

## Waves and high nutrient loads jointly decrease survival and separately affect morphological and biomechanical properties in the seagrass *Zostera noltii*

Yayu A. La Nafie,<sup>a,b,c,\*</sup> Carmen B. de los Santos,<sup>d</sup> Fernando G. Brun,<sup>d</sup> Marieke M. van Katwijk,<sup>b</sup> and Tjeerd J. Bouma<sup>a</sup>

<sup>a</sup>Netherlands Institute for Sea Research (NIOZ), Yerseke, The Netherlands

<sup>b</sup>Department of Environmental Science, Institute for Water and Wetland Research, Radboud University Nijmegen, Nijmegen, The Netherlands

<sup>c</sup>Department of Marine Science, Hasanuddin University, Makassar, South Sulawesi, Indonesia

<sup>d</sup>Department of Biology, University of Cádiz, Puerto Real (Cádiz), Spain

### Abstract

In an 8-week aquarium experiment, we investigated the interactive effects of waves (present vs. absent) and water-column nutrient level (high vs. low) on the survival, growth, morphology, and biomechanics of the seagrass, *Zostera noltii*. Survival was reduced when plants were exposed to both waves and high nutrient levels. Wave and nutrient interaction significantly reduced aboveground biomass and leaf lengths, whereas waves independently reduced growth rate, internode abundance, elongation, and appearance rates. Nutrient supply significantly reduced the strength of the leaves. Wave and nutrient interaction was the main driving force affecting survival and morphological properties of seagrass, whereas dynamical characteristics were independently affected by waves, and nutrient supply affected mainly biomechanical properties. In conclusion, this experiment revealed that the combination of exposure to waves and high nutrient levels was detrimental for *Z. noltii*, which indicates that this could be an important unexplored force involved in seagrass declines.

Being sessile organisms, seagrasses are prone to facing adverse environmental conditions from which they cannot escape that may lead to their loss, as has been observed worldwide (Waycott et al. 2009). In the current era where human use of coastal areas is intensifying (Small and Nicholls 2003) and global change processes affect the climate, the co-occurrence of adverse environmental conditions in littoral areas may be expected to increase. For example, anthropogenic activities may enhance nutrient loads via fertilizer usage and sewage release (Cabaço et al. 2008), whereas at the same time, global change process may cause increased storm frequency and wave stress (Young et al. 2011). These two abiotic factors co-exist in littoral areas and are expected to further increase in the near future (Trenberth et al. 2007); therefore, this study aims to unravel how both abiotic factors interact and may affect the seagrass *Zostera noltii*.

High nutrient levels in coastal environments are mainly due to increased anthropogenic activities (Cabaço et al. 2008). Depending on the environment, an enhanced nutrient load may sometimes have a positive (Perez et al. 1991; Brun et al. 2002) or no effect (Harlin and Thornemiller 1981; Lee and Dunton 2000) on seagrasses. However, nutrient enrichment generally has a negative effect on seagrass growth and survival either directly due to the toxic effect of nitrate (Burkholder et al. 1992) or ammonium (van Katwijk et al. 1997; Brun et al. 2002) or indirectly due to algal overgrowth, which causes light deprivation and enhanced organic matter fluxes to the sediment (Short et al. 1995; Brun et al. 2003). Hydrodynamic conditions from waves and currents are also known to have direct and

indirect effects on seagrass ecosystems. For example, increased wave energy can directly reduce survival and number of seagrass shoots (van Katwijk and Hermus 2000), while increased flow velocity can directly affect plant growth and morphometry (Schanz and Asmus 2003; Peralta et al. 2006; de los Santos et al. 2010). In addition, increased flow velocity and/or waves can have indirect effects on the photosynthetic rates and nutrient uptake by reducing boundary layers (Koch 1994). Last but not least, hydrodynamics can affect seagrasses by affecting the light availability due to self-shading or via turbidity (Koch 2001).

Although the individual effects of nutrient levels and hydrodynamic on seagrasses have both been studied in detail, their interactive effect may be difficult to predict because plant responses to individual stressors are diverse and, in some cases, opposite. For example, leaf size of *Zostera marina* was increased in response to nutrient enrichment (Short 1983), whereas *Zostera noltii* reduced leaf size under high flow velocities (Peralta et al. 2006). Hence, the aim of the present study is to assess in an aquarium experiment the interactive effects of wave forces and nutrient enrichment on the survival, morphodynamical, and biomechanical properties of seagrass *Zostera noltii*, a fast-growing species with an extensive morphological plasticity (Peralta et al. 2005). In most seagrass studies, morphodynamical responses are typically included by measuring variables such as length, appearance, and elongation rates of the different modules of the plant (i.e., shoots, rhizomes, and roots [Short et al. 1995; Lee and Dunton 2000; Peralta et al. 2005]). Biomechanical properties such as leaf strength and stiffness are, however, poorly studied on seagrasses (but see Patterson et al. 2001), even

\* Corresponding author: yayulanafie@yahoo.com

though they have been identified as important acclimation traits to the mechanical drag that imposes stress in several marine organisms (Koehl 2000) as well as in terrestrial plants (Anten et al. 2005). Waves form a major mechanical stress, so we included biomechanical properties in our study. We address the following questions: Are plants responding separately to both stressors? Or is there any combined effect of both stressors?

## Methods

**Experimental design**—In an experimental setup, *Zostera noltii* plants were exposed during 46 d to waves (present vs. absent) combined with water-column nutrient level (high vs. low) in a full factorial design: (1) wave-high nutrient (W-HN); (2) no wave-high nutrient (NW-HN); (3) wave-low nutrient (W-LN); and (4) no wave-low nutrient (NW-LN). Two replicate tanks per treatment were installed, giving a total of eight tanks, which were all filled with a 30-cm water column. Four big tanks ( $120 \times 100 \times 74$  cm<sup>3</sup>, 346 liters) were equipped with a hydraulic wave generator (operated 24 h daily), and two big tanks were split into two independent compartments to obtain four replicates without waves. Waves in the experiment were generated using a wave paddle that precisely fitted the width of the tank; the paddle was moved via a hydraulic piston from which the outward and inward movement could be controlled separately. To simulate natural waves in a small experiment is difficult because wave reflections will typically result in a standing wave. To prevent occurrence of such standing waves, we set the system to give approximately every 50 s a quick (10 s) push followed by a slow (40 s) retreat of the wave paddle. This resulted in a large wave, followed by a series of attenuating reflecting waves (Fig. 1). The reflecting waves were small enough to prevent a standing wave from forming. The resulting chaotic wave pattern does not reflect a natural system, but rather mimics a hydrodynamic stress in that the water movement causes the leaves to flap back and forth. The height of the pots was 11 cm and the water height was ~ 30 cm. The maximum wave height was ~ 4 cm (Fig. 1; Druck PTX 1830 pressure sensor). Each of the tanks contained 12 transparent pots ( $17.5 \times 11 \times 11$  cm<sup>3</sup>) with one experimental plant unit (EPU) consisting of a piece of a ramet composed of an apical and the first lateral shoot, plus two internodes in each pot (in total of 96 EPUs). The pots were surrounded by bricks acting as weights to prevent the pots from moving with the wave action. The sediment was composed of clean natural pebble (2–4-mm grain size) and sand in a 2 : 1 volume ratio. To minimize heterogeneity, the sediment was homogenized with a cement mixer. After filling the pots with the mixture, some more pebbles were placed on the surface of all the pots to avoid erosion due to wave action.

All tanks were filled with 0.2  $\mu$ m of filtered seawater from the eastern Scheld (southwest Netherlands) and the water of all the tanks was renewed twice per week. The ambient nutrient concentration of this seawater was used as low-nutrient treatment—(i.e., W-LN and NW-LN). For the high-nutrient treatments (i.e., W-HN and NW-HN),

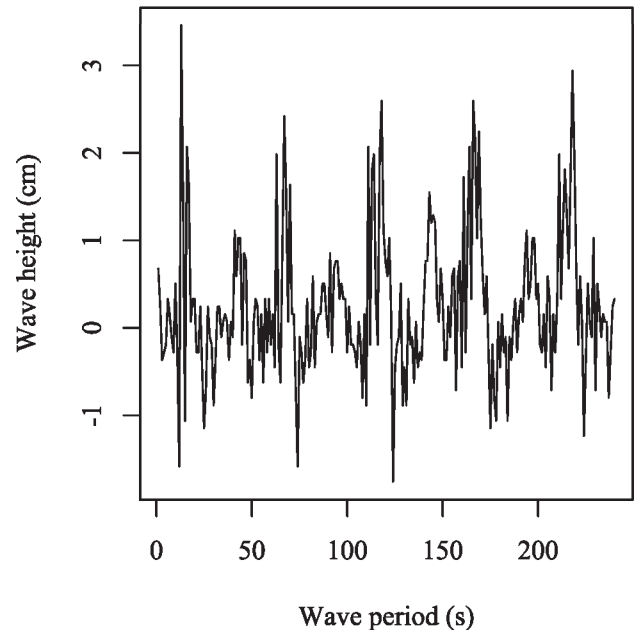


Fig. 1. Wave height in the tank, showing wave period of approximately 50 s, as a result of a quick (10-s) push followed by a slow (40-s) retreat of the wave paddle.

the water column in our treatments was enriched with  $\text{Ca}(\text{NO}_3)_2$  and  $(\text{NH}_4)_2\text{HPO}_4$  to a final desired average concentration of ~ 55  $\mu\text{mol L}^{-1}$  nitrate, 10  $\mu\text{mol L}^{-1}$  ammonium, and 10  $\mu\text{mol L}^{-1}$  phosphate. These nutrient concentrations corresponded to a moderately high eutrophication level (Burkholder et al. 1992; Valiela and Cole 2002). In addition to that, most of the cultural eutrophication (urban wastewater, agricultural run-off discharges) that affects seagrass habitats is mainly composed of nitrogen in the form of nitrate and ammonium and phosphorous in the form of phosphate (Burkholder et al. 2007; Cabaço et al. 2008). Continuous aeration was supplied to assure complete mixing of the water column. Every time the water was refreshed (twice per week), the position of the aeration systems and the pots (including plants) were changed randomly. During water refreshments, accumulated algae were removed by cleaning the pots, tanks, and leaves carefully. Amounts of algae were small and no large differences between treatments were observed. Observed dead plants and floating dead leaves were also taken out of the tanks. In addition, light irradiance was measured before water refreshment four times during the experiment with a Li-Cor LI-1000. At the same time, the chemical properties of the water column (i.e., salinity, temperature, and pH) were also measured by using a YSI multimeter sonde (Model 556).

Light was supplied by 14 Son-T lamps (400 Watts each) and the irradiance did not vary among and within treatments during the experiment. The average surface irradiance was 273  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (ranging from 248  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  to 294  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). Photoperiod was set at 18:6 light:dark period. The resulting daily integrated photon irradiance of 17.7 mol photons  $\text{m}^{-2} \text{d}^{-1}$  is known to facilitate the clonal growth in

Table 1. Morphological, dynamical, and biomechanical properties of the experimental plant units (EPUs) measured and/or calculated, at initial ( $t_0$ ) and final conditions ( $t_f$ ). Subscript  $i = 1, 2, 3, \dots$ ;  $t$  = experimental time (d).  $F$  (N) is force applied to the specimen;  $\delta$  (mm) is displacement of the specimen at the breaking point;  $L_0$  (mm) is initial length of the specimen.

Variables	Description
Survival (S), %	$S = \text{Live EPU} / \text{Initial EPU} \times 100$
Growth rate (GR), g fresh wt d <sup>-1</sup> EPU <sup>-1</sup>	$GR = (\text{Biomass}_f - \text{Biomass}_0) / (t_f - t_0)$
Aboveground biomass (AG), g dry wt EPU <sup>-1</sup>	Leaf biomass
Belowground biomass (BG), g dry wt EPU <sup>-1</sup>	Rhizome and root biomasses
Morphological properties	
Leaf length (LL), cm	Mean leaf length in an EPU
Internode length (IL), cm	Mean internode length in an EPU
Root length (RL), cm	Mean root length in an EPU
Internode abundance (IA), internodes EPU <sup>-1</sup>	Mean no. of internodes in each EPU
Root abundance (RA), roots EPU <sup>-1</sup>	Mean no. of roots in each EPU
Cross-section area (CA), mm <sup>2</sup>	$CA = \text{leaf width} \times \text{leaf thickness}$
Morphodynamical properties	
Internode appearance rate (IAR), internodes EPU <sup>-1</sup> d <sup>-1</sup>	$IAR = (IA_f - IA_0) / (t_f - t_0)$
Internode elongation rate (IER), mm EPU <sup>-1</sup> d <sup>-1</sup>	$IER = \Sigma(IL_{(t_f)} - IL_{(t_0)}) / (t_f - t_0)$
Biomechanical properties	
Specific force-to-tear ( $F_{TS}$ ), N mm <sup>-2</sup>	$F_{TS} = F_{MAX} / CA$
Young's modulus of elasticity for tension ( $E_T$ ), N mm <sup>-2</sup>	$E_T = (L_0 / CA) \times (F / \delta)$

this species (Peralta et al. 2002). Experiment-room temperature was kept constant throughout the entire experimental period (19°C). Prior to refreshment, water samples were collected to check whether nutrient uptake had occurred. At the beginning of the experiment, water samples were collected to check the applied concentrations. Ammonium, nitrate, and phosphate concentrations in water column were measured colorimetrically on an autoanalyzing system (QUAAtro).

**Biological material and measurement—*Zostera noltii*** Horneman plants were collected from an intertidal bed in Cádiz Bay Natural Park (Southern Spain, 36°29'19.79"N; 6°15'53.05"E) in May 2009, 7 d before starting the experiment. Some plants were randomly separated for initial measurements at the University of Cádiz (Cádiz, Spain). The rest of the plants were packed in moist, dark, and cool conditions to be transported the same day of collection to the Netherlands Institute for Sea Research laboratory (Yerseke, The Netherlands), where the experiment was conducted. Upon arrival at the Netherlands Institute for Sea Research (within 2 d), plants were kept in a reservoir with filtered natural seawater, aeration, and light in a mesocosm room (controlled temperature 19°C) for an acclimation period before starting the experiment. Among the pool of plants, 96 EPUs were randomly taken.

At the beginning of the experiment, we randomly separated 16 EPUs from the pool, and measured their morphological characteristics (leaf length, width, and thickness; internode length and abundance; and root length and abundance; Table 1). Prior to being transplanted, each randomly taken EPU was weighed (fresh weight [fresh wt]), and each rhizome was individually number-tagged in the youngest internode. EPUs were randomly assigned to experimental pots, and pots randomly distributed among

the treatments. During water refreshments (twice per week, i.e., 13 times), we quantified plant survival.

At the end of the experiment, all plant material was carefully harvested to keep rhizomes and roots intact. We conducted morphometric measurements on all of them, which allowed us to estimate the dynamical properties of the plants following Peralta et al. (2005; Table 1). Furthermore, living EPUs (tagged at the beginning) were individually weighed (fresh wt) to calculate the net growth rate (GR; mg fresh wt EPU<sup>-1</sup> d<sup>-1</sup>) using a linear growth model (Table 1). Finally, EPUs were divided into modules (leaves, roots and rhizomes), freeze-dried and weighed (dry weight; dry wt) to calculate the aboveground–belowground biomass ratio (AG:BG ratio).

**Biomechanical properties—**Mechanical properties of leaves were measured with a tensometer (Instron® model 3342)—at the University of Cádiz. We measured the strength and stiffness of the leaves (Patterson et al. 2001), which are scale-invariant material properties as they are standardized by leaf volume or cross-sectional area. These properties illustrate how leaves respond under the mechanical load resulting from wave action. Tearing (or tensile) tests—which measures the breaking force required to tear a tissue fragment—were conducted on a pool of 16 plants at the end of the experiment, by taking living shoots randomly from the harvested plants. The tests were always performed on the third leaf from the apical shoot of the EPUs, which was carefully cleaned of epiphytes and kept moist all the time. The chosen leaves were cut off at the junction between the sheath and the blade. Prior to the test, the width and thickness of the leaves were measured with calliper and dial thickness gauge (Mitutoyo®, precision  $\pm 0.01$  mm) respectively, to calculate the cross-section area (CA, mm<sup>2</sup>; Table 1). Then, the leaf blades were individually clamped



Table 2. Averaged nutrient concentrations before and after water refreshment for each nutrient treatment. Results of the two-sample Student's *t*-test for independent samples, showing the differences in nutrient concentration among treatments. Significantly different when  $p < 0.05$ ; superscript letters indicate significant differences between HN and LN (ns=nonsignificant).

	$\text{NH}_4^+$ ( $\mu\text{mol L}^{-1}$ )	$\text{NO}_3^-$ ( $\mu\text{mol L}^{-1}$ )	$\text{PO}_4^-$ ( $\mu\text{mol L}^{-1}$ )
After refreshment			
HN	$9.6 \pm 0.3^a$	$55.4 \pm 3.2^a$	$11.0 \pm 0.5^a$
LN	$0.7 \pm 0.1^b$	$19.3 \pm 0.5^b$	$1.2 \pm 0.04^b$
Before refreshment			
HN	$0.81 \pm 0.13^{\text{ns}}$	$6.87 \pm 3.37^{\text{ns}}$	$5.05 \pm 0.80^a$
LN	$0.84 \pm 0.11^{\text{ns}}$	$1.89 \pm 0.84^{\text{ns}}$	$0.21 \pm 0.07^b$

into the grips of the tensometer with the mountings 10 mm apart. Once clamped in the 5-Newton (N) grips (model 2712), the leaf blades were stretched at a velocity of  $10 \text{ mm min}^{-1}$ , while the extension ( $\delta$ , mm) and the force (F, N) were recorded every 0.1 s until the leaf blades broke, at which point the maximum force ( $F_{\text{MAX}}$ , N) and extension ( $\delta_{\text{MAX}}$ , mm) were recorded. As a result, we obtained the two biomechanical properties: (1) specific force-to-tear or strength ( $F_{\text{TS}}$ ,  $\text{N mm}^{-2}$ ) calculated as the maximum force ( $F_{\text{MAX}}$ , N) per cross-sectional area (CA,  $\text{mm}^2$ ), and (2) the modulus of elasticity for tension or stiffness ( $E_{\text{T}}$ ,  $\text{N mm}^{-2}$ ), which define the resistance to deformation (Niklas 1992) and is calculated by taking the initial, computer-fitted slope from the force against extension graph (F per  $\delta$ ), as well as considering the initial length ( $L_0$ ) and the cross-sectional area of the specimen (CA; Table 1).

**Statistical analysis**—In the factorial design, the experimental plots (independent tanks) were replicated twice. To demonstrate that there were no significant differences among the replicated tanks, a two-factor nested ANOVA test was conducted—where the tanks (the random factor) were nested within the treatments (the fixed factor). No significant differences were found between replicated tanks, so each plant in each pot was considered as an independent replicate when conducting further statistical tests. Plant survival was examined by Kaplan–Meier survival analysis (nonparametric) and the effects of the treatments were tested by log rank (Mantel–Cox) test with sequential Bonferroni-corrected  $\alpha$  levels. We set the  $\alpha$  values as highly significant, significant, and marginally significant when  $\alpha = 0.01$ , 0.05, and 0.1, respectively. Survival data are presented as percentage of living (survived) plants. To test the effect of waves (W), nutrients (N), and their interaction ( $W \times N$ ) on two categories of response variables (i.e., morphodynamical and biomechanical properties of the plants), we used a multivariate analysis of variance (MANOVA). The morphodynamical properties included growth rate; above- and belowground biomass; leaf, root, and internode length; internode and root abundance; internode appearance; and internode elongation rate. The biomechanical properties included strength and stiffness. The  $p$ -values of the univariate responses were used to indicate which of these separate morphodynamical variables gave the strongest response within group of morphodynamical variables. This was also applied to the group of biomechanical variables (i.e., toughness and stiffness). Those data that deviated

from normality (Kolmogorov–Smirnov's test) or homoscedasticity (Levene's test) were transformed prior to analyses to meet MANOVA assumptions. Data for these variables are presented as mean  $\pm$  1 standard error. Differences in nutrient concentrations among treatments were tested by a two-sample Student's *t*-test for independent samples.

## Results

**Water parameters**—Throughout the experiment, water temperature ( $21$ – $22^\circ\text{C}$ ), salinity ( $30$ – $31\text{‰}$ ), pH ( $8.0$ – $8.2$ ), and dissolved oxygen ( $85$ – $92\%$ ) remained fairly constant. The initial nutrient concentration after water refreshments statistically differed between HN and LN treatments (Table 2; 2-sample Student's *t*-test,  $df = 3$ ,  $p < 0.05$ ), which demonstrates that the nutrient treatment was correctly applied. The nutrient concentrations in the water before water refreshment were lower than the applied ones, indicating that there was nutrient uptake in all the treatments (Table 2). Although this uptake may have been partly due to uptake by algae, we expected that this 'sink' was limited due to (1) the high refreshment rate, (2) the frequent removal of algae, and (3) the observation that the amount of removed algae was small (not quantified).

**Biological measurements**—All plants survived during the first 4 weeks (eighth observation) independent of the treatment (Fig. 2A). It was not until the fifth week (i.e., ninth observation; Fig. 2A) when some of them started to die in the W treatments (both HN and LN), wherein many dead rhizomes were buried without aboveground biomass. Only 42% and 62% of the initial plants were alive at the end of the experiment in the W–HN and W–LN treatment, respectively. Contrastingly, plants in NW treatments remained alive until the sixth and seventh week for HN and LN, showing a final survival percentage of 79% and 92%, respectively. Results of the Kaplan–Meier survival analysis (Table 3; Fig. 2A) showed significantly lower survival under wave treatments as compared with non-wave treatments, both when comparing the high-nutrient treatments and comparing the low-nutrient treatments ( $\alpha = 0.1$ ; Table 3). In addition, results of the two-way MANOVA showed a negative influence of waves on morphodynamic properties. The strongest negative univariate responses to waves were on internode abundance and appearance rate, growth rate, and leaf length (Table 4; Fig. 2B,D,E).

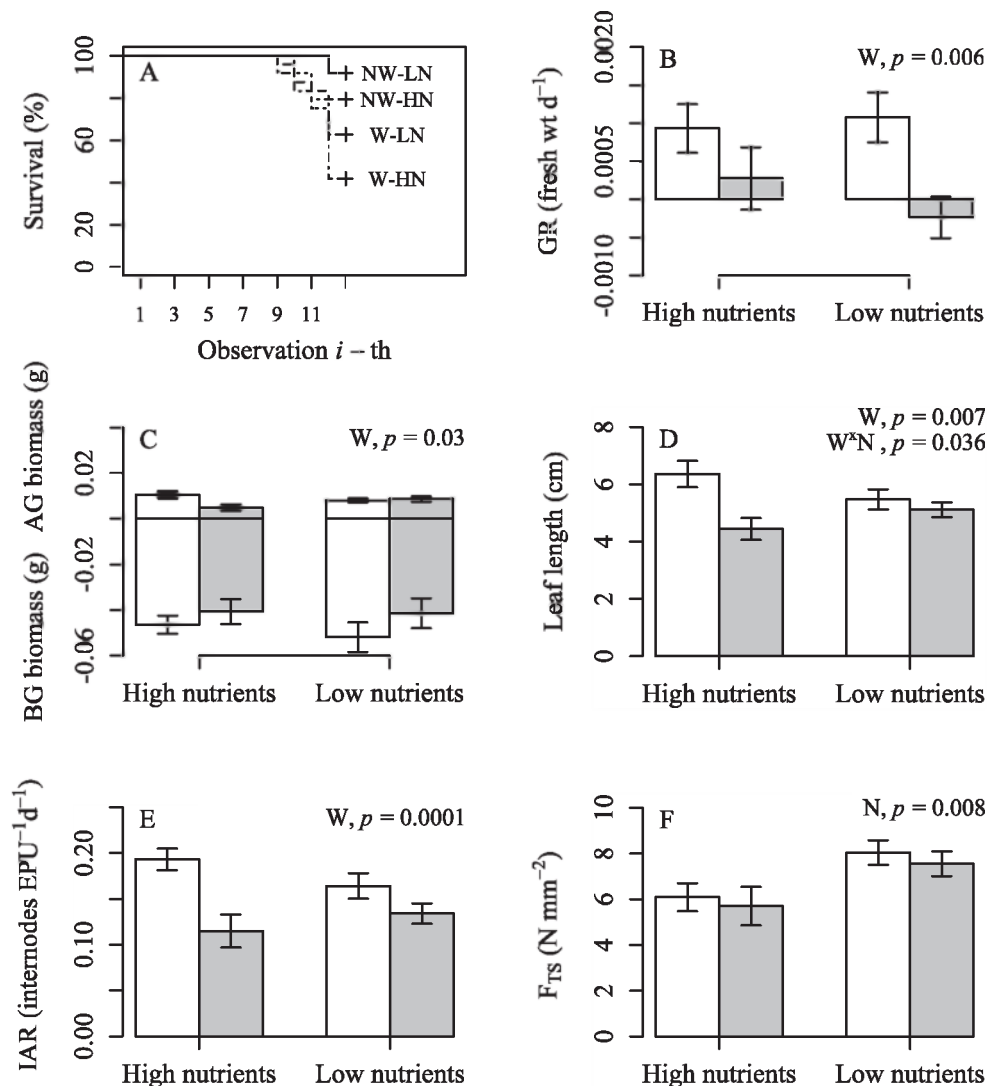


Fig. 2. (A) Kaplan–Meier survival function for each treatment, showing the percentage survival and number of observations (significances *see* Table 2). (B–E) show the univariate responses that are most related to the significant multivariate response of morphodynamical properties to waves and nutrients (i.e., [B] growth rate, [C] aboveground biomass [belowground biomass also depicted], [D] leaf length, and [E] internode abundance rate). (F) shows the univariate response of stress (or specific force-to-tear [ $F_{TS}$ ]) that is most related to the significant multivariate response of the biomechanical properties to nutrients. The  $p$ -values give an indication of the strength of the univariate response (Table 4). Error bars represent mean  $\pm$  1 SE. All blank bars correspond to no wave (NW) treatment, while grey bars correspond to wave (W). The letter W indicates waves, and N indicates nutrients.

Moreover, strong interactive effects were shown for survival and morphodynamical properties. The combination of wave treatment and high nutrients showed a significantly reduced survival as compared with the nonwave, low-nutrient treatment ( $\alpha = 0.01$ ; Table 3). Morphodynamical properties were synergistically negatively affected by the combination of waves and nutrients, with dominant univariate effects on aboveground biomass and leaf length (Table 4; Fig. 2C–D).

**Biomechanical properties**—The biomechanical properties (the combination of strength or specific force-to-tear [ $F_{TS}$ ]

and stiffness or modulus of elasticity for tension [ $E_T$ ]) were significantly negatively influenced by nutrient additions, with the strength having the strongest response (MANOVA; Table 4; Fig. 2E). The biomechanical properties were not influenced by wave presence (Table 4).

## Discussion

Increasing (anthropogenic) nutrient loads and storm frequency (due to climate change) in coastal areas may globally affect seagrass habitats. To assess the seagrass responses to such multistress conditions, we quantified the

Table 3. Kaplan–Meier survival analysis (nonparametric) showing results of log rank (Mantel–Cox) test comparing survival of EPU among treatments ( $n = 6$ ).

Treatment	Log-rank (Mantel–Cox)		
	$\chi^2$	df	$p$
W–HN–NW–HN	5.634	1	0.018*
W–HN–W–LN	1.777	1	0.182
W–HN–NW–LN	13.906	1	0.0001***
NW–HN–W–LN	1.208	1	0.272
NW–HN–NW–LN	1.685	1	0.194
W–LN–NW–LN	6.108	1	0.013*

\*  $p < 0.1$ ; \*\*  $p < 0.05$ ; \*\*\*  $p < 0.01$ .

combined effects of the presence vs. absence of waves and high vs. low nutrient load on the seagrass *Zostera noltii*. To our best knowledge, we show for the first time how waves and high nutrient loads interact and affect the survival and morphodynamical properties of a seagrass species. Furthermore, our study showed that waves mainly had a negative effect on the morphodynamical properties, whereas nutrient enrichment affected biomechanical properties on the seagrass *Zostera noltii*. However, the effect of combined stresses (waves and nutrient enrichment) was much larger than expected from assessing both factors separately.

In our experiment, survival was negatively affected by waves and by the interaction of waves and nutrient enrichment. The highest survival percentage was found in NW–LN (92%), followed by NW–HN treatment (79%); whereas, the lowest survival was by far recorded in W–HN (42%), followed by W–LN (63%; Fig. 2A). Significant (and/or marginally significant) differences resulted among W and NW treatments, which indicated that waves predominated over nutrient effects regarding plant survival. It may be speculated that toxic effects of ammonium (van Katwijk et al. 1997; Brun et al. 2002) might, in part, explain the lowest survival in W–HN-treated followed by W–LN-treated EPU, because wave-treated EPU (both HN and LN) are likely to have lower diffusive boundary layers (Koch 1994; Morris et al. 2008) and, hence, could have a higher ammonium uptake (Britto and Kronzucker 2002). Although the ammonium levels used in this experiment ( $\approx 10 \mu\text{M}$ ) are close to toxic levels (van Katwijk et al. 1997; Brun et al. 2002), we lack the data to substantiate the hydrodynamic influence on ammonium toxicity in the present study, and this should be studied in the future.

We found shorter leaves as well as lower aboveground biomass under high-wave conditions (especially in W–HN treatment) compared with those under no-wave treatments. Differences in hydrodynamic treatments are such that present results on waves cannot be directly compared with previous studies using unidirectional flow. However, the observed shorter leaves seems in line with previous studies describing the effect of waves (van Katwijk and Hermus 2000), flow velocities (Puijalon et al. 2007), and nutrient enrichment (Short et al. 1995) on leaf length (LL) in seagrass as well as on freshwater plants. This morphological

acclimation appears to be functional in reducing drag forces (Bouma et al. 2005) and thereby lowering the risk of uprooting or mechanical damage (Schutten and Davy 2000).

We observed a general negative effect of waves on the belowground dynamics, because internode abundance (IA), internode appearance rate (IAR), and root appearance rate (RAR) were lower in W treatments than in NW. Previous studies on *Z. noltii* responses to enhanced current velocity have reported contrasting effects, varying from enhanced belowground growth in order to enhance seagrass anchorage (Peralta et al. 2006; de los Santos et al. 2010) vs. findings in agreement with present results (i.e., Schanz and Asmus [2003] for *Z. noltii* and Puijalon et al. [2007] for freshwater plants). The lack of consistent results emphasizes the need for direct comparative studies on waves and flow, and a great emphasis on the effects of hydrodynamics on plant adaptation in general. In general terms, it may be speculated that low investment in belowground biomass in high energetic environments may threaten the sustainability of *Z. noltii*.

Sessile organisms (such as seagrasses) experience drag and deformation due to hydrodynamic forces. The strength of this effect depends on the biomechanical properties and the exposed surface area of the tissue (Koehl et al. 2001). Our measurements on both the material properties and size of *Zostera noltii* leaves showed that, predominantly, nutrient enrichment weakened the leaves of *Zostera noltii* by reducing their strength. This agrees with previous observations by Burkholder et al. (1992) that *Zostera marina* leaves under moderate (but chronic) nitrate enrichment seemed more brittle and broke easily. The strongest leaves were found in the treatment with the highest survival (i.e., NW–LN [Fig. 2A,E]). This suggests that having strong leaves contributes to a higher survival chance, and that biomechanical properties are an important factor to be studied in seagrass ecology.

In summary, this study shows for the first time that, when assayed together, waves and nutrient enrichment had a negative effect on *Zostera noltii* survival, aboveground biomass, and leaf length. When assayed separately, waves negatively affected survival and morphodynamical properties of the plants, whereas nutrient enrichment negatively affected biomechanical properties. These specific responses may be expected to induce negative feedbacks that may eventually lead to meadow collapses. For example, a decrease in aboveground biomass and leaf length due to waves will reduce the wave attenuation capacity of the bed, thereby resulting in more stress and, thus, a further reduction of aboveground biomass and leaf length. This negative feedback may be accelerated by an increasing risk of seagrass loss by uprooting, due to the decreased belowground growth under wave and high-nutrient conditions. Another example of a potential negative feedback may originate from the reduced strength of the seagrass leaves under nutrient loading. The latter may lead to increased plant losses under even relatively mild hydrodynamic regimes. Obviously, in combination, these negative feedbacks enforce each other even further, accelerating the potential collapse. Though the threshold concentration of

Table 4. Results of two-way MANOVA based on categorical data, showing the effects of nutrients and waves on the morphodynamical and biomechanical properties of the EPU's. The morphodynamical properties include growth rate; above- and belowground biomass; leaf, root, and internode length; internode and root abundance; internode appearance; and internode elongation rate. The biomechanical properties include strength and stiffness. Univariate responses are shown as mean per treatment  $\pm$  SE, with  $n$ -sample (mean(SE) $n$ ). To show an indication of the strength of the response,  $p$ -values are included in the table. Data transformation is specified when conducted.

Variables†	Treatments				Data transformation	p-value			
	NW-HN	W-HN	NW-LN	W-LN		W	N	W × N	
Morphodynamical									
Biomechanical									
GR	0.0009(0.0003)19	0.0003(0.0004)10	0.001(0.0003)22	0.0002(0.0003)15	—	0.002*	0.141	0.004*	
AG	0.010(0.002)19	0.005(0.001)10	0.008(0.001)22	0.009(0.001)15	—	0.801	0.032*	0.980	
BG	0.046(0.004)19	0.041(0.005)10	0.052(0.006)22	0.042(0.006)15	10(log x+3)	0.006	0.597	0.351	
AG:BG	0.22(0.022)19	0.13(0.031)10	0.16(0.013)22	0.22(0.03)15	(√x) <sup>-1</sup>	0.03	0.188	0.008	
					—	0.179	0.914	0.757	
						—	—	—	
Morphological properties									
LL	6.36(0.46)19	4.44(0.38)10	5.47(0.35)22	5.11(0.25)15	10log x	0.007	0.942	0.036	
IL	6.12(0.50)19	7.98(1.5)10	6.77(0.65)22	7.06(0.55)15	(x+1) <sup>-1</sup>	0.168	0.524	0.595	
RL	2.51(0.18)19	2.70(0.24)10	2.94(0.18)22	2.61(0.20)15	—	0.750	0.413	0.214	
IA	10.89(0.53)19	7.30(0.83)10	9.55(0.63)22	8.13(0.52)15	√x	0.0001	0.866	0.055	
RA	24.42(2.91)19	17.40(3.39)10	21.33(2.04)22	17.40(1.58)15	10log x	0.020	0.998	0.294	
Morphodynamical properties									
IAR	0.19(0.012)19	0.12(0.018)10	0.17(0.014)22	0.13(0.011)15	10(log x+1)	0.0001	0.760	0.100	
IER	1.18(0.12)19	0.77(0.09)10	1.02(0.07)22	0.91(0.08)15	—	0.012	0.947	0.149	
Biomechanical properties									
F <sub>TS</sub>	6.09(0.61)6	5.70(0.84)6	8.04(0.54)6	7.55(0.54)6	—	0.501	0.008	0.939	
E <sub>T</sub>	137.79(10.66)6	134.87(15.31)6	159.17(7.71)6	152.07(6.20)6	—	0.640	0.083	0.845	

\*  $p < 0.05$ ; \*\*  $p < 0.01$ .

† Variables measured are growth rate (GR, g fresh wt EPU<sup>-1</sup> d<sup>-1</sup>); aboveground biomass (AG, g dry wt EPU<sup>-1</sup>); belowground biomass (BG, g dry wt EPU<sup>-1</sup>); leaf length (LL, cm); internode length (IL, cm); root length (RL, cm); internode abundance (IA, internodes EPU<sup>-1</sup>); root abundance (RA, roots EPU<sup>-1</sup>); internode appearance rate (IAR, internodes EPU<sup>-1</sup> d<sup>-1</sup>); internode elongation rate (IER, mm EPU<sup>-1</sup> d<sup>-1</sup>); specific force-to-tear (F<sub>TS</sub>, N mm<sup>-2</sup>); and Young's modulus of elasticity for tension (E<sub>T</sub>, N mm<sup>-2</sup>).



nutrients as well as hydrodynamics may vary with geographic region, our study shows that restoration and conservation managers should be well aware of the increased risk of collapse when two such omnipresent stressors as high level of nutrients and waves are interacting.

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