Combined nutrient and macroalgae loads lead to response in seagrass indicator properties

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ABSTRACT

Excess nutrients are potential factors that drive phase shifts from seagrasses to macroalgae. We carried out a manipulative field experiment to study the effects of macroalgae *Ulva pertusa* loading and nutrient addition to the water column on the nitrogen (N) and carbon (C) contents (i.e., fast indicators) as well as on the morphology and structure (i.e., slow indicators) of *Zostera marina*. Our results showed rapid impact of increased macroalgae and nutrient load on *Z. marina* C/N ratios. Also, macroalgae addition resulted in a trend of decreasing belowground biomass of seagrasses, and nutrient load significantly decreased above to belowground biomass ratio. Although some morphological/structural variables showed relatively fast responses, the effects of short-term disturbance by macroalgae and nutrients were less often significant than on physiological variables. Monitoring of seagrass physiological indicators may allow for early detection of eutrophication, which may initiate timely management interventions to avert seagrass loss.

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1. Introduction

Seagrass meadows have been widely acknowledged as highly important coastal systems that support high biodiversity and productivity, and high trapping and storage of nutrients and carbon (Duarte et al., 2010; Hemminga and Duarte, 2000; Orth et al., 2006). Decline of seagrass meadows due to human activity has been reported in recent years throughout the world (Orth et al., 2006; Waycott et al., 2009). One of the most important threats to seagrasses is eutrophication, which could result from increased fertilizer use and marine cultivation (Burkholder et al., 2007; Orth et al., 2006). Nutrient enrichment can accelerate seagrass loss through direct effects, such as toxicity, increased system respiration and sediment anoxia (Burkholder et al., 2007; van Katwijk et al., 1997), but also through indirect effects via algal proliferation that could cause shading and/or smothering (Burkholder et al., 2007; Hauxwell and Valiela, 2004; van Katwijk et al., 2010).

Seagrass decline in temperate estuaries under high nutrient enrichment often coincides with high macroalgae biomass (Burkholder et al., 2007; Hauxwell et al., 2001; Short and Burdick, 1996; Thomsen et al., 2012; Valiela et al., 1997). The negative effects of macroalgal blooms on seagrasses have been documented in many areas of the world, such as Australia, Japan, America and Europe (e.g. Cummins et al.,

2004; Huntington and Boyer, 2008; Martínez-Lüscher and Holmer, 2010; Sugimoto et al., 2007). Small-statured seagrass species are more impacted than larger species, and the effect is often proportional to the biomass of macroalgae (Thomsen et al., 2012). Shading by the macroalgae causes light reduction and is one of the most common mechanisms resulting in seagrass decline (Burkholder et al., 2007), which is indicated by reduction in the depth of the meadows, lowered shoot densities, poor recruitment, slower growth rates and decreased overall production of seagrasses (Krause-Jensen et al., 2000; McGlathery, 2001). Additionally, decomposition of macroalgae mats may decrease oxygen content in eutrophicated waters and further abate seagrass survival (McGlathery et al., 2007). Furthermore, high sulfide concentration due to the anoxia from macroalgae decomposition can decrease the photosynthetic rate of seagrasses, reducing growth and even resulting in mortality (Holmer and Nielsen, 2007; Koch et al., 2007; Pedersen et al., 2004; van der Heide et al., 2012). With average temperatures rising on a global scale, blooms of green algae such as *Ulva pertusa* may be expected to increase (Sousa-Dias and Melo, 2008), which could in turn further increase the competitive advantage of green algae over some seagrasses (Koch et al., 2013 and references therein).

Fewer studies have investigated the effect of nutrients on the interaction between seagrasses and macroalgae beyond the effect of light reduction (but see Burkholder et al., 2007; Vonk et al., 2008). In general, when nutrient concentrations are low in seawater, seagrasses are dominant over macroalgae (Fourqurean et al., 1995), due to the competitive advantage conferred by roots and rhizomes in absorbing nutrients from

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sediment porewater (Hemminga, 1998; Vonk et al., 2008). In contrast, the competitive interaction is reversed under high nutrient concentrations in seawater because macroalgae can absorb nutrients more effectively through the thallus than seagrasses can through their leaves, almost irrespective of the form of nitrogen (N) (Burkholder et al., 2007; Vonk et al., 2008). In many developing countries, high levels of nutrients are discharged into coastal areas due to fertilizer use and untreated sewage or waste loading, either within catchment areas or directly into the seawater during mariculture (de Lacerda et al., 2006; Edinger et al., 1998; Lin et al., 2005).

The accelerated seagrass loss due to nutrient enrichment, either via direct effects or indirect effects, may eventually result in a phase shift from seagrass-towards macroalgae-dominated systems (Hauxwell et al., 2001; Montefalcone et al., 2007; Orfanidis et al., 2003). Most studies about such phase shifts from seagrasses to macroalgae have been conducted in highly eutrophic systems, where the shift to macroalgal dominance had already occurred (Cardoso et al., 2004; Montefalcone et al., 2007; Short and Burdick, 1996). However, few studies have directly assessed the combined effects of macroalgae cover and high nutrients on seagrass performance during macroalgae blooms before the phase shift from seagrasses to macroalgae, i.e. in systems where seagrasses and macroalgae still co-occur. Examining this stage of the interaction is highly relevant, especially for rapidly developing countries, where nutrient run-off towards seagrass meadows is rapidly increasing.

Human activities and the rapid economic development in China have already resulted in increasing nutrient loads on the surrounding seas. For example, dissolved inorganic nitrogen in the Yellow Sea has been increasing since 1976 (Lin et al., 2005), and has exceeded 14 μmol/L in more than 50% of the areas sampled since 2003 (State Oceanic Administration, 2008-2012). The world's largest macroalgae blooms during the period 2008–2012 occurred in the Yellow Sea, and over one million tons of wet macroalgae were removed from the coast in 2008 (Liu et al., 2013). Eutrophication by release of nutrients from wastewater, agriculture, and aquaculture has fostered macroalgae blooms in this area (Liu et al., 2013). In recent years, seagrasses have rapidly declined in China (Han et al., 2007; Huang et al., 2006). In some areas of the north coast of China, seagrass meadows have disappeared and been replaced by macroalgae mats (personal observations from Qiuying Han). At other areas, macroalgae mats cover seagrass *Zostera marina* meadows during extended algae blooms every summer from June to July. However, few studies have investigated the causes of seagrass degradation in these areas.

We aim to quantify experimentally in the field how both algae cover and nutrients interactively affect the remaining seagrasses, which has been often suggested but not empirically tested, in a factorial experiment. In order to evaluate the response of seagrasses to macroalgae cover, nutrients, and their combination, two types of indicators differing in their response time (fast vs. slow) were used: physiological (fast), morphological/structural (slow).

Physiological variables, such as nitrogen (N) and carbon (C) contents of seagrass tissues, are normally used as fast indicators (days to weeks) of nutrient availability and light reduction (Burkholder et al., 2007; Fourqurean et al., 1992, 1997; van Katwijk et al., 2011). Seagrass morphological and structural characteristics (e.g. number of leaves per shoot, blade width, leaf length, biomass and above to belowground biomass ratio) are slow indicators of variations in nutrient enrichment compared to the nitrogen and carbon contents of seagrass tissues (Burkholder et al., 2007; Lee et al., 2004; Roca et al., 2016). To evaluate the responses of both (fast and slow) types of indicators, we conducted a manipulative field experiment to investigate macroalgae *U. pertusa* cover and nutrient effects on the tissue nitrogen and carbon contents, biomass, and morphology of the seagrass *Z. marina* during a 6-week experiment. A period of 6 weeks was chosen in order to mimic the time scale of typical algae blooms encountered in the *Z. marina* meadows in this area, which are usually short and sudden. Our results will contribute to the knowledge base that may assist in the monitoring

and conservation of seagrasses under situations of macroalgae blooms and/or nutrient enrichment worldwide.

2. Materials and methods

2.1. Experimental field location

An in situ manipulative experiment was conducted in Swan Lake (N36°43′-37°27′, E122°09′-122°42′), located in the eastern part of the Shandong peninsula on the north coast of the Yellow Sea of China (Fig. 1). Swan Lake is a 4.8 km^2 lagoon, connected via an inlet to the north Yellow Sea. The seaweed *Laminaria japonica* is cultured on a mass scale in an area of about 6600 ha around Swan Lake and about 200 t of the bivalve *Ruditapes philippinarum* are also harvested each year. Two seagrass species can be found in mostly separate continuous assemblages in the lagoon, *Z. marina*, which is the dominant species in extended meadows, and *Zostera japonica*, which become mixed only at the edge of each seagrass meadow. More than 10,000 swans inhabit the lake, particularly in winter, and feed on *Z. marina* (Dong et al., 2007). In the past 10 years, *Z. marina* seagrass biomass has decreased, reportedly through the process of eutrophication (Dong et al., 2007). *U. pertusa* is the dominant macroalgae species in Swan Lake and blooms of this species often occurs during summer, especially in June (Zhang et al., 2014).

At the beginning of this study, seawater samples were collected from Swan Lake, put into refrigeration boxes in the field, and transferred to the laboratory for nutrient analysis. Samples were then filtered through cellulose acetate membranes (Whatman, 0.45 μmol/L). Nutrient analysis was conducted using a Flow Injection Analysis system (AA3, Bran + Luebbe, Norderstedt Germany) to measure the following parameters: ammonium, nitrite, nitrate and soluble reactive phosphorus (SRP). Nutrient analysis was executed according to the WOCE Methods Manual (Gordon et al., 1993). Dissolved inorganic nitrogen (DIN) was calculated as the total of ammonium + nitrite + nitrate. Mean values (±SE) for soluble reactive phosphate (SRP), nitrate, ammonium and DIN concentration of natural seawater were found to be 0.28 ± 0.19, 3.74 ± 1.02, 5.22 ± 1.57 and 9.41 ± 2.60 μmol/L, respectively. Using a YSI 30 portable meter, the following environmental parameters were directly measured in the field: seawater temperature, pH, dissolved oxygen concentration and salinity. Mean water column temperature, pH, dissolved oxygen concentration and salinity were 21.85 ± 3.79 °C, 6.61 ± 0.43, 5.6 ± 0.69 mg/L and 30.8 ± 0.53, respectively at the beginning of the field experiment.

2.2. Experimental design

We tested the combined effects of macroalgae in cages ("HA" = high algae: 1800 g/m^2 fresh macroalgae biomass was applied at the beginning of the experiment to mimic a relatively high macroalgae loading, "NA" = no algae: algae were removed at the beginning of the experiment) and nutrients ("HN" = high nutrient addition: 565 g osmocote slow-release fertilizer each week (g/g ratio N:P:K; 26:11:11), "NN" = no nutrient added) addition on *Z. marina* meadows in the field. The high algae treatment resembles the ambient loads to some extent. The initially applied amount of algae is approximately four times higher than the ambient fresh mean biomass of *U. pertusa* macroalgae in June (490 ± 170 g/m^2, n = 10), as it decomposes quickly 23 ± 17 g/m^2 of fresh biomass per day (n = 30), without new input in the cages. Plots assigned to the "High nutrients" treatment were fertilized with osmocote slow-release fertilizer each week. The osmocote fertilizer was inserted into nylon stockings hung above the sediment using bamboo poles in the middle of the cage, which enabled the nutrients to discharge continuously into both the water column and sediment porewater. Average loading rates of osmocote fertilizer were 204 mmol N m^{-2} day^{-1} and 92 mmol P m^{-2} day^{-1}. A total of four treatments, i.e. HA–HN, NA–HN, HA–NN, and NA–NN (control), with 5
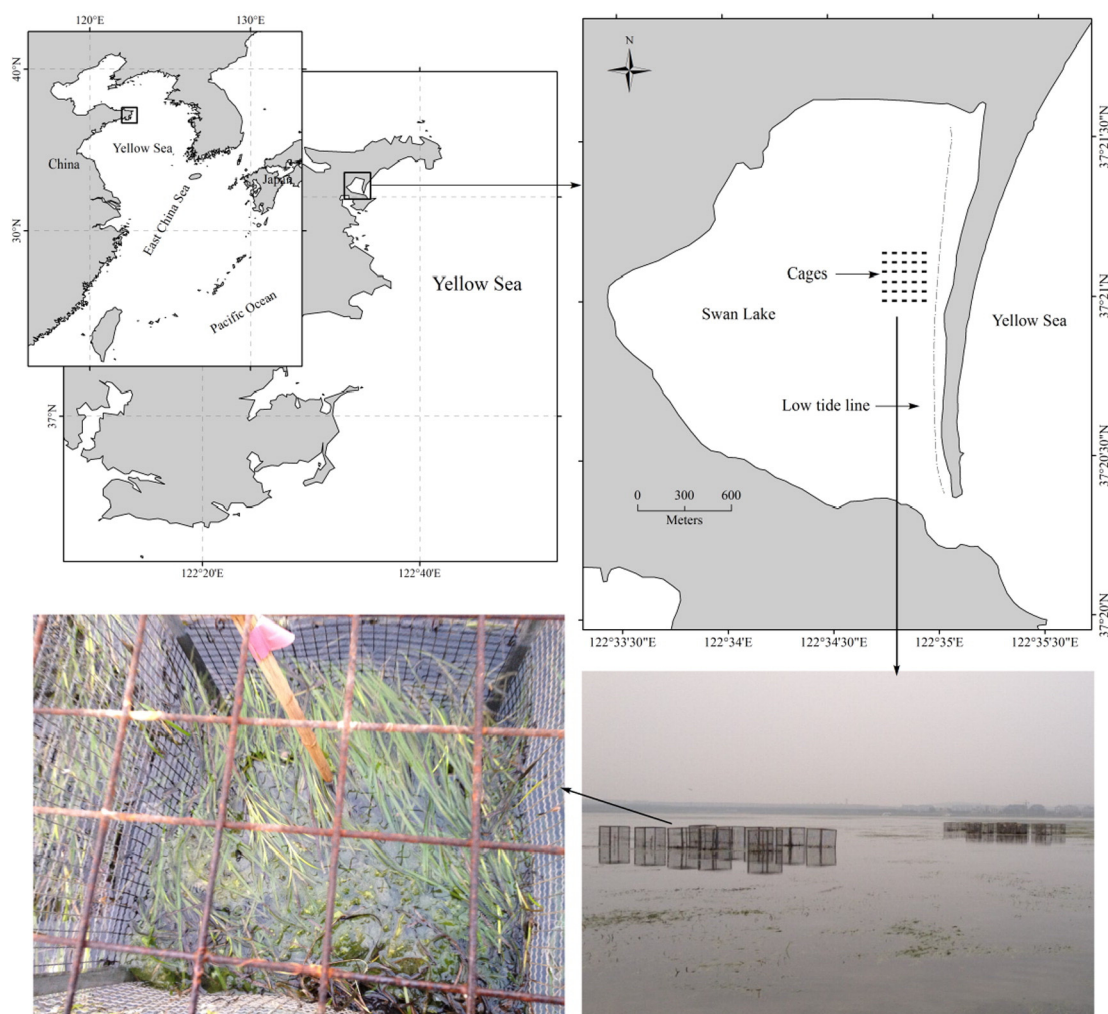
Fig. 1. Map of field experiment locations and photographs of enclosures.

replicates each were implemented on apparently homogeneous *Z. marina* seagrass substrate, with a minimum distance of 5 m between plots with the same nutrient treatment to prevent nutrient cross-contamination and a minimum distance of 30 m between different nutrient treatments. In Swan Lake, hydrodynamics are weak due to the effects of the dam wall and tidal range is limited (about 1 m) (Zhang et al., 2014; personal observations from Qiuying Han). This, and rapid consumption by seagrasses and macroalgae in the plots are assumed to be sufficient to warrant sufficient independence between the treatments (which is also supported by the results showing nutrient effects.). The experiment was set up in an area of Swan Lake with minimal differences in water depth (30 cm) and hydrodynamics at low tide. The experiment started in June 2012 and lasted for 6 weeks.

2.3. Macroalgae addition

In order to enclose the macroalgae *U. pertusa* and prevent it from floating out of treatment plots, cages consisting of nets wrapped around a solid base and framework (70 cm length, 70 cm width and 100 cm height) were used for all treatments, including controls. The mesh size around the sides of the cages was 2 cm × 2 cm, which was small enough to also prevent macroalgae from outside the plots entering into the cages. The mesh size of the top of cages was 5 cm × 5 cm in order to maximize light availability. The cage framework consisted of stainless steel wires of 0.5 mm in diameter. Four steel poles inserted into the four corners of the base were used to anchor the cages. In the cages,

macroalgae covered the top of the seagrass leaves during experiments according to our observation (Fig. 1). In the HA treatment, we added 952 g of algae biomass per cage (0.49 m^2).

2.4. Sampling and measurement

After the 6-week treatment period, all seagrasses and macroalgae were harvested from inside the cages. Seagrass (leaves with rhizomes and roots) samples were collected using a spade (digging deep into 50 cm of sediment) and then cleaned. Epiphytes were carefully removed from seagrass leaves. Macroalgae were collected manually and then cleaned. All samples were kept in a refrigeration box and transported to the laboratory at the Yantai Institute of Coastal Zone Research in Yantai, China. In the laboratory, the morphological properties of seagrasses (leaf length, leaf width, rhizome diameter, rhizome length, root length) were measured within three days following the harvest. Seagrass and macroalgae samples were frozen and dried using a Christ ALPHA 1–4 LD plus freezing drier. Dry biomass of macroalgae, and seagrass leaves, rhizomes and roots was weighed in the laboratory. The above to belowground biomass ratio of seagrasses was also calculated. Leaves, rhizomes and roots of seagrasses were separated, mixed and ground, and the macroalgae was ground as well. Lastly, the C and N contents of macroalgae and seagrass tissues (leaf, root and rhizome) were analyzed using an element analyzer (Vario Macro cube, Elementar company, Germany), and the C/N ratio of macroalgae and seagrass tissues was calculated.

Sediment porewater samples were collected directly from the sediment for each plot by using syringes connected to a Rhizon MOM 5 cm female luer (19.21.22F) (Rhizosphere research product, Wageningen, the Netherlands), then filtered with pinhole filters (25 mm in diameter, 0.45 μm in pore size). All samples were kept in a refrigeration box, transported to the laboratory at Yantai Institute of Coastal Zone Research and kept frozen till further analyses using the Flow Injection Analysis system (AA3, Bran + Luebbe, Norderstedt Germany) as above-mentioned. The following parameters were measured: ammonium, nitrite, nitrate, and soluble reactive phosphorus (SRP). Dissolved inorganic nitrogen (DIN) was calculated as above-mentioned.

2.5. Statistical analysis

We used a two-way ANOVA to test the factorial effects of the macroalgae and nutrient treatments (M, N and M×N) on C and N contents; C/N ratio of the seagrass leaf, rhizome and root, seagrass total dry biomass; aboveground biomass; belowground biomass; above to belowground biomass ratio; and seagrass morphological properties. The morphological properties included leaf length, leaf width, rhizome diameter, rhizome length and root length. Data that deviated from normality (Kolmogorov–Smirnov's test) or homoscedasticity (Levene's test) were transformed prior to analyses to meet the assumptions of two-way ANOVA. The effects of nutrients on fresh weight, C and N contents, and C/N ratio of macroalgae were analyzed using one-way ANOVA and post hoc tests. Normality and homogeneity of the data were also previously checked. If necessary, data were transformed to comply with ANOVA assumptions.

3. Results

3.1. Fast (physiological) indicators: N, C contents of seagrasses

Nutrient addition significantly increased N content of seagrass leaves, rhizomes and roots (Fig. 2A, B & C). Macroalgae addition significantly increased N content of seagrass leaves and roots (Fig. 2A, C). The

highest N content value of seagrass leaves (1.8 ± 0.1%), rhizomes (1.0 ± 0.1%) and roots (1.3 ± 0.2%) was observed in the HA–HN treatment. No significant interaction effects were found.

Our results showed that both macroalgae and nutrient addition significantly decreased C content of seagrass leaves and rhizomes (Fig. 3A, B). C content of seagrass rhizomes, however, was reduced by the combination of macroalgae and nutrient addition. The lowest C content value of seagrass leaves (23.6 ± 1.1%), rhizomes (22.6 ± 0.4%) and roots (25.7 ± 0.8%) was found in the HA–HN treatment.

Consequently, nutrient addition decreased the C/N ratio of seagrass leaves, rhizomes and roots (Fig. 4A, B & C). Macroalgae addition decreased C/N ratio of seagrass leaves and rhizomes (Fig. 4A, B). The C/N ratio of seagrass leaves and rhizomes was even more reduced by the combination of macroalgae and nutrient addition (Fig. 4A, B). The lowest C/N ratio value of seagrass leaves (13.1 ± 0.6), rhizomes (22.6 ± 2.6) and roots (20.7 ± 2.0) was in the HA–HN treatment (Table 1).

3.2. Slow indicators: morphology and structure of seagrasses

The addition of macroalgae and nutrients did not significantly affect leaf length, leaf width, rhizome length and root length of seagrasses (two-way ANOVA, Table 1). Rhizome diameter was only weakly correlated with nutrient addition ($p = 0.089$), leading to wider rhizome diameter in the nutrient addition treatments (3.6 ± 0.7 mm) than in the control treatments (3.2 ± 0.4 mm) (Table 1). Leaf length of seagrasses showed an increasing trend with macroalgae addition (Table 1).

Belowground biomass of seagrasses was significantly reduced by macroalgae addition with the highest value (10.4 ± 1.8 g/m² dry weight) recorded for the controls; the lowest value (4.8 ± 2.6 g/m² dry weight) was recorded in the HA–NN treatment (Table 1; Fig. 5A). Nutrient addition significantly decreased the above to belowground biomass ratio of seagrasses and the lowest value (3.1 ± 0.4) was found in the NA–HN treatment (Table 1; Fig. 5B). No significant interaction effects were found.
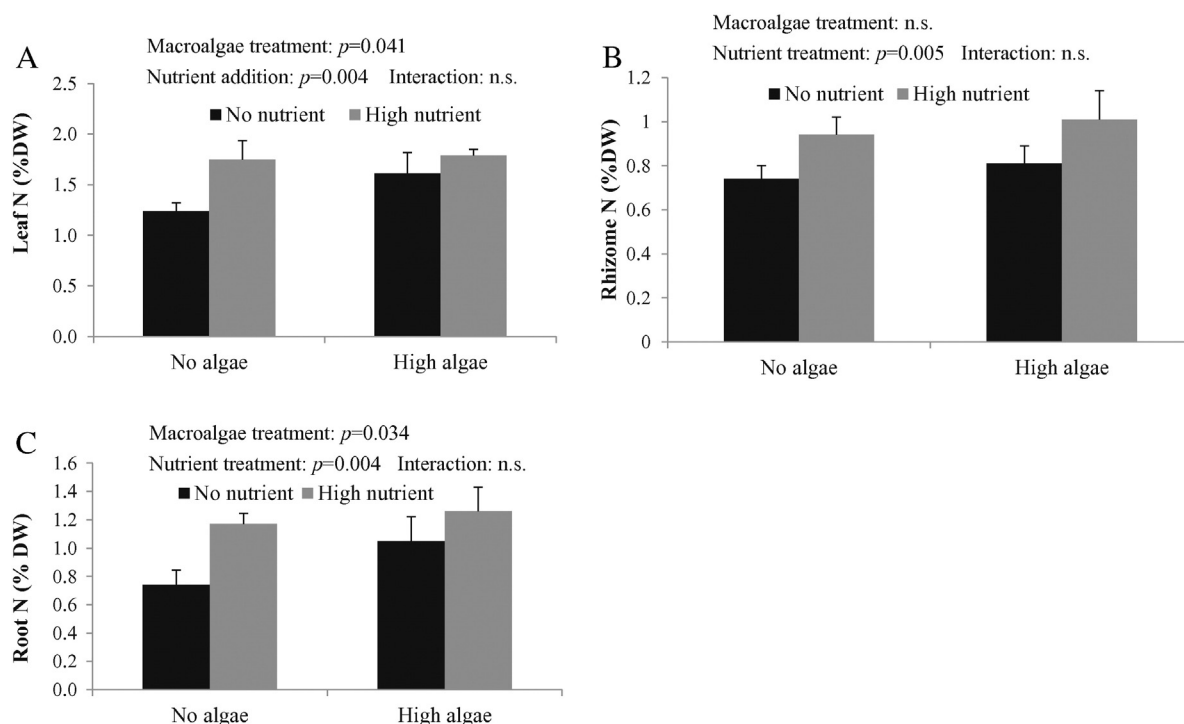

Fig. 2. Responses of seagrass nitrogen content (mean and standard error) to macroalgae and nutrient addition. The *p*-values of the two-way ANOVA (see Table 1) are indicated above the graph.

Fig. 3. Responses of seagrass carbon content (mean and standard error) to macroalgae and nutrient addition. The *p*-values of the two-way ANOVA (see Table 1) are indicated above the graph.
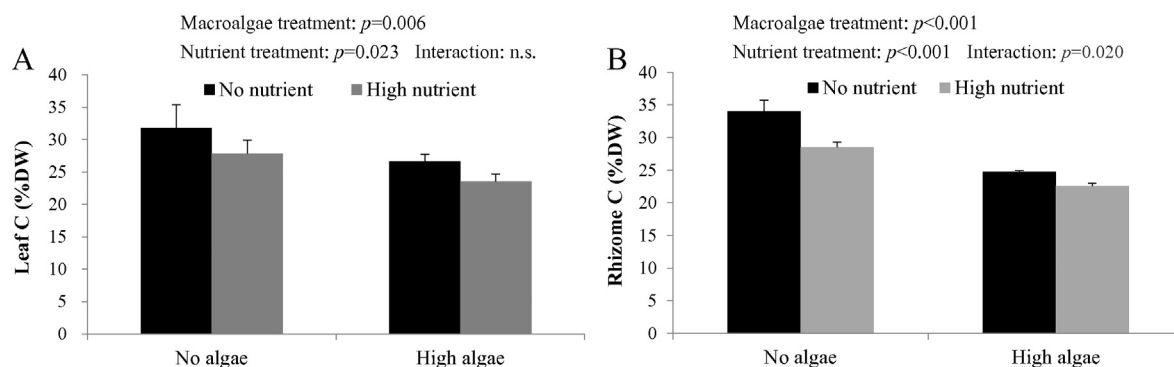
3.3. Fresh biomass, N and C contents of macroalgae

The average fresh biomass of macroalgae at the end of the experiment (6 weeks) was lower than the original fresh biomass, and it was greater in the high nutrient treatment (234.6 ± 117.6 g/m^2) than in the low nutrient treatment (195.9 ± 124.4 g/m^2) (Table 1). The first result may be due to macroalgae decomposition during the experimental period and the latter could be because the macroalgae benefitted from the nutrient addition. However, the fresh biomass of macroalgae was not significantly affected by the nutrient treatments ($F = 0.255$, $p = 0.627$).

The N content ($F = 1.137$, $p = 0.346$), C content ($F = 0.118$, $p = 0.748$) and C/N ratio ($F = 0.945$, $p = 0.386$) of the macroalgae were not significantly different between the nutrient treatments (Table 1).

3.4. Sediment porewater

Macroalgae addition significantly increased the DIN concentration in sediment porewater (Fig. 6A). Nutrient addition significantly increased the ammonium, nitrate and SRP concentration in sediment porewater (Fig. 6B, C & D); especially the ammonium concentration in the porewater was very high (120 ± 64 μmol/L) in the HA–HN treatment (Table 1). Nitrate concentration in sediment porewater was affected by the combination of macroalgae and nutrient addition, which was reduced by macroalgae addition in the nutrient addition treatments (Fig. 6C).

4. Discussion

Increasing input of anthropogenic nutrients into coastal areas and resultant blooms of macroalgae (Burkholder et al., 2007; Hauxwell et al., 2001) may lead to seagrass ecosystem degradation. The present study demonstrated the negative effects of adding green algae *U. pertusa* and inorganic nutrients on *Z. marina*. In a 6-week period, the addition of macroalgae and nutrients strongly and additively affected fast physiological indicators, such as N and C contents, and C/N ratio of seagrasses. The slow indicators, seagrass belowground biomass and the above to belowground biomass ratio, had already responded to
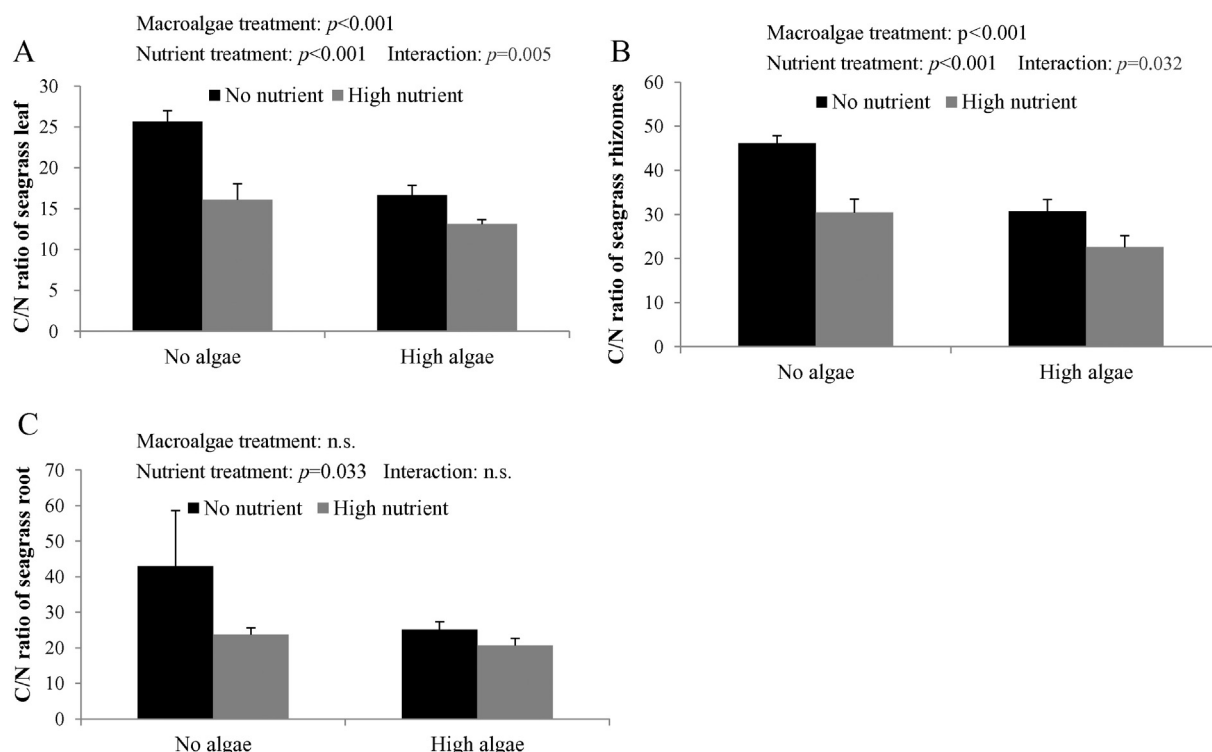

Fig. 4. Responses of seagrass C/N ratio (mean and standard error) to macroalgae and nutrient addition. The *p*-values of the two-way ANOVA (see Table 1) are indicated above the graph.

Table 1

ANOVA results showing the effects of macroalgae and nutrient addition on seagrass, macroalgae and environment variables. Univariate responses are shown as means per treatment (mean ± SE). Both the *p*-values of the two-way ANOVA for seagrasses and environment variables, and *p*-values of the one-way ANOVA for macroalgae variables are shown.

Variables	NA-NN	NA-HN	HA-NN	HA-HN	*p*-Value		
					Macroalgae	Nutrient	M & N
Dry biomass of seagrass (g/m^2)	49.9 ± 12.0	34.4 ± 12.1	28.7 ± 18.5	31.8 ± 5.3	0.137	0.534	0.744
Aboveground dry biomass of seagrass (g/m^2)	39.5 ± 10.6	26.0 ± 9.6	23.9 ± 16.0	24.4 ± 4.3	0.223	0.412	0.834
Belowground dry biomass of seagrass (g/m^2)	10.4 ± 1.8	8.4 ± 2.6	4.8 ± 2.6	7.4 ± 1.4	0.010*	0.751	0.056
Above to belowground biomass ratio	3.8 ± 0.7	3.1 ± 0.4	4.8 ± 0.8	3.3 ± 0.5	0.059	0.003**	0.221
Fresh biomass of macroalgae (g/m^2)			195.9 ± 124.4	234.6 ± 117.6		0.627	
Leaf length (mm)	322.8 ± 57.7	93.6 ± 67.9	394.7 ± 99.9	456.2 ± 109.4	0.105	0.193	0.361
Leaf width (mm)	6.4 ± 0.6	6.1 ± 0.9	6.3 ± 1.1	6.7 ± 0.4	0.793	0.894	0.244
Rhizome diameter (mm)	3.2 ± 0.4	3.6 ± 0.7	3.2 ± 0.1	3.9 ± 0.5	0.719	0.089	0.534
Rhizome length (mm)	16.2 ± 1.5	18.6 ± 1.4	15.9 ± 1.8	15.2 ± 7.5	0.446	0.718	0.508
Root length (mm)	51.4 ± 6.6	44.8 ± 21.8	60.5 ± 23.4	65.8 ± 25.3	0.883	0.957	0.795
C of leaf (%DW)	31.8 ± 3.6	27.9 ± 1.1	26.7 ± 2.1	23.6 ± 1.1	0.006**	0.023*	0.737
N of leaf (%DW)	1.2 ± 0.1	1.8 ± 0.2	1.6 ± 0.2	1.8 ± 0.1	0.041*	0.004**	0.096
C/N of leaf	25.6 ± 1.3	16.1 ± 2.0	16.7 ± 1.2	13.1 ± 0.6	<0.001**	<0.001**	0.005**
C of rhizome (%DW)	34.0 ± 1.7	28.5 ± 0.5	24.7 ± 0.7	22.6 ± 0.4	<0.001**	<0.001**	0.020*
N of rhizome (%DW)	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	0.224	0.005**	0.962
C/N of rhizome	46.1 ± 1.7	30.5 ± 3.0	30.7 ± 2.7	22.6 ± 2.6	<0.001**	<0.001**	0.032*
C of root (%DW)	30.7 ± 6.2	27.6 ± 2.2	26.3 ± 3.0	25.7 ± 0.8	0.171	0.420	0.562
N of root (%DW)	0.7 ± 0.1	1.2 ± 0.1	1.1 ± 0.2	1.3 ± 0.2	0.034*	0.004**	0.186
C/N of root	42.9 ± 15.6	23.6 ± 2.0	25.1 ± 2.2	20.7 ± 2.0	0.055	0.033*	0.148
C of macroalgae (%DW)			33.1 ± 1.4	32.5 ± 2.7		0.748	
N of macroalgae (%DW)			2.3 ± 0.1	2.5 ± 0.4		0.346	
C/N of macroalgae			14.7 ± 0.4	13.0 ± 1.1		0.386	
DIN in pw (μmol/L)	5.9 ± 1.6	114 ± 114	65.5 ± 41.3	125 ± 67	0.033*	0.332	0.492
Ammonium in pw (μmol/L)	3.4 ± 1.4	110 ± 113	53.1 ± 36.2	120 ± 64	0.522	0.048*	0.445
Nitrate in pw (μmol/L)	2.2 ± 0.4	3.6 ± 2.1	11.7 ± 6.4	4.3 ± 3.8	0.147	0.022*	0.042*
SRP in pw (μmol/L)	0.8 ± 0.2	11.6 ± 11.3	4.1 ± 1.8	10.2 ± 10.1	0.810	0.046*	0.557

* $p < 0.05$.
** < 0.01.

macroalgae and nutrient addition after 6 weeks, although their responses were weaker than those found for the physiological variables.

4.1. Fast indicator responses of seagrasses

Nutrient addition significantly increased the N content in the seagrass leaves, rhizomes and roots (Table 1; Fig. 2A, B & C), which is supported by previous studies (Burkholder et al., 2007; Duarte, 1990). N content of seagrass leaves, rhizomes and roots was lower than or equal to 1.8% of the dry weight (Table 1), indicating that these seagrasses were probably nutrient-limited at the start of the experiment (Duarte, 1990), which may explain why the meadows were able to persist under 6 weeks of macroalgae and nutrient additions. Macroalgae addition increased the N content of seagrass leaves and roots as well (Fig. 2A & C), probably due to the decomposition of macroalgae mats releasing nutrients into the seawater and further supporting the nutrient demand of seagrasses (McGlathery et al., 2007). The consistent response of tissue nitrogen to both macroalgae presence and nutrient addition renders this variable an ideal fast indicator for increasing eutrophication.

The presence of macroalgae decreased the C content of seagrass leaves and rhizomes (Table 1; Fig. 3A & B). The C content of *Z. marina* in macroalgae and nutrient loading treatments, and particularly for their combined treatment, was much lower than in the control treatment and literature values taken from Europe (leaves: 34.9–40.3%; root-rhizomes: 33.6–36.0%) (Pedersen and Borum, 1992) and North America (leaves: 29.0–40.9%) (Fourqurean et al., 1997), which might indicate that if the stress was prolonged, it could cause seagrass systems to collapse. Macroalgae cover may reduce the C content of seagrasses for two reasons. First, light reduction by macroalgae mats may contribute to a decrease in photosynthetic activity of seagrasses (Brun et al., 2003; Huntington and Boyer, 2008; Peralta et al., 2002; Ralph et al., 2007). This can cause a subsequent reduction in root and rhizome growth of seagrasses (Hemminga, 1998), as rhizomes and roots are heterotrophic tissues depending on basipetal translocation of photosynthates and oxygen from active photosynthesizing leaves to persist (Zimmerman and Alberte, 1996). Second, decomposition of macroalgae
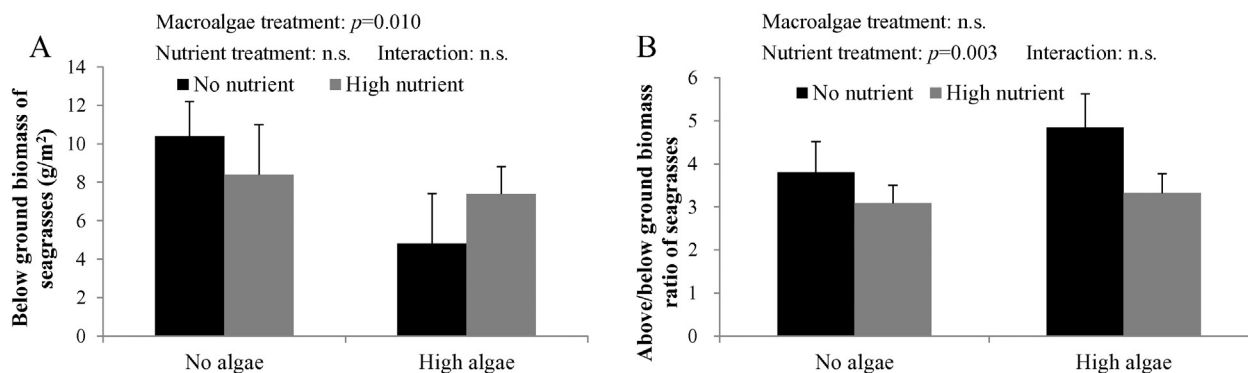

Fig. 5. Responses of morphological variables, seagrass belowground biomass and above to belowground biomass ratio (mean and standard error), to macroalgae and nutrient addition. The *p*-values of the two-way ANOVA (see Table 1) are indicated above the graph.

Fig. 6. Responses of nutrients in porewater (mean and standard error) to macroalgae and nutrient addition. The *p*-values of the two-way ANOVA (see Table 1) are indicated above the graph.

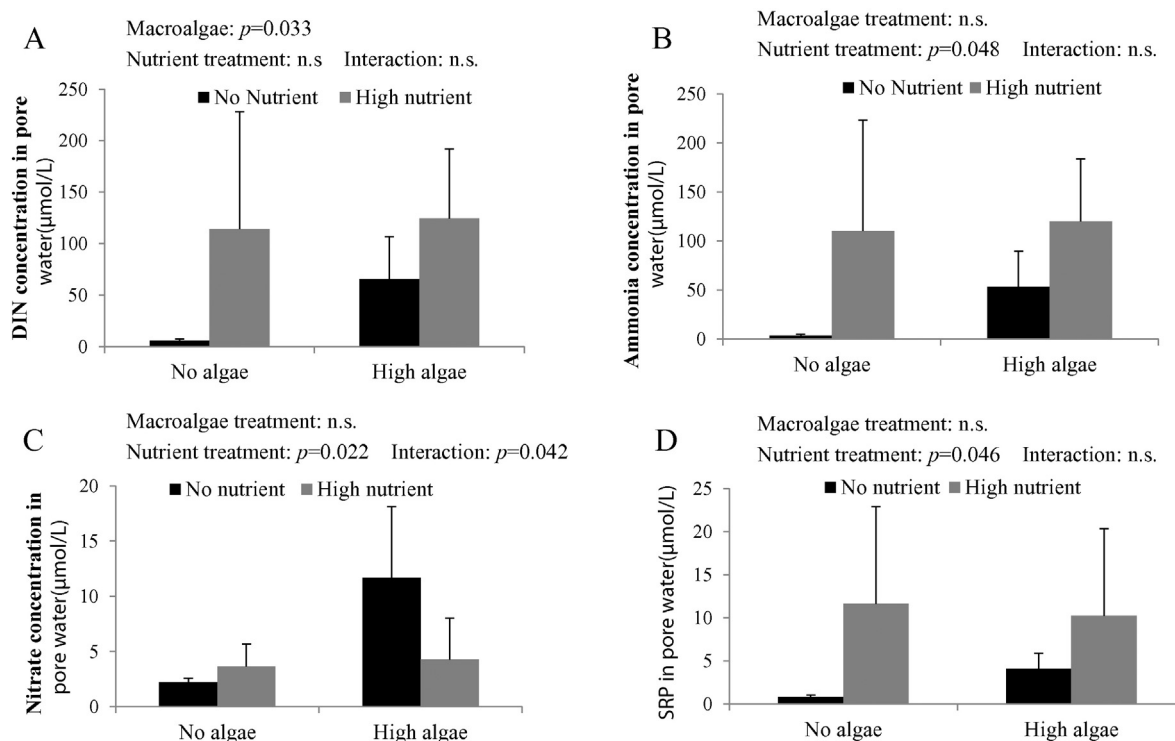mats may decrease oxygen content in waters and sediments and, thereby, constrict growth of rhizomes and roots (Hemminga, 1998; McGlathery et al., 2007). Although seagrasses can store enough carbohydrates to maintain metabolic processes and support seagrasses survival over a short period, light reduction, either or not due to macroalgae canopy may result in mortality over longer time spans (Moore et al., 1997; Alcoverro et al., 2001; Brun et al., 2003). Some *Ulva* species can survive for 2 weeks in the dark (Kamermans et al., 1998). Also *U. pertusa* was shown to tolerate darkness for 9 days, rapidly sporulate and increase in the size after being transferred from darkness to increased irradiance (Han et al., 2003). In our study, the seagrasses and *Ulva* were generally still alive after the experimental period in all treatments.

Environmental stressors such as nutrient enrichment can change the carbon demand of seagrasses (Brun et al., 2008; Christianen et al., 2011; van Katwijk et al., 2011). The C/N ratio of seagrass leaves is a function of light as well as nutrient availability (Burkholder et al., 2007; Grice et al., 1996). In our study, the C/N ratio of *Z. marina* leaves and rhizomes was additively reduced by the combination of macroalgae and nutrient addition (Fig. 4A, B); our values were lower than those found in other studies (e.g. Fourqurean et al., 1997 (19.7); Pedersen and Borum, 1992 (>20)). This could be due to the effects of low photosynthetic rates at the low light conditions under macroalgae mats, which lowers the depletion rates of nutrient supplies (Burkholder et al., 2007; Grice et al., 1996). This potential interaction effect between algal mats and nutrient supply has not been separately tested in other eutrophication studies. The strong reduction of the C/N ratio of *Z. marina* leaves and rhizomes demonstrates that macroalgae and nutrients loading change the balance between carbon and nutrients in seagrasses, which may reduce the competitive advantage of seagrass over macroalgae (Burkholder et al., 2007). Consequently, a phase shift from seagrass-dominated to macroalgae-dominated ecosystem may be induced if macroalgae and nutrient loading are prolonged.

4.2. Slow indicator responses of seagrasses

Belowground biomass was not significantly reduced by nutrient enrichment (Table 1), in contrast to the study by Christianen et al. (2012), who found that nutrient additions decreased belowground biomass of seagrasses. This difference might be due to the short term duration of our experiment (6 weeks) and nutrients being still limiting in our study, as suggested by the low %N in the seagrass leaves (1.2–1.8) compared with the study of Christianen et al. (2012, %N approximately 2.2%). Nutrient addition also had no significant effect on the aboveground biomass, but did significantly decrease the above to belowground biomass ratio of seagrasses (Table 1, Fig. 5B). Nutrient enrichment of sediment drives plants to increase photosynthetic C fixation to meet the increased demand for C. However, if photosynthetic C fixation in leaf and rhizome tissues cannot meet the increased C demand, stored C in plant leaf tissues may be used to support metabolic processes (Burkholder et al., 1992; Irlandi et al., 2004; Lee and Dunton, 1999). This may have led to the shift in balance between aboveground and belowground biomass in our study.

Macroalgae addition resulted in reduced belowground biomass of seagrasses (Table 1; Fig. 5A). Light reduction due to macroalgae shading is one of the most common mechanisms resulting in seagrass decline (Burkholder et al., 2007), which may reduce photosynthetic activity and C fixation of seagrasses, therefore decrease overall production of seagrasses (Han and Liu, 2014 and references therein; Krause-Jensen et al., 2000; McGlathery, 2001). Carbon translocation between leaf and belowground tissue and storage capacity of belowground tissue can affect the growth of seagrasses (Han and Liu, 2014 and references therein). Under the reduced light conditions encountered during macroalgae blooms, carbon translocation from aboveground to belowground tissues may be negatively impacted. These combined effects may lead to reduced belowground biomass. In addition, decomposition of macroalgae mats may release dissolved organic matter into the sediment, leading to anoxia

and further constriction of belowground biomass growth in seagrasses (McGlathery et al., 2007).

Morphometric changes of seagrasses can be used as an indicator of changes in the environmental variables as well (Peralta et al., 2005). In our study, leaf length of *Z. marina* tended to increase with *U. pertusa* addition, which might indicate shading stress. Several studies have demonstrated that leaf length increases with reduced light intensity but decreases with nutrient addition (e.g. Lee et al., 2007; Short et al., 1995). In our study increased nutrients did not significantly impact the leaf length of seagrasses, but this may have been due to nitrogen limiting growth (the low %N in the seagrass leaves).

5. Conclusions

Phase shifts from seagrass-dominated to macroalgae-dominated ecosystems have been well documented in different areas worldwide, such as in Tomales Bay, California, USA (Huntington and Boyer, 2008) and the Mondego estuary, Portugal (Cardoso et al., 2004; for a review see Han and Liu, 2014). The present study showed a rapid (i.e. within 6 weeks), significant negative impact from increased macroalgae abundance and nutrient loads on *Z. marina* in the seagrass meadow. Our study indicated that physiological variables (such as C and N contents of seagrass tissues) are good indicators for macroalgae and nutrient loading, consistently responding to both variables, separately and in combination. Morphological and structural variables responded as well to our treatments, although responses were weaker and did not show synergistic effects. Thus, we advise that monitoring of seagrasses should include physiological indicators to allow for early detection of eutrophication consisting of macroalgae blooms, nutrient enrichment or both occurring together. Early detection may initiate management interventions in time to avert loss of valuable seagrass ecosystems.

Acknowledgments

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