The response of the seagrass *Halodule wrightii* Ascherson to environmental stressors

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Abstract

Seagrasses are subjected to intense levels of anthropogenic disturbance as a result of the shallow nearshore waters they inhabit. Some seagrasses are known to have dynamic growth patterns, enabling them to colonize unstable shallow environments and adapt to a range of disturbances. This can result in high levels of variability in morphological and physiological attributes. The seagrass *Halodule wrightii* is known to be a fast-growing pioneering species with a large geographic range. The present study examines *Halodule wrightii* in a region under intense anthropogenic stress in order to determine what are the main environmental drivers affecting the morphology, physiology and status of these habitats. Parameters of plant morphology, physiology and status were measured either at the meadow scale (e.g. biochemistry) or at a higher frequency shoot scale (e.g. shoot width). We assigned an impact assessment index to a series of seagrass sites over a gradient of anthropogenic disturbance and found this to be explanatory of a number of the seagrass parameters measured including epiphyte cover, stable isotope $\delta^{15}N$ and ETR_{max} however, it did not clearly explain shoot density, a commonly used bioindicator of environmental stress. At the shoot scale, Principal Component Analysis identified epiphyte and leaf width to have the strongest association. At the meadow scale this was shoot density, dry weight and E_k, albeit with the most impacted sites showing highest shoot density. Stable isotope ($\delta^{15}N$) and leaf length were most significant in explaining the variation between sites and impact category, providing a direct link between anthropogenic sources of nutrients to seagrass meadow density.

1. Introduction

The coastal and estuarine waters where seagrasses grow are some of the most heavily impacted regions of the marine environment (Cabaço et al., 2008a; Short and Wyllie-Echeverria, 1996). The anthropogenic pressures from urban and industrial developments occurring in proximity to these areas has resulted in Worldwide declines of seagrass meadows (Short and Wyllie-Echeverria, 1996; Waycott et al., 2009). The biggest threat to seagrasses from coastal development is attributed to poor water quality resulting in the direct or indirect reduction of light availability (Biber et al., 2009; Burkholder et al., 2007; Duarte, 2002; Hemminga, 1998; Jiang et al., 2013). However, in some locations the direct physical damage and removal of seagrass from port/harbour developments and boat anchoring could arguably pose the most critical risk (Grech et al., 2012, 2011).

Seagrasses will exhibit various changes in morphology and physiology in order to adapt to changes in light environment and numerous shading studies have shown decreases in leaf length, leaf width, leaves per shoot and shoot growth (Bertelli and Unsworth, 2018; Biber et al., 2009; Collier et al., 2012; Olesen and Sand-jensen, 1993; Yaakub et al., 2013). The reduction in above-ground biomass reduces respiratory demand of the plant but decreases its photosynthetic capacity and carbon uptake (Campbell and Miller, 2002; Collier et al., 2012; Ralph et al., 2007). Light reduction is rapidly reflected in the photosynthetic performance of seagrasses resulting in a decrease in electron transport rates and carbon capture which can be measured *in situ* using chlorophyll fluorescence (Bité et al., 2007; Ralph and Gademann, 2005). Events which frequently reduce light to levels below the minimum light requirement (MLR) can lead to plant mortality and meadow die-off. Responses have been found to be species-specific in some cases (Bité et al., 2007; Collier et al., 2016; Silva et al., 2013) with factors such as seagrass plant size (Roca et al., 2016), life history, habitat and meadow form found to be significant in response time and sensitivity to environmental stressors (Kilminster et al., 2015). The

morphological and physiological changes exhibited by seagrasses in response to changes in light availability make seagrasses good ecological indicators of water quality (Grice et al., 1996; McMahon et al., 2013). However, our knowledge of these seagrass responses to water quality is limited to certain species and localities (McMahon et al., 2013). Nutrient availability is exhibited by the long-term uptake within seagrass tissue, and the ratio of leaf nutrients (C:N:P) has been successfully shown to be an indicator of nutrient enrichment in some seagrass species (Burkholder et al., 2007; Carruthers et al., 2005; Fourqurean et al., 1997; Orth et al., 2006). Nutrient inputs can lead to increases in productivity where waters are oligotrophic, but continued nutrient loading has also been found to reduce shoot density (Carruthers et al., 2005; Fourqurean et al., 2003, 1995; Tomasko et al., 1996). The evidence of reduction in shoot density and biomass from nutrient loading highlights the importance of recognising sources of nutrient inputs in seagrass meadows to allow for the management of these habitats for health and resilience. The use of stable isotopes of nitrogen (δ^{15}N) and carbon (δ^{13}C) are being increasingly used as environmental tracers within marine ecosystems (Jennings et al., 1997; Lepoint et al., 2004), and have been used to provide insight into the sources of nutrients in seagrass meadows (Carruthers et al., 2005; Fourqurean et al., 1997; Jones et al., 2018; Lepoint et al., 2004).

Halodule wrightii (Ascherson) is a common shallow and intertidal seagrass in the Tropical Atlantic Bioregion with its southern limit occurring in Brazil. Populations near the southern limit have rarely been recorded reproducing sexually, making them somewhat borderline and spatially discrete (Creed, 1997). *H. wrightii* beds have been found to show large amounts of inter-population variation in morphology as well as shoot density and biomass (Creed, 1997). This suggests environmental factors are affecting the morphology and the development or expansion of these seagrasses. Factors such as high sediment instability, low temperatures, strong wave action at low tides or exposure to air have been described as some of the potential factors that limit the existence of *H. wrightii* (Barros et al., 2013), although such drivers remain poorly explored (Creed, 1997; Creed and Amado Filho, 1999; Pitanga et al., 2012). Direct observations of environmental effects on Brazilian seagrasses are limited

(Barros et al., 2013), with data such as leaf nutrient content and photophysiological attributes lacking in the literature. Decline and die-off of *H. wrightii* meadows have also been attributed to anchor damage and overgrowth of epiphytic algae (Creed and Amado Filho, 1999; Papini et al., 2011; Sordo et al., 2011) likely caused by excess nutrients (Balata et al., 2010; Burkholder et al., 2007). Although *H. wrightii* has been found to tolerate eutrophic waters more so than other competing species (Fourqurean et al., 1995; Lapointe et al., 1994; Powell et al., 1989), nutrient enrichment will greatly increase epiphytic biomass which effects seagrass productivity (Wear et al., 1999).

The aims of the present study were to examine the anthropogenic and natural environmental drivers that modify *H. wrightii* at a shoot and a meadow scale in order to describe and define what are the major factors affecting the development of these seagrasses. By better understanding these drivers, it is possible to compare the shoot and meadow scale responses of this tropical species to other seagrasses and ascertain whether these meadows could be under threat from these impacts.

2. Method

2.1 Study sites

Anecdotal evidence indicates seagrass meadows in Brazil are extensive, yet knowledge of seagrasses in the southwest Atlantic still remains poor (Barros et al., 2013). Recent evidence suggests that although these tropical coastal meadows are of key importance to fisheries (Nordlund et al., 2016) they are under threat, requiring concerted conservation action (Copertino et al., 2016). The coast of Brazil is subjected to intense environmental threats due to the high levels of urbanisation and frequent periods of prolonged intense rainfall (Copertino et al., 2016; Marques et al., 2004). Many areas along the coast are affected by high levels of nutrients from untreated sewage and industrial discharges as well as waters laden with suspended solids diverted from surrounding basins suffering from soil erosion (Marques et al., 2004). This can cause areas of eutrophication where flushing from fresh seawater is reduced and residence time is high such as in coastal lagoons and inevitably have a direct

impact any seagrass meadows present. The ecological and environmental characteristics of ten seagrass meadows along the coast of Rio de Janeiro state and São Paulo were quantified within the month of April, in 2017. The study sites were, in Rio de Janeiro: Praia de Manguinhos and Praia dos Ossos (Armação dos Búzios); Saco do Céu and Praia do Abraãozinho (Ilha Grande); Praia Grande and Praia do Catita (Ilha de Itacuruçá); Ilha do Japonês and Praia dos Anjos (Cabo Frio); and in São Paulo: Siriúba and Praia do Sino (Ilhabela) (figure 1).
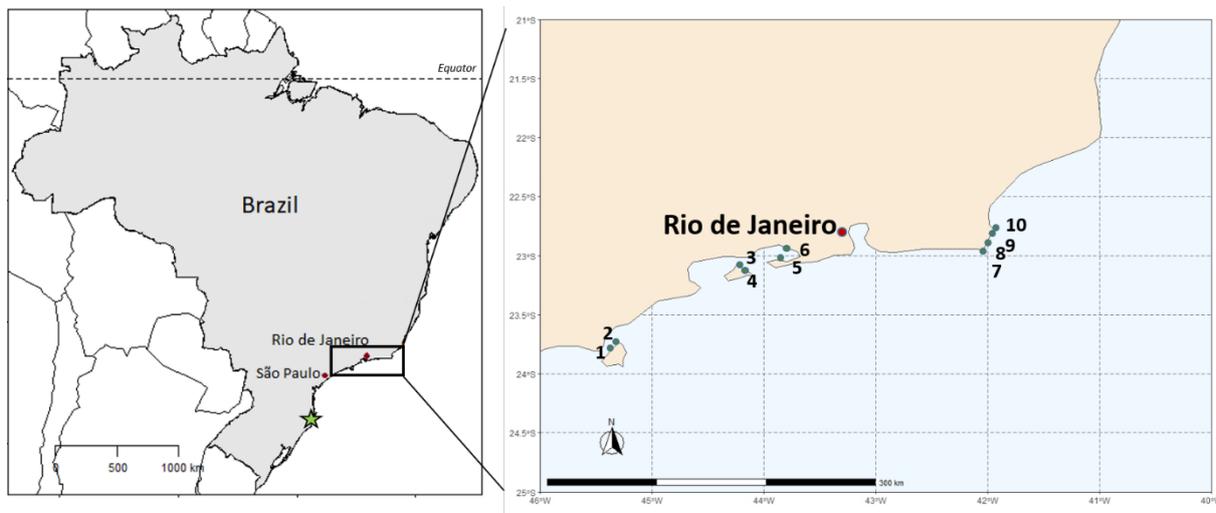

Figure 1. Left side shows distribution of seagrass sites along the coast in relation to Rio de Janeiro on the map of Brazil on the right. The sites range from from São Paulo; Siriúba (1) and Praia do Sino (2) to Rio de Janeiro; Saco do Céu (3), Praia do Abraãozinho (4), Praia do Catita (5), Praia Grande (6), Praia dos Anjos (7), Ilha do Japonês (8), Praia de Manguinhos (9) and Praia dos Ossos (10). The green star represents the southernmost extent of *H. wrightii* on the South American coast (Copertino et al., 2016).

2.2 Environmental Impacts

Each of the seagrass meadow sites were categorized in terms of environmental impacts. This used a scoring system developed by the authors based on methods and evidence identified from previous studies to provide an index (Creed and Oliveira, 2007; Jones and Unsworth, 2016; Oigman-Pszczol and Creed, 2011; Pitanga et al., 2012) including information collected based on site observations. The

anthropogenic impacts that were included in the calculation of this index were as follows, included vicinity to highly urbanized area, population, industry, agriculture, boat activity, tourism, freshwater input/sewage outfall, turbidity and enclosed water body/proximity to open sea. Grazing pressure was also included as an environmental stressor. This is because turtle grazing has been found to significantly modify seagrass meadows (Lal et al., 2010), with *H.wrightii* found to be the most important food source for juvenile green turtles found in southern Brazil (Guebert-Bartholo et al., 2011). Each impact was scored from 0 (no impact) to 3 (high impact) and added up to give an overall value. An impact assessment index was calculated by dividing the total score for each site by the maximum possible score giving a value between 0-1 (Table A.1, Fig. 2). Impact index scores were then divided up into levels - low-medium (<0.4, SS01-SS03), medium-high (0.4-0.5, SS04-SS07) and high (>0.51, SS08-SS10) categories for statistical analysis.
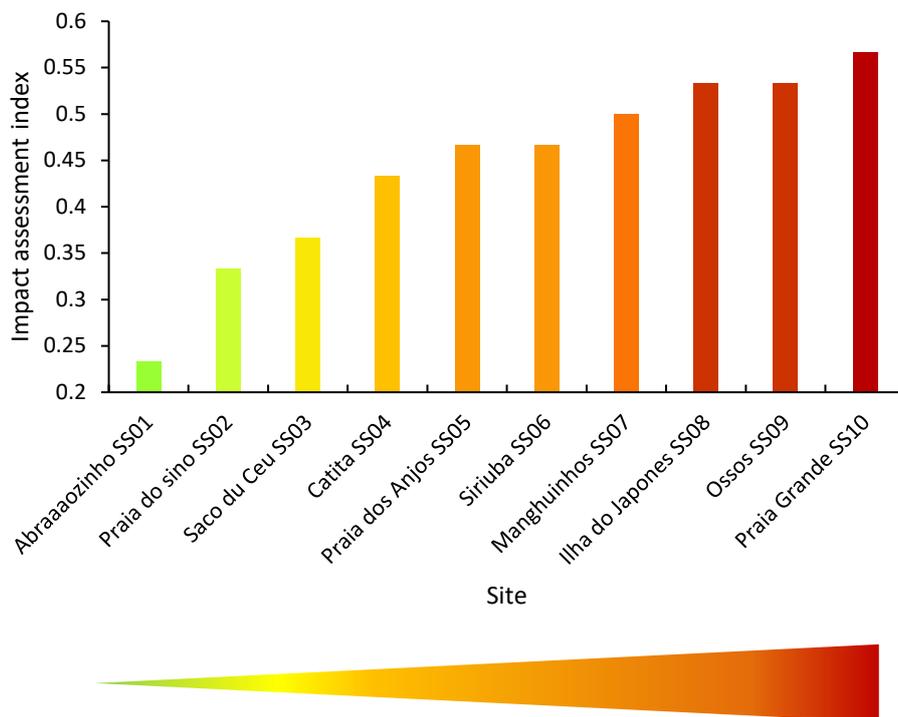

Figure 2. Environmental impact index calculated from perceived impacts scored for each seagrass site creating a gradient of increasing impact. Sites are ordered from lowest to highest perceived impact index and labelled SS01-SS10. The perceived impacts included vicinity to industry, tourism, vicinity to

highly urbanized area, population, agriculture, boat activity, freshwater input/sewage outfall, turbidity, enclosed water body/proximity to open sea and evidence of grazing.

2.3 Environmental data

At each site Hobo light loggers (Onset Hobo UA-002-64) were used to measure light availability and temperature in the middle of the seagrass bed and at the deepest edge to provide light levels at maximum depth threshold. Another light logger was placed in a location on land to record irradiance at or near the sea surface so that the percentage of surface irradiance (%SI) reaching the deep edge could be calculated to represent minimum light requirements. At each site a Secchi disk was used horizontally to estimate turbidity and salinity was recorded using a calibrated refractometer.

2.4 Meadow characteristics

The position of mid-meadow and meadow edges were identified by snorkelling along transects throughout each site. Percentage coverage was measured using a 50 cm x 50 cm quadrat. Twelve quadrats were randomly placed along a single transect line (50 -10 m depending on size of meadow) running parallel to the shore through the middle of the seagrass meadow. This was repeated along the outer edge of the meadow. Cores of seagrass were collected using a PVC corer (78.5 cm^2 to a depth of 15 cm) within six of the twelve quadrats (every other quadrat) to obtain other seagrass parameters (Howard et al., 2014; Mills and Berkenbusch, 2009). Within each core sample, the number of shoots were counted, and the length and width of each leaf was measured using a ruler and calipers. An epiphyte cover score was recorded for each leaf based on the index developed for wasting disease (Burdick et al., 1993) and adapted for use for long-term monitoring of epiphyte cover on seagrass (Bull and Kenyon, 2015; Cook, 2011; Cook and Paver, 2007) (Table A.2). Where shoot density was very high, a maximum number of 16 shoots were measured per core and the total number of shoots was recorded. The core samples were washed, dried and then weighed to provide a total dry weight measurement (Table A.2).

2.5 Photo-physiological parameters

Pulse Amplitude Modulated (PAM) fluorometry using a Diving-PAM (Waltz), was used to measure chlorophyll fluorescence for assessing photosynthetic activity. Photosynthetic parameters were obtained by performing rapid light curves using the internal stepwise function of the PAM fluorometer once attached to the mid part of *H. wrightii* leaves using a leaf clip. Rapid light curves (RLCs) were started immediately after attachment of the clip to enable the measurement of effective quantum yield (ϕpsII = (Fm'-F)/Fm'), where F is fluorescence yield and Fm' is maximal fluorescence yield of the light adapted leaf at each irradiance step (Collier et al., 2009b; Ralph and Gademann, 2005). Steps ranged from 0-2300 µmol photons $m^{-2}s^{-1}$ and were carried out close to midday, between 11am and 1pm. The stepwise RLCs were fitted to the non-linear least-squares regression model by Eilers and Peeters (Eilers & Peeters 1988) using the WinControl software (Waltz) to give photosynthetic parameters for the ETR_{max} (maximum rate of photosynthesis), α (light harvesting efficiency) and E_k (minimum saturating irradiance).

2.6 Leaf nutrient analysis

Samples of seagrass were taken randomly from within each of the sites. Leaves were separated, scraped free of epiphytes, and dried. Of the dried plant material collected and transported back to the UK for analysis, unfortunately there was only enough material to provide one sample per meadow once ground up due to the small leaf size of the plants. The dried seagrass was ground up with a pestle and mortar to a fine homogenous powder. Samples were sent to OEA laboratories Limited for analysis of the % composition of Carbon, Nitrogen and Phosphorus by weight using a continuous flow isotope ratio mass spectrometer (Sercon 20-20 IRMS coupled to Thermo EA1110 elemental analyser). The ratios of stable isotope ^{13}C to ^{12}C (δ^{13}C) indicates the deviation of the isotopic composition relative to the Vienna PeeDee Belemnite (VPDB) standard. The ratios of stable isotope ^{15}N to ^{14}N (δ^{15}N) indicates the deviation of the isotopic composition relative to the international standard of air. The elemental ratio of C:N was calculated on a mole:mole basis using atomic weights (C=12.011, N=14.007)

(McKenzie et al., 2011). These factors were determined to give values which indicate nutrient availability, anthropogenic sources of nutrients (Jennings et al., 1997; Lepoint et al., 2004) and light availability (Cabaço et al., 2008b; Collier et al., 2009; Grice et al., 1996; McMahon et al., 2013).

2.7 Statistical analysis

All the data was divided into measurements collected on a shoot scale and measurements taken at a meadow scale from quadrat data to perceive the environmental effects at these two levels. Parameters measured at shoot scale include average leaf length, leaf width and epiphyte cover. Meadow scale variables included shoot density, dry weight and the photosynthetic parameters Alpha, ETR_{max} and E_k.

All measurement data was analysed using two-way ANOVA tests with impact category and site as a random factor (Brown et al., 1974; McDonald, 2014). In cases where data residuals did not follow a normal distribution, ANOVA was used but with significant p-values set to 0.01 to minimize risk of Type I error (Collier et al., 2012; McDonald, 2014; Underwood, 1997). Tukey HSD post-hoc multi comparison test was used for comparing ANOVA data.

Principal Component Analysis (PCA) (Clarke and Warwick, 2001) was performed on all quadrat level measurements at both shoot and meadow scale to illustrate the correlations between various shoot response parameters and effects of the perceived impacts upon them. Principal components with eigenvalues greater than 1 and eigenfactors or variable coefficients $\leq$ -0.3, or $\geq$ 0.3 were selected.

General Additive Models (GAMs) (Zuur et al., 2009) were carried out in order to identify any non-linear patterns with other possible explanatory factors including abiotic measurements, leaf nutrient content and stable isotopes to determine if these are affecting the abundance of seagrass at sites. Variance inflation factor analysis (VIF) was used to identify collinearity between explanatory variables and those with the highest collinearity were removed from the model. These included %C, %N, E_k,

Secchi distance and leaf width. A base GAM model was then created using shoot density as the response variable as it most commonly used factor for monitoring changes in meadows (Burton et al., 2015; Krause-jensen et al., 2005; Mckenzie et al., 2016; Short et al., 2006; Taylor et al., 2003). Alpha, dry weight, leaf length, C:N, δ^{13}C, δ^{15}N, and impact index as explanatory factors and site as a random variable. All covariates were analysed as smooth variables (f) apart from impact index which was treated as a factor variable (F) and nutrient data owing to small sample sizes.

(1) E[shoot density] = f(Alpha) + f(dry weight) + f(leaf length) + f(C:N) + f(δ13C) + f(δ15N) + f(impact index) + F(site)

The best explanatory models were chosen using manual stepwise selection based on the adjusted R^2 and deviance explained values (table A.7).

All statistics were carried out using RStudio (R version 3.5.1) using R packages *stats*, *devtools*, *gamm4, lme4, mgvc, ggplot2, car* and *carData* (R Core Team, 2018) except for PCA analysis using Primer-e V.6 (Clarke and Gorley, 2006).

3. Results

3.1 Shoot scale responses

Shoot data showed large variation between sites with average leaf length across sites found to be 25.5 mm (± 14.16), average leaf width 0.43 mm (± 0.22) and average epiphyte score 1.41 (± 0.92).

The results of the two-way ANOVA shows significant interactions between leaf width (F=91.42, *p*=<0.001), leaf length (F=14.74, *p*=<0.001) and epiphytes (F=3.52, *p*=0.03) with impact category and site as a random factor (table A.4). For leaf width and length, the post-hoc pairwise comparison shows significant variation between plants in med-high vs high, and med-high vs low-medium impact categories (Fig. 3, table A.5), although length was found to increase with increasing impact and widest leaves found in med-high impacted meadows. The mean widths of *H. wrightii* ranged from 0.24mm (±0.07) at SS03, to 0.76mm (±0.13) at SS07. Average leaf length also varied greatly between meadows

(Fig. 3), from 17.33mm (±9.26) at SS09, to 40.84mm (±24.01) at SS04. Epiphyte cover also varied with the lowest mean score found at SS01 (0.63 ± 0.83) which was significantly lower than all other sites, and the highest score at SS08 (1.86 ± 1.43). Epiphyte cover was found to increase with increasing impact however it was not found to vary significantly between impact categories.
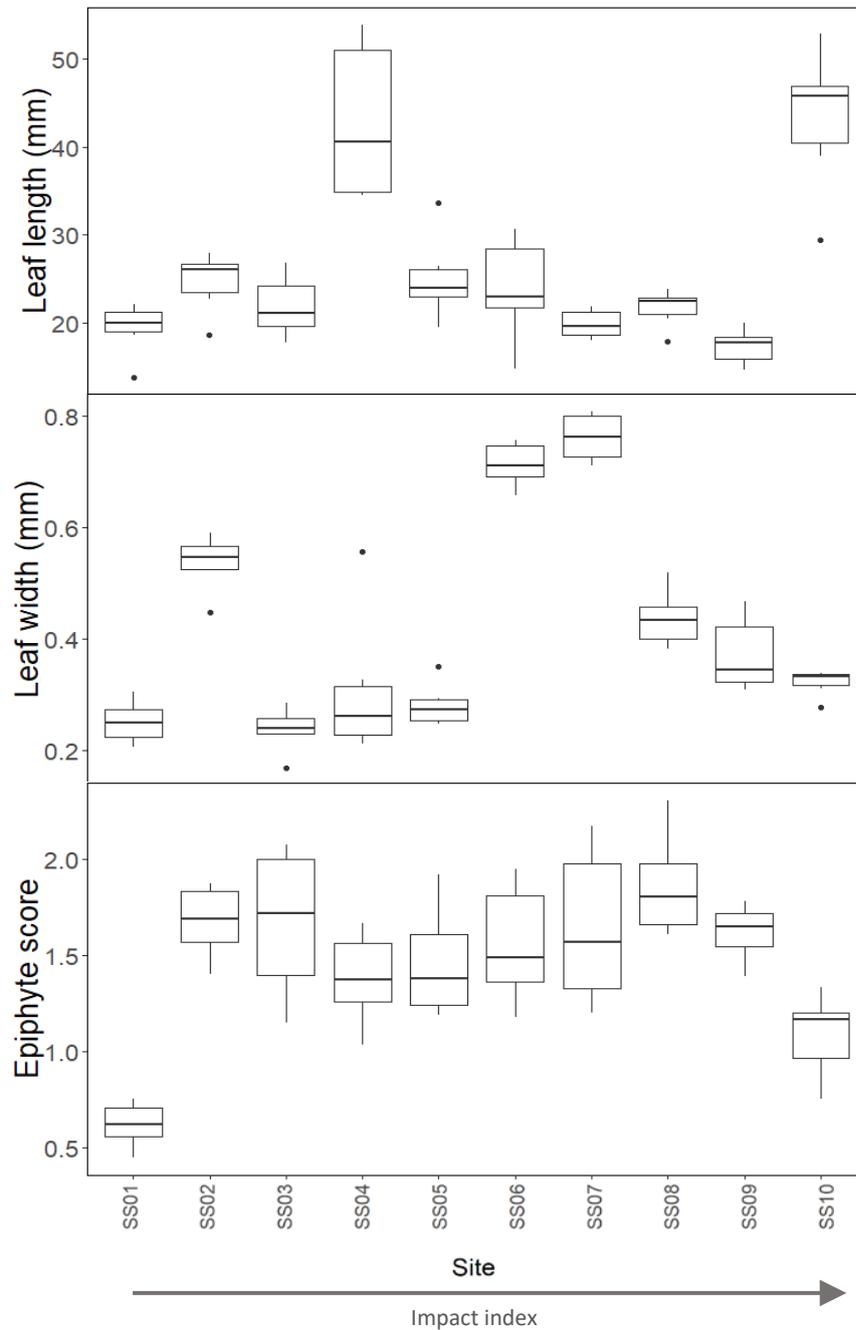
Figure 3. Boxplots to show comparison of shoot-scale responses of average leaf length, leaf width and epiphyte cover between seagrass sites (n= 55-96 per site). Median is indicated by the solid line, outliers indicated by dots. Site order from lowest impact index score (SS01) on left to highest (SS10) on the right.

3.2 *Meadow scale responses*

Meadow scale variables also showed high variation across sites, with average shoot density ranging from 764.33 (±575.36) to 16645.44.7 (±3668.85) shoots/m^2 and dry weight from 3.74 (±1.27) to 104.46 (±20.4) g/m^2.

The statistical tests showed significant interactions between dry weight (F=50.08, p=<0.001) and shoot density (F=66.51, p= <0.001) with impact category and site as a random factor (table A.4). The post-hoc pairwise comparison shows that shoot density varies significantly between each of the impact categories (*p adj*=<0.001) with shoot density actually increasing with impact. For total dry weight, low-medium vs high and low-medium vs med-high showed significant differences (both *p adj*=<0.001, table A.5), again increasing with impact, not as expected.

Dry weight was highest in SS04 and SS09 with an average of 104.46 (±20.4) and 91.78 (± 19.78) g/m^2 respectively and significantly higher than all other meadows. SS01 had the lowest dry weight at 3.74 (±1.27) g/m^2. SS09 had the highest shoot density, with an average of 16645.44 (± 3668.85) shoots/m^2 (Fig. 4). The meadows with the lowest shoot density were SS10 (764.33 ± 575.37 shoots/m^2) and SS03 (1273.89 ± 369.21 shoots/m^2).

3.3 *Photo-physiological responses*

Photosynthetic parameters also showed high levels of variation between sites, with light harvesting efficiency (Alpha) ranging from 0.26 (±0.04) to 0.39 (±0.06), the maximum rate of photosynthesis (ETR$_{max}$) from 203.01 (±18.65) to 365 (±47.1) and minimum saturating irradiance (E$_k$) ranging from 588.83 (±91.09) to 1083.62 (±131.42).

Not all photo-physiological parameters showed significant interactions with impact category only ETR_{max} (F=8.62, *p*=<0.001), however they all showed significant variation between sites (table A.4). The post-hoc pairwise comparison shows only significant variation in ETR_{max} between low-medium vs high (*p adj* = <0.001) and low-medium vs med-high (*p adj*=0.007) impact categories (table A.5) with ETR_{max} decreasing with increasing impact as would be expected.

The Alpha value recorded for seagrasses at SS04 was significantly higher than several of the other sites (0.39 ± 0.06), followed by SS10 (0.35 ± 0.05) suggesting light limitation at these sites. The sites with the lowest Alpha value were SS08 (0.26 ± 0.04) and SS05 (0.25 ± 0.03) (Fig. 4). For ETR_{max}, SS07 and SS03 seagrass showed the highest rates, 365.99 (± 47.43) and 340.3 (± 29.28) respectively. These sites were significantly higher than all other sites. SS10 and SS05 had the lowest values overall (203.01 ± 18.65 and 203.86 ± 33.08 respectively). For the E_k values, the sites that significantly varied from one another included SS08, SS07 and SS03 which had the highest levels (1128.77 ± 122.78, 1183.61 ± 131.42 and 1067.25 ± 131.42 respectively), and SS10 and SS04 which had the lowest levels (588.83 ± 91.09 and 644.71 ± 91.29 respectively, Fig. 4).
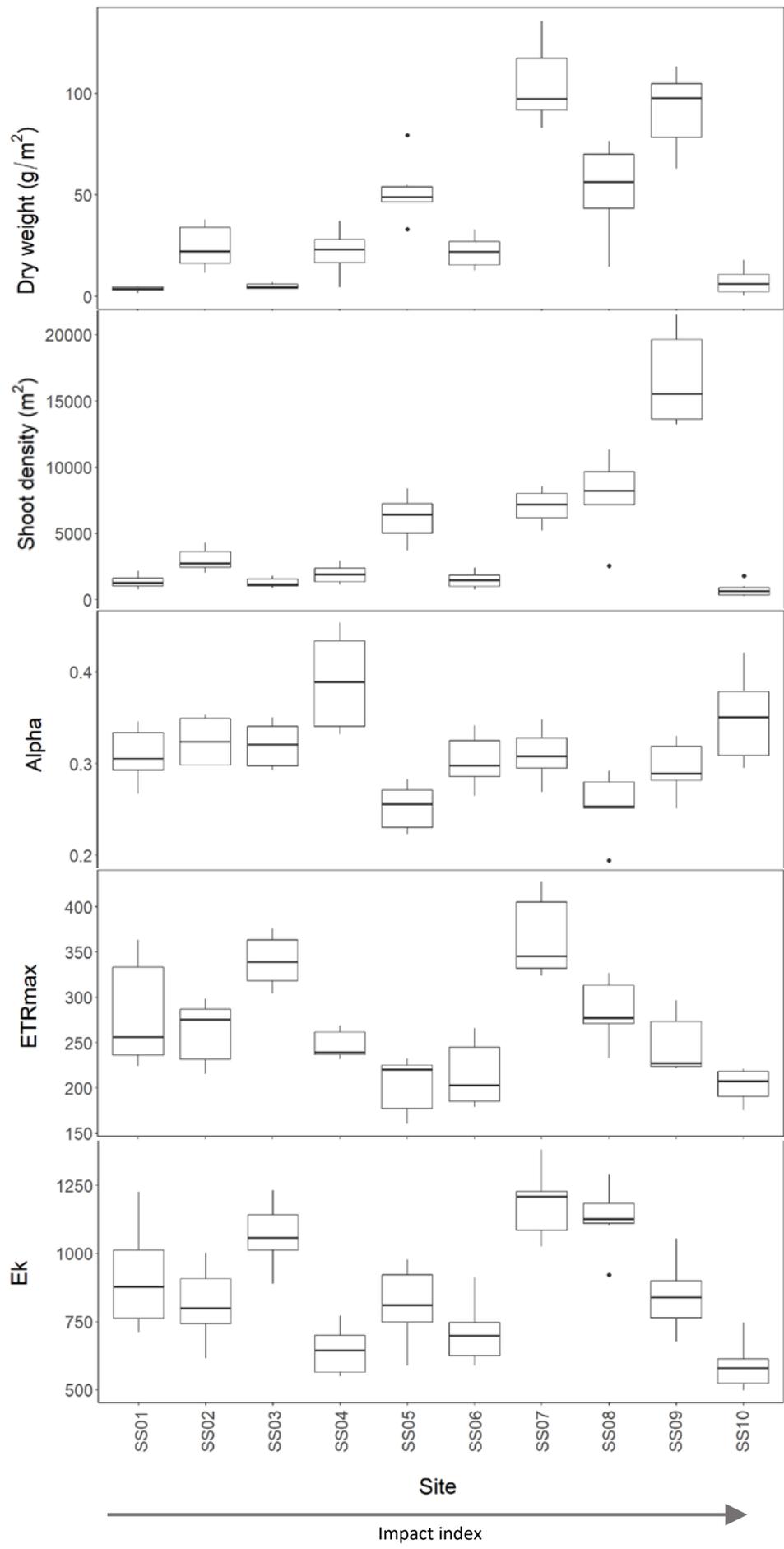
Figure 4. Boxplots to show comparison of the meadow and photo-physiological parameters, dry weight, shoot density, Alpha, ETR_{max} and E_k between seagrass sites (*n*=6). Shoot density and dry weight are shown as per m^2. Median is indicated by the solid line, outliers indicated by dots. Site order from lowest impact index score on left to highest on the right.

3.3 Principal Component Analysis

For shoot scale responses (PCA A, Fig. 5), PC1 accounted for 48.3% of the variance with an eigenvalue of 1.45. Leaf width and epiphyte cover showed the strongest responses with eigenfactors of -0.668 and -0.613 respectively, showing a negative relationship compared to leaf length (0.422). PC2 accounted for 30.7% of the variance with and eigenvalue of 0.922. Leaf length showed the strongest level of response with an eigenfactor of 0.876, followed by epiphyte cover with 0.464 (Table A.5).

For meadow scale responses (PCA B, Fig. 5), PC1 accounted for 48.8% of the variance with an eigenvalue of 2.44. All the seagrass variables included showed strong responses (eigenfactors over 0.3, less than -0.3), although the highest variables were E_k (-0.534), dry weight (-0.504) and shoot density (-0.434) all showing negative association. PC2 explained 28.2% of the variance (eigenvalue 1.41) with all variables showing strong responses except Alpha at 0.246, just below the level of selection (Fig. 5, Table A.5).
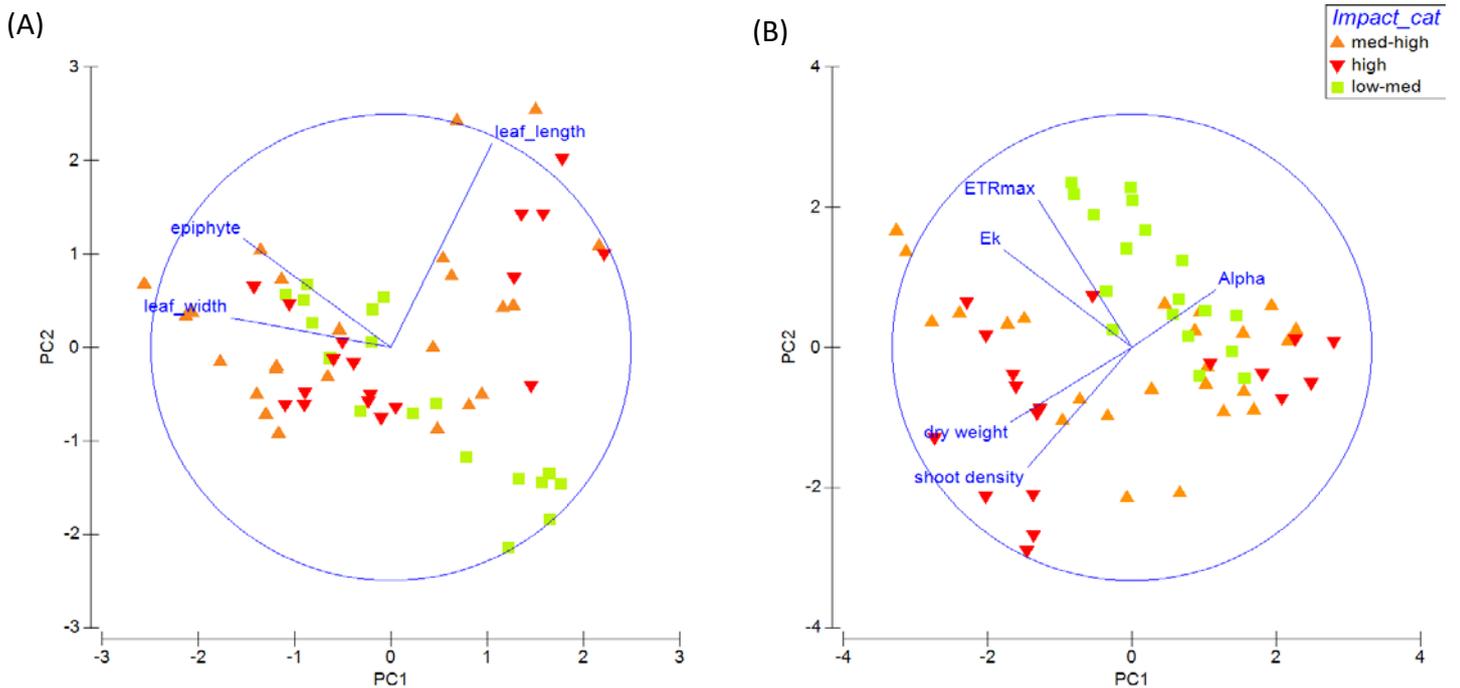
Figure 5. Principal Component Analysis of (A) shoot data (leaf width, leaf length and epiphyte cover) variation with impact score as a factor and (B) Principal Component Analysis of meadow-scale data (shoot density, dry weight and photosynthetic characteristics Alpha, ETR$_{max}$ and E$_k$). Legend separates sites by relative levels of impacts determined by scores and divided into low-medium (<0.4), medium-high (0.4-0.5) and high (>0.5).

3.4 Leaf nutrient analysis

Results from the tissue nutrient analysis of *H. wrightii* shoots show that the highest percentage of nitrogen was found in SS10 (3.39%) and SS03 (3.38%), with the average of all sites found to be 3.04 ±0.23 %. The highest percentage of phosphorus was found in the seagrass from SS03 (0.41%) and SS08 (0.40%), with the site average of 0.36 ± 0.04 %. Of the sites, SS03 displayed the lowest C:N ratio (10.44). The highest C:N ratios were found at SS07 and SS05 (12.35 and 12.37 respectively) with the study average found to be 11.63 ± 0.58 (table 2), suggesting these sites are subjected to higher light levels than others.

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The values for $\delta^{15}N$ were highest in seagrass tissue from SS10 (7.56‰) and SS04 (7.13‰), followed by

SS09 (5.74‰) and SS07 (4.99‰) all relatively higher than the study average (4.15 ± 2.3‰). Sites SS03,

SS08 and SS01 had the lowest $\delta^{15}N$ ratios (1.0, 1.68 and 1.72 ‰ respectively) indicating substantially

lower anthropogenic nutrient input at these sites than average (table 2).

Table 2. Results from the elemental analysis of *H. wrightii* leaf tissue taken from the study sites. The

stable isotope values for $\delta^{15}N$ (‰), $\delta^{13}C$ (‰).

Site	$\delta^{15}N$	$\delta^{13}C$	%N	%P	C:N
SS01 (low-med)	1.72	-10.95	3.04	0.38	12.85
SS02 (low-med)	4.14	-10.95	3.07	0.38	13.81
SS03 (low-med)	1.00	-10.22	3.38	0.41	12.17
SS04 (med-high)	7.13	-9.04	2.70	0.27	13.65
SS05 (med-high)	4.70	-11.09	2.84	0.38	14.43
SS06 (med-high)	2.87	-11.16	3.10	0.36	13.48
SS07 (med-high)	4.99	-10.43	2.72	0.34	14.40
SS08 (high)	1.68	-7.59	3.12	0.40	13.20
SS09 (high)	5.74	-9.40	3.07	0.37	13.32
SS10 (high)	7.56	-10.30	3.34	0.31	13.67
Study averages	*4.15*	*-10.11*	*3.04*	*0.36*	*13.50*
S.D.	2.30	1.13	0.23	0.04	0.68
Range	1.0-7.56	-11.16--7.59	2.7-3.38	0.27-0.41	12.17-14.43

The ratio of $\delta^{13}C$ in *H. wrightii* shoots averaged -10.11 ± 1.13‰ with little variation between all sites.

The lowest $\delta^{13}C$ was found in Siriuba (-11.16‰) and Praia dos Anjos (-11.09) and the highest $\delta^{13}C$

found in the seagrass at Ilha do Japonês (-7.59) (Table 2).

3.5 Generalized Additive Models to describe shoot density

After removal of collinear variables and those that were non-significant or would not converge within

the GAM models owing to unbalanced sample size, the final model consisted of $\delta^{15}N$, leaf length and

impact index:

(2) *E[shoot density$_i$]=f(impact)+f($\delta^{15}N$)+ f(leaf length)+F(site)*

Basic models were compared for best fit. All variables that were deemed insignificant, having little effect were removed. Site as a random factor and Impact level were also removed to test effect on model fit with results of the final comparisons in table A.7.

The first GAM model provides evidence that the impact level (low), alpha, leaf length and shoot $\delta^{15}N$ are highly significant in explaining shoot density between impact categories, with highest deviance explained (96.5%, R-sq adj. 0.978) and lowest GVC of all models tested (table A.7). The tissue $\delta^{15}N$ increases with impact level as expected, however shoot density and leaf length also increase with increasing impact levels. Model 5 which takes out impact level shows leaf length is highly significant as well as site at explaining shoot density, however $\delta^{15}N$ could not be included at this level owing to small sample size.

4. Discussion

The plasticity of seagrass meadows to environmental stressors enables them to be bioindicators of ecological change in coastal waters. The present study demonstrates that although such responses occur and do indicate the majority of seagrasses in a region of Brazil to be under anthropogenic stress, their responses are multifaceted and often difficult to interpret with respect to assessing the environmental status of seagrass meadows. One of the major attributes considered for assessing seagrass health and resistance is abundance (Unsworth et al., 2015), with increasing abundance potentially leading to increased resilience (Mckenzie et al., 2016). The present study indicates that this assumption may not always be correct as reduced shoot density and biomass did not always reflect increasing anthropogenic impact. Other indicators were found to be more robust to identifying environmental risks. Epiphyte score, and leaf nutrient content ($\delta^{15}N$, %N, %P and C:N) data supported what were assessed to be the low-medium impacted sites (SS01 and SS03), and leaf nutrients were found to be highest in plants from the site predicted to be of high impact (SS10), indicating nutrient enrichment. Other meadow scale responses also supported the case that SS10 is likely to be the most

impacted site displaying the lowest shoot density, ETR_{max} and E_k of all sites, and second highest Alpha. However, other meadow-scale responses specifically the low shoot density and biomass at SS01, do not support the low-medium impact assessment index attributed. The input of naturally occurring nutrients from adjacent mangroves or potential for higher levels of exposure to wave action and recent storm events could have resulted in a decrease in shoot density at this site.

The shoots with the longest leaves were found at SS04 (med-high) and SS10 (high), although leaf widths at these sites were relatively narrow. The seagrass within medium-high impact sites (SS06 and SS07) were found to have the widest leaves, but also amongst the shortest leaves measured. It is expected that nutrient enrichment within the water-column results in increased turbidity and light attenuation, caused by an increase in epiphytic algae growth and eutrophication. Light limitation often results in a reduction of above-ground biomass exhibited by shorter, narrower leaves, with fewer leaves per shoot and becoming less dense at a meadow scale (Bertelli and Unsworth, 2018; Biber et al., 2005; Collier et al., 2012; Ochieng et al., 2010; Olesen and Sand-jensen, 1993; Yaakub et al., 2013). However, reduction in leaf length and elongation which is exhibited in many seagrass species as a response to light limitation does not always hold true for *H. wrightii*. This study has found the sites with the longest leaves were also found to have highest levels of shoot $\delta^{15}N$, Alpha, turbidity and lowest shoot density and E_k indicating poor water quality and low light conditions. Creed (1999) also found that shoot density to be highest in meadows with shorter leaves. Previous studies have found that *H. wrightii* can show little change in leaf elongation from shading experiments (Czerny and Dunton, 1995), and *in situ, H. wrightii* growing in shade can have longer leaf lengths than neighbouring plants growing under higher light conditions (Shafer, 1999). Leaf length could also have been affected by the presence of turtles observed by the authors, the effect of which has been observed to cause *H. wrightii* in this area to grow wider leaves (Creed, pers. Comm., 2017). There was found to be a relationship between wider leaves of *H. wrightii* and evidence of grazing (turtle presence and cropped leaves, see A.1). However this response also appears to be species specific and the converse of

findings from other studies that found grazing to have the opposite effect (Fourqurean et al., 2010; Lacey et al., 2014; Lal et al., 2010).

There is a wide body of evidence which shows that light limitation and shading results in a reduction in shoot production and shoot density overall for *H. wrightii* (Biber et al., 2009; Shafer, 1999) and other seagrass species (Lee and Dunton, 1997; Longstaff et al., 1999; Olesen et al., 2002), highlighting this response as a major indication of light stress at a meadow-scale. According to the meadow-scale attributes measured (density, biomass, and photosynthetic stress), SS10 (high) is likely to be the most light-stressed, followed by SS04 (med-high). These sites are located within the semi-enclosed, shallow Sepetiba Bay, with lower flushing, into which flow several rivers draining the catchment of the highly urbanised and industrial city of Rio de Janeiro. This is also supported by the high turbidity and temperatures recorded *in situ*. SS06 also showed similar meadow-scale indicators of stress to SS04 and SS10 which could also be explained by the high level of turbidity found at the site and the low % SI reaching the seagrass (Table A.3). This site is within protected and enclosed São Sebastião Channel, also with lower flushing. Shoot density and dry weight show similar patterns for the sites as expected, which are also reflected in the values for alpha which are highest in those meadows which appear to be the most impacted.

The *H. wrightii* at SS10 was found to have the second highest level of nitrogen and the highest $\delta^{15}N$ indicating nutrient enrichment affecting the site is likely to include anthropogenic sources. Seagrass from SS03 was also found to have a high proportion of nitrogen, however its low $\delta^{15}N$ isotopic ratio indicates that there are lower anthropogenic inputs at this site. This site is also within an enclosed embayment, part of the Ilha Grande State Park, where nutrient inputs likely come from leaf litter from the adjacent preserved Atlantic rainforest and mangrove systems rather than from the sparse human habitations. The carbon to nitrogen ratio found within seagrass leaves has been found to be a robust indicator of light stress which responds over a longer time-scale than photosynthetic characteristics (McMahon et al., 2013). The results from this study suggest that SS07 and SS05 (med-high) are

subjected to a higher light environment overall, and the site subjected to the lowest light levels over time is likely to be SS03 (low-medium). However, it should be considered that the overall C:N ratios between sites showed little variation suggesting all the sites are subjected to relatively low light conditions and high nutrient status. With global averages for other seagrass species found to be considerably higher at around 20 (Atkinson and Smith, 1983; Duarte, 1990; Grice et al., 1996; Jones and Unsworth, 2016), the low site averages found in this study support such a premise although more samples should be taken in the future to support this. Increasing irradiance has also been found to lower isotopic discrimination of carbon uptake during photosynthesis in seagrasses and therefore lower $\delta^{13}C$ depletion within the plants. This suggests that the seagrass meadow at SS08 (high) is subjected to the highest light levels of all sites measured in this study which is supported with it being the shallowest meadow and highest % surface irradiance levels recorded (Tables 2, A.3). To the author's knowledge this is the first study that has analysed the C:N and stable isotopes of *H. wrightii* using modern methods of mass spectrophotometry, and despite low sample number, does provide important evidence for this seagrass species. A previous study by Powell *et al*. (1989) quantified the nitrogen and phosphorus content of *H. wrightii* plants before and after nutrient enrichment with results showing concentrations lower than those found at all ten sites measured for this study. Monitoring reports describe seagrasses with tissue nutrient ratios (C:N) below 15 as very poor (Mckenzie et al., 2016; McKenzie et al., 2011) which suggests that all sites in this study are nutrient enriched. The opportunistic and pioneering nature of *H. wrightii* allows it to grow in relatively unstable conditions compared to other seagrass species (Creed and Amado Filho, 1999; Lapointe et al., 1994; Tomasko and Lapointe, 1991; Wear et al., 1999). These unstable conditions can result in sedimentation and burial, a factor not measured in the present study, but has been found to be one of the most important causes of localised loss (Cabaço et al., 2008b; Ceccherelli et al., 2018). *H. wrightii* has a higher nutrient demand than other seagrasses (Powell et al., 1989; Wear et al., 1999) and so can better tolerate eutrophication (Lapointe et al., 1994) and has been known to displace *Thalassia testudinum* under conditions of prolonged nutrient enrichment (Fourqurean et al., 1995).

Yet, enrichment has been found to significantly decrease blade turnover and rhizome growth in other species (Wear et al., 1999), and coupled with sedimentation from anthropogenic caused run-off or natural storm events can cause considerable decline (Ceccherelli et al., 2018). Nevertheless it is expected that the variation in morphology, density and physiology of *H. wrightii* meadows is caused by differences in localised environmental conditions which have been found to be greater at its southern distributional limit (Creed, 1997; Sordo et al., 2011).

5. Conclusion

This study assessed seagrass indicators at shoot and meadow-scales across a range of seagrass meadows, and the environmental parameters which are modifying them. The results demonstrate substantial morphological and physiological variation to occur.

The results of this study have shown that although perceived impacts and undesirable environmental conditions are the cause of variation between these relatively isolated populations, it is not straight forward to identify which sites are most impacted in the case of *Halodule wrightii*. The impact assessment index developed in this study identified SS10 as the most impacted site which is reflected in the meadow scale responses; lowest shoot density, ETR_{max} and E_k of all sites, and second highest alpha. The shoot nutrient analysis also confirms that SS10 is probably one of the most eutrophic sites owing to the highest %N content of the leaves. However, the impact index perceives SS01 as the least impacted site but the low shoot density, biomass and leaf widths at this site go some way to contradict this. Other processes that have not been recorded could be responsible for the condition of the seagrass here, its location on the northern side of Ilha Grande is more exposed and potentially subjected to more storm events which could cause burial. It has also previously been rated as the most heavily impacted site within the Baía da Ilha Grande (Creed and Oliveira, 2007). The impact index has been shown to work for assessing the most impacted sites but has not been so useful at determining the least impacted, most likely due to naturally occurring drivers that were not measured.

Of the variables measures, the stable isotope of Nitrogen and leaf length were most significant in explaining the differences in shoot density between sites. Taking more samples for shoot C:N and stable isotope analysis is highly recommended to substantiate this trend. All the seagrass sites used in this study are relatively impacted, with some more than others although *H. wrightii* may also naturally better tolerate more borderline environmental conditions that other seagrasses do not. This highlights the importance of identifying the most species appropriate factors to measure when attempting to assess the health status of seagrass meadows, which could result in the development of monitoring protocols that incorporate species specific responses to site specific threats.

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Appendix

Table A.1. Table of impact assessment scores attributed to each seagrass site for each perceived impact from 0-3. The impact assessment index is calculated by divided the total score per site by the maximum possible score.

Site	Population	Industry	Tourism	Anchoring/ boat activity/ ports	Catchment	Agriculture	Freshwater input/ sewage outfall	Turbidity	Open-sea /Lagoon	Evidence of grazing	Total score	Impact assessment index
Abraaaozinho	1	1	2	1	1	0	0	0	1	0	7	0.233333
Praia do sino	1	0	2	1	1	0	2	1	1	1	9	0.333333
Saco du Ceu	1	1	1	2	1	0	1	2	3	0	12	0.4
Catita	1	3	0	1	3	1	0	2	2	0	13	0.433333
Praia dos Anjos	2	1	2	3	2	0	0	1	1	2	14	0.466667
Siriuba	1	0	2	2	1	0	2	3	1	2	14	0.466667
Manghuinhos	2	0	2	2	2	0	1	2	1	3	15	0.5
Ilha do Japones	3	1	2	0	3	0	0	2	3	2	16	0.533333
Ossos	2	0	3	2	2	0	2	1	1	3	16	0.533333
Praia Grande	1	3	1	2	3	1	1	3	2	0	17	0.566667

Table A.2. Description of epiphyte cover scores used for assessing coverage based on methods developed by Burdick et al. (1993), and described by Cooke & Paver (2007) for use for determining epiphyte scores in long-term seagrass monitoring programmes.

Score	Description	Percentage cover
0	Uninfected leaf	0
1	Minimal cover apparent	0-2
2	Up to a quarter of leaf covered	3-25
3	Up to half the leaf covered	26-50
4	Over half of all leaf covered	51-75
5	Almost all of leaf covered	76-100

Table A.3. Table of abiotic factors measured at each seagrass site in April 2017. Average light intensity for each site is taken from light recorded within the meadow (middle), % SI is taken as percentage of surface irradiance reaching meadow edge, measured using Hobo light loggers (in lux).

Site	Average light intensity mid-meadow (lux)	SD light intensity	Max light intensity during day (lux)	% SI at deep edge	Max temp (°C)	Min temp (°C)	Secchi distance (m)	Salinity (‰)
Abraãozinho	3835	6129	34445	15.3	27.17	21.09	5.5	38
Praia do sino	3524	5562	35823	13.6	26.98	25.71	3.5	34
Saco du Céu	6692	14608	220446	23.7	29.65	23.87	2.5	36
Catita	5280	13379	198401	11.9	33.43	26.68	2.5	32
Praia dos Anjos	3651	5046	24800	13.2	24.84	22.24	5	37
Siriuba	1409	2231	12400	6.7	26.59	25.61	0.75	35
Manghuinhos	3815	6550	126756	13.8	26.88	23.77	2.5	39
Ilha do Japonês	6555	10472	170846	36.2	32.09	21.19	3	39
Ossos	5522	8202	52356	11.7	26	23.97	3.75	39
Praia Grande	2204	7541	187379	14.0	33.43	26.10	0.5	35

Table A.4. Results from Analysis of variance with shoot, meadow and photo-physiological responses to impact index and site included as random factor.

		Df	F-value	Pr(>F)
Width	Impact score	2	91.42	<0.001
	as.factor (Site)	7	209.28	<0.001
Ave.leaf.length	Impact score	2	14.74	<0.001
	as.factor (Site)	7	48.56	<0.001
Epiphyte	Impact score	2	3.52	0.03
	as.factor (Site)	7	13.42	<0.001
Shoot density	Impact score	2	66.51	<0.001
	as.factor (Site)	7	43.62	<0.001
Dry weight	Impact score	2	50.08	<0.001
	as.factor (Site)	7	36.99	<0.001
Alpha	Impact score	2	1.32	0.278
	as.factor (Site)	7	9.83	<0.001
ETRmax	Impact score	2	8.62	<0.001
	as.factor (Site)	7	14.14	<0.001

Ek	Impact score	2	2.77	0.072
	as.factor (Site)	7	17.27	<0.001

Table A.5. Results from the post-hoc Tukey HSD pairwise comparison test for each variable measured.

Width	diff	lwr	upr	p adj
low-med-high	-0.01120476	-0.03842546	0.016016	0.598286
med-high-high	0.11367441	0.08931648	0.138032	0
med-high-low-med	0.12487917	0.09965718	0.150101	0
Ave leaf length				
low-med-high	-1.347614	-3.918443	1.223215	0.435291
med-high-high	3.770645	1.470187	6.071104	0.000377
med-high-low-med	5.118259	2.736196	7.500322	1.7E-06
Epiphyte				
low-med-high	-0.21339175	-0.40533217	-0.02145	0.024946
med-high-high	-0.13007459	-0.30162185	0.041473	0.176817
med-high-low-med	0.08331716	-0.09456508	0.261199	0.514472
Shoot density				
low-med-high	-51.38889	-62.348165	-40.4296	0
med-high-high	-33.5	-43.751464	-23.2485	0
med-high-low-med	17.88889	7.637425	28.14035	0.000303
Dry weight				
low-med-high	-0.311016667	-0.39909479	-0.22294	0
med-high-high	-0.004573611	-0.08696315	0.077816	0.990139
med-high-low-med	0.306443056	0.22405352	0.388833	0
Alpha				
low-med-high	0.018111111	-0.01028242	0.046505	0.280933
med-high-high	0.013763889	-0.01279583	0.040324	0.428903
med-high-low-med	-0.004347222	-0.03090694	0.022213	0.917598
ETRmax				
low-med-high	49.65061	19.47277	79.82845	0.000655
med-high-high	12.95864	-15.27015	41.18743	0.513189
med-high-low-med	-36.69197	-64.92076	-8.46319	0.007855
Ek				
low-med-high	76.99044	-29.48394	183.4648	0.198266
med-high-high	-16.52768	-116.12534	83.06998	0.915404
med-high-low-med	-93.51813	-193.11579	6.079538	0.069808

Table A.6. Table to show results of Principal Component Analysis of shoot scale data (PCA1) and meadow-scale data (PCA2).

PCA1	PC1	PC2
Summary Values		
Eigenvalues	**1.45**	**0.922**
Percent variation	48.3	30.7
Cumulative percent variation	48.3	79.0
Seagrass variables		
Leaf length	**0.422**	**0.876**
Leaf width	**-0.668**	0.127
Epiphyte	**-0.613**	**0.464**

PCA2		
Summary Values	PC1	PC2
Eigenvalues	**2.44**	**1.41**
Percent variation	48.8	28.2
Cumulative percent variation	48.8	77.0
Seagrass variables		
Shoot density	**-0.434**	**-0.513**
Dry weight	**-0.504**	**-0.319**
Alpha	**0.348**	0.246
ETR_{max}	**-0.390**	**0.632**
E_k	**-0.534**	**0.418**

Table A.7. Results from the generalised additive models used to describe shoot density. Models 1-4 showed the best fit by way of deviance explained and R^2 value.

Model 1 = gam(shoot_density ~ as.factor(Impact_cat) + Alpha + leaf length + N15)				
	Estimate	Std. Error	t-value	p-value
(Intercept)	12.065	1.142	10.563	0.0005***
Impact med-high	-0.226	0.366	-0.619	0.570
Impact high	-0.338	0.427	-0.790	0.474
Alpha	-11.925	3.934	-3.031	0.039*
Leaf length	-0.093	0.015	-6.234	0.003**
δN^{15}	0.659	0.114	5.765	0.004**
R-sq. (adj) = 0.978, deviance explained = 96.5%, GVC=**1054.1**				
Model 2 = gam(shoot_density ~ as.factor(Impact_cat) + leaf length + N15)				
(Intercept)	8.914	0.626	14.238	3.08e-05***
Impact med-high	0.166	0.547	0.304	0.773
Impact high	0.285	0.618	0.462	0.664
Leaf length	-0.104	0.022	-4.654	0.006**

δN[15]	0.445	0.137	3.255	0.023*

R-sq. (adj) = 0.918, deviance explained = 89.1%, GVC=2104.6

Model 3 = gam(shoot_density ~ as.factor(Impact_cat) + s(Alpha) + s(leaf length)+ F(Site)

(Intercept)	7.001	0.211	33.169	<2e-16***
Impact med-high	0.243	0.235	5.296	3.02e-06***
Impact high	1.953	0.255	7.660	7.85e-10***
Significance of smooth terms		Ref.df	F-value	p-value
s(Leaf length)	edf	8.957	7.756	1.56e-07***
s(Alpha)	1.00	1.00	0.581	0.450
s(Site)	8.64	1.00	0.000	0.539

R-sq. (adj) = 0.794, deviance explained = 79.5%, GVC=**1410.3**

Model 4 = gam(shoot_density ~ as.factor(Impact_cat) + s(leaf length)+F(Site)

(Intercept)	7.056	0.198	35.737	<2e-16***
Impact med-high	1.180	0.219	5.385	2.11e-06***
Impact high	1.857	0.221	8.393	5.33e-11***
Significance of smooth	edf	Ref.df	F-value	p-value
s(Leaf length)	8.64	8.957	7.756	8.01e-08***
s(Site)	6.256e-06	1.00	0.000	0.822

R-sq. (adj) = 0.794, deviance explained = 79.2%, GVC=1374.6

Model 5 = gam(shoot_density ~ s(Alpha) + s(leaf length)+F(Site)

(Intercept)	7.046	0.220	31.98	<2e-16***
Significance of smooth	edf	Ref.df	F-value	p-value
S(Alpha)	1.000	1.000	0.327	0.57
s(Leaf length)	1.000	1.000	32.687	3.12e-07***
s(Site)	0.976	1.000	46.213	2.06e-09***

R-sq. (adj) = 0.634, deviance explained = 61.9%, GVC=1874.8