (Brissac 2009)</td>
<td>26 – 29</td>
<td>Trop.</td>
<td>Thalassia</td>
<td>Lucina</td>
<td>p</td>
</tr>
<tr>
<td>South America</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bahia de Nenguane, Columbia (Diaz 2003)</td>
<td>26 – 29</td>
<td>Trop.</td>
<td>Thalassia, Syringodium</td>
<td>Lucina, Anodontia</td>
<td>p</td>
</tr>
<tr>
<td>Santiago de Tolú, Columbia (Otero Otero and Romani Lobo 2009)</td>
<td>26 – 29</td>
<td>Trop.</td>
<td>Thalassia</td>
<td>Lucina</td>
<td>p</td>
</tr>
<tr>
<td>Parracho de Maracaújia, Brazil (Martinez 2008)</td>
<td>25 – 28</td>
<td>Trop.</td>
<td>Halophila, Halodule</td>
<td>Codakia, Divalicella</td>
<td>p</td>
</tr>
<tr>
<td>Abrolhos Bank, Bahia Brazil (Dutra et al. 2005)</td>
<td>23 – 27</td>
<td>Trop.</td>
<td>Halodule</td>
<td>Codakia, Ctena, Parvilucina</td>
<td>p</td>
</tr>
<tr>
<td>Ilha do Japonês, Brazil (Marques and Creed 2000, Creed and Kinupp 2011)</td>
<td>18 – 26</td>
<td>Trop.</td>
<td>Halodule</td>
<td>Codakia, Divalicella</td>
<td>++++</td>
</tr>
<tr>
<td>Ilha do Mel, Paranaçu, Brazil (Couto and Savian 1998)</td>
<td>18 – 26</td>
<td>Trop.</td>
<td>Halodule</td>
<td>Lucina</td>
<td>p</td>
</tr>
<tr>
<td>Europe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Western Atlantic, Norway (Fredriksen et al. 2010)</td>
<td>6 – 13</td>
<td>Temp.</td>
<td>Zostera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skagerrak, Atlantic, Norway (Fredriksen et al. 2010)</td>
<td>4 – 17</td>
<td>Temp.</td>
<td>Zostera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baltic, Finland (Bostrom and Bonsdorff 1997)</td>
<td>1 – 16</td>
<td>Temp.</td>
<td>Zostera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sylt, Wadden Sea (Reise 1985)</td>
<td>4 – 18</td>
<td>Temp.</td>
<td>Zostera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>South England (Dando et al. 1986)</td>
<td>8 – 17</td>
<td>Temp.</td>
<td>Zostera</td>
<td>Lucinoma</td>
<td>+</td>
</tr>
<tr>
<td>South Ireland (Dale et al. 2007)</td>
<td>9 – 17</td>
<td>Temp.</td>
<td>Zostera</td>
<td>Lucinoma</td>
<td>+++</td>
</tr>
<tr>
<td>Africa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Banc d'Arguin, Mauritania (van der Geest et al. 2011)</td>
<td>18 – 26</td>
<td>Subtr.</td>
<td>Cymodocea, Halodule, Zostera</td>
<td>Loripes</td>
<td>+++/+****</td>
</tr>
<tr>
<td>Mahé, Seychelles (Taylor and Lewis 1970)</td>
<td>26 – 30</td>
<td>Trop.</td>
<td>Thalassia</td>
<td>Anodontia, Codakia, Ctena</td>
<td>+</td>
</tr>
<tr>
<td>Langebaan lag., South-Africa (Siebert and Branch 2005)</td>
<td>15 – 19</td>
<td>Subtr.</td>
<td>Zostera</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Area (source)</td>
<td>Temp.</td>
<td>Clim.</td>
<td>Seagrass genus</td>
<td>Lucinid genus</td>
<td>Density</td>
</tr>
<tr>
<td>--------------</td>
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<td>---------</td>
</tr>
<tr>
<td><strong>Asia/Pacific</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>United Arab Emirates (Feulner and Hornby 2006)</td>
<td>21 – 33</td>
<td>Subtr.</td>
<td>Halodule, Halophila</td>
<td>Anodontia, Pallucina</td>
<td>+++</td>
</tr>
<tr>
<td>Oman (this study)</td>
<td>25 – 28</td>
<td>Trop.</td>
<td>Halodule, Halophila</td>
<td>Pallucina</td>
<td>+++</td>
</tr>
<tr>
<td>Palk Bay, India (Gophinadhapillai and Appukuttan 1980)</td>
<td>27 – 30</td>
<td>Trop.</td>
<td>Cymodocea, Halodule, Syringodium, Thalassodendron</td>
<td>Codakia, Lucina</td>
<td>p</td>
</tr>
<tr>
<td>Posyet Bay, Sea of Japan (Kharlamenko et al. 2001)</td>
<td>2 – 21</td>
<td>Temp.</td>
<td>Zostera</td>
<td>Pallucina</td>
<td>+++</td>
</tr>
<tr>
<td>Tokyo, Bay of Japan (Whanpetch 2011)</td>
<td>16 – 26</td>
<td>Subtr.</td>
<td>Zostera</td>
<td>Luncinidae</td>
<td>p</td>
</tr>
<tr>
<td>Guangxi, China (Huang 2008)</td>
<td>20 – 29</td>
<td>Trop.</td>
<td>Halodule, Halophila, Zostera</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Guangdong, China (Huang 2008)</td>
<td>21 – 29</td>
<td>Trop.</td>
<td>Halodule, Halophila</td>
<td>Pallucina</td>
<td>p</td>
</tr>
<tr>
<td>Hainan, China (Huang 2008)</td>
<td>22 – 29</td>
<td>Trop.</td>
<td>Cymodocea, Enhalus, Halodule, Halophila, Thalassia</td>
<td>Pallucina</td>
<td>p</td>
</tr>
<tr>
<td>Tubbataha Reefs, Philippines (Yamaguchi 1999)</td>
<td>27 – 30</td>
<td>Trop.</td>
<td>Halodule, Halophila, Thalassia</td>
<td>Epicodakia</td>
<td>p</td>
</tr>
</tbody>
</table>

| **Oceania** | | | | | |
| Rottnest Island, Australia (Barnes and Hickman 1999) | 19 – 23 | Subtr. | Posidonia | Wallucina | +++/++++ |
| South-West Australia (Hutchings et al. 1991) | 16 – 20 | Subtr. | Amphibolis, Posidonia | Anodontia | p |
| New South-Wales, Australia (Gibbs et al. 1984) | 19 – 24 | Subtr. | Halophila | Wallucina | p |
| New South-Wales, Australia (McKinnon et al. 2009) | 17 – 23 | Subtr. | Halophila, Zostera | 0 | |
| Western Port, Victoria, Australia (Watson et al. 1984, Edgar et al. 1994) | 13 – 18 | Temp. | Halophila, Zostera | 0 | |
| Tasmania (Edgar et al. 1999a, Edgar et al. 1999b) | 12 – 16 | Temp. | Heterozostera, Ruppia, Zostera | Wallucina | ++/++++ |
| New Caledonia (Glover and Taylor 2007) | 24 – 28 | Subtr. | Cymodocea, Halodule, Thalassia | Anodontia, Codakia, Ctena | p |
| Slipper Island, New Zealand (Schwarz et al. 2006) | 15 – 21 | Subtr. | Zostera | Divaricella | p |
Table S7.2 Overview of the statistical output from the analyses of the data presented in Figures 7.2, 7.3, and S7.4.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>df</th>
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<tr>
<td>Sulfide measurements (Figure 7.2a; repeated measures ANOVA)</td>
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<tr>
<td>Zostera</td>
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<tr>
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<tr>
<td>Oxygen measurements (Figure 7.2b; ANOVA)</td>
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<tr>
<td>Zostera</td>
<td>1</td>
<td>39.3</td>
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</tr>
<tr>
<td>Loripes</td>
<td>1</td>
<td>125.0</td>
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<tr>
<td>Sulfide</td>
<td>1</td>
<td>8.9</td>
<td>0.066</td>
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<tr>
<td>Zostera * Loripes</td>
<td>1</td>
<td>48.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zostera * Sulfide</td>
<td>1</td>
<td>0.0</td>
<td>0.862</td>
</tr>
<tr>
<td>Loripes * Sulfide</td>
<td>1</td>
<td>0.3</td>
<td>0.578</td>
</tr>
<tr>
<td>Zostera * Loripes * Sulfide</td>
<td>1</td>
<td>0.5</td>
<td>0.505</td>
</tr>
<tr>
<td>Error</td>
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</tr>
<tr>
<td>Zostera shoot biomass (Figure 7.3a; ANOVA)</td>
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<tr>
<td>Loripes</td>
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<td>Zostera root biomass (Figure 7.3b; ANOVA)</td>
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<tr>
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<tr>
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<td>Loripes fitness (Figure 7.3c; ANOVA)</td>
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<td>Sulfide</td>
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<td>Error</td>
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<td></td>
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<tr>
<td>Ammonium (Figure 7.4a; ANOVA)</td>
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<tr>
<td>Zostera</td>
<td>1</td>
<td>59.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Loripes</td>
<td>1</td>
<td>505.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sulfide</td>
<td>1</td>
<td>35.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zostera * Loripes</td>
<td>1</td>
<td>57.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zostera * Sulfide</td>
<td>1</td>
<td>73.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Loripes * Sulfide</td>
<td>1</td>
<td>39.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zostera * Loripes * Sulfide</td>
<td>1</td>
<td>68.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error</td>
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<td></td>
</tr>
<tr>
<td>Phosphorus (Figure 7.4b; ANOVA)</td>
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<td></td>
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</tr>
<tr>
<td>Zostera</td>
<td>1</td>
<td>58.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Loripes</td>
<td>1</td>
<td>562.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sulfide</td>
<td>1</td>
<td>19.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zostera * Loripes</td>
<td>1</td>
<td>55.1</td>
<td>&lt;0.001</td>
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<tr>
<td>Zostera * Sulfide</td>
<td>1</td>
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<td>0.888</td>
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<tr>
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<td>0.000</td>
</tr>
<tr>
<td>Zostera * Loripes * Sulfide</td>
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</tr>
<tr>
<td>Error</td>
<td>32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
When two ecosystem engineers share the same natural environment, the outcome of their interaction will be unclear if they have contrasting habitat-modifying effects (e.g. sediment stabilization vs. sediment destabilization). The outcome of the interaction may depend on local environmental conditions such as season or sediment type, which may affect the extent and type of habitat modification by the ecosystem engineers involved. We mechanistically studied the interaction between the sediment-stabilizing seagrass Zostera noltii and the bioturbating and sediment-destabilizing lugworm Arenicola marina, which sometimes co-occur for prolonged periods. We investigated (1) if the negative sediment destabilization effect of A. marina on Z. noltii might be counteracted by positive biogeochemical effects of bioirrigation (burrow flushing) by A. marina in sulfide-rich sediments, and (2) if previously observed nutrient release by A. marina bioirrigation could affect seagrasses. We tested the individual and combined effects of A. marina presence and high porewater sulfide concentrations (induced by organic matter addition) on seagrass biomass in a full-factorial lab experiment. Contrary to our expectations, we did not find an effect of A. marina on porewater sulfide concentrations. A. marina activities affected the seagrass physically as well as by pumping nutrients, mainly NH$_4^+$ and PO$_4^{3-}$ from the porewater to the surface water, which promoted epiphyte growth on seagrass leaves in our experimental set-up. We conclude that A. marina bioirrigation did not alleviate sulfide stress to seagrasses. Instead, we found synergistic negative effects of the presence of A. marina and high sediment sulfide levels on seagrass biomass.
INTRODUCTION

Ecosystem engineers are “organisms that directly or indirectly modulate the availability of resources to other species, by causing physical state changes in biotic or abiotic materials. In doing so, they modify, maintain and create habitats” (Jones et al. 1994). The intertidal zone is a dynamic area, which is subject to many stressors such as waves and currents when submerged, but also to desiccation stress when exposed. A wide range of ecosystem engineers inhabit the dynamic intertidal flats (Passarelli et al. 2013), which may cause different and sometimes opposing habitat modifications (Bouma et al. 2009). In general terms, benthic engineering species living on the intertidal flats can be divided into epibenthic engineers, which live on top of the sediment, and endobenthic engineers, which mainly live within the sediment. Epibenthic ecosystem engineers, such as seagrasses and oyster reefs, typically modify the sedimentary habitat mainly by affecting hydrodynamics and sediment dynamics with their physical structures, which protrude into the water column (Bouma et al. 2005, Bos et al. 2007, Koch et al. 2009). In contrast, endobenthic engineers, such as several polychaete species, typically modify the sedimentary habitat by bioturbation (sediment reworking) and bioirrigation (burrow flushing) (Cadée 2001, Reise 2002, Meysman et al. 2006).

In recent decades, there has been a growing interest in the way in which ecosystem engineers can benefit other species (Bertness and Leonard 1997, Bruno et al. 2003), partly because such interactions can be highly relevant for coastal restoration projects (Crain and Bertness 2006, Van Katwijk et al. 2009). More recently, researchers have emphasized the importance of negative engineering interactions from an ecological and restoration perspective (Van Wesenbeeck et al. 2007, Suykerbuyk et al. 2012). This raises the questions how ecosystem engineers can interact, whether the engineering might have positive and negative effects at the same time, and how the outcomes depend on environmental conditions. In this study, we addressed this issue using sediment-stabilizing seagrasses (Zostera noltii) and bioturbating and bioirrigating lugworms (Arenicola marina) as model organisms.

Seagrasses are epibenthic ecosystem engineers that can improve their own growing conditions by stabilizing the sediment (Fonseca 1989, Bos and van Katwijk 2007, Christianen et al. 2013) and by attenuating waves and currents (Fonseca and Cahalan 1992, Peterson et al. 2004, Peralta et al. 2008), resulting in the accumulation of small sediment particles and suspended organic matter (Granata et al. 2001, Van der Heide et al. 2011). These ecosystem characteristics have also been identified for our model species Zostera noltii (Bouma et al. 2005, Brun et al. 2009). On the other hand, the entrapment of suspended organic matter and the production of organic matter by seagrasses often results in high sulfate reduction rates in the sediment, leading to the production of sulfides (Jørgensen et al. 2009). On the other hand, the entrapment of suspended organic matter by seagrasses often results in high sulfate reduction rates in the sediment, leading to the production of sulfides (Jørgensen and the production of organic matter by seagrasses often results in high sulfate reduction rates in the sediment, leading to the production of sulfides (Jørgensen et al. 2009). On the other hand, the entrapment of suspended organic matter by seagrasses often results in high sulfate reduction rates in the sediment, leading to the production of sulfides (Jørgensen et al. 2009). On the other hand, the entrapment of suspended organic matter by seagrasses often results in high sulfate reduction rates in the sediment, leading to the production of sulfides (Jørgensen et al. 2009).
Ecosystem engineering by *A. marina* might thus potentially have positive as well as negative effects on the seagrass *Z. noltii*, and it is still unclear how the effect may depend on the environmental conditions. The physical effects of bioturbation on seagrasses have been relatively well studied (Philippart 1994, Reise and Kohlus 2007, Suykerbuyk et al. 2012), but its biogeochemical effects on seagrasses still remain to be elucidated. Hence, we investigated how the combination of altered biogeochemistry and physical disturbance by *A. marina* bioturbation could affect the seagrass *Z. noltii* in sediments with contrasting organic matter contents. We hypothesized that *A. marina* could have positive effects on *Z. noltii* growth by alleviating sulfide toxicity in organically enriched sediments through sediment aeration and pumping to the overlying water column. However, the same flushing could lead to an increased release of nutrients to the water column, which might potentially cause toxic effects (NH$_4$) or epiphyte blooms (NH$_4$ and PO$_4$).

**MATERIALS AND METHODS**

**Experimental design**

To test the individual and interacting effects of *A. marina* bioturbation & bioirrigation and sulfide toxicity (organic matter addition) on *Z. noltii* biomass, we designed a full factorial experiment, in which aquaria were provided with organic matter (OM), *A. marina* (Ar), seagrass (Sg), or a combination of these treatments, resulting in a total of 8 different treatments: Control, OM, Ar, Sg, OMAr, OMSg, SgAr and OMSgAr. For the OM treatment, 2 g L$^{-1}$ organic matter (1 treatments, resulting in a total of 8 different treatments: Control, OM, Ar, Sg, OMAr, OMSg, SgAr and OMSgAr. For the OM treatment, 2 g L$^{-1}$ organic matter (1

**Experimental design**

To test the individual and interacting effects of *A. marina* bioturbation & bioirrigation and sulfide toxicity (organic matter addition) on *Z. noltii* biomass, we designed a full factorial experiment, in which aquaria were provided with organic matter (OM), *A. marina* (Ar), seagrass (Sg), or a combination of these treatments, resulting in a total of 8 different treatments: Control, OM, Ar, Sg, OMAr, OMSg, SgAr and OMSgAr. For the OM treatment, 2 g L$^{-1}$ organic matter (1 g starch + 1 g cellulose L$^{-1}$ sediment) was mixed into the sediment to stimulate sulfide production (Peralta et al. 2003, Govers et al. 2014), before the start of the experiment. We used 5 replicates per treatment, resulting in 40 experimental units, which were randomly placed in a water bath. During the experiment, the synthetic seawater was completely refreshed twice a week.

The experiment was conducted in 24 L glass aquaria (l x w x h=20x20x60 cm), which were filled with a 30 cm sediment layer (12 L) and a 25-30 cm water layer, which was prepared from deionized water and Tropic Marin® synthetic sea salt; salinity levels were comparable to levels measured in the field (27-29 psu). An aquarium pump and aquarium bubbler in each aquarium aerated the surface water. Aquaria were placed in a large water bath at 20°C. Light intensity was set at 300 μmol m$^{-2}$ s$^{-1}$, with a day-night cycle of 14-10 hours, which is comparable to light conditions in the field (Isaksen and Finster 1996). The experiment lasted for 28 days and all treatments were run simultaneously.

**Sample collection and analysis**

During the experiment, sediment porewater samples were collected on seven occasions (t=0, 3, 7, 14, 19, 24, 28 days), using vacuumed flasks connected to soil moisture samplers (Rhizons, Eijkelkamp Agrisearch Equipment, Giesbeek, the Netherlands). 10 mL of porewater was collected at each sampling occasion for sulfide analysis, and 20 mL was sampled at the end of the experiment for both sulfide and nutrient analysis. The rhizons were placed horizontally, in the central part of all aquaria, at a depth of 5 cm in the sediment, which is the average root depth of *Z. noltii*. Similar porewater samples were simultaneously taken at depths of 10 cm and 20 cm. These porewater samples were immediately used to measure sulfide concentrations in a mixture of 50% sample and 50% Sulfide Anti-Oxidation Buffer (SAOB) (Lamers et al. 1998) using a calibrated ion-specific silver-sulfide electrode. At the end of the experiment (t=28 days), we used the same method to collect porewater samples for nutrient concentration measurements (20 mL), which were frozen and later analyzed in the lab. Surface water samples were collected through rhizons completely submerged in the water layer, in order to filter the water samples prior to analysis. Porewater and surface water ammonium and ortho-phosphate concentrations were measured colorimetrically (Skalar and Seal autoanalyzer), using ammonium-molybdate and salicylate. Nitrate was determined by sulfanilamide, after reduction of nitrate to nitrite in a cadmium column (Wood et al. 1967). All nutrients were measured at the analytical lab of the Royal Netherlands Institute for Sea Research (NIOZ) in Yerseke, The Netherlands.

**Origins of sediment and biological materials**

Sediment, seagrass (*Z. noltii*) and lugworms (*A. marina*) were obtained from the mudflats of the Oosterschelde area (51°39’ N, 4°01’ E), The Netherlands. Sandy sediment, with a median grain size (D50) of 180 μm and 0.6% organic matter, was collected on a single day at the exposed Dortsman mudflats (Suykerbuyk et al. 2012), transported to the laboratory in Nijmegen (The Netherlands), sieved (5 mm) to remove coarse material and macrobenthos and subsequently mixed to homogenize the sediment. This meant that mud snails (*Hydroida ulvae*), which were smaller than 5 mm, were present (289±25 m$^{-2}$) in the experiment (Figure 8.4), providing a source of epiphyte grazing. We found no treatment effect on Hydrobia spp. densities (P=0.288) and the observed densities were not very high compared to the field densities of >4000 m$^{-2}$ (personal observations) and 20,000 m$^{-2}$ (Grilo et al. 2012). *Z. noltii* was collected in the field at the start of the growing season, and was immediately planted in the prepared sediment in the lab, at densities of 50 shoots per aquarium (1250 m$^{-2}$), which represents the average density at the start of the growing season (Vermaat and Verhagen 1996). *A. marina* were obtained from a professional collector (‘t Zeepiertje, Yerseke). On the day of collection, adult *A. marina* with a length of 20-25 cm were put in the aquaria, at densities of two individuals per aquarium (50 m$^{-2}$), which is similar to high adult *A. marina* densities in the field (Suykerbuyk et al. 2012). The *A. marina* were retrieved alive after the termination of the experiment.
Seagrass was harvested at the end of the experiment (t=28 days), after which epiphytes were carefully removed and plants were divided into leaves, sheaths, rhizomes and roots. Total dry weight (g DW) was determined after drying the material for 48 hours at 60°C.

**Statistical analysis**

All results are summarized as means±SE. Prior to analysis, extreme outliers were omitted based on Dixons’ Q-test (Dean and Dixon 1951), and normality of the data was tested with a Shapiro Wilk test and QQ-plots. Non-normally distributed data were log-transformed prior to analysis. We used a three-factor repeated measure ANOVA to compare the effects of the treatments on porewater sulfide concentrations. All other data were tested using a 3-way (nutrients) or 2-way (biomass) ANOVA. Relevant statistical results are presented in the figure legends and in the results section. Differences with P<0.05 were considered significant. All statistical tests were performed with IBM SPSS Statistics 20.0 and R 2.15.

**RESULTS**

We successfully induced sulfide production in the sediment by adding organic matter (OM), which enabled us to study the effects of *A. marina* on porewater sulfide concentrations (Figure 8.1a). We obtained similar results from the sediment sulfide measurements at depths of 10 cm and 20 cm, so these results are not

![Figure 8.1](image1.png)

**Figure 8.1** Porewater sulfide concentrations of treatments (a) with organic matter addition and (b) without organic matter addition. Added organic matter (OM, dashed lines) interacted with both *Arenicola marina* (Ar) and seagrass (*Zostera noltii*) (Sg) to increase sulfide concentrations in the porewater (OM x Ar P=0.009, OM x Sg P=0.049). A highly significant interaction between organic matter addition, *A. marina* and seagrass (OMAr) led to the highest porewater sulfide concentrations (P<0.001) (a). The presence of *A. marina* (Ar) did not significantly affect porewater sulfide concentrations (black symbols, P=0.075). Error bars represent SE (n=5). Note that the y-axis is displayed on a logarithmic scale.

![Figure 8.2](image2.png)

**Figure 8.2** (a) Porewater ammonium (NH₄) concentrations were significantly (P<0.001) lowered by the presence of *A. marina* (Ar), while (b) surface water NH₄ concentrations increased significantly (P=0.032) as a result of bioturbation and bioirrigation by *A. marina*. The presence of seagrass (Sg) or the addition of organic matter (OM) affected neither porewater ammonium nor surface water ammonium concentrations. (c) Porewater nitrate (NO₃) concentrations were not affected by any of the treatments, whereas (d) surface water NO₃ was significantly elevated in the OMAr treatment (P=0.019). (e) Porewater phosphate (PO₄) concentrations were not affected by any of the treatments, but (f) surface water PO₄ concentrations were highly elevated in the Ar treatments (P<0.001). Error bars represent SE (n=5).
presented separately. We observed a sulfide peak in the porewater in the OM treatments on day 7, with sulfide levels of 450-800 μmol L⁻¹ in the OM, OMAr and OMSg treatments, and >4000 μmol L⁻¹ in the OMSgAr treatment. Sulfide levels in the OM treatments (Figure 8.1a) decreased after the observed sulfide peak to levels similar to those in the other treatments (10-100 μmol L⁻¹, Figure 8.1b).

On day 21, a small sulfide peak was observed (25-250 μmol L⁻¹) due to degradation of the organic matter (0.6%), which was naturally present in the sediment. Contrary to our hypothesis, A. marina had not decreased porewater sulfide concentrations at any monitoring moment during our experiment. Similarly, the presence of Z. noltii did not decrease porewater sulfide concentrations. This was also contrary to our expectations, as seagrasses are known to leak oxygen from their roots, which can decrease sulfate reduction rates in the sediment. After the strong initial increase, especially in the OM treatments, porewater sulfide concentrations showed a decrease, very probably due to diffusion or outgassing of (hydrogen) sulfides from the sediment to the water column and subsequently to the air. Porewater NH₄ concentrations were significantly lowered in the presence of A. marina (P<0.001, Figure 8.2a), whereas surface water NH₄ concentrations were significantly increased in the presence of A. marina (P<0.05, Figure 8.2b). Nevertheless, porewater NH₄ concentrations were still more than ten times higher than surface water concentrations. Porewater NO₃ (Figure 8.2c) and PO₄ (Figure 8.2e) levels were not affected by any of the treatments, but surface water NO₃ concentrations were significantly elevated through an interaction between organic matter and A. marina (P<0.05, Figure 8.2d), and the presence of A. marina significantly increased the surface water PO₄ (P<0.001, Figure 8.2f).

Total seagrass biomass m⁻² was more than twelve times higher in the seagrass control treatment (Sg) than in the other treatments (Figure 8.3a). Both A. marina (Ar) and organic matter (OM) had strong negative effects on seagrass biomass (P<0.001 and P<0.001, respectively). The interaction of both stressors (OMAr) even led to a synergistic negative effect (P<0.001), as almost all of the seagrass died in this treatment, resulting in an extremely low (<0.1 g DW m⁻²) seagrass biomass. Additionally, the seagrass leaves in the control treatments all had a healthy green color throughout the experiment, whereas the leaves in all other treatments gradually died off as a result of epiphyte overgrowth. As a consequence of the elevated surface water nutrient levels, epiphyte biomass per leaf (Figure 8.3b) increased strongly in the A. marina treatments (P<0.001), reaching values of up to 100 times more epiphyte biomass than leaf biomass. OM addition also led to significantly more epiphytes per leaf (P=0.036), but this was the result of the absolute decrease in leaf biomass rather than of the absolute increase in epiphyte biomass (as there were no significant effects of OM addition on absolute epiphyte biomass, results not shown). Epiphyte biomass was extremely high in the A. marina treatments, which meant that the leaves were completely overgrown in these treatments (Figure 8.4).

**DISCUSSION**

We falsified our hypothesis that oxygenation of the sediment by A. marina bioturbation and bioirrigation would potentially alleviate sulfide toxicity for seagrass in temperate systems. In our experiment, A. marina had no net beneficial effect on seagrass growth through reducing porewater sulfide concentrations. Secondly, we showed that the sediment-stabilizing seagrass and the sediment-
reworking A. marina are not only physically contrasting ecosystem engineers (as is well known from the literature; e.g. Suykerbuyk et al. (2012)), but that they also display contrasting biogeochemical behavior. Whereas seagrasses are known to be important nutrient sinks (Romero et al. 2006), A. marina increased nutrient fluxes from the sediment to the surface water in our experiment, as was also observed in the field by Asmus and Asmus (1998) on bare mudflats. In our experiment, in the presence of seagrass, these fluxes promoted the growth of epiphytes on the seagrass leaves. In addition, the synergistic negative effects of organic matter addition (sulfide stress) and the presence of A. marina (physical and biogeochemical disturbance) led to the almost complete disappearance of seagrass biomass.

**Sulfide biogeochemistry**

Bioturbation is known to alter sediment biogeochemistry by increasing oxygen penetration into the sediment (Banta et al. 1999, Kristensen 2000, Timmermann et al. 2006), but also by increasing or changing the input of organic matter through sediment reworking (Hines and Jones 1985, Hansen et al. 1996, Kristensen 2000, Kristensen et al. 2012). Bioturbation is therefore expected to have two contrasting effects on sulfate reduction rates in the sediment; increased organic matter input by sediment reworking stimulates sulfate reduction (Hines and Jones 1985, Hansen et al. 1996, Holmer and Nielsen 1997, Valdemarsen et al. 2010), while on the other hand, sulfate reduction rates may also be reduced by the increased oxygen input that results from bioirrigation (Banta et al. 1999, Nielsen et al. 2003). Our nutrient measurements show that active bioirrigation by A. marina took place, as we found decreased porewater nutrient concentrations and increased surface water nutrient concentrations in the A. marina treatments. However, contrary to our expectations, A. marina did not decrease porewater sulfide concentrations. This indicates that A. marina may reduce sulfate reduction rates only locally, in a narrow zone of 5-15 mm surrounding the burrow (Nielsen et al. 2003). Additionally, the fact that we did not find decreasing sulfide concentrations in the A. marina treatments may be explained by A. marina respiration, which consumes a considerable proportion of the oxygen that is pumped into the burrow (Timmermann et al. 2006). Sediment sulfide concentrations became very high in the OMSgAr treatment (>4500 μmol L⁻¹), possibly because the combined effect of increased sulfide production and epiphyte growth resulted in seagrass roots dying off, which in turn stimulated sulfide production by providing easily degradable organic matter.

**Nutrients, seagrass and epiphytes**

As bioturbation is known to stimulate decomposition rates (Andersen and Kristensen 1992, Banta et al. 1999), one might expect an increase in the porewater nutrient concentrations (Meysman et al. 2006). However, in our experiment we found exactly the opposite in the A. marina treatments: porewater nutrient concentrations decreased while surface water nutrient concentrations increased. These results are in agreement with results found in open systems. Volkenborn et al. (2007) found that porewater NH₄ and o-PO₄ increased in plots where A. marina was excluded and Asmus and Asmus (1998) observed a release of ammonium from A. marina sandflats.

It is well known that the sediment-reworing activities of A. marina can hamper seagrass growth by burying or uprooting the plants (Cadée 1976, Philippart 1994, Valdemarsen et al. 2011, Suykerbuyk et al. 2012). However, our experiment included the biogeochemical effects of bioturbation, and showed that these could, in conjunction with physical disturbance, also negatively affect seagrass growth. We found a major decrease in seagrass biomass in all A. marina treatments, and even more so in the interactive OMSgAr treatment, which was probably due to the synergistic effects of sulfide toxicity and epiphyte bloom as a result of nutrient release. Soil sulfide toxicity may have triggered dieback of the root system, which in turn led to even higher sulfide production rates. Excessive epiphyte growth on seagrass leaves is known to reduce light intensity and decrease the uptake of carbon for photosynthesis, which may lead to reduced growth levels and eventually to seagrass leaves dying off (Sand-Jensen 1977, Tomasko and Lapointe 1991).

Several studies have also reported that A. marina bioturbation activities stimulate the growth of bacteria in the burrow system (Grossmann and Reichardt 1991, Ashforth et al. 2011), which is called ‘gardening’ (Hylleberg 1975). This gardening activity, which is an additional ecosystem engineering property of bioturbating organisms such as A. marina, might also extend to the epiphytobenthos, as A. marina might stimulate the growth of epibenthic algae by increasing the flow of nutrients to the surface, thus stimulating the growth of its own food source. We observed (but did not quantify) this in our A. marina treatments (Figure 8.4). This potential positive feedback in systems dominated by A. marina deserves further investigation.

The experiment was conducted in aquaria with a refreshment rate of twice a week. Although this is much lower than the refreshment rate in natural systems, our findings suggest that increased surface water nutrient availability could severely affect seagrasses by promoting epiphyte growth in sheltered bays and estuaries with limited water movement. And even though nutrient loads and subsequent epiphyte loads may have been aggravated by our experimental setup, we still expect similar effects, though less severe, to occur in field situations, as the nutrient efflux from the sediment passes through the canopy. In addition, in more exposed systems, with higher current velocities, A. marina can still increase the nutrient load of the system, as shown by Asmus et al. (1998) and Asmus...
and Asmus (1998), and nutrient release may even rise with increasing current velocities (Asmus et al. 1998), although these loads may not necessarily promote epiphyte blooms. Nonetheless, physical burial or smothering by A. marina may have more detrimental effects on seagrasses in the field than biogeochemical disturbance by bioturbation and bioirrigation. At the same time, however, increased epiphyte loading as a result of bioirrigation may render seagrasses more vulnerable to physical disturbance by sediment reworking. Hence, our study contributes to the unraveling of mechanisms that shape the complex interaction between seagrasses and bioturbating animals, which inhabit seagrass beds, rather than predicting the precise effect of bioirrigation by A. marina on seagrasses in a field situation.

Conclusions and implications
Contrary to our expectations, bioturbation and bioirrigation by A. marina did not alleviate sulfide stress to seagrasses in organic sediments in the present experiment. Instead, we found synergistic negative effects of stressors (i.e. A. marina activity and sulfide toxicity from organic loading, aggravated by A. marina activity). This knowledge adds to our understanding of the complex interaction between seagrasses and A. marina. Although the outcome of interactions in a particular field situation cannot be predicted from our findings, our study shows that (i) increasing A. marina numbers may represent an increased threat to seagrasses, and (ii) organic matter addition will further threaten seagrass beds. A. marina numbers in the Wadden Sea and the Dutch Delta have increased since the 1980s (Philippart 1994, Reise et al. 2008, Eriksson et al. 2010). This increase may have been caused by eutrophication, human exploitation, and/or climate change (Van Beusekom 2005, Reise et al. 2008). Our study shows that such increases in A. marina numbers, particularly in combination with organic matter loading, can seriously threaten seagrass beds. Therefore we expect mutual exclusion rather than co-occurrence of these two ecosystem engineers, even more so as each of the two, A. marina and seagrasses, displays self-facilitating positive feedbacks (Van Wesenbeeck et al. 2007, Van der Heide et al. 2011). The occasional co-occurrence of both species in the field is thus likely to be explained by disturbance or seasonal dynamics (Eklof et al. 2011), rather than being a long-term, stable outcome of biomechanical warfare or competition.

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Chapter 10

SYNTHESIS

Seagrass beds are important and valuable coastal ecosystems that provide many ecosystem services (Orth et al. 2006). They trap nutrients, organic matter and sediment, thereby promoting local nutrient cycling (Marba et al. 2006), and are highly productive, which results in high carbon sequestration rates (Fourqurean et al. 2012). In addition, seagrass beds attenuate waves and currents (Fonseca and Cahalan 1992), and stabilize the sediment, thereby providing a form of coastal protection (Christianen et al. 2013); they also function as nursery areas for commercially important fish and shellfish species (Nagelkerken 2000), and promote high biodiversity by providing food and shelter for a large array of species (Heck and Valentine 2006, Larkum et al. 2006). Finally, seagrass beds are often connected to adjacent coastal ecosystems such as coral reefs, mangroves or saltmarshes, which promotes cross-habitat utilization of mobile species and the exchange of nutrients and carbon (Nagelkerken 2010).

Unfortunately, the valuable seagrass beds are threatened by increasing human activities in coastal areas (Short and Wyllie-Echeverria 1996, Orth et al. 2006, Waycott et al. 2009). Main threats to seagrasses are eutrophication, dredging, salinity changes, erosion of terrestrial habitats, resulting in increased sediment loads, industrial pollution, and altered food web composition as a result of overexploitation (Orth et al. 2006). Many of these stressors also interact, and multiple stressors may simultaneously affect seagrass ecosystem functioning, which complicates the assessment of seagrass health.

In this thesis, we focused on the effects of nutrients, high sulfide levels and trace metal pollution on seagrass dynamics, resilience and restoration, using intertidal Zostera noltii and subtidal Thalassia testudinum as model species. In addition, we highlighted the importance of species interactions for seagrass ecosystem functioning. This chapter integrates the most important findings of this thesis, describing the effects of biogeochemical stressors on seagrass dynamics and species interactions within seagrass beds.
Biogeochemical processes shape seagrass dynamics bottom-up

Seagrass dynamics, such as disturbance-recovery processes and patch dynamics, may be affected by top-down processes such as grazing (e.g. Christianen (2013)), but we here argue that biogeochemistry also plays an important role in shaping seagrass dynamics (Chapter 2, 4). Sulfide is a common toxicant to seagrasses, which is produced during anaerobic decomposition of organic matter by sulfate reducing bacteria (Jørgensen 1984). High porewater sulfide levels may not only disturb plant physiological processes, but also affect seagrass ecosystem functioning by causing large-scale diebacks (Carlson et al. 1994).

**Figure 10.1** Graphic impression of joint detoxification of sulfide by high seagrass densities. High seagrass densities aerate the sediment by radial oxygen loss and thereby not only prevent the intrusion of toxic sulfides, but also oxidize sulfides present. This mechanism enables high seagrass growth in high densities despite high sediment sulfide production. In contrast, when a gap is created in a dense meadow, local joint-detoxification does not work anymore and sulfide levels in the gap may rise drastically compared to the surrounding bed, which complicates recovery (Chapter 4). Thickness of the arrows indicates the strength of the process.

We are the first to demonstrate empirically, that high sediment sulfide levels also affect medium-scale ecosystem processes such as patch dynamics and disturbance-recovery processes in seagrass beds. We found that seagrass patch survival and expansion are constrained by high porewater sulfide concentrations (Chapter 2). Especially patch expansion processes were affected, which hints at a higher vulnerability of low densities (expansion) to toxic substances than high densities (meadow), implying that toxicity may be a density-dependent process. We observed this density dependent toxicity for porewater ammonium in Chapter 2, and we are the first to show density-dependent toxicity of sulfide (Chapter 4). Density-dependent toxicity of sulfide, or ‘joint-detoxification’, is likely caused by high levels of radial oxygen loss in dense seagrass beds (Figure 10.1). Oxygenation of the rhizosphere can prevent intrusion of toxic sulfides, enabling seagrass growth even in high-sulfide sediments. We however showed that processes such as expansion and recovery, which are essential to seagrass meadow maintenance, are more vulnerable to sediment toxins (high ammonium and sulfide levels), due to a lack of the density-dependent feedbacks in bare sediments. This implies that seagrass beds suffering from high porewater sulfide and ammonium concentration, i.e. as a result of eutrophication, are less likely to expand or recover from disturbances, as bare zones may be biogeochemically impregnable areas. This may be especially problematic for patchy and heterogeneous seagrass beds, which are becoming more and more common due to increasing anthropogenic activities (Chapter 2). We thus illustrated that seagrass dynamics (patch expansion, gap recovery) may be shaped bottom-up due to the lack of toxin relieving density-dependent feedbacks (joint-detoxification) in bare areas.

**Eutrophication threatens seagrasses: toxic effects & identification of sources**

Nutrients are essential to seagrass growth and survival, but either a surplus or shortage of nutrients may constrain seagrass performance (Romero et al. 2006). Hence there is a precarious balance between nutrient excess and limitation. Excess nutrient availability is even one of the most serious threats to seagrasses worldwide (Burkholder et al. 2007). Elevated nutrient loads in the water layer can seriously affect seagrasses by either promoting direct toxicity (van Katwijk et al. 1997), or by decreasing light-availability by promoting plankton bloom in the water column. We demonstrated that next to toxic effects of high water column ammonium concentrations (Chapter 2), also high porewater ammonium concentrations might be toxic to seagrasses. Porewater ammonium toxicity may be important as nutrient levels may more easily increase in fine coastal sediments than in a constantly refreshed water column, due to limited exchange rates between pore- and surface water. We found that porewater ammonium concentrations only become toxic in very high concentrations (>2000 μmol L⁻¹) compared to surface water ammonium concentrations (<100 μmol L⁻¹), which
may be explained by a difference in affinity for ammonium between leaves and roots. Moreover, high porewater ammonium concentrations as a result of high decomposition rates by sulfate reducing bacteria often occur simultaneously with high porewater sulfide levels. Co-occurrence of both toxins may increase plant stress and possibly lowers levels at which both stressors become toxic. It may thus be interesting to investigate the interaction between those two porewater toxins. Additionally, it would be interesting to compare the sensitivity of different seagrass species to high porewater ammonium levels, as has been done for surface water ammonium toxicity (Van der Heide et al. 2008, Christianen et al. 2011).

Extremely eutrophic seagrass beds are clearly recognizable as a result of seagrass die-offs due to limited light availability and direct (surface and porewater) toxic effects. However, to prevent seagrass loss caused by eutrophication, it is important to first identify imminent threats from high nutrient loads. A classical way to measure nutrient availability to seagrasses is by determining surface water and porewater nutrient concentrations (NH₄, NO₃, PO₄). However, we found that high nutrient loads are difficult to observe in the water column and in the porewater due to high exchange and flushing rates (Chapter 6), even in the immediacy of a sewage discharge pipe. Although poorly detectable, high nutrient pulses may still promote algal and epiphyte bloom (Figure 1.2), and yield subsequent negative effects to seagrasses. Fortunately, we found that high nutrient loads can be detected in the leaf tissue of late successional and thus slow-growing seagrass species such as *Thalassia* sp., a genus that can be found all over the tropics. *Thalassia* leaf nutrient concentrations (%N and %P) were elevated in the vicinity of the source of eutrophication (sewage discharge, coastal residencies). Using this information we could identify possible threats to the seagrass beds of Curacao (Caribbean), which function as key nursery habitats for commercially important fish species.

**Seagrasses as first-level indicators for eutrophication, trace metal pollution, and winter survival**

We thus found that seagrasses are good bioindicators for nutrient pollution and may even be used to identify point sources of eutrophication. In addition to eutrophication, trace metal pollution may also threaten seagrass ecosystem functioning. Trace metal pollution in seagrass beds results from mining activities, sewage, agriculture and atmospheric deposition (Irvine and Birch 1998, Guzman and Garcia 2002). High trace metal levels may present a direct threat to seagrasses, by negatively affecting photosynthesis and metabolic rates (Ralph and Burchett 1998, Macfarlane and Burchett 2001), but trace metals also accumulate in leaves and rhizomes. We compiled a global benchmark for trace metal levels in seagrass leaves and conclude that seagrasses worldwide may be used as a first-level bioindicator for trace metal pollution in coastal ecosystems (Chapter 5). Seagrasses integrate ecological conditions over a longer timescale (Gonzalez-Correa et al. 2005, Madden et al. 2009) and trace metal levels in seagrass tissue therefore reflect long-term trace metal loading, which may not be measurable in the porewater or surface water (Chapter 5). More importantly, seagrasses are primary producers that provide stock food for a large variety of herbivores and are thus integrated in the coastal food web. Trace metal levels in seagrasses may therefore also be indicative of trace metal concentrations at higher trophic levels, which may be used to detect possible threats to ecosystem services such as fisheries. As most trace metals accumulate in the food web (Wang 2002), high trace metal levels in seagrass leaves may indicate serious trace metal pollution in the whole food web. We found such a relationship for lead (Pb) in the seagrass species *Thalassia testudinum* and the fish species *Haemulon flavolineatum* in the seagrass beds of Curacao (Figure 10.2). Although this fish species does...
not consume seagrass directly, it generally feeds in seagrass beds as a juvenile and sub-adult fish (Verweij et al. 2006). We stress that it is very important to investigate such relationships, as these may even be used to examine if food safety standards for certain trace metals are exceeded. Seagrasses can thus be used as first-level indicators for coastal trace metal pollution, which may indicate threats to ecosystem health and ecosystem services such as fisheries.

Next to the indicator value of seagrasses for both nutrient and trace metal pollution, we also discovered that rhizome starch may predict the winter survival of intertidal temperate seagrasses (Chapter 3). Starch is an important carbohydrate reserve, which is stored in times of excess photosynthesis and used in times of reduced photosynthetic rates, such as the winter in temperate areas. It is especially important for seagrass monitoring and restoration projects to predict the winter survival of seagrasses, as this period is usually less intensely studied, but may present a bottleneck to seagrass survival (Suykerbuyk et al. in prep). We thus found that rhizome starch was indicative of winter survival of seagrasses, knowledge that may also be used for management and restoration purposes.

The importance of species interactions for seagrass ecosystem functioning

A vast array of different species depends directly or indirectly on seagrass ecosystems – either for food, shelter or nursery habitat (Valentine and Heck 1999, Nagelkerken 2000, Williams and Heck 2001). The importance of seagrass beds for grazers is quite well-known (Williams and Heck 2001, Heck and Valentine 2006), but the importance of seagrasses for benthic animals and vice versa is relatively poorly studied. Since seagrass beds are biogeochemical hotspots, it is to be expected that benthic infauna may also affect and be affected by biogeochemical processes in the sediment where they are living. We discovered that seagrasses are globally involved in a mutualistic relationship with lucinid bivalves and their sulfide-oxidizing gill symbionts through a biogeochemical feedback loop (Figure 10.3a, Chapter 7). As this mutualism forms the foundation of seagrass ecosystems, it may be called a keystone mutualism. Keystone mutualisms are also found in other coastal ecosystems such as coral reefs, mangroves, and salt marshes (Bertness 1984, Ellison et al. 1996, Hoegh-Guldberg et al. 2007), but were previously unknown to exist in seagrass beds. Rapid environmental change may disrupt keystone mutualisms and accelerate ecosystem degradation, which has been observed in plant-pollinator interactions, coral reefs and plant-mycorrhiza relationships (Hoegh-Guldberg et al. 2007, Kiers et al. 2010, Burkle et al. 2013). Therefore, it is important to understand and recognize keystone mutualisms such as the seagrass- lucinid bivalve- sulfide-oxidizing gill-symbionts symbiosis. In Chapter 9, we showed that the breakdown of this mutualism due to desiccation stress indeed accelerates habitat degradation in seagrass beds, thus strengthening the importance of this symbiosis.

Many seagrasses in tropical (97%) and sub-tropical (90%) areas are associated with lucinid bivalves, but in temperate seagrass beds, this symbiosis seems less prevalent (56%) (Chapter 7). We therefore hypothesized that another common benthic associate of seagrasses, the lugworm Arenicola marina, might alleviate sulfide stress to temperate seagrasses by aerating the sediment through bioturbation and bioirrigation (Chapter 8). Arenicola marina activity did not, however, reduce porewater sulfide concentrations by flushing or aeration, but instead promoted harmful epiphyte bloom on seagrass leaves as a result of increased nutrient release by lugworm bioirrigation. The importance of the association between seagrasses and lucinid bivalves seems to decrease from tropical to temperate areas (97% vs. 56% presence of lucinids in seagrass beds, Chapter 7). Similarly, seagrass sensitivity to sulfide varies between species and between geographical ranges. Temperate Zostera noltii and Zostera marina seem fairly resistant to high sulfide levels (Chapter 2, Lamers et al.
Seagrass restoration in the Oosterschelde (Box 1.1) has only been successful at two out of six tidal flats, one likely as a result of nearby natural bed expansion, and the other one likely as a result of transplanting (Suykerbuyk et al. in prep). Although large areas with high densities of *Zostera noltii* have been transplanted, survival of the transplants has been limited. We therefore identified several factors that could have affected transplant success.

**Porewater biogeochemistry:** high porewater sulfide levels are toxic to seagrasses and may strongly limit seagrass patch survival and expansion (Chapter 2). In addition, lucinid bivalves do not live in the Dutch Delta, sulfide levels are thus not reduced. However, we did not measure porewater sulfide concentrations >100 μmol L⁻¹ in the transplants between 2007-2013 (Giesen et al. 2014). Hence, high sulfide levels have not affected transplant survival.

**Nutrient limitation:** seagrasses growing on terrigenous sediments may be nitrogen limited (Udy and Dennison 1997). We therefore measured seagrass nutrient content of *Zostera noltii* in the Oosterschelde and discovered that seagrass growth and survival in the Oosterschelde can not be attributed to nutrient limitation as we measured leaf %N of 3.8 %N and 0.9 %P in high summer (Chapter 2), which is high above the general limitation boundaries of 1.8 %N and 0.2 %P (Duarte 1990).

**Hydrodynamics:** High waves and high current velocities may decrease seagrass survival and expansion. We did however find successful transplants on both sheltered (Roelshoek) and exposed (Dortsman) sites. Hydrodynamics alone can therefore not be responsible for the lack of transplant success.

**Bioturbation:** Suykerbuyk et al. (2012) demonstrated that high adult lugworm densities decreased initial transplant survival, so lugworm excluding shell layers were implemented in all seagrass transplants from 2010 onwards. Furthermore, bioturbation may also have interacted with sediment dynamics (see below, Suykerbuyk et al. in prep).

**Winter survival:** We did not find any relationship between the preceding year’s transplant success and next year’s growth success (Chapter 3) and therefore looked at wintering processes. We found that rhizome starch levels can be indicative of next year’s growth success. Low rhizome starch concentrations may therefore be a bottleneck to transplant winter survival.

**Macroalgae:** Dense macroalgal layers may suffocate seagrasses and promote sulfide stress. We quantified macroalgal presence in the transplants, but seldom observed high densities of macroalgae (Giesen et al. 2012).

**Grazing:** Brent geese (Brenta bernicla) have been observed grazing on dwarf eelgrass in the Oosterschelde. Geese grazing pits have been observed in both natural seagrass beds and seagrass transplants in the Oosterschelde (Giesen et al. 2012). Although grazing may not have detrimental effects on healthy seagrass populations (Ganter 2000, van der Heide et al. 2012), vulnerable transplants may be negatively affected when heavily grazed.

**Sediment dynamics:** The implementation of the storm surge barrier in the Oosterschelde reduced the tidal regime and the supply of sediments from the North Sea into the Oosterschelde. These changes resulted in a loss of intertidal area and increased erosion of tidal flats, a process called ‘sand hunger’ (zandhonger). Due to this process, about ~50ha of intertidal habitat is lost annually, which is a catastrophe for foraging birds, but may also affect intertidal *Zostera noltii*. Dwarf eelgrass seems to have retreated to the areas with low sediment dynamics and low erosion rates (Suykerbuyk et al. In prep.). Local sediment dynamics may also have affected transplant survival.

**Genetic diversity:** High genetic diversity in seagrass beds can lead to increased resistance to disturbances such as grazing and heat stress (Williams 2001, Hughes and Stachowicz 2004, Reusch et al. 2005). Decreasing seagrass populations such as in the Oosterschelde may suffer from impoverished genotypic diversity, which may be reflected by low restoration success. We did however not investigate genotypic diversity of *Zostera noltii* in the Oosterschelde, but would recommend this for future research.

Concluding, the lack of restoration success in the Oosterschelde can probably be attributed to a complex interplay of many known and unknown factors.
10. Synthesis

Thus are poorly adapted to high sulfide concentrations. Conditions, may explain why seagrasses from those areas never experience, and thus are poorly adapted to, high sulfide concentrations.

Lessons for seagrass management and restoration

Seagrasses are important coastal ecosystems, but have been globally declining for decades (Orth et al. 2006, Waycott et al. 2009). To counteract and reduce these losses, many attempts to seagrass restoration have been undertaken, with various degrees of success (van Katwijk et al. submitted). Restoration success is related to the planting scale, with an increasing success rate with higher amounts of planted shoots, but also site characteristics, species, and planting techniques affect seagrass restoration performance (van Katwijk et al. submitted). This effect of planting scale on restoration success, may be attributed to the existence of positive feedback mechanisms in seagrass beds (Van der Heide 2009). A certain threshold size or density is needed to generate self-facilitation of seagrasses and increase restoration success (Van der Heide 2009, van Katwijk et al. submitted). However, these feedbacks are generally lacking in areas selected for restoration as a result of low or lacking seagrass densities. Restoration should thus be aimed at introducing high-enough seagrass densities to restore self-facilitating feedbacks. Furthermore, we showed (Chapter 2, 4) that especially processes important to successful restoration, such as expansion and recovery processes, are vulnerable to stressors (here: biogeochemical toxins) due to the lack of density-dependent stress-alleviating feedbacks (joint-detoxification). This again illustrates the importance of density thresholds for self-facilitating feedbacks, which should be taken into account by restoration projects, although important feedbacks should first be identified and studied.

Next to feedbacks, restoration success may also be affected by site characteristics such as background stress levels. Low, but continuous stress levels, such as elevated nutrient and sulfide levels (Chapter 2, 4, 6), trace metals (Chapter 6) and bioturbation pressure (Chapter 8) may interact with disturbance-recovery processes and thereby affect ecosystem resilience (Chapter 4) and restoration success. So in addition to identifying the presence (or lack) of feedbacks for management and restoration purposes, it is also important to identify background stress levels as they may interact with restoration attempts.

In this thesis, we reveal several tools for identifying background stress levels in seagrass beds such as high nutrient and trace metal levels (Chapter 5, 6). We found that high nutrient levels (%P and %N) in seagrass leaves are indicative of point source eutrophication. In addition, high trace metal levels in the coastal environment are easily detected by studying heavy metal concentrations of seagrass leaves, which generally integrate metal loads over a longer time scale. Although trace metals by itself may not present a threat to seagrasses, seagrass trace metal levels may indicate threats to other trophic levels connected to the seagrass habitat. Additionally, we found that seagrasses in turn are also depending on co-occurring species, such as lucinid bivalves (Chapter 7, 9), which could be less resistant to metal pollution.

Overall, we have provided several important messages and tools for seagrass conservation, management and restoration in this thesis: 1) Meadow maintenance processes such as expansion and recovery may be constrained by the lack of density-dependent feedbacks, 2) background conditions and stress levels may interact with disturbances such as a restoration attempts, 3) Rhizome starch may be used to predict seagrass survival during/after low light level events such as winter, eutrophication induced algal blooms and dredging, 4) nutrient and trace metal pollution in coastal ecosystems may easily be detected by studying concentrations of seagrass leaves, 5) species interactions and keystone mutualisms should be taken into consideration for management and restoration of seagrass ecosystems, as the breakdown of keystone mutualisms may accelerate habitat degradation.

Concluding summary

Seagrass ecosystems are threatened by eutrophication (Chapter 2, 6) and trace metal pollution (Chapter 5), as a result of increased human activities in coastal areas. We showed that such threats to seagrass ecosystem functioning can be discovered by using seagrass nutrient and metal concentrations as bioindicators. The indicator function of seagrasses may also be used to gain insight in plant-level processes, as our findings illustrated that rhizome starch can be indicative of seagrass winter survival (Chapter 3). Next to promoting plankton bloom and subsequent reduction of light availability, eutrophication also promotes the input of organic matter in seagrass beds. This organic matter is mainly decomposed anaerobically, resulting in toxic sediment sulfide levels, which may affect seagrass meadow-scale dynamics (Chapter 2, 4), due to the lack of density-dependent feedbacks in low seagrass densities. Seagrasses all over the world are coping with sulfide stress, but we discovered that a three-stage mutualism between seagrasses, lucinid bivalves and their sulfide-oxidizing gill bacteria globally enables growth of seagrasses in organic sediments (Chapter 7). The importance of this keystone mutualism is illustrated in Chapter 9, which showed that breakdown of this mutualism may lead to accelerated habitat degradation. Another benthic animal that frequently inhabits seagrass beds is the lugworm Arenicola marina. Seagrasses and lugworms are involved in a biomechanical warfare, as they display...
contrasting properties. In addition to the negative effects of lugworm bioturbation by burial, we found that lugworms may also negatively affect seagrasses by altering biogeochemistry; lugworm activity increases nutrient fluxes, promoting detrimental epiphyte bloom on seagrass leaves (Chapter 8). These examples (Chapter 7, 8, 9) emphasize the importance of including species interactions in seagrass ecosystem studies.

Based on our findings, I conclude that it is important to study all levels and aspects of biogeochemical stressors on seagrass ecosystems, from plant- to meadow-scale (Figure 10.4) and including species interactions, to gain a deeper understanding of ecosystem functioning, in order to provide useful tools for seagrass conservation, management, and restoration.

Figure 10.4 Graphical abstract of this thesis. Chapter numbers are indicated in red. Proportions of plants and animals are not based on real measures and species composition is not a realistic reproduction of a real-life seagrass ecosystem.

ACKNOWLEDGEMENTS
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Chapter 10  Synthesis

Resilience & Restoration

Chapter 2  What are the biogeochemical constraints for seagrass patch survival and expansion?

Chapter 3  What are the bottlenecks for the winter survival of Zostera noltii in the Oosterschelde?

Chapter 4  What are the effects of local environmental conditions and feedbacks on the resilience of seagrasses?

Pollution

Chapter 5  What is the overall status of trace metals in seagrass beds worldwide and in the Caribbean in particular?

Chapter 6  What is the nutrient status of the seagrass beds on Curaçao and Bonaire?

Biogeochemical species interactions

Chapter 7  How do seagrasses worldwide cope with sulfide stress in organic sediments?

Chapter 8  What is the biogeochemical effect of lugworm activity on seagrasses?

Chapter 9  What happens with the three-stage seagrass-lucinid-gill-bacteria symbiosis in seagrass beds under desiccation stress?

Question

Answer

High porewater sulfide and ammonium concentrations are toxic constraints to seagrass patch expansion and survival.

Autumn starch reserves indicate next year’s growth success of Zostera noltii.

Local environmental conditions, such as sediment grain size and organic matter level, and feedbacks affect disturbance-recovery dynamics of intertidal seagrasses.

Seagrasses are good bioindicators of trace metal pollution in coastal ecosystems worldwide. Additionally, some bays in the Caribbean are heavily polluted by trace metals, which may form a potential threat to higher trophic levels.

Eutrophication threatens important nursery habitats on Curaçao in Spanish Water Bay and Piscadera Bay.

A common mutualistic interaction between seagrasses, lucinid bivalves and their symbiotic gill-bacteria forms the foundation of seagrass ecosystems by reducing sulfide stress.

Lugworms do not relieve sulfide stress to seagrasses, but instead stimulate nutrient fluxes from the porewater to the surface water, which in turn promote detrimental algal bloom on the leaves.

A mutualism-breakdown due to desiccation stress accelerates habitat degradation in seagrass beds.

Table 10.1 Overview of questions and answers in this thesis


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Otero Otero, A. and J. Roman Lobo. 2009. Macroinvertebrados asociados a pastos marinios (Thalassia testudinum) en el golfo de Morrosquillo (Zone de Berrugas) Departamento de Sucre. Universidad de Sucre, Sincelejo, Columbia.
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Y


Z


Seagrasses are rooting and flowering plants that can form extensive meadows in shallow coastal zones all over the world. These meadows form key-ecosystems for a wide diversity of species, including some highly endangered species such as dugongs and sea turtles. Seagrass beds are among the most productive and valuable ecosystems on earth, comparable to coral reefs, salt marshes and mangroves. These often strongly inter-connected coastal ecosystems provide important ecosystem services such as carbon sequestration, nutrient cycling, coastal protection by wave attenuation and sediment stabilization, and form a nursery habitat for commercially important fish species.

Unfortunately, seagrass beds all over the world are threatened and rapidly disappearing as a result of human activities in coastal areas. Threats to seagrasses include increased nutrient loads of coastal waters, industrial pollution, increased sediment loads, dredging, and erosion. Many of these stressors may simultaneously affect seagrass beds, and in this thesis, we focused on the effects of some particular stressors: high sulfide levels, pollution by heavy metals, and the effects of high nutrient loads on seagrass ecosystems. We especially looked at the importance of species inhabiting seagrass beds and seagrass plant and meadow scale processes in relation to these stressors.

Biogeochemical processes and nutrients

Biogeochemical processes are generally chemical processes in the sediment, mainly involving nutrient cycling processes, often performed by soil bacteria. Biogeochemical processes occur in every soil, but in marine sediments, biogeochemistry is especially affected by the composition of the seawater. Seawater naturally contains a lot of sulfate, a component that can be converted to sulfide under low oxygen conditions. Sulfide is volatile and can be recognized by the characteristic rotten egg smell. This horrid smelling compound is toxic to seagrasses and has been known to cause large-scale diebacks of seagrass beds. We discovered that small-scale expanding seagrass patches, which may result from seagrass habitat degradation, are much more vulnerable to sulfide toxicity that high seagrass densities. This may be due to a process called density-dependent...
totoxicity, as high seagrass densities pump oxygen into the root zone, which forms a protective layer against toxic sulfides. Furthermore, not only sulfides are toxic to low seagrass densities, extremely high nutrient levels may also result in disturbed seagrass patch dynamics.

Although nutrients (mainly nitrogen – N – and phosphorus – P –) are essential to seagrass growth, a surplus of nutrients may have negative effects on seagrasses, not only by affecting growth of patches, but mostly by decreasing the light availability for seagrasses, which need sunlight for photosynthesis, by promoting algal bloom in the water column. It is thus important to identify high nutrient loads to prevent loss of valuable seagrass beds. The seagrass beds of Curaçao and Bonaire are valuable nursery habitats for many commercially important fish species, but may be threatened by high nutrient loads (eutrophication). Hence, we evaluated the nutrient status of these seagrass beds and discovered that although nutrient loads could not be measured in the water layer, seagrass leaf tissue of *Thalassia testudinum* accumulated nutrients (N and P) in the vicinity of a nutrient source. Using this information, we could identify possible threats to the seagrass beds of Curaçao and Bonaire.

**Heavy metal pollution**

In addition to high nutrient levels, heavy metals (or trace metals) such as mercury (Hg), lead (Pb), cadmium (Cd), chromium (Cr), originating from mining activities, sewage or agriculture, may also threaten seagrass beds and their inhabitants. We found that seagrasses can indicate long-term high nutrient loads as they apparently integrate ecological conditions over a longer time-scale. We also tested this for heavy metal pollution in seagrasses worldwide, and found that seagrass tissue also reflects long-term heavy metal pollution, which may not be measurable in the water layer. This is important, as seagrasses form the base of the food chain, and heavy metals may accumulate in the higher levels of the food chain. Heavy metal accumulation in seagrasses may thus be used as a first-level indicator for coastal metal pollution, indicating potential threats to fisheries.

**The importance of species interactions for seagrass beds**

Many species depend directly or indirectly on seagrass beds, not only animals, but also algae, and even bacteria inhabit seagrass beds. Seagrass beds are ecosystems with high biogeochemical activity and some animals living in seagrass beds may affect the biogeochemical processes in the sediment. We discovered that seagrasses are globally involved in a specialized partnership – ‘mutualism’ – with clams from the Lucinidae family, where the clams do not only benefit from the presence of seagrasses, but seagrasses in turn do need the clams to survive. This extraordinary relationship is based on another mutualism as the lucinid clams have special bacteria, living in their gills, which consume the toxic sulfides produced in seagrass sediments. This symbiotic partnership enables seagrasses to grow in areas where they would have died from sulfide stress if the clams had not been present. The clams in turn need the seagrasses as they pump oxygen into the soil, which they need to cope with toxic sulfides. This mutualism may be called a keystone mutualism, as it forms the foundation of seagrass ecosystems, on which many other species depend. Although this mutualism forms a strong foundation of seagrass ecosystems, we found that breakdown of this mutualism may accelerate ecosystem collapse. This is important to recognize, as many other important ecosystems depend on keystone mutualisms such as plant-pollinator interactions, coral-zooxanthellae relationships and plant-mycorrhiza symbioses. The important seagrass-lucinid clam-gill bacteria symbiosis, which enables seagrasses to grow in toxic soils, is less common in temperate areas. However, as sulfide may still pose a threat to seagrasses in those areas, we thought that perhaps another interaction between seagrasses and the common lugworm *Arenicola marina*, which produces characteristic castings on beaches and mudflats, could relieve sulfide stress for seagrasses. As these worms move around sediment (bioturbation) to feed on organic particles and algae, they mix oxygen into the soil, potentially decreasing toxic sulfide levels. However, in our experiment, we did not find any positive effects of lugworm activity on seagrasses. Lugworms did not relieve sulfide stress, but instead buried seagrasses and additionally promoted algal bloom on seagrass leaves by pumping nutrients from the sediment to the water layer. Thus, there might still be an alternative mechanism that relieves sulfide stress to temperate seagrasses, but it has yet to be discovered.

**Lessons for seagrass management and restoration**

Seagrass beds are very important ecosystems, but have been globally declining for decades. Therefore, many attempts to restore seagrass beds have been undertaken, with varying degrees of success. Lack of restoration success may sometimes be attributed to too low planting densities, as seagrasses generally need high-enough densities to restore self-facilitating feedbacks. We found that especially processes important to restoration such as expansion and recovery are vulnerable to stressors such as sulfide and high nutrient concentrations, due to the lack of density-dependent stress-alleviating feedbacks. Next to feedbacks, background stress levels such as heavy metal pollution, high nutrient levels, sulfide and bioturbation pressure may also affect seagrass restoration success. It is thus important to identify those background stress levels before attempting to restore seagrass beds. Furthermore, we found that rhizome starch levels may be used to predict next year’s transplant success, which was previously hard to predict based on the preceding summer’s seagrass density. Finally, as we discovered that a keystone mutualism forms the foundation of seagrass ecosystems, we stress that this mutualism should be taken into account when restoring seagrass beds. All these discoveries may be used to improve seagrass management and restoration efforts.
Conclusion
We conclude that seagrass beds are threatened by multiple stressors such as high nutrient levels, heavy metal pollution and sulfide, as a result of human activities in coastal areas. These stressors may interact and synergistically affect seagrass health and functioning. It is therefore important to detect potential threats to seagrass ecosystems by using seagrass nutrient and heavy metal concentrations as bioindicators of long-term pollutant loads. We discovered that stressors such as sulfide and high nutrient levels may affect meadow-scale dynamic processes, due to the lack of self-facilitating feedbacks in low seagrass densities. However, we also found that seagrasses have a mechanism to protect themselves from sulfide stress: A keystone mutualism between seagrasses, lucinid clams and their gill bacteria, enables seagrasses to grow in sulfide-rich soils all over the world. This illustrates the importance of studying all levels and aspects of biogeochemical stressors on seagrass ecosystems, including species interactions, to gain a deeper understanding of ecosystem functioning, which may also provide useful tools for seagrass conservation, management, and restoration.
Zeegrassen zijn wortelende, bloeiende planten die uitgestrekte velden kunnen vormen in ondiepe kustwateren over de hele wereld. Zeegrasvelden vormen sleutel-ecosystemen, voor een brede diversiteit aan soorten, waaronder bedreigde diersoorten zoals zeeschildpadden en zeekoeien. Zeegrasvelden behoren tot de meest productieve en waardevolle ecosystemen op aarde, vergelijkbaar met koraalriffen, mangroves en kwelders. Deze kustecosystemen zijn vaak sterk met elkaar verbonden en leveren belangrijke ecosysteemdienssten, zoals koolstofopslag, recycling van voedingsstoffen, kustbescherming door golfdamping en stabilisatie van de bodem, en ze dienen als kraamkamer voor commercieel belangrijke vissoorten. Helaas worden zeegrasvelden over de hele wereld bedreigd en het areaal aan zeegras gaat snel achteruit door toegenomen menselijke activiteiten in kustgebieden. Zeegrassen worden o.a. bedreigd door sterk verhoogde concentraties van meststoffen in kustwateren, industriële vervuiling, baggerwerkzaamheden en erosie. Veel van deze stressoren kunnen, apart of tegelijkertijd, een negatief effect hebben op zeegrassen en op de algen en dieren die afhankelijk zijn van zeegrasvelden.

In dit proefschrift ligt de nadruk op een aantal specifieke stressoren: hoge sulfide concentraties, vervuiling door zware metalen, en de effecten van verhoogde niveaus van meststoffen op zeegras. Biogeochemische processen en voedingsstoffen

Biogeochemische processen en voedingsstoffen

Biogeochemische processen zijn chemische processen die in de bodem plaatsvinden. Ze worden vaak uitgevoerd door bodembacteriën. Biogeochemische processen vinden plaats in iedere bodem, maar in zoute (mariene) bodems worden deze processen sterk beïnvloed door de samenstelling van zeewater. Zeewater bevat van nature veel sulfaat, een stof die onder zuurstoffloze omstandigheden in de bodem kan worden omgezet in sulfide. Sulfide is een vluchtige stof die gemakkelijk te herkennen is aan de typische ‘rotte
eieren’ lucht. Dit vies ruikende goedge is erg giftig voor zeegrassen. Een hoge sulfideproductie in de bodem heeft zelfs op sommige plekken geleid tot massale zeegrassterfte.

Wij hebben ontdekt dat kleinschalige, zich uitbreidende zeegrasveldjes, die het resultaat zijn van versnipperde aaneengesloten zeegrasvelden, veel gevoeliger zijn voor giftig sulfide dan hoge zeezadidgehden. Dit zou kunnen worden veroorzaakt door een proces dat ‘dichtheidafhankelijke toxiciteit’ wordt genoemd. Hoge zeezadidgehden pompen namelijk veel zuurstof in de bodem. Dat vormt een natuurlijk beschermend laagje tegen giftig sulfide. Lage zeezadidgehden kunnen niet zoveel zuurstof de bodem inpompen, waardoor het sulfide door kan dringen in de wortels. Bovendien is niet alleen sulfide giftig voor lage zeezadidgehden, ook hoge concentraties meststoffen in de bodem kunnen kleinschalige zeegrasveldprocessen verstoren.

Meststoffen, oftewel voedingsstoffen (voornamelijk stikstof – N – en fosfor – P –) zijn essentieel voor de groei van zeegrassen. Echter, de aanwezigheid van teveel voedingsstoffen kan juist negatieve effecten hebben op zeegrassen. Deze negatieve effecten worden vooral veroorzaakt doordat een teveel aan voedingsstoffen in het water door het stimuleren van algenbloei de beschikbaarheid van licht, dat zeegrassen nodig hebben voor fotosynthese, negatief beïnvloed. Ook kunnen te hoge concentraties voedingsstoffen rechtstreekse giftige effecten hebben op zeegrassen. Om verder verlies van waardevolle zeezadigeveld te voorkomen is het van groot belang hoge belasting van meststoffen in kustwateren op te sporen. De zeegrasvelden van Curacao en Bonaire zijn belangrijke kraamkorsten voor commercieel belangrijke vissoorten, maar worden mogelijk bedreigd door te hoge voedingsstofbelasting (eutrofiëring). Daarom hebben we de voedingsstof-status van deze zeegrasvelden geclassificeerd en ontdekt dat, hoewel hoge concentraties voedingsstoffen niet in het water konden worden gemeten, deze waardes wel terug te meten waren in het blad van de zeegrassoort Thalassia testudinum. Vooral in de nabijheid van een bron van voedingsstoffen, zoals een rioleringspijp of huizen, waren de concentraties voedingsstoffen in de planten sterk verhoogd. Gebruikmakend van deze informatie waren we in staat om mogelijke bedreigingen te identificeren voor zeegrassen op Curacao en Bonaire.

Vervuiling door zware metalen

Naast verhoogde concentraties voedingsstoffen kan het voortbestaan van zeezadigevelden ook bedreigd worden door zware metalen zoals kwik (Hg), lood (Pb), cadmium (Cd), en chroom (Cr), afkomstig van mijnactiviteiten, rioolwater en landbouw. Zeegrassen kunnen gebruikt worden als indicator voor lange-termijnbelasting door hoge concentraties voedingsstoffen. Wij hebben getest of dit ook geldt voor zware metalen, en vonden dat zware metalen concentraties in zeegrasweefsel (blad en wortelstok) wereldwijd ook lange-termijnbelasting door zware metalen weergeven. Dit is een belangrijk gegeven omdat zeegrassen de basis vormen van de voedselketen en zware metalen zich mogelijk opstapelen in de hogere niveaus van de voedselketen. Dit vormt een mogelijk risico voor mensen als het consumptievis betreft. Zware metalen concentratie in zeegras kan dus mogelijk worden als een eerste indicatie voor zware metalen vervuiling in kustgebieden en de mogelijke gevaren hiervan voor de visserij.

Het belang van soorteninteracties voor zeegrasbedden

Veel soorten zijn direct of indirect afhankelijk van zeegrasbedden. Niet alleen diersoorten, maar ook algen en zelfs bacteriën bewonen zeezadigevelden. Zoals eerder genoemd zijn zeezadigevelden een omgeving met een hoge biogeochemische activiteit en sommige dieren die in het zeegras leven kunnen deze processen mogelijk beïnvloeden, al is hier nog in zeer beperkte mate onderzoek naar gedaan. Wij hebben ontdekt dat zeegrasvelden wereldwijd betrokken zijn in een gespecialiseerd partnerschap – ‘mutualisme’ – met tweekleppige schelpdieren uit de Lucinidae familie, waarbij de schelpdieren niet alleen profijt hebben van de aanwezigheid van zeegras, maar waarbij zeegrassen op hun beurt ook de schelpdieren nodig hebben om te overleven. Deze bijzondere relatie is gebaseerd op een andere symbiose; de schelpdieren hebben namelijk speciale bacteriën in hun kieuwen, die het giftige sulfide dat in zeegrassbodems wordt geproduceerd kunnen gebruiken voor hun energievoorziening. Dit mutualisme tussen zeegrassen, schelpdieren, en hun kieuwbacteriën, heeft er voor gezorgd dat zeegrassen kunnen groeien in een omgeving waar ze anders waren doodgegaan door sulfidestress. Deze symbiose kan een ‘sleutelmutualisme’ worden genoemd, omdat het de fundering vormt voor het functioneren van zeegrassosystemen, waar ook veel andere soorten van afhankelijk zijn. We vonden echter dat, hoewel dit mutualisme een stevige basis vormt voor zeegrassosystemen, de afbraak van dit mutualisme, door bijvoorbeeld een externe stressor, juist de degradatie van een ecosysteem versnelt. Dit is belangrijke informatie, omdat over de hele wereld ecosystemen afhankelijk zijn van soortgelijke sleutelmutualismes, die onder druk staan door o.a. klimaatverandering en vervuiling. Voorbeelden van soortgelijke sleutelmutualismes zijn plant-bestuiver interacties, koraalriften die afhankelijk zijn van hun symbiose met algjes (zooxanthellae) en plant-mycorrhiza interacties (bodemschimmels).

De belangrijke symbiose tussen zeegrassen, schelpdieren en kieuwbacteriën, die het voor zeegrassen mogelijk maakt om in sulfiderijke bodems te groeien, is echter minder algemeen in de gematigde streken. Omdat ook in deze streken sulfidestress kan voorkomen, dachten wij dat mogelijk een andere interactie tussen zeegras en bodemdieren verantwoordelijk zou kunnen zijn voor een verlaging van sulfidestress. Hierbij dachten wij aan de veelvoorkomende wadpier, Arenicola marina, die kan worden herkend aan de typische wadpierhoopjes die...

Leren voor zeegrasmanagement en -herstel

Zeegrasvelden zijn belangrijke ecosystemen die al jaren een sterke achteruitgang vertonen. Daarom hebben er al veel pogingen plaatsgevonden om zeegrasvelden te herstellen, met verschillende gradaties van succes. Het gebrek aan restauratiesucces kan soms worden toegeschreven aan te lage plantdichtheden. Zeegrassen hebben over het algemeen hoge dichtheden nodig om zelffaciliterende terugkoppellings mechanismen, zoals het remmen van golven, in stand te houden. Wij vonden dat juist processen die van belang zijn voor herstel, zoals uitgroei en herstel, gevoelig zijn voor stressoren door het ontbreken van deze stressverlagende, zelffaciliterende terugkoppellings mechanismen. Naast terugkoppellings mechanismen zijn ook achtergrondstressniveaus, zoals concentraties van zware metalen, voedingsstoffen, sulfide en bioturbatiedruk van invloed op zeegrasrestauratiesucces. Dus is het belangrijk om deze achtergrondstressoren te identificeren, voordat restauratie daadwerkelijk plaatsvindt. Tevens hebben we ontdekt dat hoewel zeegrasrestauratiesucces na de winter moeilijk te voorspellen is op basis van zeegras dichtheid in het voorgaande jaar, het wel mogelijk is om een voorspelling te doen over zeegrasrestauratiesucces na de winter op basis van zetmeel niveaus in de wortelstokken van zeegrass vóór de winter. Tot slot willen we benadrukken dat het sleutel-mutualisme tussen zeegrassen, schelpdieren en hun kieuwbacteriën, de basis vormt van zeegras ecosystemen en daarom ook in acht moet worden genomen bij zeegras herstel. Al deze ontdekking kunnen worden gebruikt om zeegrasmanagement en herstel te verbeteren.

Conclusie

We concluderen dat zeegrasvelden wereldwijd worden bedreigd door veel verschillende stressoren zoals hoge concentraties voedingsstoffen, zware metalen vervuiling en sulfide, die het gevolg zijn van menselijke activiteiten in kustgebieden. Deze stressoren kunnen interacteren en gezamenlijk de gezondheid en het functioneren van zeegrasbedden negatief beïnvloeden.
Les herbiers de végétation aquatique sont composés de plantes marines (angiospermes marines) qui s’enracinent dans le sédiment et fleurissent pour former de vastes prairies dans les zones côtières peu profondes partout dans le monde. Ces prairies forment des écosystèmes clé ainsi que de véritables niches écologiques permettant le développement d’une grande diversité d’espèces, y compris certaines espèces très menacées comme les dugongs et les tortues marines. Les herbiers marins sont parmi les écosystèmes les plus productifs et les plus précieux sur terre, comparables aux récifs coralliens, aux marais salants ou aux mangroves. Ces écosystèmes côtiers souvent fortement interconnectés fournissent des services écosystémiques importants, tels que la séquestration du carbone, le cycle des nutriments, la protection du littoral par l’atténuation des vagues et la stabilisation des sédiments, et forment une zone d’alevinage pour les espèces de poissons à grande importance commerciale.


Les processus biogéochimiques et les nutriments
Les processus biogéochimiques sont généralement définis comme des processus chimiques dans les sédiments, affectant principalement le cycle des nutriments, et souvent effectués par des bactéries présentes dans le sédiment. Ces processus biogéochimiques ont lieu dans tous les types de sols, cependant dans les
sédiments marins, ils sont particulièrement affectés par la composition de l'eau de mer. L'eau de mer contient naturellement un grand nombre de sulfates, un composant qui peut être converti en sulfure dans des conditions de faible teneur en oxygène. Le sulfure est un composant volatile et peut être reconnu par son odeur d'œuf pourri caractéristique. Ce composé à l'odeur fétide est aussi toxique pour les plantes d'herbiers marins et a été reconnu comme l'un des facteurs pouvant provoquer le dépérissement à grande échelle des herbiers marins. Nous avons constaté que l'expansion des zones d'herbiers à faible densité, qui pourraient résulter de la dégradation progressive de ces herbiers, est bien plus vulnérable à la toxicité des sulfures que celle (l'expansion) des herbiers de plus forte densité. Cela peut être dû à un processus caractérisé par une dépendance « toxicité-densité », étant donné que de fortes densités de zostères peuvent permettre aux plantes de pomper plus d'oxygène dans la zone racinaire, et ainsi de former une couche protectrice contre les sulfures toxiques. En outre, non seulement les sulfures sont toxiques pour les herbiers de faible densité, mais les concentrations extrêmement élevés en nutriments peuvent également entraîner des perturbations en terme d'évolution spatiale des herbiers (dynamique de patchs).

Bien que les nutriments (principalement l’azote - N - et le phosphore - P -) soient essentiels à la croissance herbiers, un excès de nutriments peut avoir des effets négatifs sur les herbiers, non seulement en affectant la croissance des « patchs » d’herbiers, mais surtout par la diminution de la luminosité disponible pour ces herbiers, qui ont besoin de lumière pour la photosynthèse, en favorisant la prolifération d'algues dans la colonne d'eau. Il est donc important d'identifier les charges en éléments nutritifs pour prévenir la perte des herbiers. Les herbiers de Curacao et de Bonaire sont des habitats d’alevinage rares pour de nombreuses espèces de poissons commercialement importants, mais peuvent être menacés par des charges élevées en nutriments (eutrophisation). Par conséquent, nous avons évalué l’état nutritionnel de ces herbiers et découvert que, bien que les charges en nutriments soient difficilement mesurables dans la colonne d'eau, les tissus végétaux provenant des feuilles de Thalassia testudinum peuvent accumuler des nutriments (N et P) à proximité d’une source de nutriments. En utilisant cette information, nous avons pu identifier d’éventuelles menaces pour les herbiers de Curacao et Bonaire.

La pollution par les métaux lourds

En plus des niveaux élevées en nutriments, les métaux lourds (ou métaux traces) comme le mercure (Hg), le plomb (Pb), le cadmium (Cd), le chrome (Cr), originaires des activités minières, des rejets d'eaux usées ou de l'agriculture, peuvent aussi menacer les herbiers et leurs habitants. Nous avons constaté que les herbiers permettent d’indiquer l’existence de fortes charges en nutriments à long terme car ils semblent pouvoir intégrer les conditions écologiques sur une échelle de temps plus longue. Nous avons également testé cela pour la pollution par les métaux lourds dans des herbiers du monde entier, et nous avons constaté que les tissus des plantes reflètent également la pollution par les métaux lourds à long terme, pollution qui ne semble pas être mesurable dans la colonne d'eau. Ceci est important étant donné que les herbiers forment la base de la chaîne alimentaire, et que les métaux lourds peuvent s'accumuler dans les niveaux supérieurs de cette même chaîne alimentaire. L'accumulation de métaux lourds dans les herbiers peut donc être utilisée comme un indicateur de premier niveau de pollution côtière par les métaux lourds, signalant les potentielle menaces de pollution pour la pêche.

L'importance des interactions entre les espèces pour les herbiers

De nombreuses espèces dépendent directement ou indirectement des herbiers de végétation marine, non seulement les animaux, mais aussi certaines algues, et même des bactéries qui demeurent dans les herbiers. Les herbiers marins forment des écosystèmes ayant une activité biogéochimique intense et certaines espèces d'animaux qui vivent dans ces herbiers peuvent affecter les processus biogéochimiques des sédiments. Nous avons découvert que les herbiers sont globalement impliqués dans un partenariat spécialisé de «mutualisme» avec des bivalves de la famille Lucindae, où les bivalves non seulement bénéficient de la présence d’herbiers, mais les herbiers à leur tour ont besoin de ces bivalves pour survivre. Cette relation extraordinaire est basée sur un autre mutualisme : les bivalves Lucindae disposent de bactéries spéciales, vivant dans leurs branchies, et qui consomment les sulfures toxiques produites dans les sédiments des herbiers marins. Ce partenariat symbiotique permet aux herbiers de croître dans des zones où ils auraient dépéris en raison du stress des sulfures si les bivalves n’avaient pas été présents. Les bivalves ont à leur tour besoin des herbiers qui pompent l’oxygène dans le sol pour faire face aux sulfures toxiques. Ce mutualisme peut être appelé un « mutualisme clé de voûte », car il constitue le fondement des écosystèmes de prairies sous-marines, desquels dépendent de nombreuses autres espèces. Bien que ce mutualisme constitue une base solide des écosystèmes d’herbiers sous-marins, nous avons constaté que la rupture de ce mutualisme peut accélérer l’effondrement de ces écosystèmes. Il est important de discerner ce mutualisme, tout comme pour de nombreux autres écosystèmes qui dépendent de tels mutualismes clés comme les interactions plantes-pollinisateurs, les relations entre coraux et zooxanthelles ou encore les symbioses plantes-mycorhizes.

La symbiose entre herbier-bivalve - branchies-bactéries, qui permet aux herbiers de se développer dans des sols toxiques, est moins fréquente dans les régions tempérées. Cependant, comme le sulfure peut encore constituer une menace pour les herbiers dans ces régions, nous avons pensé que peut-être une autre
interaction entre les herbiers et l’arénicole commun *Arenicola marina*, qui produit des figures caractéristiques (cônes ou buttes) sur les plages et les vasières, pourrait soulager le stress dû aux sulfures dans les herbiers. Comme ces vers de vase perturbent le sédiment (bioturbation) pour s’alimenter en particules organiques et en algues, de l’oxygène se mélange dans le sol, ce qui peut diminuer les taux de sulfure toxiques. Cependant, dans notre expérience, nous n’avons trouvé aucun effet positif dû à l’activité des arénicoles sur les herbiers. La présence d’*Arenicola marina* ne diminue pas le stress dû à la présence de sulfures, mais leur activité enfouis les plantes d’herbiers et favorise la prolifération d’algues sur les feuilles des plantes marines par pompage des nutriments depuis les sédiments vers la colonne d’eau. Un autre mécanisme diminuant le stress dû à la présence de sulfure pourrait exister dans les herbiers de zones tempérées mais reste à découvrir.

**Leçons pour la gestion des herbiers et leur restauration**

Les herbiers marins constituent d’importants écosystèmes mais leur distribution a considérablement diminué à l’échelle mondiale au cours des dernières décennies. Afin d’y remédier de nombreuses tentatives de restauration des herbiers marins ont été entreprises, avec plus ou moins de succès. Ce manque de succès peut parfois être attribué à des densités de plantation trop faibles, comme les herbiers ont généralement besoin de densités suffisamment élevées pour rétablir les processus rétroactifs d’auto-facilitation. Nous avons observé que les processus de restauration particulièrement important tels que l’expansion et la récupération des herbiers sont sensibles à des facteurs de stress comme par exemple de trop hautes concentrations en sulfure ou en nutriments, en raison de l’absence de processus rétroactifs et dépendant de la densité qui pourraient atténuer le stress. En dehors de ces processus rétroactifs, les niveaux de stress initialement présents tels que la pollution par les métaux lourds, les niveaux de nutriments élevés, la forte présence de sulfure et la pression des organismes bioturbateurs peuvent également affecter les chances de succès de restauration des herbiers. Il est donc important d’identifier les niveaux de stress présents avant de tenter de restaurer les herbiers. En outre, nous avons constaté que les teneurs en amidon contenu dans les rhizomes des plantes peuvent être utilisés pour prédire le succès de la transplantation de l’année suivante. Succès qui était auparavant difficile à prédire en se basant uniquement sur les mesures de densité de plantes de l’été précédent. Enfin, comme nous avons découvert qu’un mutualisme clé était à la base des écosystèmes de prairies sous-marines, nous soulignons que celui-ci devrait être pris en compte lors de la restauration des herbiers. Toutes ces découvertes peuvent être utilisées afin d’améliorer la gestion des herbiers ainsi que les efforts de restauration.

**Conclusion**

Nous concluons que les herbiers marins sont menacés par de multiples facteurs de stress tels que les niveaux élevés en nutriments, la pollution par les métaux lourds et le sulfure, tout ceci en raison de la présence d’activités humaines dans les zones côtières. Ces facteurs de stress peuvent interagir et affecter simultanément l’état de santé des herbiers et leur fonctionnement. Il est donc important de détecter les menaces potentielles sur les écosystèmes des herbiers marins en utilisant les valeurs de concentrations en nutriments et métaux lourds comme bio-indicateurs de pollution à long terme. Nous avons découvert que les facteurs de stress tels que les niveaux élevés en nutriments ou en sulfures, peuvent affecter les processus dynamiques à l’échelle des herbiers, en raison de l’absence de processus rétroactifs d’auto-facilitation dans les herbiers à faible densité de plantes. Cependant, nous avons également constaté que les plantes marines bénéficient d’un mécanisme de protection contre le stress dû à la présence de sulfures: un mutualisme clé de voûte entre les herbiers, les bivalves Lucinidae et leurs bactéries branchiales, et qui permet aux herbiers de pousser dans des sols riches en sulfures dans le monde entier. Cela illustre l’importance de l’étude des herbiers à tous les niveaux et pour tous les aspects de stress biogéochimiques, y compris les interactions entre les espèces, afin d’acquérir une meilleure compréhension du fonctionnement des écosystèmes, qui peuvent également fournir des outils indispensables pour la conservation des herbiers, leur gestion et leur restauration.
Het schrijven van een proefschrift en al het onderzoek dat voorafgaat aan het daadwerkelijke schrijfproces is een hele opgave. Daarom wil ik graag iedereen die actief en/of op de achtergrond bij mijn leven betrokken is geweest gedurende deze periode hartelijk bedanken. Zonder jullie steun en vertrouwen had ik deze klus niet kunnen klaren!

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List of publications

Published Papers


Submitted papers

**Govers LL**, Bouma TJ, Van der Ent E, Suykerbuyk W, Godet L, Asmus R, Van der Heide T, Van Katwijk MM (submitted) Feedbacks and local environmental settings affect seagrass persistence and resilience

**Govers LL**, Leon PM Lamers, Alfons JP Smolders, Peter MJ Herman, Evelyn van der Ent, Tjeerd J Bouma, Marieke M van Katwijk (submitted) First experimental evidence of priming in seagrass beds


Suykerbuyk W, Van Oven P, Govers LL, Giesen K, Giesen WBJT, De Jong DJ, Bouma TJ, Van Katwijk MM (submitted) Living in the intertidal; desiccation reduces seagrass growth, but shading, high salinity or population of origin have no additional effect

Non peer-reviewed reports


Laura Leone Govers was born in ‘s–Hertogenbosch, the Netherlands, on September 16, 1985. The sea and its hidden treasures have always fascinated her, and she started snorkeling in the turbid waters of the North Sea when she was about 9 years old. This turned out to be quite disappointing due to poor visibility. However, these first encounters with the underwater world did not discourage her from loving the sea and its interesting inhabitants such as sea mammals and corals. So aged 11, she decided to become a marine biologist (instead of a sea mammal vet). This ambition has stuck to her ever since, and after graduating high school (Gymnasium Beekvliet, Sint-Michielsgestel) in 2003, the only logical choice was to start studying Biology at Radboud University Nijmegen. In her third year she followed a course on marine biology at the University of Amsterdam. This course, which included a field excursion to Mallorca, strengthened her interest in Marine biology and marine ecology in particular. After obtaining her BSc. degree in 2006, she started with the masters program ‘Limnology & Oceanography’ at the University of Amsterdam. After following courses in Amsterdam, she switched back to Nijmegen where she conducted her master’s research projects. After a first research topic on the food web structure of marine lakes in the indo-pacific region, she went to Curaçao for 5 months of fieldwork, investigating migration mechanisms of nursery fish species. As she could not get enough of tropical marine ecology, she went subsequently to a remote island in Indonesia (Derawan) to study top-down and bottom-up effects of sea turtle grazing in seagrass beds. After graduation in 2009 (Cum Laude), she became enthusiastically involved in seagrass research in the Netherlands and from June 2009 – June 2014 she carried out her PhD project, focusing on the effects of biogeochemical stressors in seagrass ecosystems, at the departments of Environmental Science and Aquatic Ecology and Environmental Biology of the Radboud University Nijmegen. This research was carried out in close collaboration with the Spatial Ecology group from NIOZ Yerseke, the Marine Ecology group from NIOZ Texel, and the Community and Conservation Ecology department from the University of Groningen. In this period, she supervised 5 students, was involved in 4 different BSc. and MSc. level courses, presented her research on international conferences in Brazil and the USA and participated in international expeditions to Mauritania (Banc d’Arguin), Australia (Shark Bay), and the USA (Rhode Island). She is currently doing postdoctoral research on the effects of Phytophthora (plant disease) on the seed germination of seagrasses, for a restoration project in the Wadden Sea.