



Landscape-scale variation in a sulfur-based sediment stress indicator for the seagrass *Thalassia testudinum* in Florida Bay, USA

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ABSTRACT: Intrusion of sediment-derived hydrogen sulfide into above-ground tissues of seagrasses is correlated with reduced growth and has been linked to large-scale die-offs of *Thalassia testudinum* in Florida Bay, USA. In May of 2019, leaves from *T. testudinum* short shoots at 350 sites within 13 basins across Florida Bay were collected to investigate bay-wide variation in a novel, stable sulfur isotope-based indicator of hydrogen sulfide intrusion [$(\delta^{34}\text{S}_{\text{leaf}} + 30)/\text{total sulfur content}$]. The components of this sediment stress indicator (SSI), total sulfur content (% dry weight) and $\delta^{34}\text{S}$, were negatively correlated ($R^2 = -0.24$), indicating greater sediment sulfide exposure in plants with higher sulfur content. Generalized additive model selection revealed that SSI was best predicted by a model which included *T. testudinum* cover, sediment depth, and *Halodule wrightii* cover ($R^2 = 0.24$, weight = 0.48). Macrophyte communities dominated by dense *T. testudinum* climax communities and with deep sediments, which are characteristics associated with die-off, had the lowest SSI values, indicating greatest sulfide intrusion. Sites within the area of a recent (2015) seagrass die-off had significantly higher SSI values than nearby, non-die-off-affected sites (mean \pm SE: 44.1 ± 1.5 vs. 40.2 ± 1.6 , Wilcoxon $p < 0.05$). The recent die-off sites also had lower *T. testudinum* cover and higher *H. wrightii* cover than the non-die-off sites, indicating they were in the midst of recovery and at an early successional stage. Our findings indicate that SSI may provide a minimally destructive indicator of chronic sulfide intrusion in *T. testudinum* in Florida Bay.

KEY WORDS: Seagrass · Stress indicator · Die-off · Sulfide · Florida Bay

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1. INTRODUCTION

Seagrasses are marine angiosperms that form extensive communities in shallow lagoons and estuaries. These plants are considered ‘ecosystem engineers’ because they modify environmental conditions and create habitats by stabilizing sediments, sequestering nutrients, and providing shelter for commercially and recreationally important faunal species (Barbier et al. 2011). Turtle grass *Thalassia testudinum* is a large-bodied, late-successional seagrass species common in tropical coastal environments. This species has become the dominant macro-

phyte in Florida Bay, USA, as a result of the large (60%) reduction in historic freshwater input from the Everglades (Fourqurean & Robblee 1999). The reduction in freshwater inflow largely resulted from the construction of levees and water control structures in the 1950s that diverted water flows for human land use and development. This diversion caused a transition away from a more heterogeneous community consisting largely of *Halodule wrightii* and *Ruppia maritima*, mixed with *T. testudinum*, among other seagrass and macroalgae species (Fourqurean & Robblee 1999, Fourqurean et al. 2002). *Thalassia*-dominated seagrass beds in the central

and northwestern sections of Florida Bay have undergone repeated mass mortality events, in 1974–75 (Schmidt, 2002), 1987–89 (Robblee et al. 1991), and 2015 (Hall et al. 2016). These mass mortality events have been attributed to an interaction of high temperatures, salinities, and seagrass densities that created hypersaline, hypoxic-to-anoxic bottom waters, and high porewater sulfide levels. This multi-stressor environment is thought to overwhelm the physiological tolerances of *T. testudinum* and result in fatal intrusion of sediment sulfide (Koch et al. 2007b).

Florida Bay is located at the southern tip of the Florida peninsula. This roughly triangular area is bordered by mainland Florida to the north, the Florida Keys to the east and south, and the Gulf of Mexico to the west. Highly variable biogenic carbonate sediment accretion over a karst limestone platform has created many mud banks and mangrove islands that segment the bay into about 50 distinct shallow (0.5 to 3.0 m) basins (Lee et al. 2016). The shallow, fragmented nature of the bay, coupled with a reduction in water exchange with the Atlantic Ocean caused by the construction of dredged material islands in the Florida Keys, results in little water exchange among basins, long hydrological residence times, and high spatial variability in water quality (Lee et al. 2016).

Distribution of *T. testudinum* in Florida Bay is primarily controlled by sediment depth and phosphorus availability (Fourqurean et al. 2002, Herbert et al. 2011). In the current high-salinity condition of Florida Bay, *T. testudinum* is most abundant in the western basins of Florida Bay where the high phosphorus concentrations and deep sediments support the large amount of below-ground biomass that typifies the species (Tomlinson & Vargo 1966, Zieman et al. 1989, Fourqurean et al. 2002). Variability in sediment accretion within basins often results in hard-bottom habitats in basin centers incapable of supporting dense *T. testudinum* beds, and deeper sediments around basin margins where *T. testudinum* grows more densely (Zieman et al. 1989).

Shoal grass *Halodule wrightii* was historically the most commonly found seagrass in Florida Bay, but abundance decreased in the decades following the reduction of freshwater flow from the Everglades (Fourqurean et al. 2002, Herbert et al. 2011). It is an early-colonizing seagrass most commonly found in areas of the bay that are subject to frequent disturbance. It also co-occurs with *T. testudinum*, albeit at much lower biomass. *H. wrightii* became dominant in affected basins in the years immediately following *T. testudinum* die-off in the late 1980s and early 1990s

(Durako et al. 2002). These recovering, early successional communities had sparser seagrass cover, but were more diverse than *T. testudinum* climax communities, with several genera of macroalgae (*Batophora*, *Dasycladus*, *Caulerpa*, *Laurencia*, etc.) also acting as early colonizers before *T. testudinum* gradually recolonized.

Recovery of *T. testudinum* seagrass beds to pre-die-off state densities can be very slow (Durako et al. 2002, Hall et al. 2016). Algal blooms and high turbidity caused by late 1980s die-off resulted in poor growth conditions for 3–5 yr (Durako et al. 2002). Following a short recovery period after the 2015 die-off, the category 4 Hurricane Irma passed over western Florida Bay in September 2017. Although Hurricane Irma did not result in immediate major losses of seagrasses in the western basins, the physical disturbance from Irma was linked to multi-year states of heightened turbidity and chlorophyll *a* concentrations (Wilson et al. 2020), conditions that are associated with post-die-off related seagrass losses in Florida Bay (Durako et al. 2002). Longer-term negative hurricane effects are supported by recent reports of declines in *T. testudinum* cover, persistently high turbidities, chronic phytoplankton blooms, and increasing *H. wrightii* cover in Johnson Key basin, in western Florida Bay, since the passage of Hurricane Irma (M. S. Koch, M. O. Hall, B. T. Furman pers. comm.).

In response to seagrass die-offs in Florida Bay, an active area of research has focused on developing early-warning indicators of physiological stress in seagrasses (Durako et al. 2007). However, these efforts have yet to find a metric that is temporally and spatially consistent across Florida Bay. *T. testudinum* in Florida Bay exhibits a high level of phenotypic plasticity among basins and spatial variation in morphological metrics, such as leaf width and length, shoot density, and biomass, reflect environmental variability across the bay (Hackney & Durako 2004, 2005). Short-shoot demographics estimated from reconstructive aging also indicate variation in recruitment and mortality among basins in response to seagrass die-off (Durako 1994). Unfortunately, both of these approaches require destructive sampling and large numbers of short shoots. In more recent years, pulse amplitude modulated (PAM) fluorometry has been used to non-invasively measure photosynthetic quantum efficiencies and electron transport rates in *T. testudinum* in the bay (Durako & Kunzelman 2002, Belshe et al. 2007, Durako 2012, Genazzio & Durako 2015). However, this technique can be unreliable when irradiances are very high, which is the case in many shallow areas of the bay (Beer & Axelsson

2004, McPherson et al. 2011), and photosynthetic yields can exhibit high spatial variability when measuring shoots in die-off patches (Durako & Kunzelman 2002). In addition, significant diurnal variations in photosynthetic quantum efficiencies confound inter-basin and temporal analysis, making PAM fluorometry ineffective when assessing populations at the landscape level, which requires measurements over diurnal time periods (Belshe et al. 2007, Genazzio & Durako 2015).

High concentrations of hydrogen sulfide (H_2S) are toxic to seagrasses (Koch & Erskine 2001). Isotope ratio mass spectrometry allows the use of stable sulfur isotopes to identify sediment-derived sulfide in seagrass tissues (Holmer & Hasler-Sheetal 2014). When sulfate is reduced to sulfide by bacteria, sulfur stable isotopes become fractionated, resulting in more negative $\delta^{34}S$ values due to lower ^{34}S relative to ^{32}S in sediment and porewater sulfide than in water column sulfate. Thus, tissues with decreased $\delta^{34}S$ values indicate intrusion and assimilation of sediment sulfide. Studies of *Zostera marina* and *Halophila ovalis* indicate that the $\delta^{34}S$ value of leaf-tissue sulfur to total leaf sulfur ($(\delta^{34}S + 30)/\text{total sulfur}$) is an effective indicator of plant health (Kilminster et al. 2014). This sediment stress indicator (SSI) is positively correlated with seagrass growth in *H. ovalis*. High total sulfur and low $\delta^{34}S$ (high proportion of total sulfur and sulfide coming from the sediment) in leaves result in low SSI values, which correlate with reduced shoot growth. Thus, the presence of sediment sulfide in leaves serves as an indicator of plant sulfide stress. Sample collection for this technique is rapid and minimally destructive (leaf tissues from 1–2 shoots per site). Geographic differences in SSI in *H. ovalis* remained stable across seasons (Kilminster et al. 2014), making SSI a potentially excellent ecoinicator candidate for annual sampling programs. However, *H. ovalis* is a small-bodied seagrass capable of rapid turnover and extensive sexual reproduction. This contrasts with the large-bodied, long-lived, and mostly vegetatively reproducing *T. testudinum*; *H. ovalis* does not produce long-lived, leaf-replacing vertical short shoots. Thus, the effectiveness of SSI in representing sulfide stress may vary between the 2 species.

The goal of this study was to determine the efficacy of SSI in *T. testudinum* leaves collected during the yearly spring Fisheries Habitat Assessment Program (FHAP) sampling by examining spatial variation within basins across Florida Bay and comparing die-off versus non-die-off locations. FHAP, established in 1995 in response to the 1987–1989 die-off, is a yearly sampling regime designed to monitor status and

trends of macrophytes across Florida Bay (Durako et al. 2002). Correlations among the components of SSI, and correlations between SSI and potential stressors were tested. We also examined 3 hypotheses: (1) that SSI will decrease in Florida Bay from east to west, coinciding with increases in *T. testudinum* cover and sediment depth, factors known to facilitate sulfate reduction; (2) that those areas historically vulnerable to sulfide-induced die-off will have lower SSI values than non-die-off locations (although recent die-off sites may have higher SSI values because they have not fully recovered); and (3) that macrophyte communities dominated by *T. testudinum* will have lower SSI values than mixed-species communities with sparse *T. testudinum*. Our overall objective was to determine if SSI for *T. testudinum* leaves can be used as an effective, relatively nondestructive tool for managers to identify die-off-susceptible areas and potentially take management actions (e.g. increasing freshwater inflow to targeted areas) to reduce or prevent future mortality events.

2. MATERIALS AND METHODS

2.1. FHAP sampling design

Landscape-level sampling was performed during annual FHAP sampling in May 2019. FHAP uses a systematic random sampling design wherein each sampled basin is segmented into 28–31 tessellated hexagons and a random centripetal algorithm is used to select a station location in each hexagon. For this study, *Thalassia testudinum* samples were obtained within 13 Florida Bay basins (see Table 1 for basin

Table 1. Fisheries Habitat Assessment Program (FHAP) names and corresponding abbreviations for basins in Florida Bay, USA

Basin	Abbreviation
Barnes Sound	BAR
Blackwater Sound	BLK
Calusa Key Basin	CAL
Crane Key Basin	CRN
Duck Key Basin	DUC
Eagle Key Basin	EAG
Johnson Key Basin	JON
Madeira Bay	MAD
Manatee Bay	MAN
Rankin Lake	RAN
Rabbit Key Basin	RKB
Twin Key Basin	TWN
Whipray Bay	WHP

names and abbreviations), for a total of approximately 390 sampling stations (Fig. 1). At stations where *T. testudinum* was present, 2 adjacent shoots were collected. Shoots were rinsed in seawater to remove attached sediments, then rinsed in freshwater, placed in a labeled plastic bag, and stored on ice for the duration of the sampling day. Each evening, the length of the longest leaf of each shoot was measured and the youngest and second youngest (rank 1 and 2) leaves were removed from the shoots at sheath level, then scraped with a glass slide to remove epiphytes. Cleaned leaves were placed in plastic scintillation vials with silica gel desiccant for transport back to the University of North Carolina Wilmington Center for Marine Science (UNCW CMS).

At each FHAP station, basic water quality parameters (salinity, water temperature [°C], and pH) were recorded using YSI instruments. In addition, water depth (cm) and Secchi depth (cm) were determined using a graduated PVC pole with a 20 cm diameter Secchi disk attached to the end; attenuation of photosynthetically active radiation $K_d(\text{PAR})$ (m^{-1}) was calculated from light profiles using LI-COR scalar quantum sensors positioned 0.5 m apart. Sediment depth (cm) at each station was measured by inserting a graduated metal pole into the sediments until it contacted the limestone substrate. Macrophyte cover was estimated visually within 50 cm × 50 cm quadrats ($n = 8$), haphazardly distributed within a 10 m radius of the anchored vessel, at each station using a modified Braun-Blanquet method (Furman et al. 2018). The method assigns a score from 0 to 5 to each quadrat that can be converted into percent cover for statistical analysis (van der Maarel 2007, Furman et al. 2018). Leaf plastochrone intervals for *T. testudinum* in Florida Bay average between 13 and 18 d, with an average of 3.7 leaves shoot⁻¹ (Durako 1994, 1995). This results in estimated leaf life spans ranging from 48–66 d. Time series of water temperature and salinity in the 2 mo preceding the FHAP sampling (15 March – 15 May) were created using hourly data from National Data Buoy Center (NBDC) sampling stations to examine patterns that could have contributed to stress over the lifespan of the rank 1 and 2 leaves that were collected during the May sampling.

2.2. Sulfur isotope analyses

At UNCW CMS, leaves were oven dried to a constant weight at 60°C. The dried material was ground to a fine powder using a Fisherbrand Bead Mill 4 with 2 ml ceramic bead tubes and 2.8 mm ceramic beads. A 6.5 mg ground sample was placed into a tin cup with 13 mg of vanadium pentoxide prior to analysis. Total sulfur (TS as % dry weight, DW) and $\delta^{34}\text{S}$ analyses were performed with a continuous flow elemental analyzer isotopic ratio mass spectrometer (Thermo Delta V plus IRMS and a Thermo Flash HT Plus Elemental Analyzer) using procedures outlined by Monaghan et al. (1999). $\delta^{34}\text{S}$ was calculated with ISODAT software using the following equation: $\delta^{34}\text{S}$ (‰) = $(R_{\text{sample}} / R_{\text{VCDT}} - 1) \times 1000$, where $R = {}^{34}\text{S}/{}^{32}\text{S}$ and R_{VCDT} is the reference standard Vienna Cañon Diablo Troilite. The $\delta^{34}\text{S}$ values were then used to calculate the SSI: $(\delta^{34}\text{S}_{\text{leaf}} + 30) / \text{TS}$.

2.3. Statistical analyses

The statistical program R (version 4.0.3; R Core Team 2020) was used for all data analyses. Maps of the study area were created using the 'leaflet' package (Cheng et al. 2021) and publicly available satellite imagery (Esri). Generalized additive models

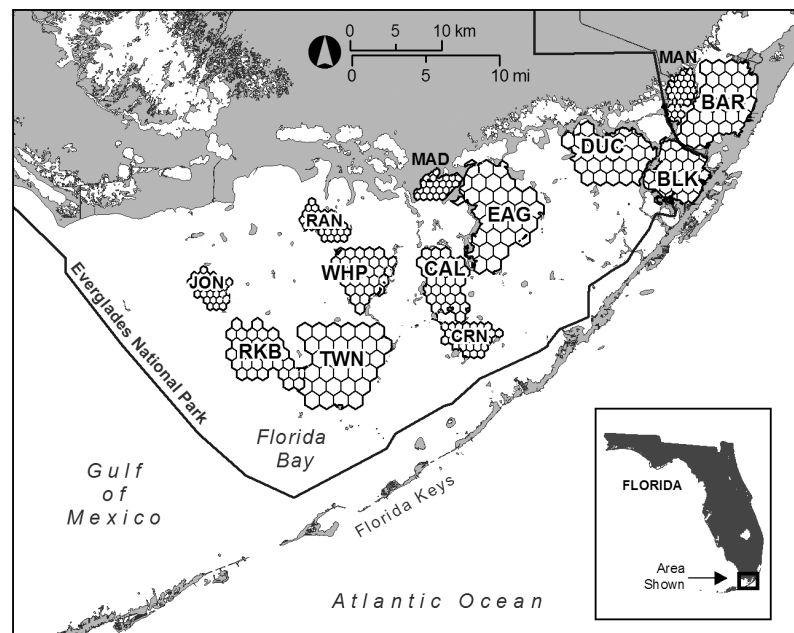


Fig. 1. Florida Bay, USA, showing the 13 Fisheries Habitat Assessment Program basins and sampling hexagons sampled during May 2019. See Table 1 for basin names corresponding with the 3 letter abbreviations (adapted from Peñalver et al. 2020, with permission)

(GAMs) with Gaussian distributions and identity links were used to examine correlations between the SSI components and among SSI and potential predictor variables. Raw data were used for these calculations, with the exception of *T. testudinum* and *Halodule* cover, which were log transformed. Non-parametric Kruskal-Wallis and Wilcoxon ranked-sum tests were used to determine differences among groups due to varying group sample size and potential heteroscedasticity.

A correlation matrix of all untransformed FHAP predictor variables was formed prior to multivariate analyses. Variables with greater than 0.5 Pearson's R-values (water depth, leaf length) were then excluded to reduce covariation. PCA was performed using the 'FactomineR' package (Le et al. 2008). The PCA included all sites with sulfur isotope data (N = 348) using SSI, sediment depth, salinity, *T. testudinum* cover, *Halodule* cover, total macroalgae cover, and Shannon-Wiener diversity of the macrophyte community. All variables were transformed for normality using Tukey's ladder of powers, then scaled and zero-centered prior to analyses (Mangiafico 2020). Hierarchical clustering of principal components (HCPC) was conducted on the results of the PCA with the 'FactoMineR' package using Ward clustering.

Multivariate GAMs of SSI as a function of the physical and biological predictors included in the PCA were constructed and compared using Akaike's Information Criterion corrected for small sample size (AICc) with the package 'AICcmodavg' (Mazerolle 2020) and a separate 10-fold cross validation (Kuhn 2020). Covariation among predictors was examined using variance inflation factors with a threshold of 3.0 for exclusion (Zuur et al. 2009). No predictors exceeded the threshold in the original model selection. An additional selection was performed with basin identity included as a factor. One variable in this analysis, *T. testudinum* cover, had a variance inflation factor of 3.54, exceeding the threshold. Due to the importance of this variable, it was not removed. Values are reported as means \pm SD. Statistical significance was assessed at $\alpha = 0.05$.

3. RESULTS

3.1. SSI components

Mean leaf TS content for *Thalassia testudinum* leaves collected from 13 basins across Florida Bay in May 2019 was $0.80 \pm 0.14\%$ DW and mean $\delta^{34}\text{S}$ was

$4.33 \pm 6.28\%$. This resulted in an overall SSI of 44.6 ± 13.3 . Leaf $\delta^{34}\text{S}$ generally decreased in a linear fashion with increasing TS (Fig. 2, $R^2 = 0.24$). The strength of this relationship varied among basins. JON (see Table 1 for full basin names) was a notable outlier, having the highest average residual value for the GAM regression (5.19 ± 4.21 ; Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m670p033_supp.pdf). This indicates that a smaller than predicted proportion of tissue sulfur is derived from sediment sulfide in JON, based on the bay-wide regression. Sites at basin margins had lower residual values of $\delta^{34}\text{S}$ as a function of TS than sites at basin centers (Fig. S1), revealing that sediment sulfide makes up a larger proportion of leaf TS at margin sites. Thus, given equal TS, leaves at deeper-sediment margin sites had more sulfide intrusion than basin-center sites.

SSI had a negative, non-linear correlation with TS, the denominator in the equation for SSI (Fig. 2, $R^2 = 0.73$, EDF = 4.52). Increases in TS in leaves with low S content resulted in steeper declines in SSI than in leaves with high S content. SSI increased as the numerator variable, $\delta^{34}\text{S}$, increased (Fig. 2, $R^2 = 0.76$, EDF = 7.92). Although the overall correlation is non-linear, increases from low to moderate $\delta^{34}\text{S}$ values correlated linearly with SSI. Higher $\delta^{34}\text{S}$ and SSI values had a more complex relationship where the slope flattened near 10‰ and steepened at values above 12‰. The flattening is a result of the previously mentioned sites in JON that have abnormally high $\delta^{34}\text{S}$ values relative to their high TS (Fig. S2), which results in lower than predicted SSI based on their high $\delta^{34}\text{S}$. The sharp increase in slope at sites with very high $\delta^{34}\text{S}$ corresponds to sites in BAR (the easternmost basin), which have very low TS and abnormally high $\delta^{34}\text{S}$ based on their low TS.

3.2. Bay-scale variation in SSI

SSI values generally decreased along a bay-wide, east to west longitudinal gradient ($R^2 = 0.15$; Fig. 3; Table S1), roughly coinciding with increases in *T. testudinum* cover ($R^2 = 0.48$) and sediment depth ($R^2 = 0.39$). Within-basin variation was high, and basin margins, which have high *T. testudinum* cover and relatively deep sediments, tended to have lower SSI values than basin centers (Fig. 4). This was especially evident in basins that contained sites with very shallow sediment depths at their centers (DUC, EAG, CAL). Despite high within-basin variation, SSI still exhibited significant among-basin variation (Kruskal-

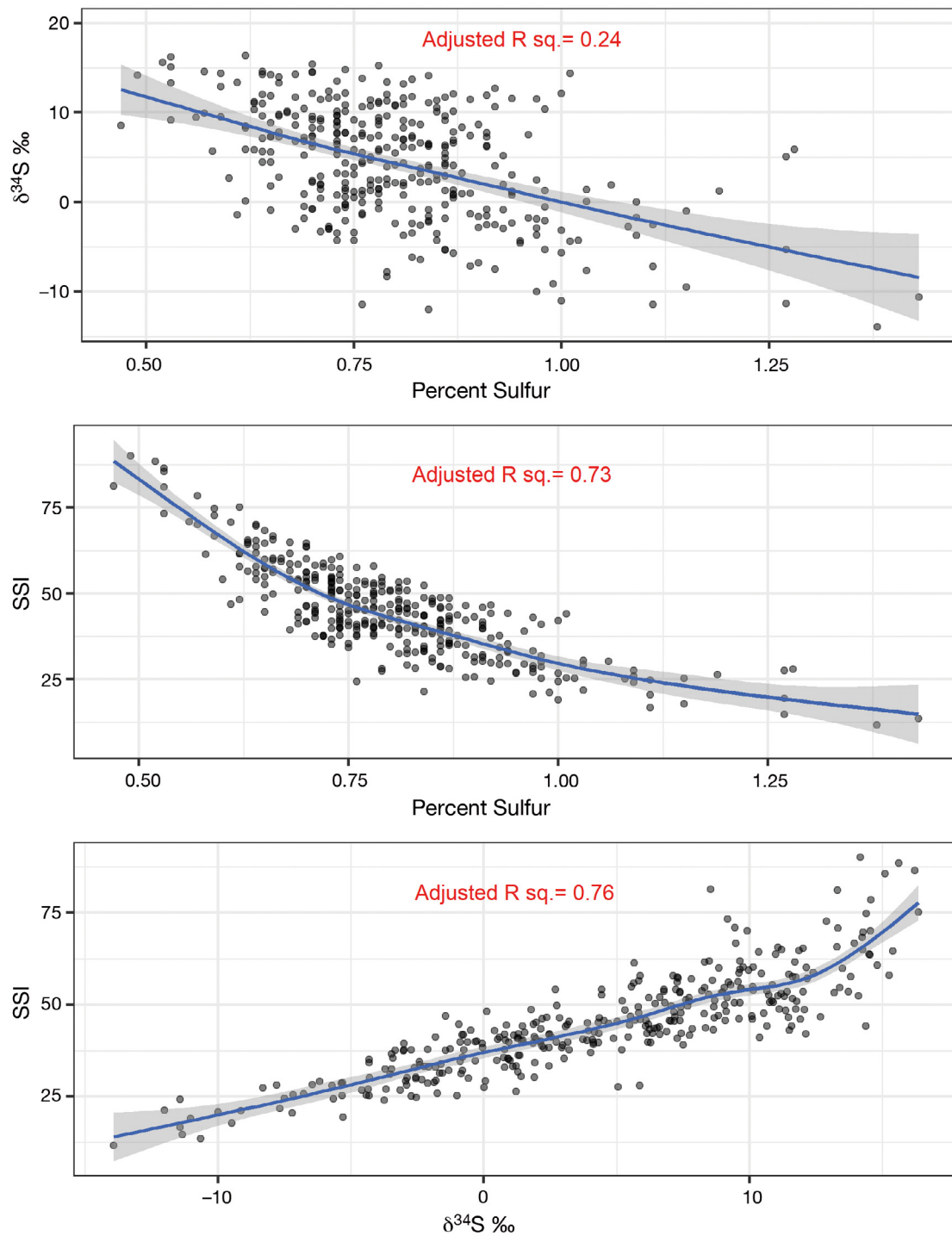


Fig. 2. Generalized additive models of the components of the sediment stress indicator (SSI) in *Thalassia testudinum* leaves across Florida Bay. Red text denotes $p < 0.0001$. Shaded area represents the 95% confidence interval

Wallace $p < 0.0001$; Fig. 5; Table S1). Mean SSI values of the western basins RAN (39.3 ± 7.0), RKB (38.1 ± 9.0), and TWN (37.9 ± 16.1) were significantly lower than the eastern basins BLK (54.0 ± 10.0) and BAR (58.9 ± 17.8). An obvious outlier to the east to

west SSI trend was JON (Fig. 5), the western-most basin sampled with deepest sediments (Table 2) and location of severe 1990s and 2015 seagrass die-offs. The SSI values in JON were significantly higher (51.7 ± 9.1) than the other western basins, RAN, RKB,

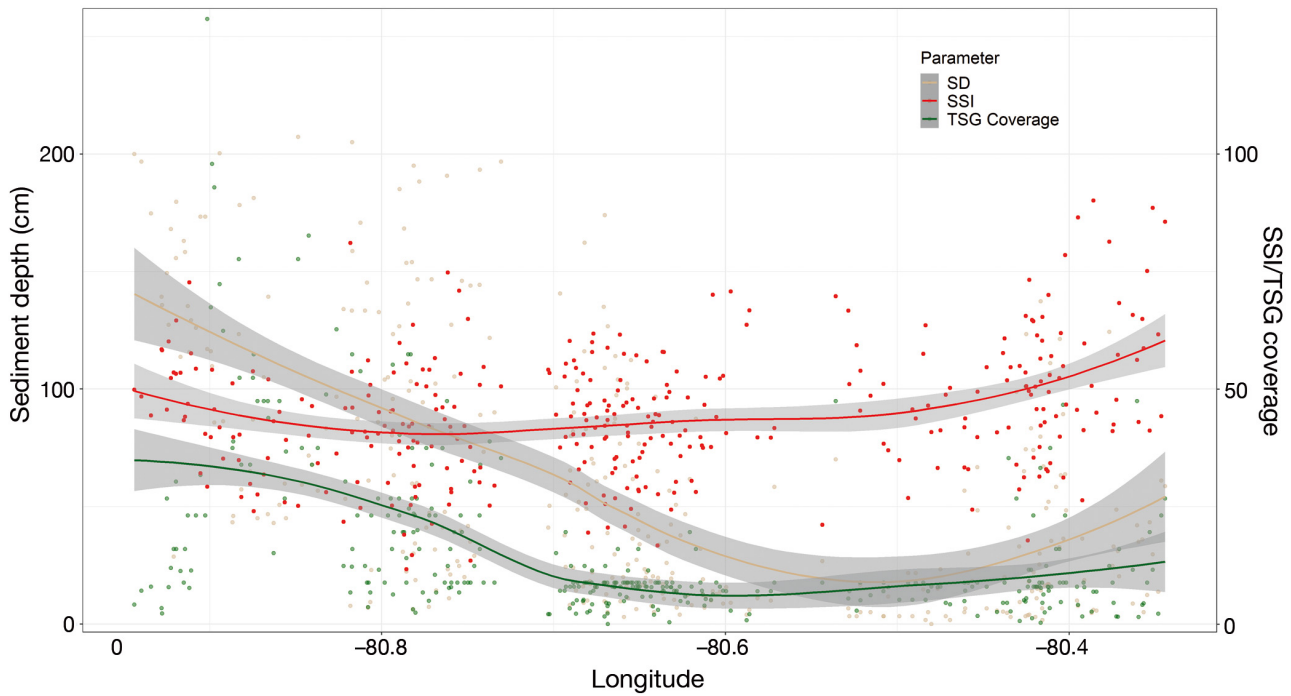


Fig. 3. Generalized additive models of sediment depth (SD, cm), total seagrass cover (TSG Coverage, %), and the sediment stress indicator (SSI) along a longitudinal gradient across Florida Bay. Shaded area represents the 95% confidence interval

and TWN (Table S1). When JON sites were excluded, there was a consistent decline from eastern to western sites (Fig. S3).

RAN and RKB had consistently deep sediments, while TWN and RKB had the highest and third

highest median *T. testudinum* covers (Table 2); both deep sediments and high seagrass cover were associated with lower SSI values. TWN had the largest range of sediment depths (6–205 cm), which may have contributed to the large range of SSI values observed within this basin (Fig. 5).

Despite a low mean SSI value, RAN had few extreme SSI values, with the smallest standard deviation (7.0) of all basins (Fig. 5). BAR and BLK, the basins with the highest mean SSIs, had relatively shallow, although variable, sediment depths. These 2 basins also had the highest median water depths, macroalgae cover, Shannon-Wiener diversity, and lowest *T. testudinum* cover (Table 2). Among-basin differences in *T. testudinum* cover, sediment depths, and macrophyte diversity, in addition to geographic factors such as proximity to freshwater and nutrient inputs, may contribute to the differing SSI values of the eastern versus western basins.

Sites within the geographic range of extensive die-off in 2015 had higher SSI values, sediment depth, and *Halodule* cover, but lower *T. testudinum* cover than adjacent non-die-off sites

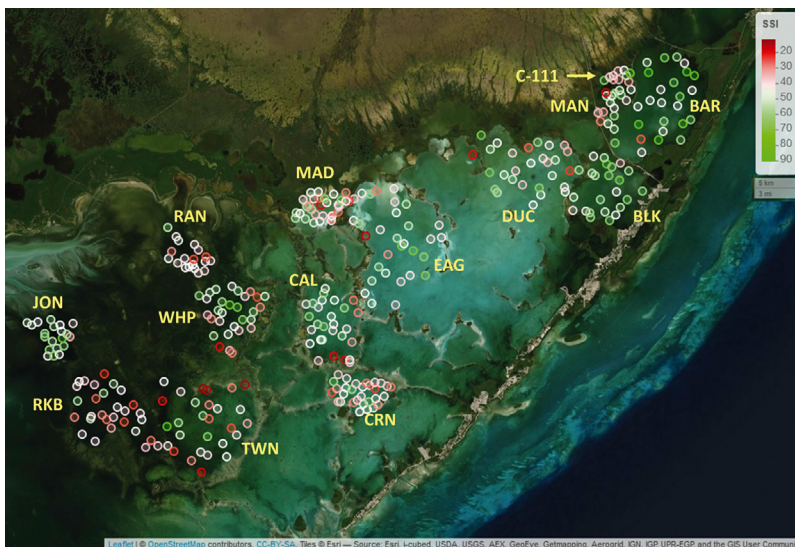


Fig. 4. Sediment stress indicator (SSI) values across the study area. White represents values close to the mean (44.6). Red and green represent increasingly low (= high stress) and high (= low stress) SSI values, respectively. The C-111 canal (arrow) is a major source of freshwater and nutrient input into northeastern Florida Bay

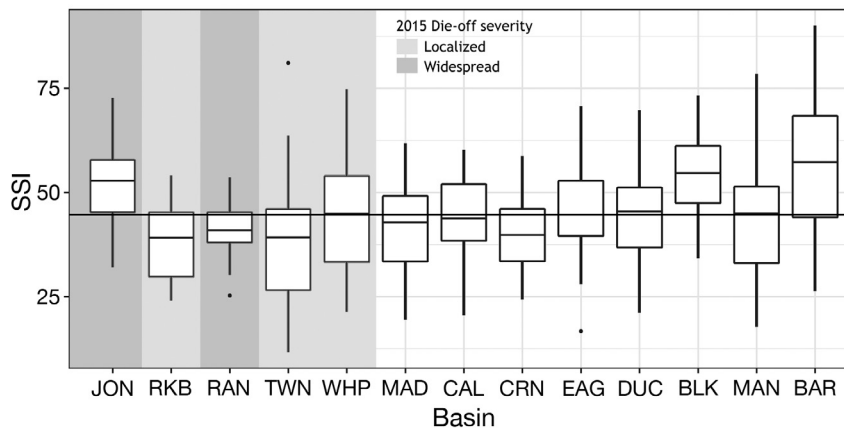


Fig. 5. Basin-level variations in the sediment stress indicator (SSI) across Florida Bay. Basins are oriented from west to east along the x-axis. Shaded portions indicate the relative severity of the 2015 die-off (from Hall et al. 2016). Boxes represent the basin interquartile ranges, vertical lines extend to each maximum and minimum, horizontal lines indicate basin medians, and dots represent potential outliers. The horizontal line running the length of the plot indicates bay-wide mean SSI of 44.6

Table 2. Median values of the sediment stress index (SSI) and associated variables by basin in Florida Bay. S-W: Shannon-Wiener

Basin	SSI	Depth (cm)		Salinity	Cover (%)			S-W diversity
		Sedi-ment	Water		<i>Thalassia</i>	<i>Halodule</i>	Macroalgae	
BAR	57.3	29.7	295.0	40.1	4.4	0.1	6.8	1.3
BLK	54.6	20.0	240.0	38.4	4.5	0.0	6.8	1.2
JON	52.8	152.5	136.0	37.4	6.3	5.2	0.3	0.8
DUC	45.4	6.7	152.0	38.4	7.0	0.3	5.2	1.0
MAN	45.0	34.2	163.0	40.4	7.9	0.3	5.4	1.1
WHP	44.9	89.5	160.0	41.9	17.2	1.5	1.4	0.6
EAG	44.6	12.3	175.0	37.7	7.0	0.5	1.6	1.0
CAL	43.8	19.0	211.0	40.8	7.3	0.0	0.8	0.4
MAD	42.8	84.0	111.0	42.0	8.8	0.9	0.9	0.7
CRN	39.8	26.5	225.0	40.6	7.8	0.0	5.3	1.0
RAN	39.7	104.0	116.0	39.7	6.6	6.1	4.9	1.0
TWN	39.2	30.4	230.0	39.2	12.4	0.0	5.0	1.0
RKB	39.2	83.3	200.0	36.5	47.5	0.5	4.6	0.5
Overall	43.8	44.2	175.0	39.8	8.0	0.4	3.5	0.9

(Fig. 6). These physical and biological characteristics indicate that these sites may be in early succession or recovery phases after the die off, which are usually characterized by initial rapid increases in *Halodule* cover followed by slower increases in *T. testudinum* (Thayer et al. 1994, Durako et al. 2002).

3.3. SSI predictors

Generalized additive models of SSI as a function of potential univariate predictors revealed weak, but significant, negative, non-linear correlations with *T.*

testudinum cover (Fig. S4, $R^2 = 0.10$) and sediment depth ($R^2 = 0.07$). SSI had significant, positive correlations with water depth ($R^2 = 0.08$), Shannon-Wiener diversity ($R^2 = 0.07$), *Halodule* cover ($R^2 = 0.04$), and *T. testudinum* leaf length ($R^2 = 0.02$). Macroalgae cover, salinity, and *T. testudinum* leaf number did not correlate with SSI (Fig. S4). The large sample size, heterogeneity of Florida Bay, unmeasured factors such as nutrient input, and frequent collinearity among predictors (Fig. S5) likely contributed to the low correlation coefficients of the univariate models and unexpected significant correlations (leaf length, diversity). However, SSI still correlated with variables expected to contribute to chronic sulfide intrusion (*T. testudinum* cover and sediment depth).

PCA indicated that PC1 accounted for 31.4% of the variance and PC2 accounted for 19.4% of the variance among SSI and the potential predictor variables (Fig. 7). Shannon-Wiener diversity was the largest contributor to PC1 and *H. wrightii* cover the largest contributor to PC2. The ordination suggests that SSI is most strongly and negatively correlated with *T. testudinum* cover, with evidence of weaker positive relationships with Shannon-Wiener diversity and *H. wrightii* cover.

Ten-fold cross validation and AIC model selection of multivariate GAMs using the physical and biological predictors included in the PCA revealed that SSI was likely best predicted by *T. testudinum* cover, *H. wrightii* cover,

and sediment depth ($r^2 = 0.24$, weight = 0.48). Models including these predictors in addition to Shannon-Wiener diversity or macroalgae cover were also supported, with weights of 0.19 and 0.17, respectively. No models excluding *T. testudinum* cover, *H. wrightii* cover, or sediment depth received more than 2% support (Table 3). Adding basin identity (ID) as a factor to the selection model, increased predictive power and model support over the previous best model ($r^2 = 0.34$, $\Delta AIC > 30$, Table S2), demonstrating that basin ID is linked to unrecorded factors that influence SSI, potentially phosphate and light availability. The variance inflation factor for

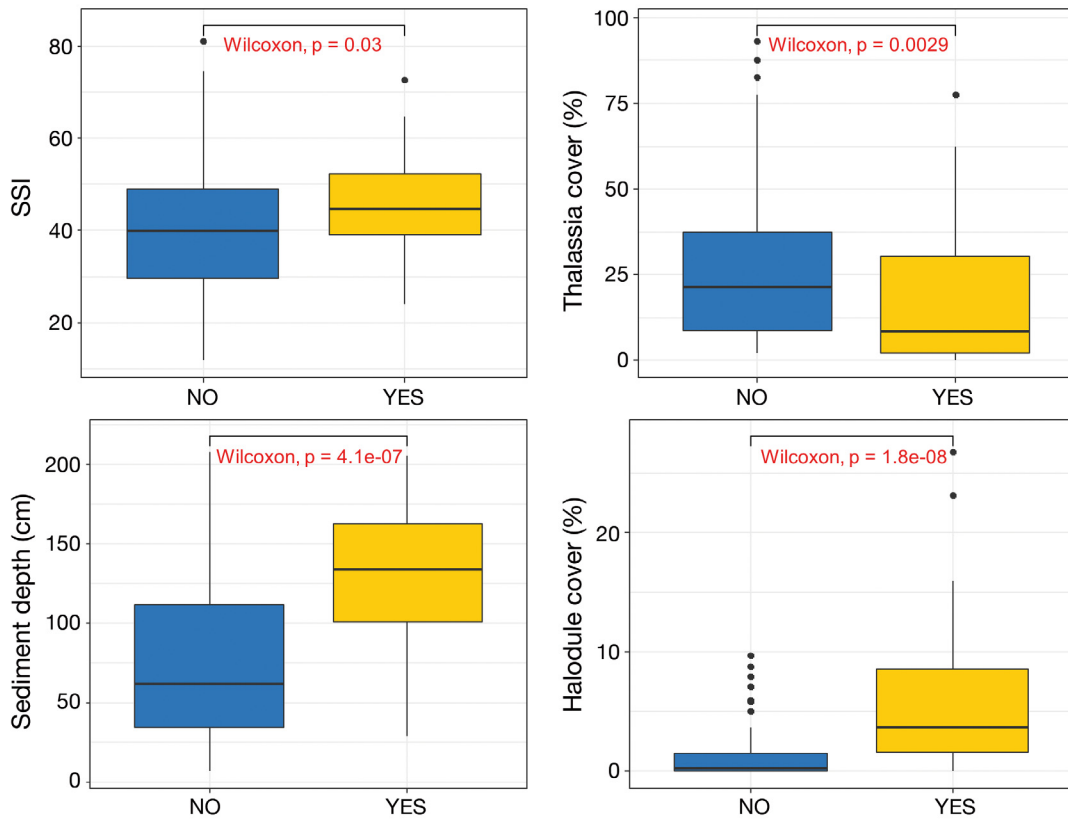


Fig. 6. Wilcoxon ranked sum test results of the sediment stress indicator (SSI), *Thalassia testudinum* cover, *Halodule wrightii* cover, and sediment depth between non-die-off (NO) and 2015 die-off (YES) sites in western basins (JON, RAN, RKB, TWN, WHP; see Table 1 for full basin names). Refer to Fig. 5 for details of boxplot parameters

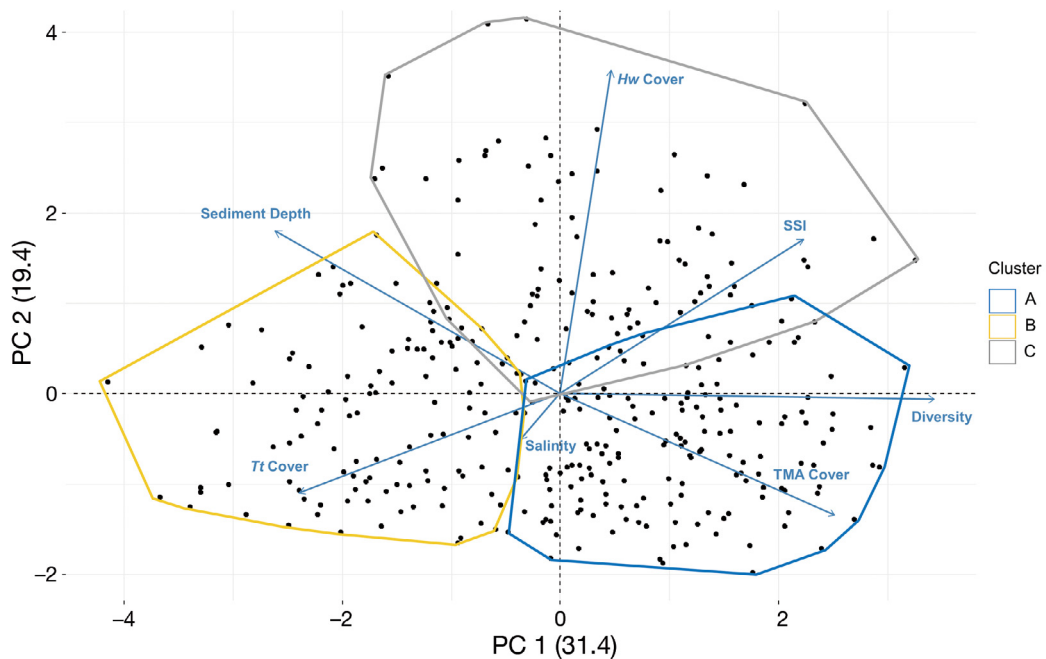


Fig. 7. PCA and hierarchical clustering of principal components (HCPC). Points represent individual sites, vectors represent the direction and magnitude of variable effects on ordination, and polygons delineate HCPC clusters. Orthogonal vectors suggest weak/no correlation, while collinear or opposite facing vectors indicate stronger correlation. The sediment stress indicator (SSI) appears to have the strongest correlation with *Thalassia testudinum* (Tt) cover. Hw: *Halodule wrightii*; TMA: total macroalgae

Table 3. Akaike's information criterion (AIC) selection table of null model and generalized additive models receiving a weight of at least 0.01 for sediment stress index (SSI) as a function of non-linear potential predictors. + indicates inclusion in the model. *Tt*: *Thalassia testudinum*; *Hw*: *Halodule wrightii*; S-W: Shannon-Wiener; TMA: total macroalgae; AICc: AIC corrected for small sample size

Variables included						Model scores				
<i>Tt</i> cover	<i>Hw</i> cover	Sediment depth	S-W diversity	TMA cover	Salinity	df	LogLik	AICc	ΔAIC	Weight
+	+	+				14	-1338.71	2707.32	0.00	0.48
+	+	+	+			14	-1339.30	2709.16	1.84	0.19
+	+	+		+		15	-1338.48	2709.37	2.05	0.17
+	+	+	+	+		15	-1339.35	2710.86	3.54	0.08
+	+	+	+	+	+	19	-1335.52	2712.03	4.71	0.05
	+	+				12	-1343.63	2713.78	6.46	0.02
	+	+	+			13	-1343.31	2714.82	7.50	0.01
						2	-1393.71	2791.46	84.14	0.00

T. testudinum cover in this model is relatively high (3.64), indicating that basin ID is closely linked to *T. testudinum* cover, so inclusion of both predictors may be redundant. Furthermore, a model using only basin ID, *H. wrightii* cover, and sediment depth had slightly more support than the basin ID, *T. testudinum*, *H. wrightii*, and sediment depth model (35 vs. 28%).

HCPC using the PCA results of SSI and potential predictors revealed 3 clusters (Figs. 8 & 9). Cluster A has, among other characteristics, low seagrass (*T. testudinum* and *H. wrightii*) cover, shallow sediments, deep water, and high SSI values. This cluster largely represents eastern sites and the centers of

central and western basins (Fig. 8). There are almost no sites within the region of the 2015 die-off in cluster A. Cluster B has dense *T. testudinum*, sparse *H. wrightii*, deep sediments, shallow water, and low SSI values (high sulfide stress), representing western basins with isolated die-offs, and sites at the margins of central and eastern basins (Fig. 8). Cluster C has deep sediments, shallow water depths, low *T. testudinum* cover, high *H. wrightii* cover, and the highest SSI values, suggesting low sulfide stress. Many sites in cluster C correspond to sites within the region of most severe die-off in 2015 (RAN and JON) and thus may be in an early successional or recovering phase.

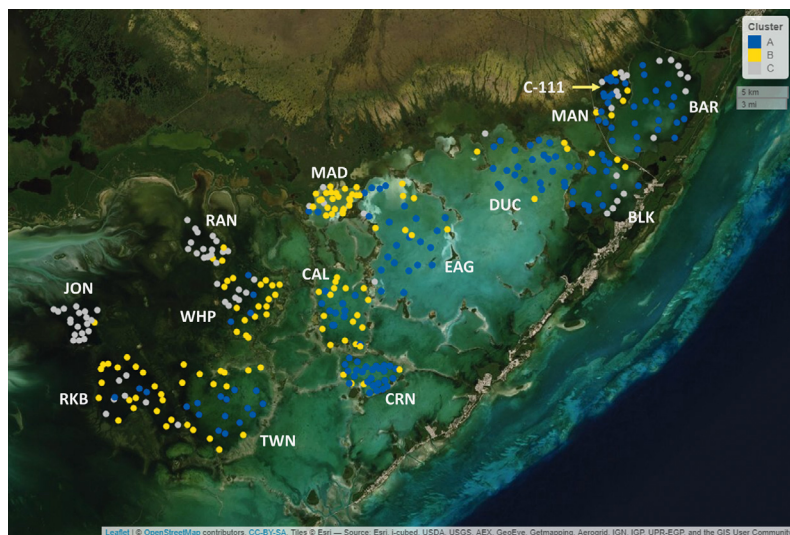


Fig. 8. Sampling sites colored by hierarchical clustering of principal components (HCPC) clusters from Fig. 7. Cluster A is largely comprised of central/eastern bay sites, and sites near the center of each basin. Basins subject to widespread die-off in 2015 (RAN, JON; see Table 1 for full basin names), and sites at the margins of eastern basins form the majority of cluster C. Other western basins, less severe die-off sites, and sites at basin edges make up cluster B. Cluster A contains few sites within the range of the 2015 die-off

4. DISCUSSION

4.1. SSI components

The negative linear correlation between $\delta^{34}\text{S}$ values and percent sulfur ($R^2 = 0.24$) indicates that sulfur accumulation in *Thalassia testudinum* leaf tissue in Florida Bay reflects assimilation of sulfur from a combination of water column sulfate and increasing proportions of sediment sulfide. Conversely, $\delta^{34}\text{S}$ values and percent sulfur are positively correlated in *Posidonia oceanica* leaves ($R^2 = 0.36$) and negatively correlated in the roots and rhizomes, tissues in close proximity to sediment sulfide, of *Zostera marina* ($R^2 = 0.51$, $R^2 = 0.41$) and *P. oceanica* ($R^2 = 0.12$, $R^2 = 0.15$; Frederikson et al. 2007, Holmer & Hasler-Sheetal 2014). Additionally,

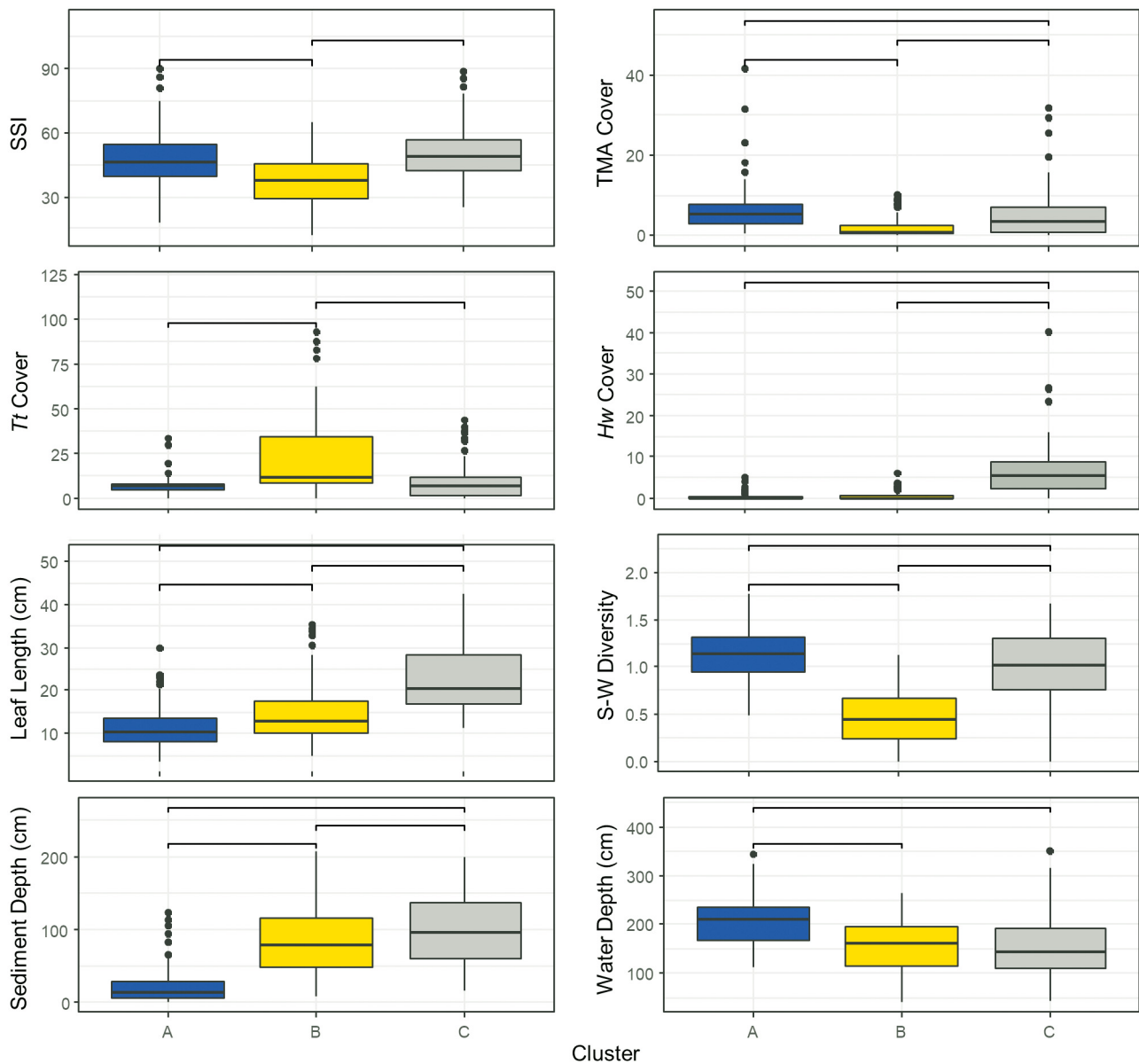


Fig. 9. Kruskal-Wallis tests of biotic (e.g. *Thalassia testudinum* [*Tt*] leaf length, Shannon-Wiener [S-W] diversity) and abiotic (sediment and water depth) variables across hierarchical clustering of principal components (HCPC) clusters from Fig. 7. Lines between clusters indicate $p < 0.05$. Lack of a line indicates $p > 0.05$. Refer to Fig. 5 for details of boxplot parameters. SSI: sediment stress indicator; TMA: total macroalgae; *Hw*: *Halodule wrightii*

mean leaf TS for our study was 0.8% DW, almost 2-fold higher than the reported mean TS of 0.5% for *T. testudinum* in the Virgin Islands (Holmer & Hasler-Sheetal 2014), and we observed a mean $\delta^{34}\text{S}$ value of 4.3‰, less than half of the Virgin Island mean of 11.7‰. Together these patterns indicate that *T. testudinum* in Florida Bay is subject to higher levels of sulfide intrusion and assimilation than other large-bodied seagrass species across the world and conspecifics of nearby carbonate systems.

4.2. Bay-scale trends

In generally decreasing from east to west, SSI followed other bay-scale spatial trends; western basins typically have deeper sediments, higher phosphorus availability, and denser seagrass than eastern basins (Zieman et al. 1989) and are historically more vulnerable to sulfide-induced die-off (Koch et al. 2007b, Johnson et al. 2018). However, there were low SSI values at sites across the entire study area, often

reflecting within-basin gradients in sediment depth and *T. testudinum* cover. For example, CAL, a basin with highly variable sediment depths that range from 3 cm mid-basin to 162 cm along the basin margins, had a relatively strong linear correlation ($R^2 = 0.48$) between sediment depth and SSI. This correlation reflects the relationships among seagrass biomass, sediment depth, and sulfate reduction within Florida Bay. Deeper sediments enable growth of denser *T. testudinum* beds and larger amounts of belowground biomass (Zieman et al. 1989, Fourqurean et al. 2002). The additional plant material results in more organic matter in sediments, which supports higher levels of sulfate reduction (Holmer & Nielsen 1997). Unrecorded factors that varied among basins may have also contributed to changes in SSI. Including basin identity in the GAM selection increased predictive power by 40% (Table S2), and although basin identity covaried with *T. testudinum* cover, the large increase in explained variance indicates there are other factors encompassed by basin identity. Hydrology, nutrient and organic matter input, and hurricane impacts all vary among basins and could potentially influence sulfide intrusion.

Of specific interest is the difference in SSI between the western basins RAN and JON. They are similar in environmental characteristics (Fig. 8) and macrophyte assemblage, and were both subjected to widespread die-off in 2015 (and 1987–89). Yet, *T. testudinum* leaves in JON had considerably higher SSI values than those in RAN. Although *T. testudinum* in JON exhibited a year of post 2015 die-off recovery from 2016 to 2017, there have been 2 years (2018 and 2019) of consecutive declines in *T. testudinum* cover following the passage of Hurricane Irma near western Florida Bay in the fall of 2017 (Peñalver et al. 2020). In contrast, *T. testudinum* cover has steadily increased since 2015 in the more central RAN. Although Hurricane Irma did not result in immediate loss of seagrass in JON (Wilson et al. 2020), hurricane impacts to Florida Bay are linked to multi-year states of elevated turbidity and chlorophyll *a* concentrations (Cole et al. 2018). If SSI is a good indicator of sulfide stress, then it is possible that the surviving, but declining, seagrass patches sampled in JON in 2019 were still too sparse to facilitate high levels of sulfate reduction and H₂S intrusion and the ongoing mortality may be the result of secondary effects (light limitation and physical disturbance) rather than sulfide-induced die-off. This is supported by recent reports of persistently high turbidities, chronic phytoplankton blooms, and increasing *H. wrightii* cover in this basin (M. S. Koch, M. O. Hall, B. T. Furman pers. comm.).

Sites within the region of the 2015 die-off did not have lower SSI than geographically similar non-die-off sites (Fig. 6). This initially suggests that SSI may not be useful in predicting susceptibility to die-off. However, the sampled die-off sites also had relatively sparse *T. testudinum* cover, the most reliable predictor of SSI in our study (Table 3, Fig. 7; Fig. S4), and denser *H. wrightii*, an early successional seagrass in Florida Bay. *H. wrightii* rapidly increases in cover following die off of *T. testudinum* and is an indicator of recovering communities in Florida Bay (Thayer et al. 1994, Durako et al. 2002). If the 2015 die-off sites were in the midst of active recovery or at an early successional stage when we sampled, they may have been less vulnerable to sulfide-induced die-off than nearby sites in TWN, RKB, and WHP, as they did not have the dense *T. testudinum* required to facilitate high sediment H₂S concentrations.

4.3. Predictors of SSI

Negative correlations between SSI and *T. testudinum* cover were detected by the univariate GAMs, multivariate GAM selection, and PCA (Table 3, Figs. 7–9). This relationship is consistent with previous studies linking dense seagrass cover, sulfate reduction, and sulfide intrusion (Holmer & Hasler-Sheetal 2014) and supports the efficacy of SSI as an indicator of sulfide stress. Sediment depth had a significant negative correlation with SSI in the univariate GAM and was among the best predictors in multivariate GAM selection, but did not appear to correlate strongly with SSI in the PCA ordination. Although sediment depth limits seagrass growth in Florida Bay, and correlates with phosphorus availability and sulfate reduction (Fourqurean et al. 2002), these relationships seem to plateau once sediments become deep enough (about 50 cm; Fig. S4) to support dense *T. testudinum*. Additionally, in our analyses, JON, the basin with the deepest sediments, had anomalously high SSI values and relatively low *T. testudinum* cover, which weakened the bay-scale sediment depth–SSI relationship.

The relationship between Shannon-Wiener diversity and SSI remains unclear. It is possible that diverse community assemblages either result from, or contribute to, lower sulfide stress conditions in *T. testudinum*. However, *T. testudinum* cover dominated the diversity index calculations and was the strongest predictor of SSI. The second largest contributor to diversity, macroalgae cover, had no univariate correlation with SSI (Fig. S4) and only weak

evidence of correlation in multivariate GAMs (Table 3), indicating that the correlation may be caused by covariance with *T. testudinum* cover and sediment depth. The likely explanation is physiographic, as many macroalgae species in Florida Bay are limited to locations with exposed hardbottom or very shallow sediments (Landry 2005) that are not deep enough to support dense *T. testudinum* and high rates of sulfate reduction. Thus, in areas with more diverse algal-dominated communities there is relatively little sulfide intrusion into *T. testudinum* tissues.

Water depth decreases as sediment depth increases in Florida Bay, and sediments can approach the surface along mud banks because the bay is a largely flat karst platform with variable sediment accumulation (Zieman et al. 1989). This relationship may be the cause of the trend in the GAM of SSI as a function of water depth (Fig. S4). A flattened curve at shallow water depths where sediments are deep enough to support dense seagrass and a steeper, positive-slope curve begins as the water deepens and diminishing sediment depths begin to limit seagrass growth and sulfate reduction. There was no relationship between SSI and salinity in our samples. Salinity is one of the primary stressors that contributes to die-off (Koch et al. 2007a, Hall et al. 2016, Johnson et al. 2018, 2020). However, we expected a lack of a significant relationship, as salinities were moderate and did not approach levels (>65) that facilitate sulfide intrusion (Johnson et al. 2020), during our sampling (Table 2) nor during the 2 mo prior to collection in May (Fig. S6). Although dissolved oxygen, pH, and temperature were recorded during FHAP and are known stress factors (Koch et al. 2007a, Holmer & Hasler-Sheetal 2014), they were excluded from analyses due to large diurnal variation. NDBC time series data and our daily FHAP data did not indicate conditions outside of typical spring ranges (Fig. S7).

The HCPC results indicated that there are 3 types of macrophyte communities within Florida Bay based on SSI: climax communities with low *T. testudinum* cover (Cluster A), climax communities with high *T. testudinum* cover (Cluster B), and early successional/recovering communities (Cluster C). Cluster A was composed of basin centers and eastern sites with shallow sediments incapable of supporting dense seagrass growth and contains almost no sites with histories of die-off. This cluster likely represents sites that have reached a steady-state climax community without dense enough seagrass cover or the required environmental conditions to facilitate sufficient sul-

fate reduction and subsequent sulfide intrusion to cause die-offs. These sites may be the least vulnerable to sulfide-induced die-off. Cluster B was composed of basin margins and western sites, contained sites in basins that had localized mortality in 2015 (WHP, RKB), and had some sites along the eastern edges of basins (JON, RAN) with widespread die-off. These sites have deep sediments, dense *T. testudinum* cover, and low SSI values, representing climax communities dominated by *T. testudinum* with sulfide-rich sediments and greater sulfide intrusion. Given the appropriate environmental conditions of high water temperatures and high salinities, these sites may be the most vulnerable to sulfide-induced die-off in the immediate future. In the near future, changes in SSI values of Cluster B sites will likely be the most informative for management decisions regarding freshwater flow into Florida Bay with the goal of mitigating hypersaline conditions to prevent sulfide-induced die-off. Cluster C contained sites capable of supporting dense *T. testudinum* (deep sediment, basin margins), but mainly had western sites with low *T. testudinum* cover, high *H. wrightii* cover, high diversity, and high SSI values. This cluster mostly contains sites within the region of widespread 2015 die-off. The species composition of these sites and high SSI suggests that this cluster represents early successional and/or recovering die-off sites. Altered bathymetry, salinity, and nutrient regimes from Hurricane Irma in 2017 may have also facilitated growth of *H. wrightii* and other macrophyte species at these sites. Water exchange among these basins could have also been increased by 'blow-outs' of sediment banks separating basins caused by the strong winds and wave action of Irma resulting in hydrological conditions more conducive to diverse macrophyte assemblages (Loria 2019). Despite past vulnerability to die-off, sites in this cluster may be less susceptible to sulfide-induced die-off than cluster B in the immediate future, as they have yet to reach climax communities of *T. testudinum* dense enough to facilitate high levels of sulfate reduction.

4.4. Efficacy of the SSI

Overall, SSI values in our samples correlated and clustered with variables expected to increase sulfide stress (Koch et al. 2007b, Holmer & Hasler-Sheetal 2014), indicating that even spring-time SSI values may be reliable indicators of chronic sulfide intrusion in *T. testudinum*. Chronic sulfide stress is suspected

to be a carbon drain for seagrasses (Erskine & Koch 2000), and high concentrations of sediment sulfide are strongly linked to reductions in growth in multiple seagrass species (Holmer & Hasler-Sheetal 2014). However, healthy *T. testudinum* grows uninhibited in 10 mM sediment sulfide concentrations under typical water quality conditions and only exhibits die-off when high sulfide concentrations are coupled with high temperatures and/or high salinities, which typically occur in late summer/early fall (Koch et al. 2007b, Johnson et al. 2020). Conversely, *T. testudinum* also does not exhibit mortality when exposed to thermal and osmotic stress without H₂S present (Koch & Erskine 2001, Koch et al. 2007a). Kilminster et al. (2014) also found that the ranking of site SSI values for *Halophila ovalis* did not change across seasons. Thus, we expect that *T. testudinum* plants in Florida Bay with evidence of high sulfide intrusion (low SSI) in the spring and winter months will be vulnerable to possible die-off when exposed to more environmentally stressful conditions during the summer. This indicates that chronic sulfide intrusion quantified using SSI is a potentially valuable ecoindicator for assessing the susceptibility of *T. testudinum* to die-off, even when used within the landscape-scale FHAP sampling framework.

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