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Rhizome starch as indicator for temperate seagrass winter survival

Laura L. Govers [a,*], Wouter Suykerbuyk [a,b], Jacqueline H.T. Hoppenreijs [a], Kris Giesen [a,b], Tjeerd J. Bouma [b], Marieke M. van Katwijk [a]

[a] *Department of Environmental Science, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands*
[b] *Department of Spatial Ecology, NIOZ Yerseke Royal Netherlands Institute for Sea Research, P.O. Box 140, 4400 AC Yerseke, The Netherlands*

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ABSTRACT

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.

1. Introduction

Seagrass beds are important coastal ecosystems, as they are highly productive, sequester carbon, 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., 2015; 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, 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

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[*] Corresponding author at: Department of Environmental Science, Radboud University Nijmegen, Faculty of Science, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands. Tel.: +31 63 057 1679; fax: +31 24 355 3450.
E-mail addresses: lauralgovers@gmail.com, l.govers@science.ru.nl (L.L. Govers).

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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, 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 cm^2 cm^{-2} day^{-1} versus loss rates of -0.110 cm^2 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 (Fig. 1) with a winter leaf removal field experiment, using *Z. noltii* as a model species.

2. Materials and methods

2.1. Location

We studied the winter survival of the natural *Z. noltii* beds and transplanted *Z. noltii* beds in the Oosterschelde Delta, The Netherlands (Fig. 1a, 51°39′ N, 4°01′ E). The Oosterschelde has a surface area of 351 km^2, 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 *Z. 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 *Z. 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 submersed) is around 3–4.5 m Secchi disc visibility in summer (Wetsteyn and Kromkamp, 1994).

2.2. 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 10 × 10 m or 15 × 15 m plots on several mudflats in the Oosterschelde (for details, see Suykerbuyk et al. (2012)) (Fig. 1). Plots were monitored in spring and summer from spring 2009 to 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 each spring (end of April/start of May) and each autumn (end of October, start of November). Simultaneously, samples for starch analysis were taken from a number of natural seagrass beds in the Oosterschelde. In addition several abiotic characteristics (sediment grain size, sediment organic C, leaf %C, leaf %N, porewater NH_4, NO_3, PO_4, and H_2S) were determined every summer (Table 1). For detailed methodological details on these measurements, please see Govers et al. (2014b).

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.

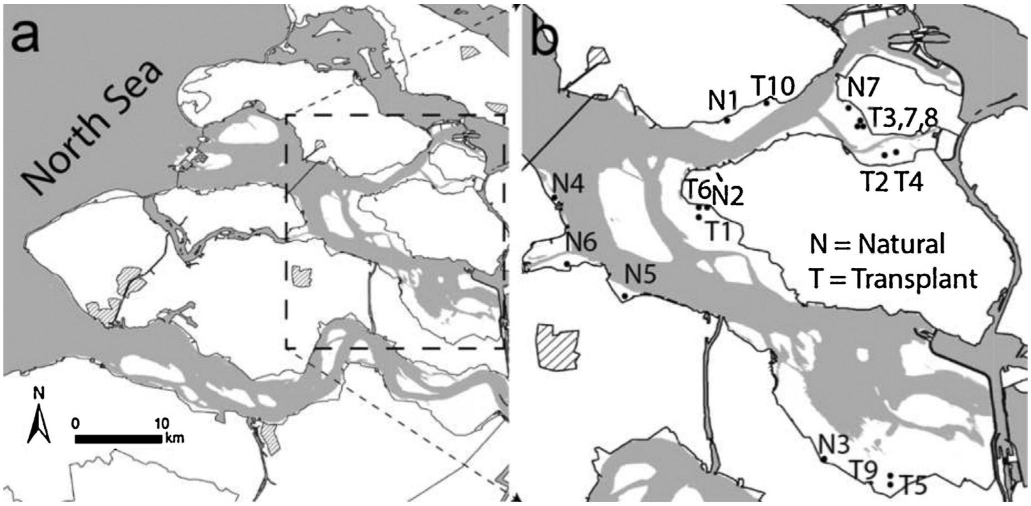

Fig. 1. Maps of (a) the Dutch Delta, and (b) the locations of the transplant sites (T1–10) and natural seagrass beds (N1–7) in the Oosterschelde.

Table 1
Abiotic characteristic of the natural populations, successful transplants and unsuccessful transplants. Both mean values and standard errors (in italics) of all parameters are displayed. Parameters units are: sediment median grain size (um), sediment organic C (%), leaf total nitrogen (% DW), leaf total carbon (% DW), porewater ammonium concentrations (umol L^{-1}), porewater nitrate concentrations (umol L^{-1}), porewater phosphate concentrations (umol L^{-1}), and porewater sulfide concentrations (umol L^{-1}). Hydrodynamic exposure was categorized into three categories: exposed, intermediated, and sheltered. Statistical differences are indicated by letters (a,b), and based on Tukey post-hoc tests.

Site	Grain size		Sediment C		Leaf %N		Leaf %C		NH_4		NO_3		PO_4		H_2S		Hydrodynamic Exposure
Natural populations	115^a	*4.6*	0.44^a	*0.06*	3.33^a	*0.06*	36.1^a	*0.4*	55^a	*11*	2.0^a	*0.8*	13.16^a	*1.8*	1.49^a	*1.0*	Exposed to sheltered
Successful transplants	133^a	*7.9*	0.24^b	*0.02*	3.28^a	*0.08*	36.6^a	*1.2*	88^a	*17*	47.1^b	*22.2*	17.97^a	*2.6*	22.97^b	*11*	Exposed to sheltered
Unsuccessful transplants	122^a	*4.7*	0.26^b	*0.03*	3.32^a	*0.04*	36.3^a	*0.9*	89^a	*9*	26.7^b	*3.6*	17.46^a	*1.0*	26.65^b	*12*	Exposed to sheltered

2.3. Leaf removal experiment set-up

Winter survival of *Z. noltii* occurs by a short rhizome with a single shoot, which encloses an active meristem (Vermaat and Verhagen, 1996) (Fig. 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, Fig. 1b N2), 38.8 mg g^{-1} DW in Oostdijk (OD – medium starch, Fig. 1b N3), and 18.8 mg g^{-1} DW in KATS (Kats – low starch, Fig. 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 2.

On each site, we selected five paired plots of 50 × 50 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 to 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 30 × 30 cm square was harvested.

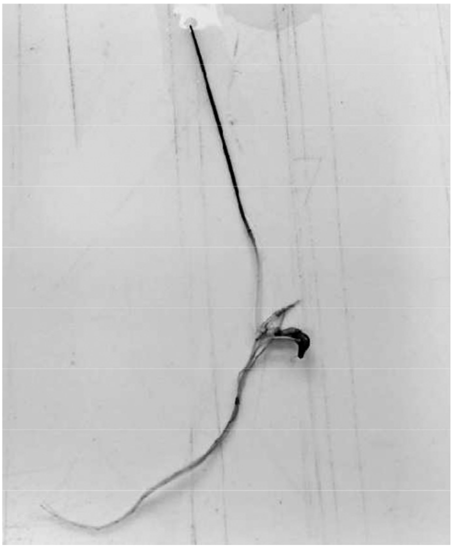

Fig. 2. Photograph of wintering unit of *Z. noltii* with one single shoot and an active meristem.

2.4. 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–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 ($>0.7\ F_v\ F_{m^{-1}}$; 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 1 h 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 ($(Fm - F_0)/Fm$). Fm is the maximum phyto-fluorescence after dark-adaptation and F_0 is the minimum fluorescence of the chlorophyll. Photosynthetic yield was measured on the middle part of each leaf (in the middle between the leaf tip and leaf base).

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 up into aboveground and belowground biomass, frozen and subsequently freeze-dried, after which their dry weight was determined. Harvested samples were treated in the same way after termination of the experiment.

2.5. Starch analysis of transplants, natural beds and experiment

Freeze-dried rhizomes were ground for the starch measurements, and soluble sugars were extracted in ethanol. Subsequently, starch was extracted from the ethanol-insoluble fraction by hydrolysis in 5% HCL and boiled at 100° C for 30 min. Next, both soluble sugars and starch were measured by anthrone assay standardized to sucrose (Yemm and Folkes, 1954). All samples were measured in duplicate and a new duplicate calibration curve was prepared for every series of measurements.

2.6. Statistical analysis

Normal distribution of the data was tested on the data with the Shapiro Wilk test, and data were log-transformed prior to testing if assumptions were violated. Outliers were detected with Dixon's Q test (Dean and Dixon, 1951), and removed from the data. Abiotic differences between natural population sites, successful, and unsuccessful transplant sites were determined by one-way

Table 2
Characterization of the leaf experiment sites.

Location	Code map	Starch[a]	Starch characterization	Grain size	Sediment C	Leaf %N[a]	NH_4[b]	NO_3[b]	PO_4[b]	H_2S[c]	Hydrodynamic exposure
DMN	N2	57.3	High	103	0.49	3.1	119.97	0.85	19.67	11.56	Exposed
OD	N3	38.8	Medium	78	0.44	2.8	22.75	0.00	10.55	23.48	Sheltered
Kats	N4	18.8	Low	117	NA	3.0	26.75	0.70	7.65	0.00	Intermediate

Abbreviations: DMN = Dortsman Noord, OD = Oostdijk, Kats = Kats. Sed. = sediment. Units are: starch (mg g^{-1} DW), sediment median grain size (D50, um), sediment organic C (%), leaf total nitrogen (% DW), and porewater NH_4, NO_3, PO_4 and H_2S were measured in the porewater.

[a] Sampled and measured in November 2012.

[b] Sampled and measured in September 2012.

[c] Sampled and measured in September 2013.

ANOVAs with a Tukey HSD post-hoc test. PAM yield, shoot density and rhizome starch were tested with a two factor (time, location) repeated measures ANOVA in IBM SPSS Statistics 21.0. Experimental effects and transplant effects were tested with linear mixed effect models using the lme function with a Tukey HSD post-hoc test and location or time as a random term. Data were tested in the R environment (version 2.15).

3. Results

3.1. 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 ($R^2 = 0.02$, Fig S1). 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 (Fig. 3). 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 were not mutually different (Fig. 3). 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 1).

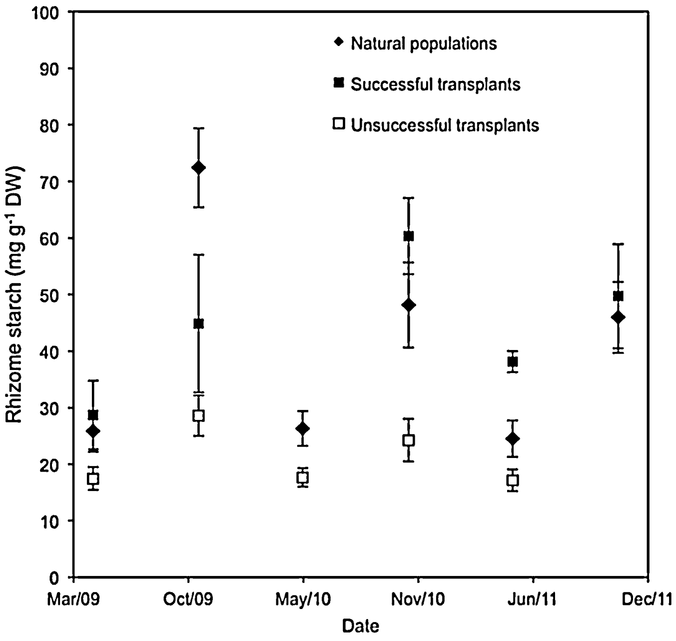

Fig. 3. Rhizome starch concentration in spring and autumn in the period 2009–2011 of natural populations, successful transplants and unsuccessful transplants in the Oosterschelde. Displayed values are means and error bars represent standard errors (SE).

3.2. 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 (Fig. 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) (Fig. 4b). Shoot density did not differ significantly between the latter two locations (Fig. 4b). Shoot density also decreased during winter ($F_{1,78} = 7.496$, $P = 0.019$), 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 (Fig. 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 (Fig. 4a) and shoot densities started to increase again around March (2013), in DMN (high) and OD (medium) (Fig. 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.

Leaf removal treatments resulted in lower shoot densities at DMN (high) (Linear mixed model, $F_{1,53} = 7.547$, $P = 0.008$) and OD ($F_{1,53} = 23.58$, $P < 0.001$), 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) ($F_{1,53} = 0.607$, $P = 0.439$), where standing biomass was very low to begin with (Fig. 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,

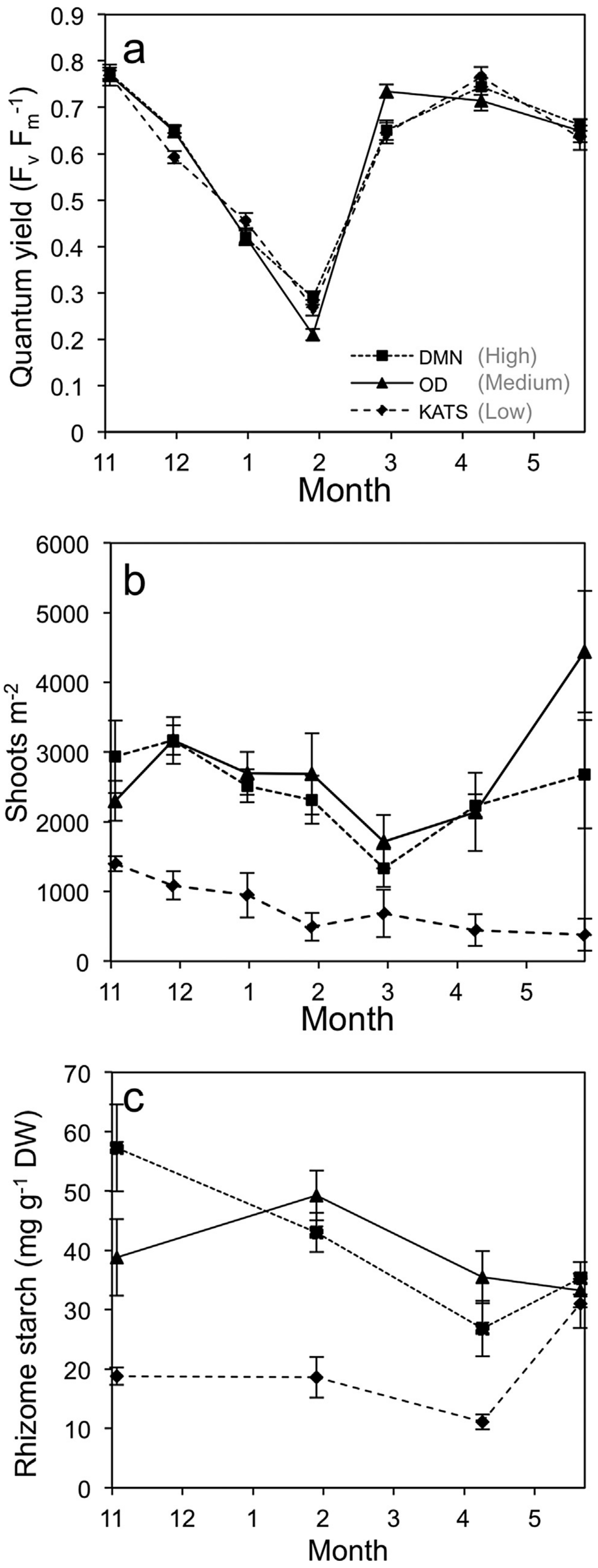

Fig. 4. (a) PAM yield, (b) shoots m^{-2}, and (c) rhizome carbohydrate concentration of *Z. noltii* on three different locations. Displayed values are means and error bars represent standard errors (SE).

$F_{1,26} = 0.861$, $P = 0.362$) (Fig. 5a). Seagrass biomass differed significantly between all three locations (ANOVA, $F_{1,27} = 31.68$, $P < 0.001$), with biomass OD (medium) > DMN (high) > Kats (low) and Kats having a total biomass m^{-2} that was 10× lower than at OD (medium) (Fig. 5a). Rhizome starch concentration, however, did not differ anymore between locations in June 2013 (ANOVA, $F_{2,26} = 3.207$, $P = 0.057$), despite the differences in autumn. Leaf removal treatment did not affect rhizome starch concentration in June 2013 (Linear mixed model, $F_{1,25} = 1.81$, $P = 0.191$) (Fig. 5b).

Rhizome starch concentration in November 2012 was positively related to shoot density in June 2013 ($R^2 = 0.51$, $P = 0.006$) (Fig. 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 (Fig. 6):

$$y = 67.69x - 723.43 \ (1) \qquad (1)$$

In this equation (Eq. (1)), x describes rhizome starch levels in November (in mg g^{-1} DW) and y describes shoot densities (# m^{-2}) the following June.

4. 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.

4.1. 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 *Z. noltii.* By solving Eq. (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

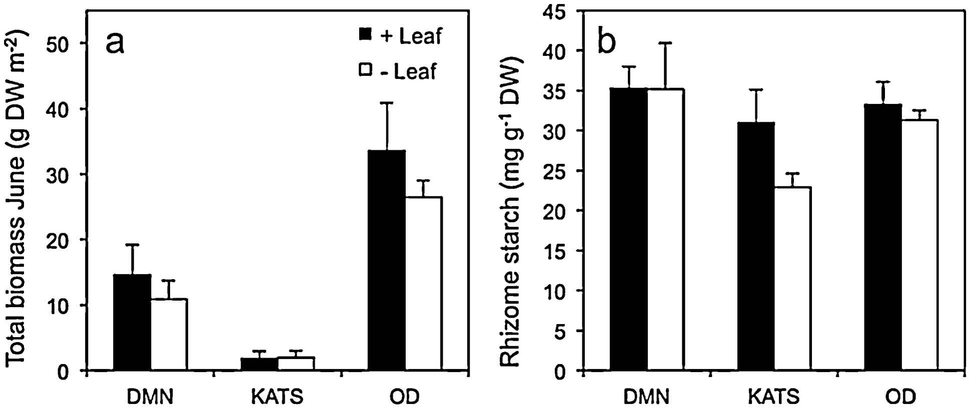

Fig. 5. Effects of the leaf removal treatment (+leaf and −leaf) on (a) seagrass biomass, and (b) rhizome carbohydrate concentrations in June, at the end of the experiment on three locations: DMN (high), Kats (low), and OD (medium). Displayed values are means and error bars represent standard errors (SE).

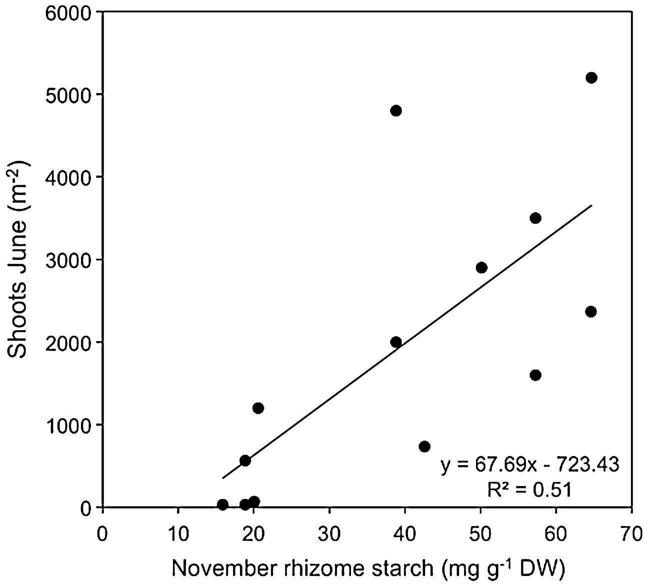

Fig. 6. The relationship between rhizome carbohydrate concentration in autumn and next year's shoot density in June.

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 *Z. 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.

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

We found that photosynthetic performance of *Z. 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.

5. 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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecolind.2014.10.002.

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