LIMNOLOGY and OCEANOGRAPHY

Variation in sediment and seagrass characteristics reflect multiple stressors along a nitrogen-enrichment gradient in a New England lagoon

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Abstract

We examined concentrations of organic carbon, dissolved sulfides, total sediment sulfur, and stable sulfur isotope ratios in seagrass leaf tissues across a nitrogen-enrichment gradient in a coastal marine ecosystem (Cape Cod, Massachusetts) in 2007–2010 and 2017–2019. We also measured seagrass aboveground and belowground biomass, epibiota biomass, and leaf chlorophyll content. Seagrasses were present at all sites in the former period but were lost at our most nitrogen-impacted site (Snug Harbor) by 2011. In 2007–2010, sediment organic carbon and dissolved sulfides were highest in Snug Harbor and decreased along the gradient; leaf tissues depleted in ^{34}S also indicated higher sulfide intrusion into seagrass tissues in more eutrophic areas. By 2017–2019, sediment organic carbon and pore-water soluble sulfides had decreased in Snug Harbor, but had increased at the intermediate site, to levels found at the most impacted site prior to the seagrass die-off. Again, leaf tissue ^{34}S depletion reflected this pattern, indicating seagrasses were exposed to the highest sulfides at the intermediate site. The decreases in sediment organic carbon and soluble sulfides in Snug Harbor years after the loss of the seagrasses illustrate a feedback between high organic matter in seagrass beds and increasing stressors like elevated soluble sulfides in nutrient-enriched systems. We found significant relationships between sediment conditions and seagrass responses, including greater aboveground to belowground biomass ratios, epibiota biomass, and ^{34}S-depleted leaves at sites with high pore-water sulfide and highly organic sediments. Our research suggests that the reduction of anthropogenic nitrogen entering the harbor is necessary for improving sediment quality and preventing seagrass mortality.

Eutrophication of estuaries often leads to greater levels of sediment organic matter accumulation (Nixon 1995) and pore-water sulfide (Morgan et al. 2012), loss of seagrass biomass and percent cover (Nixon et al. 2001), and reduced light intensity reaching the benthos (Jerlov 1976). Hydrogen sulfide, which can be present at high levels in anoxic sediments, is toxic to eukaryotic organisms (Cooper and Brown 2008), including seagrasses (Lamers et al. 2013). Seagrasses have evolved mechanisms to persist in high-sulfide, anoxic sediments due in part to passive diffusion of oxygen (O_2) from their roots as well as internal sulfide detoxification using photosynthetically derived O_2 (Hasler-

Sheetal and Holmer 2015, Holmer 2019). Despite this, sulfide stress may still occur in environments where seagrasses are subject to high nutrient loads and low light levels (Goodman et al. 1995). In N-enriched systems, epibiota are abundant and reduce light reaching plant tissue (Nelson 2017). Reduced photosynthetic output under light-limited conditions lowers the amount of oxygen produced by the plant, interfering with internal sulfide detoxification pathways (Brodersen et al. 2015; Hasler-Sheetal and Holmer 2015). Seagrasses suffering from light stress produce more leaf chlorophyll (Dennison and Alberte 1986). Eutrophic conditions also influence ratios of aboveground to belowground biomass in seagrass, which has been used as a predictor of survivability with higher values associated with poor survival outlook (Nixon et al. 2001).

In highly nutrient-enriched estuaries, sediment organic carbon may be 3- to 4-fold higher than in a mesotrophic or oligotrophic system (Nixon 1995; Dell'Anno et al. 2002). In seagrass-dominated estuaries, sediment organic carbon concentrations

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are higher in beds when compared to unvegetated areas (Fonesca et al., 1982; Gacia et al. 2002). Increased organic matter drives benthic microbial respiration, particularly sulfate reduction, which can comprise as much as 70–90% of the microbial respiration in the sediments of productive estuaries (Howarth 1984). High levels of sulfate reduction can deplete the pore-water sulfate pool relative to conservative ions in seawater, such as chloride (Stumm and Morgan 1981). Sulfide may accumulate in pore waters (Canfield 1989) and can diffuse into seagrass tissues where radial root oxygen leakage is suppressed (Brodersen et al. 2015).

The δ^{34}S ratio, which measures the ratio of two stable sulfur isotopes, ^{34}S and ^{32}S, in a substance relative to a standard (Vienna-Canyon Diablo Troilite) in seagrass tissues can be an indirect measure of the level of sulfide the plants are exposed to, with *Zostera marina* shoots in high sulfide conditions demonstrating isotopically lighter δ^{34}S values than plants in low sulfide conditions (Fraser and Kendrick 2017). Seawater sulfate has an average δ^{34}S of +21‰ (Rees et al. 1978; Böttcher et al. 2007), while pore-water sulfide δ^{34}S values in sediments underlying *Z. marina* meadows range as low as −22 to −30‰ (Frederiksen et al. 2006). Pore-water sulfide has been found to be the source of as much as 68% of the sulfur found in leaf tissues growing in highly organic sediments (Holmer and Hasler-Sheetal 2014).

Healthy seagrass meadows tend to occur in sediments where organic matter comprises less than 6% of sediment dry weight (DW), possibly due to increased sulfate reduction in more highly organic sediments (Hemminga and Duarte 2000). In addition to sediments influencing seagrass, seagrass influences sediments as well: seagrass canopy presence increases the height of the diffusive boundary layer above the sediment–water interface and slows the diffusion of oxygen from the water column to the sediment (Koch et al. 2007). In addition, seagrasses trap organic matter by increasing drag on and reducing the buoyancy of particles entering the seagrass meadow (Van Katwijk et al. 2010), and by adding organic matter directly to sediments via root exudation and production of both seagrass and associated epibiota biomass (Kennedy et al. 2010). Seagrass particle trapping is beneficial in nutrient-scarce, oligotrophic conditions, but may be detrimental in more nutrient-enriched basins where water column organic matter is high. We hypothesize that under such conditions, trapping of fine particles, in addition to primary production by seagrass and associated epibiota, leads to highly organic sediments and high levels of pore-water soluble sulfide, which then intrudes into seagrass tissues, and impacts meadow success. We investigated this process along a gradient of N-enrichment in West Falmouth Harbor (WFH), a shallow estuary in Massachusetts, USA.

Methods
Study site

West Falmouth Harbor is a shallow lagoon (average depth at mean high tide of 1.9 m), adjoining Buzzards Bay on Cape Cod, Falmouth, Massachusetts, USA (Fig. 1). For a detailed description of the WFH system, *see* Hayn et al. (2014) and Howarth et al. (2014). WFH has received elevated N inputs for the past two decades from an aquifer contaminated by a wastewater treatment plant upgradient of the lagoon. The N from this contaminated aquifer enters largely through groundwater in the Snug Harbor portion of WFH, as shown in Fig. 1 (Howarth et al. 2014). WFH receives an N load of approximately 4.2 mmol N m^{-2} d^{-1} from this and other watershed and atmospheric sources (Hayn et al. 2014). We have collected water column nutrient, sediment, and seagrass data at multiple sites in WFH annually since 2005. Information on additional site factors such as water depth and seagrass density can be found in Supporting Information Table S2.

Between 2005 and 2010, subtidal seagrass meadows consisting of monospecific *Z. marina* were present in three sub-basins within WFH (which we denote Snug Harbor, Middle Harbor, and Outer Harbor). These three basins exist along an N-enrichment gradient, since two-thirds of the total N load to WFH enter through Snug Harbor due to input from the contaminated aquifer. Consequently, while the average N load across the entire harbor is estimated as 4.2 mmol N m^{-2} d^{-1}, the load to Snug Harbor is 17 mmol N m^{-2} d^{-1} (Howarth et al. 2014). WFH exchanges water with Buzzards Bay through a single outlet on the western edge of the Outer Harbor, which makes the Outer Harbor better flushed than the harbor as a whole. Because so much of the total N load enters through Snug Harbor and because the Outer Harbor is better flushed with coastal water that has lower N concentrations, we believe that N availabilities are greatest in Snug Harbor and lowest in the Outer Harbor, with the Middle Harbor being intermediate. Concentrations of dissolved inorganic N during the growing season are low across all of WFH, probably due to rapid biological uptake, but are on average lowest in the Outer Harbor, intermediate in the Middle Harbor, and significantly higher on average in Snug Harbor (Hayn et al. in prep.). Total N concentrations, which are composed largely of organic N, are more than an order of magnitude greater than dissolved inorganic N throughout WFH and are much higher in Snug Harbor than in the Outer Harbor (Jakuba et al. 2021; Hayn et al. in prep.).

In 2010, seagrass meadow covered 20% of the sediments in Snug Harbor at mean water, before succumbing to a major mortality event in July 2010 (Hayn 2012; Howarth et al. 2014). Very low density beds persisted in this area in the spring of 2011 but were gone by the end of July 2011 and have not reestablished. As of June 2018, 0% of Snug Harbor, 60% of the Outer Harbor, and 68% of Middle Harbor sediment area at mean water were colonized by subtidal *Z. marina*, with northward expansion of meadow area in the Middle Harbor basin occurring between 2010 and 2017. Occasional *Ruppia maritima* can be found in isolated patches in parts of the Middle Harbor as well as in the low salinity southern end of the embayment, outside of our study area. All vegetated sampling
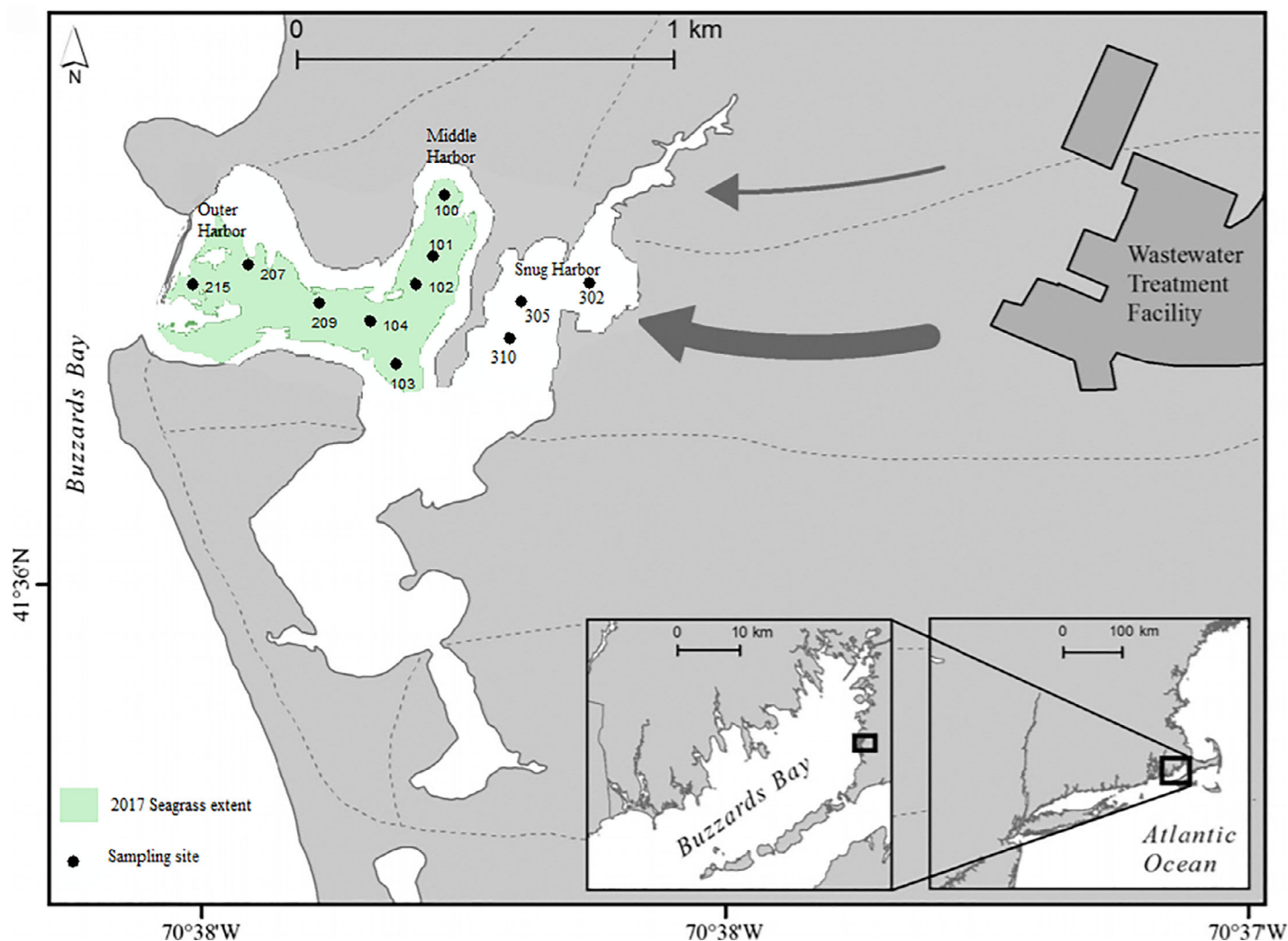
Fig. 1. The 2017–2019 sediment sampling sites and seagrass extent in West Falmouth Harbor, with the flowpath and relative magnitude for the dominant groundwater nitrate inputs to West Falmouth Harbor indicated by arrows. Figure adapted from Howarth et al. (2014). Seagrass extent shown in green, with sediment sampling sites denoted by black dots.

sites are between 2.7 and 3.1 m depth at mean higher high water.

Sediment analyses

In 2018 and 2019, we extracted 6.4 cm diameter sediment cores using acrylic tubes to ~ 12 cm sediment depth at three sites in Snug Harbor, five sites in the Middle Harbor, and three sites in the Outer Harbor ($n = 11$, Fig. 1). Many of these sites have been assessed annually since 2005 for seagrass aboveground biomass and seagrass tissue isotopic composition (see *Seagrass analyses* section). Within 2 h of core extraction, we sliced cores into 2-cm sections and put samples in a drying oven at 100°C. Sections remained in the oven for 48+ h; we then ground the samples using a mortar and pestle and measured organic matter in each core section by loss on ignition (LOI). A subset of each core section was rinsed to remove

soluble forms of S such as residual seawater sulfate, and was then measured for total solid-phase S using an Elementar Vario CNS analyzer. We sampled a subset of these samples for organic C using the CNS analyzer as well, and derived an equation describing the relationship between LOI and C in WFH, where organic matter is made up of 42% organic carbon (Supporting Information Fig. S1, $p < 0.0001$, $R^2 = 0.97$). The relationship developed through this analysis matched the relationship derived earlier in the project (2007) for samples taken across the harbor. All organic carbon values presented here are derived from LOI measurements using this equation.

Pore-water analyses

To measure pore-water concentrations of total soluble sulfide, sulfate, and chloride, we deployed four pore-water "peepers" (Teasdale et al. 1995) each in July 2018, July 2019,

and early August 2019 at vegetated locations at least 20 m from the nearest bed edge, except in Snug Harbor where seagrass is absent. Pore-water peepers were also deployed in WFH in 2007 in a vegetated portion of Snug Harbor, prior to the loss of seagrass there. The peepers, ~3 6 cm in length, are PVC wedges each with 14 wells (each well is spaced 1.5 cm apart for the first 12 cm, and 2 cm thereafter) covered with a 0.2-μm pore size semi-permeable amphoteric, nylon membrane (Supporting Information Fig. S2). We filled each well with 18.2 MΩ ultrapure distilled, deoxygenated water in a 100% N_2-atmosphere glove bag and deployed the peepers below the sediment surface for 2 wk. At the time of deployment, divers noted which wells were above the sediment–water interval, to properly estimate depth. Peepers stayed in anoxic conditions before and during deployment, as well as during pore-water sample extraction in the lab, described below. Peepers were collected by divers using double zip-top bags flushed and filled with N_2 gas to prevent oxygenation of the pore waters.

To assay total soluble sulfides, we used the colorimetric methylene blue method of Gilboa-Garber (1971) as adapted by Howarth et al. (1983). We allowed color to develop in the dark for 2 h before spectrophotometric analysis at an absorbance of 670 nm. Sulfate and chloride were measured using a Dionex ion chromatograph after a dilution of 1 : 200 in ultrapure distilled water.

Seagrass analyses

From 2005 to 2009 as part of an NSF Biocomplexity project, our research group, along with our colleagues at the Marine Biological Laboratory and the University of Virginia collected grasses in WFH every year during mid-summer, and we continued this work from 2010 to 2019. Plant morphometry data (biomass and leaf area) were collected from 24 stations, including 10 stations in Snug Harbor prior to the 2010–2011 die-off. Total shoot density was assessed by divers using a 0.25-m^2 quadrat, counting all live shoots present at a subset of these stations. We also analyzed seagrass leaf tissue δ^{34}S annually at 17 of these sites (7 from the Outer Harbor, 4 from the Middle Harbor, and 6 from Snug Harbor). For this analysis, we carefully scraped epibiota off the youngest blade (denoted blade 1), and second- and third-youngest blades (denoted blade 2 + 3) from 10 representative plants, pooled the blades, and dried the leaf tissues at 60°C for 48+ h. We chose the youngest blade so that we could assess the source of the sulfur incorporated in the most recently grown tissue, and the second- and third-youngest blades to assess older, fully grown and not yet senesced plant tissue as an integrative measure of the sulfur environment it was in while growing. We then ground the tissue in a mortar and pestle containing liquid nitrogen and redried immediately before δ^{34}S tissue analysis at the Marine Biological Laboratory Stable Isotope Lab at the Ecosystems Center. Analytical accuracy of isotopic results showed a

standard deviation of ≤ 1.2‰ both within and across analysis runs.

In 2018–2019, we selected eight sites for enhanced seagrass sampling due to their distribution along a gradient that spanned the nitrogen-enriched Middle Harbor region to the better flushed, less nutrient-impacted Outer Harbor (Fig. 1). In addition to morphometry, isotopic analysis, and shoot density, from 2018 onwards, we added quantification of aboveground to belowground biomass ratios, chlorophyll content, and epibiota biomass as described below.

To measure aboveground and belowground biomass, at each site, we carefully harvested five terminal shoots with their full rhizome attached. For each plant, we then measured 7 cm of horizontal rhizome, and all live aboveground biomass attached to that portion of rhizome was processed (Short et al. 2006). Because these terminal shoots are larger than average, we report our measurements in biomass per shoot, rather than biomass per m^2 (*see* "Results" for further discussion). For context, areal biomass measured at 15 sites annually across the Middle and Outer Harbors from 2015 to 2019 ranged from 63 to 374 g m^{-2} with a mean of 167 g m^{-2}. Plants with large portions of aboveground biomass missing due to leaf breakage during sampling were excluded from analysis, except in cases where this was characteristic of nearly all plants at a site, which was the case in the northern Middle Harbor (sites 100 and 101). This observed breakage could be due to softening of leaf tissue as a result of sulfide toxicity, boat propellers, or herbivory by geese. For these sites, we included only plants with the least amount of breakage possible. We separated aboveground from belowground biomass and then scraped blades and meristems to remove epibiota and carefully cleaned roots and rhizomes in a distilled water bath. We then measured the DWs after drying at 60°C for 72 h.

In 2019, we collected epibiota from the blades by carefully bagging 7–8 additional individual plants in the field at each site. We sealed bags underwater around each plant, and clipped the plants at the sediment–water interface. In the lab, we filtered the water within each bag on preweighed Whatman glass microfiber (GF/C) filters to collect the weight of any epibionts, which had fallen off the blades during transport. We then carefully scraped the epibiota from both sides of intact blades with a razor blade and dry-weighed them. We established an estimate of epibiota biomass per double-sided leaf area. Note that this method, which includes epiphytes sloughed off during field sampling and transport, produced 2- to 3-fold greater measurements of epibiota biomass at some sites than estimates where epibiota biomass was assessed by scraping alone. After drying and weighing epibiota, we ashed samples in a muffle furnace at 450°C for 4 h to determine the organic portion of epibiota biomass.

After removing epibiota, we froze plants at −80°C for 2 months, after which we measured chlorophyll content using the method established by Dennison (1990), assessing

chlorophyll $a + b$ content on the top 10 cm^2 of each blade. We chose to assess the top 10 cm^2 of the blade after assessing different locations along a seagrass blade for chlorophyll content (top, top-middle, bottom-middle, and base) and determining that the blade tip best represented average chlorophyll content along the blade.

Statistical analyses

All statistical analyses were carried out in the R computing environment. We used linear models to examine how rhizosphere sulfide depended on organic carbon concentration, and included basin as a block effect. We log-transformed the response variable to meet model assumptions of normality and homoscedasticity. We used the same methodology to examine relationships between sulfide and leaf tissue sulfur isotopic composition, as well as sulfide and aboveground-belowground biomass ratio.

Relationships were visualized using estimated marginal means (EMMs) using the "emmeans" package for R (Lenth 2020). Differences in variables between basins were analyzed using a combination of Tukey's HSD test (where three basins were compared), Wilcoxon rank-sum test (where two basins were compared and data were non-normally distributed), and t-tests (where two basins were compared and data were normally distributed). For further details, *see* Supporting Information Tables S3–S6.

Results

Sediment and pore-water data

Sediment organic carbon was highest in the Middle Harbor, with measurements averaging 4.5% organic carbon; intermediate in Snug Harbor at 3.6% average organic carbon; and lowest in the Outer Harbor at 2.0% average organic carbon (Fig. 2). The differences in organic carbon between the Middle Harbor than the Outer Harbor ($p < 0.001$), and between Snug Harbor and the Outer Harbor ($p = 0.03$) were significant, while the difference between Snug Harbor and the Middle Harbor was less so ($p = 0.09$). Organic carbon at all sites generally declined with sediment depth.

Total solid-phase sulfur was greater in Snug and Middle Harbor than in the Outer Harbor (Supporting Information Table S1) in 2018. The Outer Harbor had much lower levels of total solid-phase sulfur ($p < 0.001$), with an average value of 0.58% by weight, compared to the other two basins; Snug Harbor and the Middle Harbor did not differ in total solid-phase sulfur, with average values of 0.92% and 0.86% by DW, respectively. Total solid-phase sulfur showed no consistent pattern with depth throughout the length of the cores.

Total soluble sulfides were strikingly higher in the Middle Harbor than the other basins in both 2018 and 2019 ($p < 0.001$), with values in the top 6 cm reaching 2.0–2.7 mM (Fig. 3). At 20 cm depth at site 104 in the Middle Harbor, soluble sulfide values reached the highest value of any of our
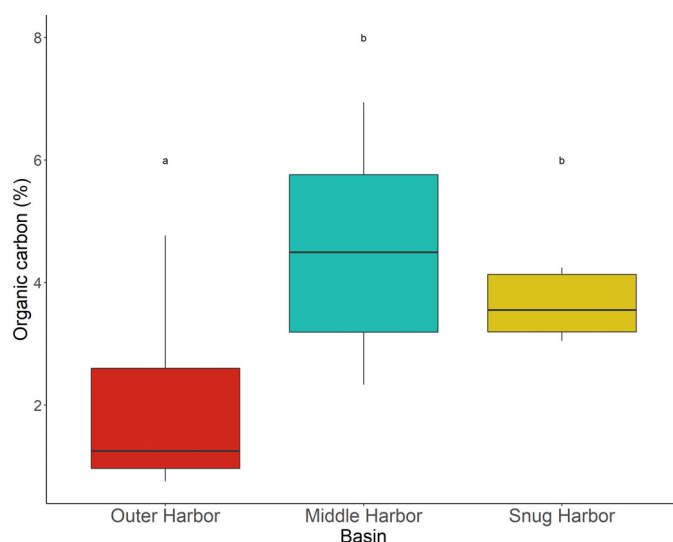

Fig. 2. Organic carbon values from cores collected in 2018 and 2019, averaged for all core sections in the top 12 cm of sediment. Boxes represent median, first and third quartile, with whiskers spanning the rest of the values. Different letters denote significant differences by Tukey's multiple comparison of means test.

measurements at 6.7 mM. Concentrations in the other basins were far lower, 30 μM to 1.5 mM in the Outer Harbor and 200–700 μM in Snug Harbor (Fig. 3). In the sandiest vegetated region of the Outer Harbor (site 207), soluble sulfide concentrations were the lowest we saw in WFH at 30–100 μM sulfide, increasing with sediment depth. Sulfide increased with depth in all pore-water profiles except for site 101 in the Middle Harbor, where it remained uniformly high across depth, reaching its peak at 2.5 mM within the seagrass rooting zone at 4–6 cm sediment depth. Pore waters sampled in Snug Harbor in 2007 prior to the loss of seagrass (using the same methodology as above) averaged 2.3 mM sulfide in the rooting zone (Fig. 4). However, pore-water sulfide values in that region in 2018 and 2019 (8 years after the loss of the seagrass bed there) average an order of magnitude lower, with concentrations comparable to values seen in the sandy, low organic C regions of the Outer Harbor. Similarly, Snug Harbor organic carbon values have decreased over these 8 years, from 4.4% organic carbon at a vegetated site in 2007 to 3.6% at the same site, now without vegetation, in 2018.

The molar ratio of sulfate to chloride ($SO_4^{2-} : Cl^-$) in the pore water decreased with depth at all sites and was lowest in the Middle Harbor (Supporting Information Fig. S3). In surface sediments near the Middle Harbor, $SO_4^{2-} : Cl^-$ was 0.05, virtually identical to the seawater global average $SO_4^{2-} : Cl^-$ ratio of 0.051, and decreased to 0.02 by 14 cm depth. The Middle Harbor had significantly lower $SO_4^{2-} : Cl^-$ at all depths than the Outer Harbor and Snug Harbor (Middle and Outer Harbor, $p = 0.0003$; Middle and Snug Harbor, $p = 0.005$), while Snug Harbor and the Outer Harbor did not differ ($p = 0.89$).
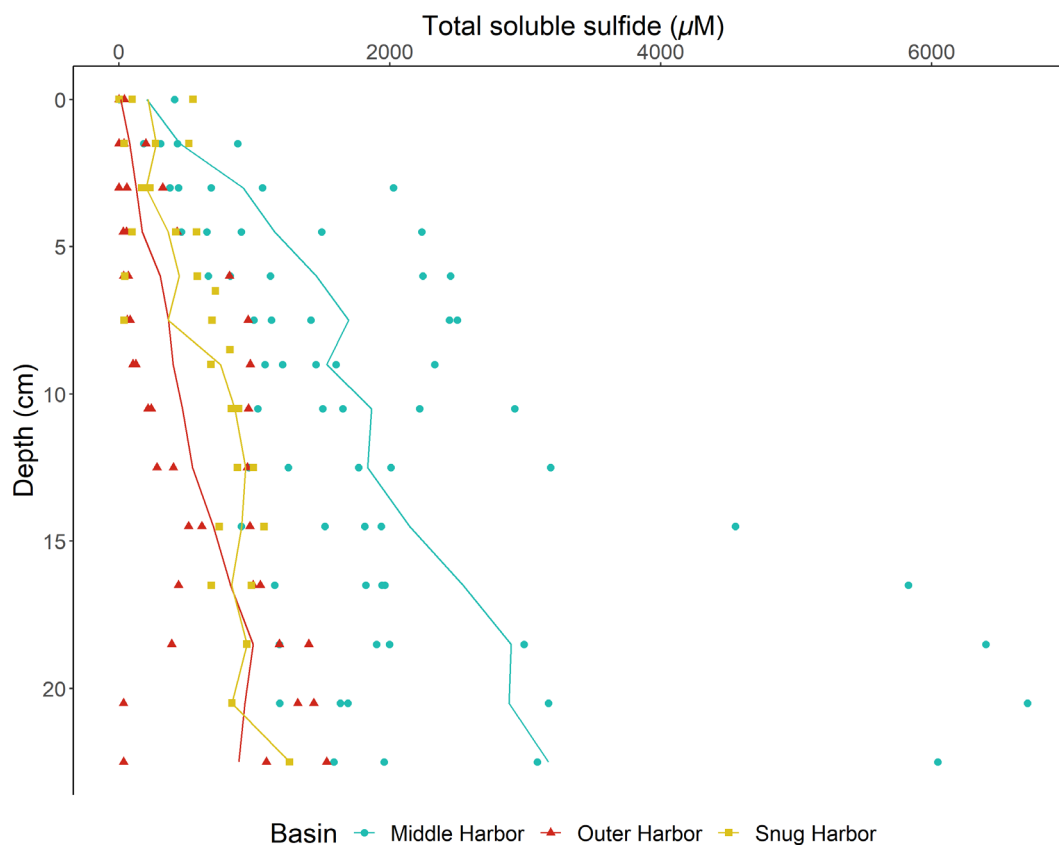
Fig. 3. Total soluble sulfides at all WFH sites plotted with sediment depth, with color and shape denoting basin. All measurements are shown, with each point representing one pore-water measurement at one depth/site. Highest overall values were found in Middle Harbor. Lines represent average value at each depth per basin. Samples collected in July of 2018 and 2019.

Seagrass data

Seagrass aboveground biomass of terminal shoots in the Middle Harbor in 2018 was on average 1.3 ± 0.5 g DW per shoot, while Outer Harbor aboveground biomass was 1.0 ± 0.4 g DW per shoot. Belowground biomass in the Outer Harbor was 0.26 ± 0.06 g DW per shoot, and in the Middle Harbor was 0.28 ± 0.08 g DW per shoot in 2018. The ratio of aboveground to belowground biomass was higher in the Middle Harbor ($p = 0.03$). In July 2019, biomass values were lower at all sites for both categories, with aboveground biomass in the Middle Harbor averaging 1.0 ± 0.3 g DW per shoot and 0.7 ± 0.2 g DW per shoot in the Outer Harbor. Belowground biomass in both the Middle and Outer Harbor averaged 0.21 g DW per shoot. Aboveground to belowground biomass ratio remained higher in the Middle Harbor in 2019. ($p = 0.03$).

Total epibiota biomass in the Middle Harbor in July 2019 averaged 1.76 ± 0.63 mg epibiota per cm^2 leaf area. In the Outer Harbor, epiphyte biomass averaged 1.52 ± 1.38 mg epibiota per cm^2 leaf area. The organic portion of epibiota biomass in the Middle Harbor averaged 0.55 ± 0.27 mg epibiota per cm^2 leaf area. In the Outer Harbor, organic epibiota biomass averaged 0.52 ± 0.24 mg epibiota per cm^2 leaf area.

Neither organic epibiota biomass or total epibiota biomass differed across the two basins ($p = 0.76$, $p = 0.66$).

Plant average chlorophyll content did not significantly differ on a basin level across both years (2018: $p = 0.45$, 2019: $p = 0.18$), and in 2018 averaged 14.2 μg chlorophyll per cm^2 leaf area in the Middle Harbor and 13.2 μg/cm^2 in the Outer Harbor. Across both basins, chlorophyll content corresponded closely to blade age, with the second-youngest blade showing consistently high values.

We found several patterns in leaf tissue $\delta^{34}S$ in blades 2 and 3 between 2005 and 2019 in all three basins of WFH (Fig. 5). The record is continuous for the Outer Harbor, while we have data from Snug Harbor from 2005 until the mortality event in 2010/2011, and we only began collecting annual data from the Middle Harbor after this event, in 2013, with one additional collection in 2005. Plants from the earlier period in Snug Harbor show $\delta^{34}S$ values very similar to plants in the Middle Harbor (both with means of $+ 0.3$‰), and both these sites show an overall depletion of the heavier S isotope compared with plants from the Outer Harbor (mean of $+7.6$‰ over the entire record). The Outer Harbor has shown signs of the beginning of a decline in recent years, with lower median $\delta^{34}S$ values in 2017–2019 than any previous year since 2005,
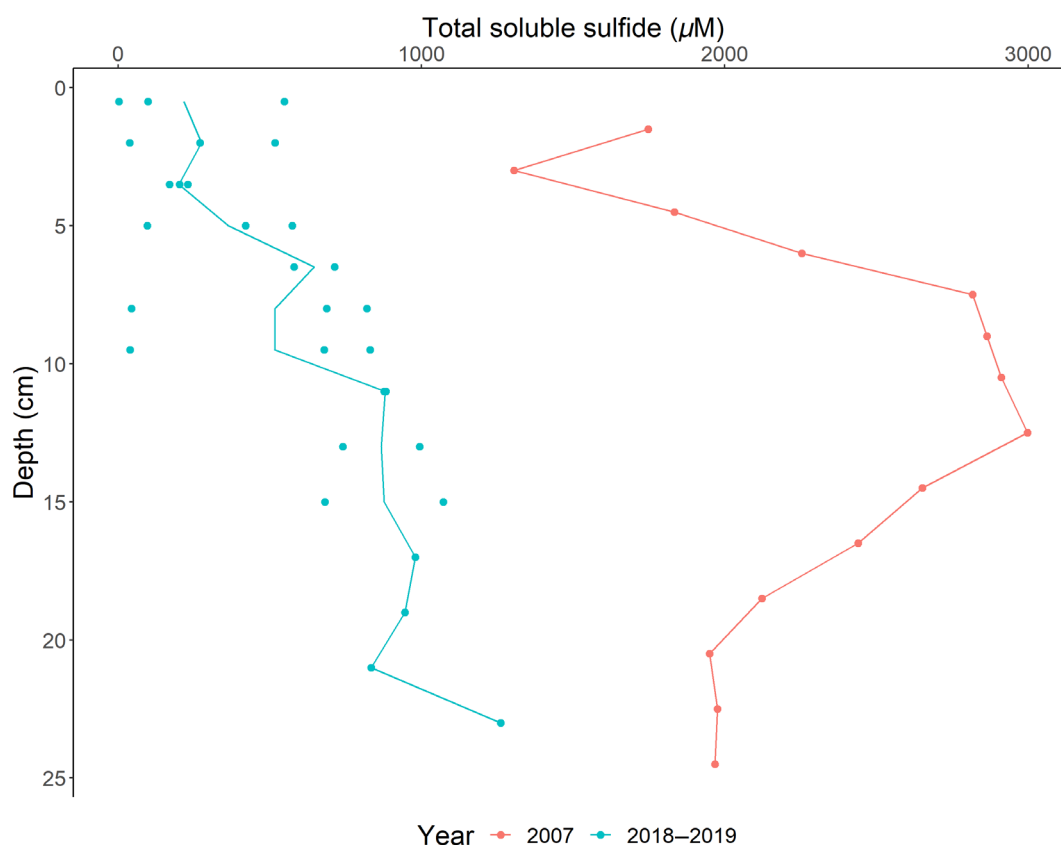
Fig. 4. Total soluble sulfide depth profiles in Snug Harbor over time at one site (305); 2007 values averaged 2300 μM, while 2018–2019 values averaged only ~ 700 μM sulfide. In 2007, this site was vegetated, and as of mid-2011, all vegetation had died-off and has not reestablished. Points represent individual pore-water measurements. Note that 2007 represents only one peeper deployment, while 2018–2019 represent three peeper deployments in July and early August at one site across the 2-yr period.

and a much lower mean for this period (+2.8‰ for 2017–2019, compared with +9.1‰ for 2005–2016). We also observed a pattern in $\delta^{34}S$ values with blade age. At each sampling site in both the Outer and Middle Harbors from 2013 to 2019, the younger blade (blade 1) had a more depleted $\delta^{34}S$ signal than the older blades (Fig. S4, $p < 0.001$).

Relationships between variables

Across all sediment depth classes and sites, rooting zone organic carbon and pore-water sulfide had a strong positive relationship, where the log of sulfide concentration increases with organic carbon content (Fig. 6). Depths group in different locations along the trend line, with samples taken at 3 cm depth having a lower intercept than samples taken at 9 cm depth, likely indicating diffusion of water column oxygen limiting sulfide accumulation in shallower sediments as well as higher sulfate reduction rates at depth, as shown by the decreased $SO_4^{2-} : Cl^-$ ratios we found at increased depths. The relationship across all depths was positive ($p < 0.001$), indicating increased sulfide production at sites with greater levels of organic carbon, with sites in the Middle Harbor having the greatest levels of carbon and sulfide. In addition, we saw

depleted $SO_4^{2-} : Cl^-$ ratios in the Middle Harbor basin relative to seawater average values, indicating drawdown of sulfate relative to the conservative ion Cl^-, and signifying increased biological sulfate reduction (or less reoxidation of sulfide) in the Middle Harbor compared to the other basins.

We found a positive relationship between pore-water soluble sulfides and seagrass leaf tissue $\delta^{34}S$ in 2018 and 2019, shown in Fig. 7 ($p = 0.006$). Plants that incorporate mostly seawater sulfate (+21‰) into their tissues as they grow have a heavier $\delta^{34}S$ signal, while those that incorporate more pore-water sulfide are more depleted in the heavier isotope, due to fractionation favoring the lighter S isotope during the sulfate reduction process in the sediments. Thus a more depleted plant tissue $\delta^{34}S$ indicates greater sulfide intrusion (Holmer and Hasler-Sheetal 2014; Fraser and Kendrick 2017). The high sulfide values seen in Snug Harbor sediments in 2007 coincided with very ^{34}S-depleted sulfide tissues in the seagrass there prior to their die off, with some samples measuring as low as −10‰. Seagrass growing in the high-sulfide Middle Harbor sediments showed more negative $\delta^{34}S$ than those growing in the Outer Harbor. In addition, older blades, which grew and incorporated sulfur into their leaf tissues from earlier
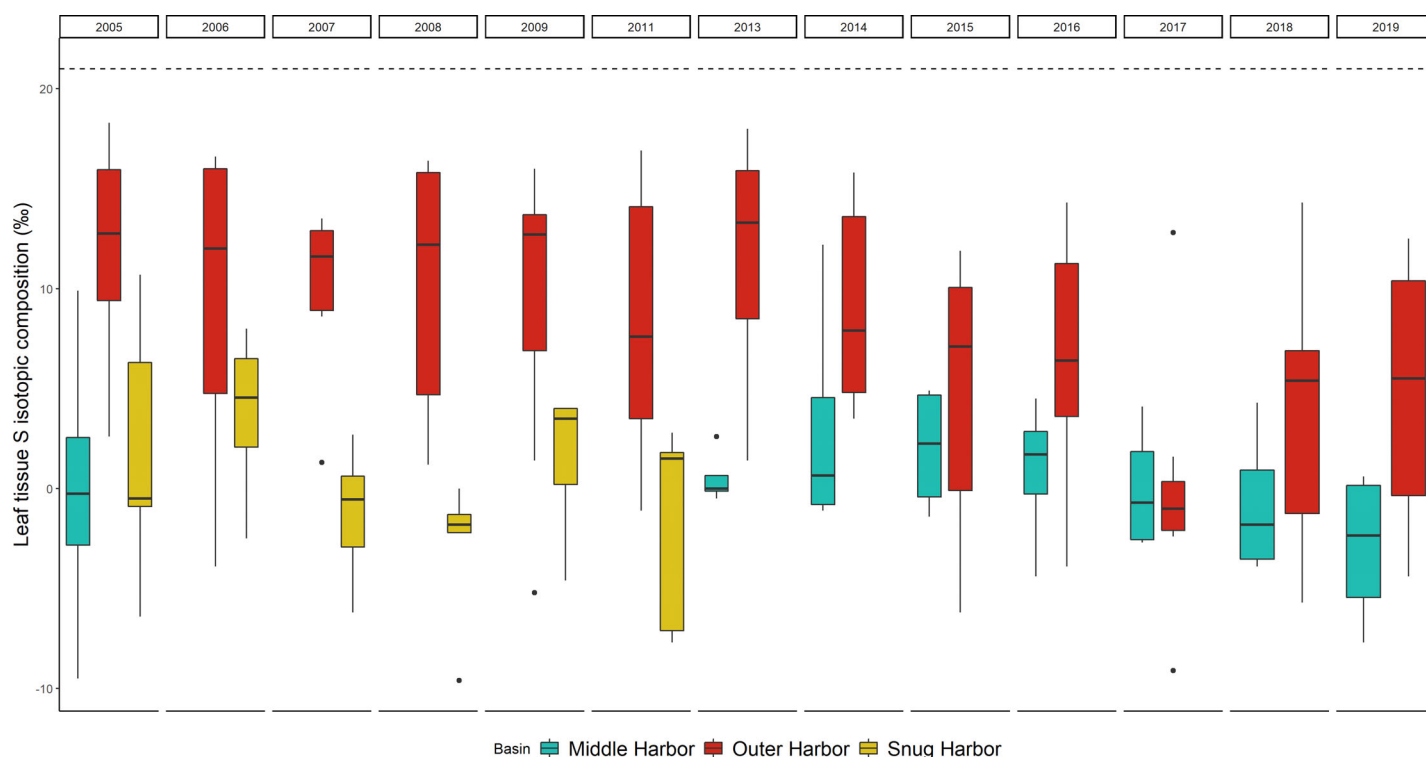
Fig. 5. Seagrass leaf tissue δ^{34}S isotope data for the second- and third-youngest blades of plants collected from multiple sites within each basin. Note that the *y*-axis spans −10 to +25‰. The dashed gray line at +21‰ represents average seawater δ^{34}S.

in the season (Kilminster et al. 2014), were less depleted in ^{34}S than younger blades which were sampled at the same time but began growing later in the season. This could either reflect a reduction in the δ^{34}S of the sediment sulfide pool over time, or a higher contribution of sulfides to the tissue sulfur content in younger blades compared with older blades of the same plants.

Discussion

Sediment organic carbon concentrations in WFH are high compared to values commonly observed for seagrass meadows. A review of over 3500 observations of organic carbon in seagrass meadows around the world by Fourqurean et al. (2012) found an average value of 2.0% sediment organic carbon and a median value of 1.4%. In the Middle Harbor, carbon values average 4.5%, greater than ~ 90% of measurements reviewed by Fourqurean et al. (2012). Snug Harbor organic carbon averages 3.6% in recent years, slightly less than the Middle Harbor. However, prior to the die-off, Snug Harbor's vegetated sediments had a higher average carbon value, averaging 4.4% carbon. Organic carbon in Snug Harbor seagrass beds was comparable to the Middle Harbor, likely due to seagrass particle trapping and high rates of primary production at this site, closer to the N-loading source. The lower carbon levels found in the post-die-off, unvegetated sediments in Snug Harbor compared to the vegetated Middle Harbor

sediments supports the idea that seagrass particle trapping and seagrass community primary productivity in eutrophic conditions can lead to organic-rich sediments. Seagrass organic matter-trapping may benefit plants in nutrient-scarce conditions, or where plants are light-limited and benefit from clearer waters (De Boer 2007). However, in nutrient-enriched conditions, this may become a stress to the plants.

Although dwarfed by Middle Harbor sulfide concentrations at 2.3 mM, soluble sulfides at 10 cm depth even in the Outer Harbor exceed 1.0 mM, well beyond the sulfide concentration of 0.6 mM where Goodman et al. (1995) and later Höffle et al. (2011) saw declines in parameters associated with seagrass health. Dissolved soluble sulfides tend to be low where seagrass beds are found, likely due to rhizosphere oxidation (Brodersen et al. 2015), with a median value of 50 μM total soluble sulfides in seagrass meadows across the globe reported in a review by Terrados et al. (1999). In that review, only 1 site out of 22 had greater than 300 μM soluble sulfide, which contrasts starkly with our average rhizosphere value at Middle Harbor site 101 of 2.3 mM, more than 7 times higher. In addition, sulfide values at site 101 in the Middle Harbor did not increase with depth like they did at other sites, and instead was greatest between 1 and 5 cm, likely resulting in high levels of sulfide stress on the belowground tissues of plants in the Middle Harbor.

A notable result in this study is the similarity between pore-water soluble sulfide concentrations in Snug Harbor in
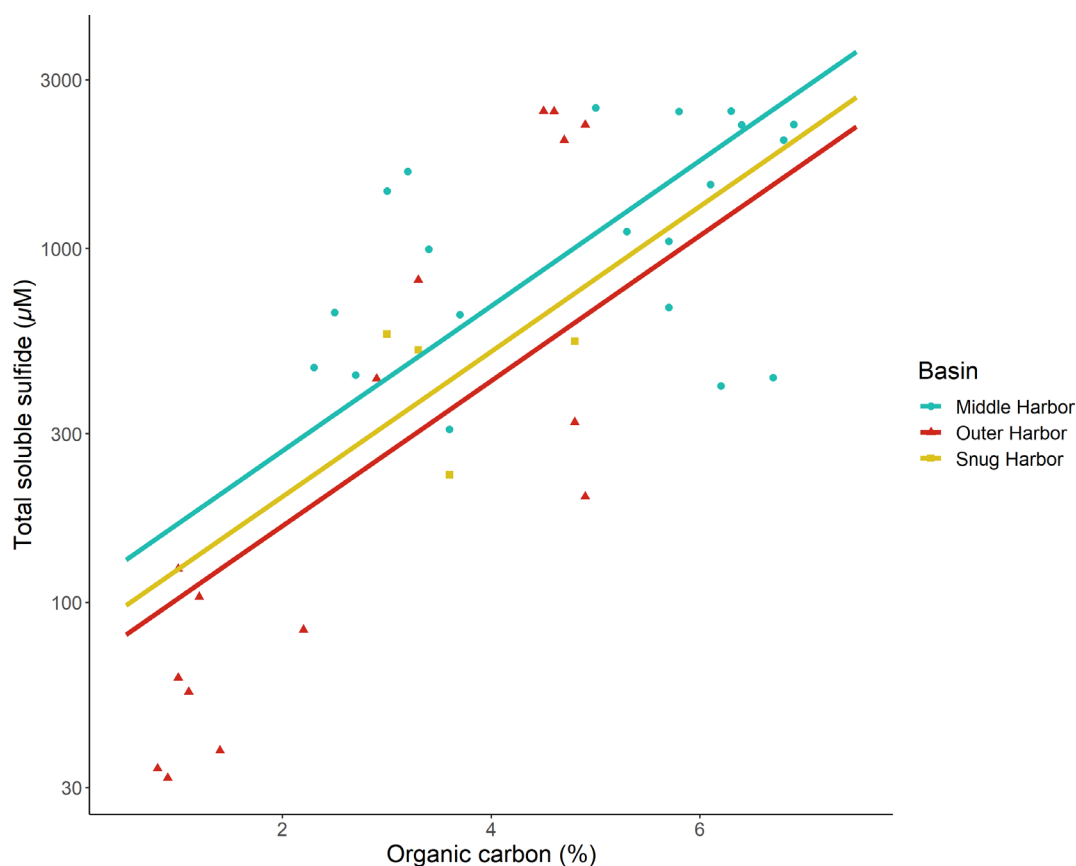
Fig. 6. Organic carbon (%) and total soluble sulfides ($p < 0.001$ across all basins) in the seagrass rhizosphere to 8 cm sediment depth, shown by point shape and color. Points represent one 2 cm core section for organic carbon, and one pore water measurement from the same depth. Solid lines represent modeled relationships for each basin between organic carbon and total soluble sulfides, calculated using EMMs. For further information, see Supporting Information Table S4.

2007 (Fig. 4) and the Middle Harbor in 2018 (Fig. 3). At these sampling times, each basin contained the closest seagrass bed to the enriched groundwater nitrogen source, and sites in the vegetated areas of these basins represent the two highest soluble sulfide concentrations in the rhizosphere (SH-305 in 2007, and MH-101 in 2018), with soluble sulfides in the vegetated area of Snug Harbor in 2007 equivalent to values in the Middle Harbor in 2018 and 2019. Between 2007 and 2018/2019, Snug Harbor concentrations had decreased substantially for both sulfides (from ~ 2.0 to 0.5 mM) and organic carbon (from 4.4% to 3.6%). This decrease over the decade following seagrass loss, without any corresponding decrease in nitrogen load, suggests the importance of the role of seagrass in elevating organic matter concentrations and pore-water sulfides.

The δ^{34}S values in West Falmouth Harbor's seagrass tissues are more depleted in ^{34}S than many plants measured in previous studies. A review by Holmer and Hasler-Sheetal (2014) of 44 leaf tissue stable sulfur isotopic observations in *Z. marina* across the globe found an average value of +4.0‰. Plants in the Middle Harbor had an average δ^{34}S value of −5.3‰ in

2019 (Fig. 5), with individual plant measurements of the youngest blades as low as −12.9‰. One site in the western portion of the Outer Harbor, where there has been a considerable decline in leaf tissue δ^{34}S since 2014, had an average δ^{34}S value of −9.7‰ in 2018; this value is isotopically lighter than 98% of observations in the review by Holmer and Hasler-Sheetal (2014). Prior to Snug Harbor's mortality event, δ^{34}S leaf tissue values were as low as −7 to −10‰, with nearly 60% of measurements taken on Snug Harbor grasses between 2007 and 2010 found to be below 0‰, implicating sulfide intrusion may have been a significant stressor contributing to the 2010 seagrass mortality event in Snug Harbor.

Seagrass in WFH with more negative δ^{34}S values have a higher ratio of aboveground to belowground biomass ($p = 0.009$, Fig. 8), the latter being a strong indication of stress (Nixon et al. 2001). In both years, this was measured, much of the variation in this ratio was driven by belowground biomass, with Middle Harbor plants having less root and rhizome biomass than Outer Harbor plants of similar canopy height (see Supporting Information Table S2), perhaps due to sulfide
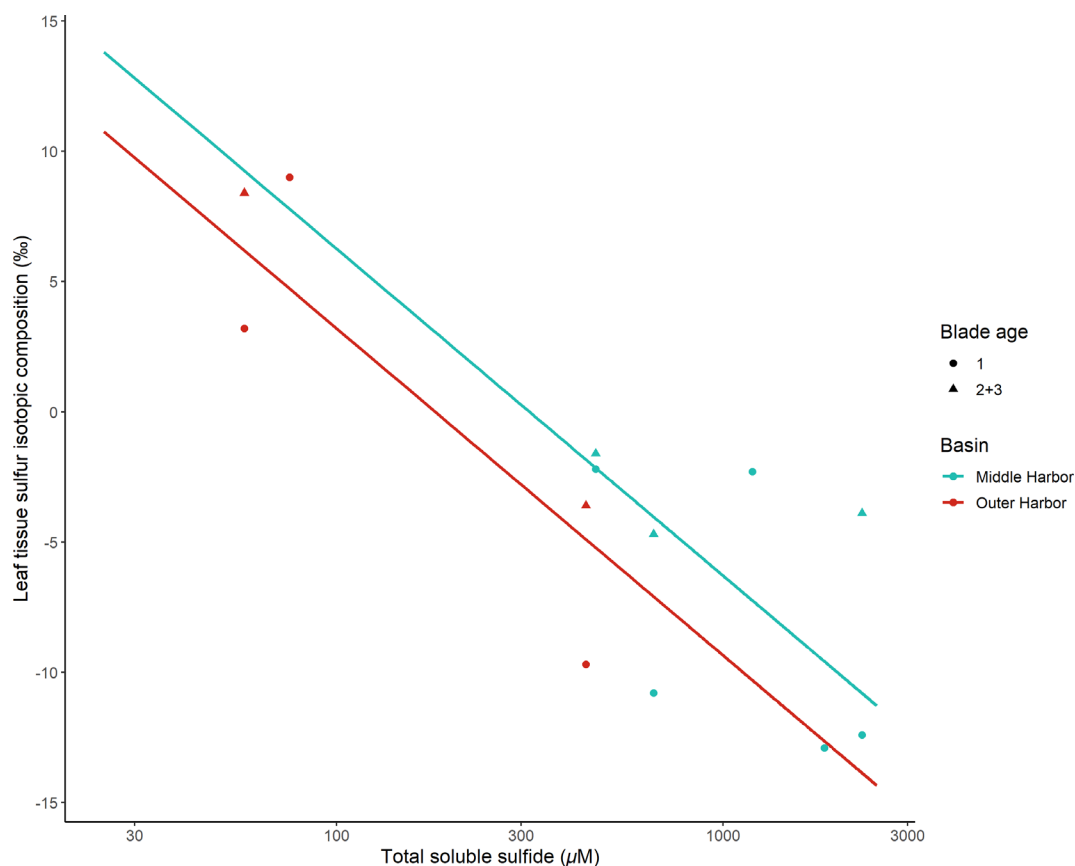
Fig. 7. Total soluble sulfides versus seagrass leaf tissue sulfur isotopic (δ^{34}S) value in West Falmouth Harbor ($p = 0.006$ across both basins), sampled in 2018 and 2019. Solid lines represent relationships between δ^{34}S and total soluble sulfides for each of the two basins, calculated using EMMs, accounting for basin as a fixed effect. Each point represents one site average value (3–9 cm) for pore water sulfide, and site-averaged measurement for δ^{34}S divided by blade age. Blade 1 (represented by circles) is the youngest blade on a plant, while blades 2 + 3 (represented by triangles) are the second- and third-youngest blades. For further information, *see* Supporting Information Table S5.

toxicity in the rhizosphere. Nixon et al. (2001) noted that seagrass mortality is increasingly likely as the aboveground to belowground biomass ratio exceeds 4. Ratios higher than this are common within WFH, particularly in the Middle Harbor where the July 2018 average of sites 101, 102, 103, and 104 was 4.7. At sites in the central portion of the Middle Harbor (101 and 102), where sulfide levels in the top 6 cm of sediment reach 2.0 mM, the average aboveground to belowground ratio exceeded 6.0. In both 2018 and 2019, 75% of sites in the Middle Harbor exceeded the 4.0 threshold reported by Nixon et al. (2001), with one site having 7-fold greater aboveground biomass than belowground biomass in 2019. In the Outer Harbor, only site 215, which also contains the highest levels of rooting zone pore-water soluble sulfide seen in the Outer Harbor at 1.0 mM, exceeded 4.0 for its aboveground to belowground biomass ratio. Researchers in other systems have found relationships between leaf tissue sulfur isotopic composition and seagrass aboveground to belowground biomass ratios, but their data show an inverse relationship to ours, where aboveground to belowground biomass ratio increases as

δ^{34}S increases (Frederiksen et al. 2008). However, sulfide values found by Frederiksen et al. in the seagrass beds they studied are much lower (maximum 0.58 mM) than the values we found in WFH, and plants there may be better able to cope with pore-water sulfide invasion into roots and rhizomes. Although it is likely that light availability also plays a role in moderating biomass allocation in WFH, we did not find a strong relationship between leaf chlorophyll content and aboveground to belowground biomass ratio ($p = 0.07$), which we would expect if seagrass biomass allocation was driven mainly by availability of light. This suggests that in WFH, diffusion of sulfide from the rhizosphere into seagrass leaf tissues potentially influences seagrass resource allocation, favoring larger leaves and smaller root and rhizome structures (Fig. 8), though further research is needed to understand additional other potential drivers of this relationship.

We found additional indications of seagrass stress in parts of WFH, such as high epibiota biomass grown on the plants, which can reduce light reaching seagrass surfaces (Nelson 2017) and compete with their seagrass hosts for
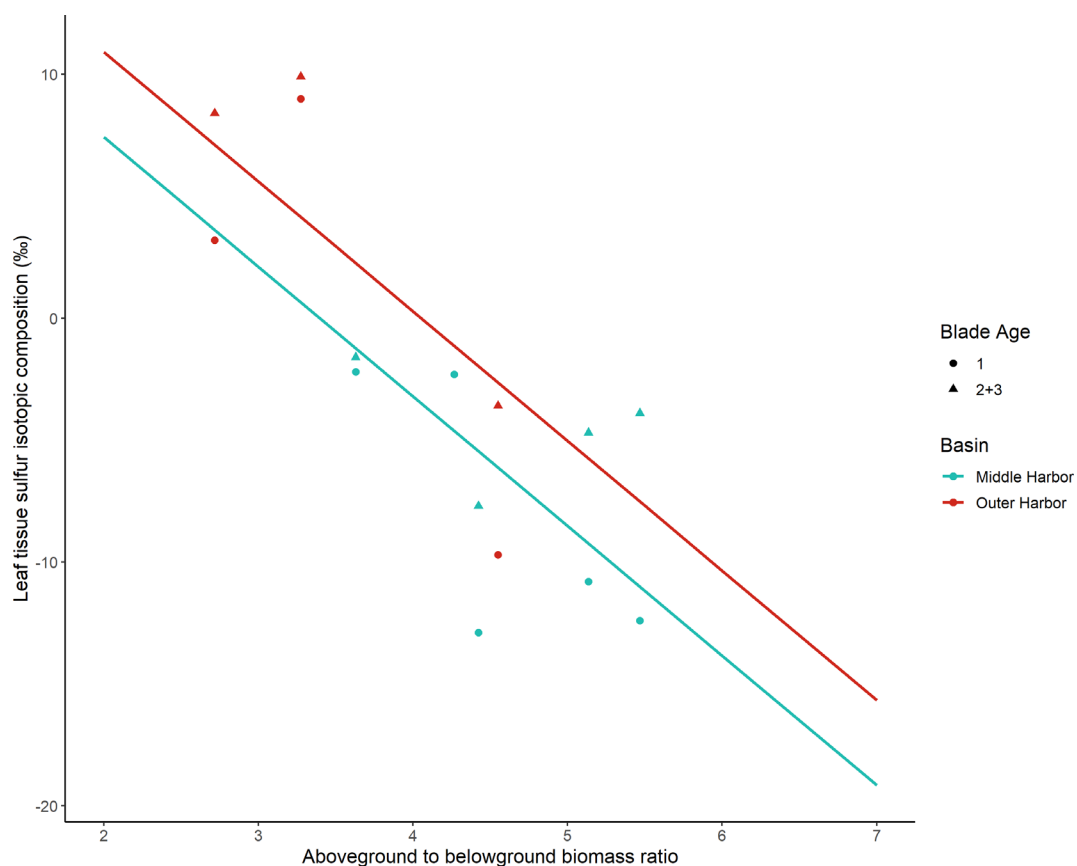
Fig. 8. Leaf tissue sulfur isotopic ratio (δ^{34}S) vs. seagrass above- to below-ground biomass ratio ($p = 0.009$ across both basins) in West Falmouth Harbor. Solid lines represent relationships between δ^{34}S and total soluble sulfides for each of the two basins, calculated using EMMs. For further information, *see* Supporting Information Table S6.

nutrients (Sand-Jensen 1977; Sand-Jensen et al. 1985). Leaves with lower leaf tissue sulfur isotopic ratio trended toward denser colonies of epibiota ($p = 0.013$). Epibiota values as high as 1.9 mg DW per cm^2 leaf area (site 101) represent an additional stressor on Middle Harbor seagrasses, as colonization at this level has been shown by Nelson (2017) to correlate to more than 50% light attenuation at the blade surface. Seagrass in light limited conditions photosynthesize at lower rates (Dennison and Alberte 1986), which reduces tissue and rhizosphere oxygen levels and may be associated with greater sulfide intrusion (Goodman et al. 1995; Brodersen et al. 2015). While epibiota and sediment sulfide concentration did not show a relationship ($p = 0.37$), the correlation between decreased δ^{34}S and denser epibiota colonies supports this potential mechanism.

A notable feature of West Falmouth Harbor's Middle and Snug Harbor sub-basins is that they are low-energy, shallow (generally less than 2 m depth) basins protected from high wave-action. Seagrass in these regions can persist with very small root and rhizome structures; in fact, seagrass here can survive with their belowground structures at the sediment–water column interface, exposed, rather than buried in the sediment. We observed this behavior in the Middle Harbor repeatedly from 2017 to 2019 and collected photographic evidence of the phenomenon each year (Supporting Information Fig. S4). In addition, we observed rhizomes growing above the sediment surface in Snug Harbor several times leading up to the 2010–2011 die-off event. Despite protecting roots and rhizomes from toxic sediments, this strategy may leave the below-ground tissue vulnerable to herbivory and uprooting in strong tides or storm-induced wave action. In WFH, we have only seen this behavior at sites with high levels of pore-water sulfide (101, 102, and Snug Harbor in 2007). Further research is needed to determine whether this phenomenon is an adaptive strategy used by plants in high-sulfide conditions, or a result of other physical effects of eutrophic conditions.

Given the high concentrations of pore-water total soluble sulfides and sediment organic matter in WFH's Middle Harbor, as well as high epibiota loads contributing to potential light limitation, seagrass in WFH face multiple environmental stressors, all of which occur as a direct or indirect result of anthropogenic nitrogen enrichment. Our research indicates

that to improve sediment condition and protect WFH's seagrasses from further mortality (particularly in the Middle Harbor basin) water quality improvements, most notably the reduction of anthropogenic nitrogen entering the harbor, are necessary. Future reductions in N-loading associated with groundwater entering the harbor are expected, due to the implementation of tertiary N-removal treatments at the upstream wastewater treatment plant, presenting an interesting opportunity for us to continue looking at long-term trends in sediment quality and seagrass health indicators as this ecosystem recovers in the future. Our findings may help inform successful management, and perhaps reintroduction, of seagrass meadow ecosystems in New England, and suggest the importance of considering sediment conditions when assessing the feasibility of seagrass restoration and success.

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Conflict of Interest

The authors declare that they have no conflicts of interest.