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Succession pattern and phylotype analysis of microphytobenthic communities in a simulated oil spill seagrass mesocosm experiment

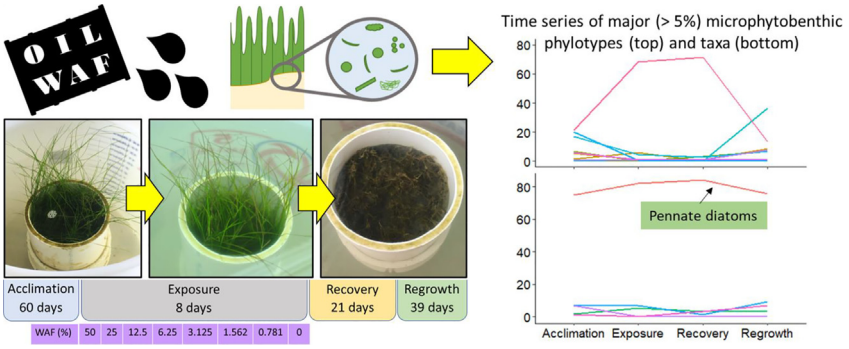
Taylor L. Hancock [a,b,c], Samantha L. Blonder [a,b], Alison A. Bury [a,b], Rachel A. Smolinski [a,b], Michael L. Parsons [a,b], Alison Robertson [b,d], Hidetoshi Urakawa [a,b,c,*]

[a] Department of Marine and Ecological Sciences, Florida Gulf Coast University, Fort Myers, FL, USA
[b] Alabama Center for Ecological Resilience (ACER), Dauphin Island, Alabama, United States
[c] School of Geosciences, University of South Florida, Tampa, FL 33620, USA
[d] Department of Marine Sciences, University of South Alabama and Dauphin Island Sea Lab, Dauphin Island, Alabama, USA

HIGHLIGHTS

- Oil did not greatly alter microphytobenthic community structure at the class level.
- Chronically oil exposed sediments exhibited increased resiliency to oil stressors.
- Microphytobenthic phylotypes that responded to oiling were identified.

GRAPHICAL ABSTRACT



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ABSTRACT

Microphytobenthic communities play a significant role in nutrient modulation, sediment stabilization, and primary production in seagrass beds, which provide various ecosystem services. We hypothesized that microphytobenthic communities in sediments of chronically oil-exposed seagrass beds will exhibit increased resiliency to stressors associated with oil exposure as opposed to seagrass beds never exposed to oil spills. We prepared 14-liter seawater mesocosms, each containing a submersed macrophyte *Ruppia maritima* collected from the Chandeleur Islands, Louisiana, and Estero Bay, Florida. Mesocosms were initially exposed to 50% water-accommodated oil fractions (WAF) and subsequently diluted by 50% with daily artificial seawater exchanges over 8 days to simulate tidal dilution. High-throughput amplicon sequencing based on 23S rRNA gene targeting cyanobacteria and chloroplasts of eukaryotic microphytobenthos was conducted to assess the impact of oiling on microphytobenthic communities with additional assessment via microscopy. High-throughput sequencing in combination with traditional microscopic analysis provided a robust examination in which both methods roughly complemented each other. Distinct succession patterns were detected in benthic algal communities of chronically oil-exposed (Louisiana) versus unexposed (Florida) seagrass bed sediments. The impact of oiling in microphytobenthos across all samples showed that benthic diatoms dominated all algal communities with sample percentages ranging from 42 to 97%, followed by cyanobacteria (2 to 50%). It is noteworthy that drastic changes in microphytobenthic community structure in terms of the larger taxonomic level were not observed, rather change occurred at the phylotype level. These results were also confirmed by microscopy. Similarity percentages (SIMPER) analysis identified seven phylotypes (Cyanobacteria, Bacillariophyceae, and Mediophyceae)

* Corresponding author at: Department of Ecology and Environmental Studies, Florida Gulf Coast University, Fort Myers, FL, USA.
E-mail address: hurakawa@fgcu.edu (H. Urakawa).

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in the Louisiana samples and one phylotype (Bacillariophyceae) in the Florida samples that increased in relative sequence abundance after oil exposure. The detailed phylotype analysis identifying sentinel microphytobenthic indicators provides a base for future research on benthic microalgae response to ecosystem disturbance.
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1. Introduction

Marine ecosystems are the largest of Earth's aquatic environments and experience constant change via natural and anthropogenic processes. Certain species of marine organisms such as corals, mangroves, kelps, and seagrasses serve as ecological engineers and can significantly affect the shape of seascapes and ecosystems. Seagrasses are among the most productive plant communities in nearshore environments (Byron and Heck, 2006; Heck and Wetstone, 1977), serving as habitat, feeding grounds, and nurseries for many marine species, including commercially important fish and shellfish (Nordlund et al., 2016). Seagrasses stabilize shallow seafloor sediments with their dense roots and rhizomes coated and strengthened by biopolymers created by benthic algal and bacterial communities (James et al., 2019). This sediment stabilization and erosion prevention is crucial during storm and hurricane events, providing economical value similar to coastal wetlands (Dewsbury et al., 2016). Many species of algae and microalgae (such as diatoms) not only play a significant role in this sediment stabilization and nutrient modulation in a seagrass ecosystem but also serve as sentinel biological indicators of water quality and ecosystem health because of their high sensitivity to nutrient enrichment and pollution (Urakawa and Bernhard, 2017; Zhang et al., 2017). The response of microphytobenthic organisms to anthropogenic disturbances, like oil spills, remains difficult to predict and often varies in a taxon-dependent manner (Kenworthy et al., 2016).

The Deepwater Horizon oil spill was the largest and most significant spill in United States history (Hester et al., 2016; McNutt et al., 2012). During this event, an oil and gas blowout at BP's Macondo Prospect (MC252) 66 km off the coast of Louisiana (Beyer et al., 2016) released an estimated total of 4.9 million barrels (779,100,000 L) of Louisiana Sweet crude oil into the Gulf of Mexico (Parsons et al., 2015; United States Coast Guard, 2011). The spill spread along 291.3 km of Louisiana, Mississippi, Alabama and Florida shoreline and reaching ecologically sensitive coastal lines, such as the Chandeleur Islands, LA, in early May 2010 (Kenworthy et al., 2017). This oil spill affected a wide range of marine organisms (Beyer et al., 2016; Kenworthy et al., 2017; Urakawa et al., 2019). Previous studies have suggested that seagrass communities become stressed when exposed to oil and may experience complete mortality (Foster et al., 1971a, 1971b; Scarlett et al., 2005). However, multiple studies examining impacts of the Deepwater Horizon oil spill on the submersed macrophyte, *Ruppia maritima*, found those growing in the northern Gulf of Mexico, the Chandeleur Islands specifically, were not severely impacted by the oil spill (Kenworthy et al., 2017; Martin et al., 2015). This could be an indication that these ecosystems have been conditioned to chronic oil exposure due to oil extraction activities since the 1970s and have established resiliency to oiling as a stressor (Colten et al., 2012; Parsons et al., 2015). It should be noted that *Ruppia maritima* can survive in a wide range of salinity (0 to 70 ppt) and not considered as truly a marine species; however, it forms seagrass beds as one cosmopolitan species of submerged vegetation found throughout the northern Gulf of Mexico (La Peyre and Rowe, 2003; Martin et al., 2015).

Resiliency can be defined as the capability of an ecosystem or the biotic components within, in this case seagrass beds and their microphytobenthic communities, to tolerate and persist through a disturbance (Falk et al., 2019; Halpern, 1988). A system, community, or taxon which is resilient to a disturbance will exhibit inertia; not readily changing due to less sensitivity to stressors induced and often having a greater ability to return to conditions present before the disturbance (Westman, 1978). A system experiencing intermediate disturbances can develop resiliency as selective pressures redistribute taxa within communities and genotypes within taxa so the most resilient have the advantage to survive. While dependent on the frequency and magnitude of the disturbance as well as other pressures over time, a resilient system can develop via a succession of resilient taxa and traits (Colten et al., 2012; Townsend et al., 1997). An understanding of such characteristics, especially in response to climatic anthropogenic disturbance events like oil spills, is vital to understanding how humans alter natural systems and how we may mitigate the negative effects of these disturbances. This is especially true for phytoplankton, in which oil exposure has been documented to negatively impact physiological and molecular processes (Garr et al., 2014). Many phytoplankton taxa, however, exhibit the genetic capacity to adapt via phenotypic acclimation and mutation (Romero-Lopez et al., 2012).

In this study, we assessed if the taxonomic and phylotypic response of microphytobenthic communities could act as sentinel biological indicators of seagrass beds to oil exposure. We also hypothesized that because of these resiliency and sensitivity characteristics, the succession pattern of microphytobenthic communities from a chronically oil-exposed seagrass bed (resilient) and a non-polluted seagrass bed (sensitive) could be differentiated in a simulated oil spill seagrass mesocosm experiment.

2. Materials and methods

2.1. Seagrass core collection and acclimation

Submerged macrophyte cores (10 cm deep) were collected from two populations of *Ruppia maritima* in the Gulf of Mexico: Estero Bay, Florida, which has never been exposed to historical oil spills, and the Chandeleur Islands, Louisiana, which has been chronically exposed to oil from the 1984 Alvenus oil spill, re-oiling caused by Hurricane Katrina (2005) and Hurricane Isaac (2012), and the Deepwater Horizon oil spill of 2010. The Florida cores were collected on June 19, 2016, and placed into seawater-filled buckets for transportation back to the Vester Marine Station at Florida Gulf Coast University by boat. The Louisiana cores were collected on June 15, 2016, and transported to the Vester Marine Station in buckets fitted with air stones by temperature-controlled ground transportation. The seagrass cores were collected in 15 cm diameter, 20 cm long PVC tubes that were then capped on the bottom to keep macrophyte roots and associated sediments contained (Fig. 1). From each population, treatment and control mesocosms (each in triplicate) were established by placing each core into a 5 gal/19 L white high-density polyethylene (HDPE) plastic bucket filled with artificial seawater (14 L) and an aerating stone. Mesocosm conditions were maintained at 25 ppt, approximately 24 °C water temperature (ambient), and approximately 500 μmol m^{-2} s^{-1} photosynthetically active radiation using two Solar Flare T5 HO 240-V fluorescent lights on a 20:4 photo/dark period corresponding with *R. maritima*'s optimal range of 400–500 μmol m^{-2} s^{-1} (Evans et al., 1986) (Fig. 1). Mesocosms were acclimated for 60 days prior to experimentation to ensure that the submerged macrophytes survived and recovered from any stressors related to sampling, transport, and mesocosm establishment.

2.2. Water-accommodated fraction (WAF) production

WAF solutions (82 L total) were prepared following the methods of Aurand and Coelho (CROSERF 2005) with the exception that preparations

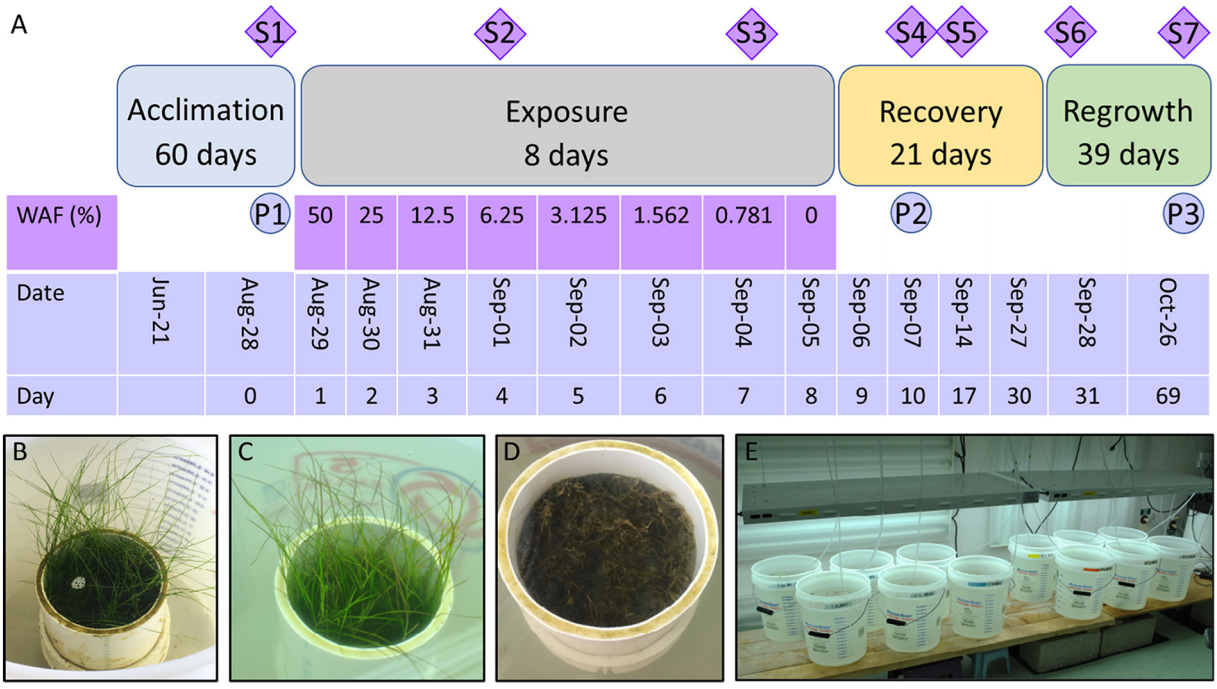

Fig. 1. Mesocosm design and study timeline in 2016. A, study timeline including the day (0–129), time period (Acclimation, Exposure, Recovery, and Regrowth), WAF concentrations, sediment collection periods (S 1–7) for DNA extraction, microelectrode measurement (S1–3), and microscopy measurement periods (P 1–3); B, seagrass sediment core mesocosm, which includes *Ruppia maritima* blades and roots during acclimation phase; C, seagrass core after initial exposure (Day 2); D, seagrass core after the exposure period (Day 8); E, experimental setup including 14 L mesocosm with seagrass core, overhead lights, and aerating tube/stone.

were done under ambient light versus the dark (to better mimic field conditions). Louisiana Sweet Crude (LSC) surrogate oil (provided by BP America Production Company) was layered on top of filter-sterilized artificial seawater (25 ppt) made with Instant Ocean in sealed glass aspirator bottles at a ratio of 1 g crude oil to 40 mL seawater. In order to avoid gas expansion issues, 20% headspace was left in each bottle. The solutions were mixed on a stir-plate using large stir bars. Mixing speed was adjusted to produce a vortex height no greater than ~10% of the total height of the mixture. After mixing for 72 h in direct sunlight, the mixture was allowed to settle for approximately 5 h before draining the WAF through the sidearm outlet at the bottom of the aspirator bottle. The crude oil supernatant that was floating on top was left behind and later discarded. The WAF solution (designated as 100% WAF) was stored in glass containers with PTFE-lined lids at −20 °C until experimentation. A parallel experiment (Tyre, 2018) analyzed the hydrocarbon constituents in WAF prepared as described above at multiple salinities by gas chromatography/mass spectrometry using selected ion monitoring as described by Turner et al. (2019). Analytical analysis (GC/MS-SIM) of 50% WAF made with 34 ppt seawater identified 42 analytes with a total concentration of 119.9 μg/L. The three most abundant analytes were naphthalene (24%), C1-naphthalenes (23%), and C2-naphthalenes (8.7%). No benzo [*a*]pyrene was detected in the 34 ppt 50% WAF material.

2.3. Oil exposure experiments

Treatment group mesocosms were exposed to 50% WAF (14 L; made as described above) and diluted approximately 50% every 24 h by removing 7 L of WAF and replacing it with 7 L of 25 ppt artificial seawater. This dilution gradient was used to simulate tidal flushing/dilution that the Chandeleur seagrass beds likely experienced after the initial oiling from the Macondo oil spill. The control mesocosms were filled with 25 ppt artificial seawater (14 L) and were similarly flushed, in which 7 L of artificial seawater was removed from the mesocosms after 24 h and replaced with fresh artificial seawater (Fig. 1). Over a period of eight days, WAF levels therefore decreased from approximately 50% to 25%, 12.5%, 6.25%, 3.125%, 1.562%, and 0.781%. As the primary purpose of these dilutions was to simulate tidal flushing rather than a specific dilution gradient, the percentages are approximate. Furthermore, the data analyses focused on three-time points (before, during, and after exposure – see below) and were therefore not dependent on specific dilution values.

After the 8-day exposure period, all WAF and/or water was removed from each mesocosm and replaced with 14 L of fresh artificial seawater. The seagrass mesocosms were then subjected to a 21-day recovery period to provide the exposed seagrasses with an opportunity to recover from the oiling impact. Over the course of the experiment, the submerged macrophyte blades exposed to oil were visibly stressed; their blades turned brown and fragmented or detached from the rhizome during the exposure phase before recovering and regrowing green blades during the respective experimental phases. Following this recovery period, the above-ground (blades) of the macrophytes were cut and weighed. The submerged macrophyte then underwent a regrowth period of 39 days to determine how much biomass was regained during this period. At the end of the experiment, below-ground (root) biomass was measured and soil samples (approximately 6 g dry weight) were taken from each core for sediment analysis. Subsamples were combusted at 500 °C to determine organic content (% loss on ignition) and sieved through a 63 μm mesh to determine silt content (% of grains <63 μm).

2.4. Sediment oxygen profiling using a microelectrode

Regular microsensor monitoring of oxygen was conducted in the acclimation (S1) and exposure phases (S2 and S3) of the experiment to monitor for changes that may be attributable to microphytobenthic metabolic activity (Fig. 1). Oxygen depth profiles were recorded with Clark-type microelectrodes OX-50 (50-μm tip diameter; Unisense, Denmark) at 500-μm resolution. Calibration of the oxygen microelectrode was performed with a two-point calibration made in air-saturated seawater (100% saturation) and at depth in anoxic sediment (0% saturation). The oxygen penetration depth was defined as the depth below which the oxygen reading dropped below 1 μM.

2.5. Sediment sample collection for DNA analysis and microscopy

Sediment samples for DNA extraction (≈ 0.5 g) were collected from each phase of the experiment, yielding one sample at the end of the acclimation phase (prior to exposure), two samples from the exposure phase on days 4 and 7, two samples for the recovery phase on days 10 and 17, and two samples for the regrowth phase on days 31 and 39. DNA was extracted using the MagAttract PowerSoil DNA KF Kit (Qiagen) according to the manufacturer's instructions. Sediment samples for microscopy were collected at the end of the acclimation phase (day 0), the recovery phase on day 10, and the regrowth phase on day 17 (Fig. 1). Samples were unable to be collected for microscopy during the exposure phase due to the presence of oil WAF.

2.6. Benthic algae identification and cell counting

A subsample of the sediment (0.16 ± 0.09 g) collected for microscopic analysis was diluted with 1 mL of 25 ppt artificial seawater and kept at 4 °C until analysis. The algal composition was determined by transferring 1 mL of sample into one well of a Nunc Lab-Tek Chamber Slide. The samples were analyzed using an Olympus IX71 Inverted epifluorescent phase-contrast microscope outfitted with an Olympus DP71 digital camera. Each sample was scanned at 200× magnification. For each sample, 100 algae units (cells or colonies) were counted (unless the entire well was scanned without reaching that target number), using a method modified from Shapiro et al. (1989). Organisms (i.e., diatoms, dinoflagellates, and cyanobacteria) were identified to the class level or lowest practical taxonomic level and enumerated. Light microscopy was supported by epifluorescent microscopy with Uvitex staining before analysis for all samples to aid in armored dinoflagellate cell enumeration using the ultraviolet excitation/emission (DAPI) filter. A Texas Red filter was used to identify and enumerate cyanobacteria based on autofluorescent signals.

2.7. High-throughput sequencing

Amplification of 23S rRNA gene targeting cyanobacteria and chloroplasts of eukaryotic microphytobenthos was conducted using a primer pair p23SrV_f1 (GGACAGAAAGACCCTATGAA) and p23SrV_r1 (TCAGCCTGTTATCCCTAGAG) (Sherwood and Presting, 2007) tagged with the Illumina i5 forward and i7 reverse sequencing primer, respectively. Each polymerase chain reaction (PCR) contained 25 μL reactions with Qiagen HotStar Taq master mix, equal amounts of forward and reverse primers (5 μM each), and 1 μL of DNA template (1–20 ng). The thermal cycling consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, annealing at 54 °C for 40 s, and extension at 72 °C for 1 min, with a final extension of 10 min at 72 °C. PCR product from the first stage was transferred to a second PCR based on qualitatively determined concentrations (gel electrophoresis) with primers for the second PCR based on the Illumina Nextera PCR primers. The second stage amplification was conducted the same as the first except for 10 cycles instead of 35 cycles. Amplicons were visualized with eGels (Life Technologies) and products were pooled equimolar with each size selected quantified using the Qubit 2.0 fluorometer (Life Technologies). Amplicons were then loaded on an Illumina MiSeq (Illumina) 2 × 300 flow cell at 10 pM (RTL Genomics). For analysis, FASTQ formatted files were merged using the PEAR Illumina paired-end read merger (Zhang et al., 2013). Prefix dereplication and clustering at a 3% divergence level were conducted using the USEARCH (Edgar et al., 2011). Operational taxonomic unit (OTU) selection was performed using UPARSE-OTU algorithm (Edgar, 2013). Chimeric sequences were detected using UCHIME algorithm and removed for further analysis (Edgar, 2010). Representative OTUs were used to determine taxonomic information through a basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI). The high-throughput sequence datasets were deposited in GenBank under BioProject number PRJNA630525.

2.8. Data analysis

To analyze sequence data, experimental phases which had two sets of data from separate sample collections (exposure, recovery, and regrowth phases) were averaged in each sample. The resulting mean sequence reads and those of the acclimation phase for each sample were normalized (scaled to 10,000) for relative comparisons. The Shannon diversity index was also calculated using these data to determine if diversity changed throughout the experiment. A Friedman test with Nemenyi post-hoc comparisons was conducted to compare the abundance of taxa from each of the experimental phases within and between the oiled and control (non-oiled) samples of the respective Louisiana and Florida samples. A Bray-Curtis dissimilarity matrix was calculated to compare each experimental phase and treatment. Similarity percentages (SIMPER) of phylotypes using Bray-Curtis dissimilarity measures were conducted in the vegan package (Dixon, 2003) for Rx64 3.6.1 (http://cran.stat.sfu.ca/) to determine which phylotypes changed the most between experimental phases. SigmaPlot 12.0 (Systat Software, San Jose, CA) was used for general statistics- including student-*t*-test and one-way analysis of variance (ANOVA).

3. Results

3.1. Sediment characteristics

The sediment organic contents estimated by the ignition loss ranged between 3.0 and 3.1% in Florida mesocosms and 4.4 to 6.6% in Louisiana mesocosms. The ratio of silt content (< 63 um) in the sediment ranged between 5.9 and 7.0% in Florida mesocosms and 20.0 to 21.7% in Louisiana mesocosms. The bottom waters were partially oxygenated in all mesocosms (198 to 212 μM) under acclimated conditions and no statistical difference was found between Louisiana and Florida mesocosms. However, the bottom water oxygen levels became significantly lower after oil exposure in the Florida mesocosms (student *t*-test, $p < 0.001$) and Louisiana mesocosms ($p < 0.001$) in comparison with their controls, falling to 124.2 ± 7.1 μM (mean ± SD) and 164.8 ± 2.6 μM, respectively. The reduction of oxygen penetration depth was observed in both Florida and Louisiana mesocosms after oil exposure. However, the pattern of control mesocosms varied (Fig. 2). Oxygen was present to a depth of 2.75 ± 0.27 and 2.42 ± 0.86 mm deep in the sediment in the acclimated phase and was reduced to 1.30 ± 0.27 mm and 1.75 ± 0.27 mm after oil exposure in Florida and Louisiana mesocosms, respectively. Overall, our oxygen microelectrode measurements showed that oil exposure reduced the bottom water oxygen levels and oxygen penetration depth in the sediment (Fig. 2).

3.2. General statistics of 23S rRNA gene amplicon sequencing

In total, 263,541 sequences were determined in this study and the mean number of analyzed reads after removal of nontarget sequences (1536 ± 107; mean ± SE) was 9412 ± 118 ($n = 16$) (Table 1). No statistically significant correlation was found between analyzed sequence reads and phylotypes (ANOVA for regression, $p = 0.138$, $n = 28$). Moderate nontarget sequence contamination in the process of gene amplification was mainly caused by specific lineages of bacteria such as *Verrucomicrobia* and *Cytophagia*, which comprised 94% of the total bacterial fraction. After removing these non-algal sequences, the samples underwent normalization (scaled to 10,000 reads) before further benthic microalgal taxa and phylotype analysis.

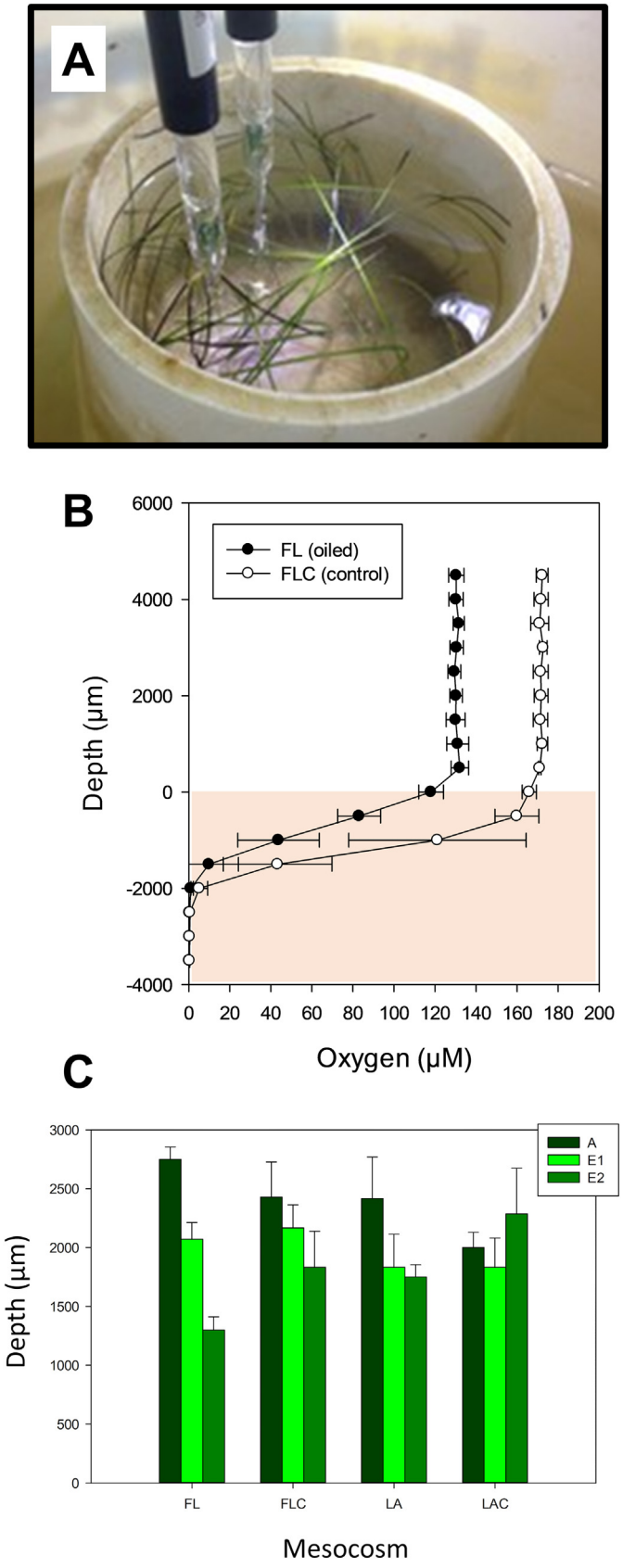

Fig. 2. Sediment oxygen profiling using a microelectrode. A, Oxygen measurement in a seagrass mesocosm core. Measurements were conducted before each water exchange. B, Two oxygen profiles of control and oiled Florida mesocosms (4 days of oil exposure). Data are means and SD ($n = 3$) C, Sediment oxygen penetration depths of each mesocosm setting. S1 (Corresponding to Fig. 1), acclimation, S2, oil exposure day 4 (6.25% WAF condition), S3, oil exposure day 7 (0.78% WAF condition). Data are means and SE ($n = 5$ to 7).

Table 1
Analyzed sequence reads and phylotypes of benthic algal communities of seagrass core mesocosm sediment.

Location (latitude, longitude)	Sample	Day collected	Experimental phase	Analyzed sequence reads	Analyzed phylotypes
Chandeleur Islands, Louisiana (29.863750°, −88.841466°)	Louisiana Oil	0	Acclimation	13,238	66
		4	Exposure	10,585	66
		7		8668	69
		10	Recovery	6396	38
		17		12,390	91
		31	Regrowth	8820	58
		69		7200	73
	Louisiana Control	0	Acclimation	11,767	85
		4	Exposure	6361	83
		7		11,977	84
		10	Recovery	9767	83
		17		5813	43
		31	Regrowth	9026	88
		69		4904	84
Estero Bay, Florida (26.381726°, −81.857501°)	Florida Oil	0	Acclimation	4345	49
		4	Exposure	1215	47
		7		7771	22
		10	Recovery	13,162	37
		17		13,848	49
		31	Regrowth	10,742	66
		69		8049	50
	Florida Control	0	Acclimation	12,054	74
		4	Exposure	12,592	73
		7		11,532	55
		10	Recovery	13,114	80
		17		13,408	65
		31	Regrowth	9651	71
		69		5146	53
			Total	263,541	
			Mean	9412	64
			± SE	±118	3

3.3. Evaluation of the acclimation phase of the benthic microphytobenthic communities in the mesocosms

One of the large challenges of these mesocosm experiments was creating a homogenate initial condition between control and experimental mesocosms. Homogeneity of each mesocosm in the acclimation phase was evaluated using microphytobenthic communities determined by 23S rRNA gene amplicon sequencing. As visualized by non-metric multidimensional scaling of Bray-Curtis dissimilarity indices, there were no statistically significant differences of community structure found in pair-wise comparisons of experimental phases between or within the Louisiana oiled and control samples (Friedman test: $p = 0.290$, $n = 8$) (Fig. 3). However, the Florida oiled sample and control exhibited statistically significant differences between pairwise comparisons of community structure composition of experimental phases within and between samples (Friedman test: $p = 0.019$, $n = 8$). The Bray-Curtis dissimilarity index of the oiled and control sediment samples in the Louisiana mesocosms was calculated as 0.079 and exhibited highly similar algal communities during the acclimation phase of the experiment (Table 2). Both were primarily dominated by Bacillariophyceae (Fig. 3). In the Florida mesocosms, oiled and control exhibited dissimilar algal communities during the acclimation phase of the experiment; Bacillariophyceae dominated in the oiled sample while cyanobacteria dominated in the control sample (Fig. 4). This initial heterogeneity of the oiled and control samples was supported by the Bray-Curtis dissimilarity index (0.507), which was higher than the Louisiana mesocosms (Table 2).

3.4. Community shifts induced by the changes of experimental stages

In the Louisiana mesocosms upon oil exposure, the oiled sample diverged from the control, becoming dissimilar from its initially nearly identical composition (Fig. 3). During the recovery phase, the oiled

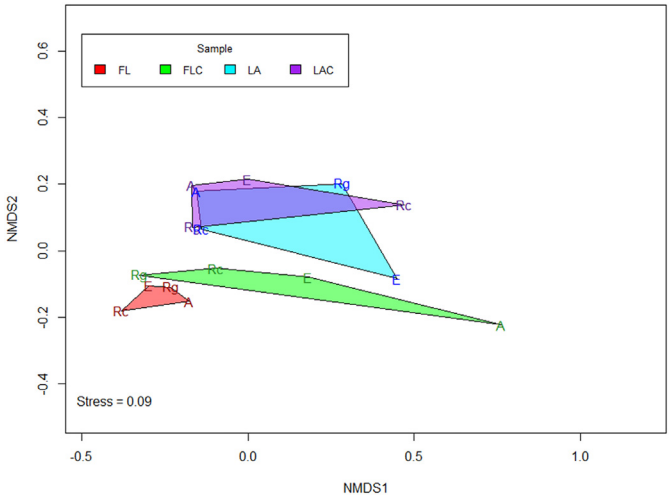

Fig. 3. Non-metric multidimensional scaling plot in two dimensions of Bray-Curtis dissimilarity matrices of taxa across samples (FL = Florida oiled, FLC = Florida control, LA = Louisiana oiled, LAC = Louisiana control) and experimental phases. Data points represent experimental phases (A = Acclimation, E = Exposure, Rc = Recovery, Rg = Regrowth) and are connected by a convex polygon for each sample.

sample returned to a similar state as in the acclimation phase before becoming more dissimilar in the regrowth phase. While the control sample also changed over time, it did not show a similar pattern. Throughout the experiment, the Louisiana oiled sample and control exhibited a dominance of benthic diatoms (mean ± SE: 82.5 ± 5.4%), followed by cyanobacteria (12.9 ± 4.6%) (Fig. 4). Pennate diatoms (pennales) occupied a larger fraction (77.1 ± 1.8%) than centric diatoms. Within pennate diatoms, Bacillariophyceae (93.2 ± 0.7%) were more abundant than Fragilariophyceae. Upon oil exposure, the relative abundance of diatoms belonging to Bacillariophyceae decreased as cyanobacteria replaced them. In the subsequent recovery phase, the phytoplankton community returned to a similar condition as in the acclimation phase initially until cyanobacteria began to become more abundant and replaced the decreasing proportion of Bacillariophyceae again during the regrowth phase.

In the Florida mesocosms, the oiled and control samples exhibited dissimilar algal communities during the acclimation phase of the experiment, and despite experiencing different experimental conditions, became more similar during the experiment (Fig. 3). The oiled sample changed little over the course of the experiment, maintaining a community composition highly similar to its initial state upon oil exposure and through to the end of the experiment. The control did not show a similar pattern, becoming steadily dissimilar from its initial composition throughout the experiment. The oiled sample exhibited a dominance of benthic diatoms (88.1 ± 0.9%) followed by cyanobacteria (3.5 ± 0.7%) for the duration of the experiment. This observed pattern was common in all other mesocosms (Fig. 4). Pennate diatoms (pennales) occupied a larger fraction (90.9 ± 2.1%) than centric diatoms. Within pennate diatoms, Bacillariophyceae (98.8 ± 0.2%) was more abundant than Fragilariophyceae. During oil exposure, Bacillariophyceae diatoms remained highly dominant and exhibited slight increases. Cyanobacteria also slightly increased in abundance during this time period while almost all other taxa decreased. In the following recovery phase, Bacillariophyceae continued this dominant trend while cyanobacteria started to decrease along with most other taxa. The regrowth phase saw a decrease in Bacillariophyceae as Mediophyceae diatoms increased to replace them along with other taxa. Throughout the experiment, the control exhibited a dominance of benthic diatoms (72.7 ± 11.2%) followed by cyanobacteria (18.4 ± 8.3%). Pennate diatoms (pennales) occupied a larger fraction (84.7 ± 4.1%) than centric diatoms. Within pennate diatoms, Bacillariophyceae (95.5 ± 0.8%) were more abundant than Fragilariophyceae. Over the course of the experiment, Bacillariophyceae steadily increased, replacing the lost proportion of decreasing cyanobacteria.

3.5. Phylotype analysis

SIMPER (similarity percentage) analysis of the Louisiana oiled samples indicated 14 phylotypes contributed to roughly 70% of the phylotype composition dissimilarity between the acclimation and exposure phases (Table 3). Of these phylotypes, cyanobacteria saw the largest increase across three Synechococcaceae phylotypes (phylotype-46: *Cyanobium* sp., 48: *Synechococcus* sp., 49: *Synechococcus* sp.), followed by three Bacillariophyceae (phylotype-84: *Nitzchia* sp., 87: *Pseudonitzschia* sp., 90: *Fistulifera* sp.) and one of Mediophyceae (phylotype-113: *Lithodesmium* sp.). However, no cyanobacteria phylotypes decreased, whereas Bacillariophyceae exhibited the largest decreases across five phylotypes (phylotype-89: *Fistulifera* sp., 102: *Rhizosolenia* sp., 103; *Rhizosolenia* sp., 85: *Nitzschia* sp., 93: *Phaeodactylum* sp., 86: *Nitzschia* sp., 168: *Phaeodactylum* sp.). These same 12 phylotypes of cyanobacteria (phylotype-48, 46, 49), Bacillariophyceae (phylotype-90, 84, 87, 89, 102, 103, 85, 93, 86, 168), and Mediophyceae (phylotype-113) were among 17 which contributed to roughly 70% of the phylotype composition dissimilarity between the exposure and recovery

Table 2
Bray-Curtis dissimilarity matrix comparing high-throughput sequencing microphytobenthos taxa of experimental phases within and between oiled and control samples.

	Louisiana	Oil				Control		
		Acclimation	Exposure	Recovery	Regrowth	Acclimation	Exposure	Recovery
Oil	Exposure	0.386	–	–	–	–	–	–
	Recovery	0.119	0.339	–	–	–	–	–
	Regrowth	0.274	0.208	0.282	–	–	–	–
Control	Acclimation	0.079	0.374	0.121	0.276	–	–	–
	Exposure	0.111	0.309	0.141	0.217	0.135	–	–
	Recovery	0.344	0.172	0.358	0.160	0.347	0.257	–
	Regrowth	0.098	0.374	0.080	0.278	0.109	0.156	0.349
	Florida	Oil				Control		
		Acclimation	Exposure	Recovery	Regrowth	Acclimation	Exposure	Recovery
Oil	Exposure	0.117	–	–	–	–	–	–
	Recovery	0.150	0.083	–	–	–	–	–
	Regrowth	0.114	0.107	0.128	–	–	–	–
Control	Acclimation	0.507	0.542	0.556	0.529	–	–	–
	Exposure	0.211	0.289	0.342	0.288	0.341	–	–
	Recovery	0.120	0.138	0.197	0.129	0.459	0.187	–
	Regrowth	0.150	0.069	0.080	0.128	0.560	0.304	0.131

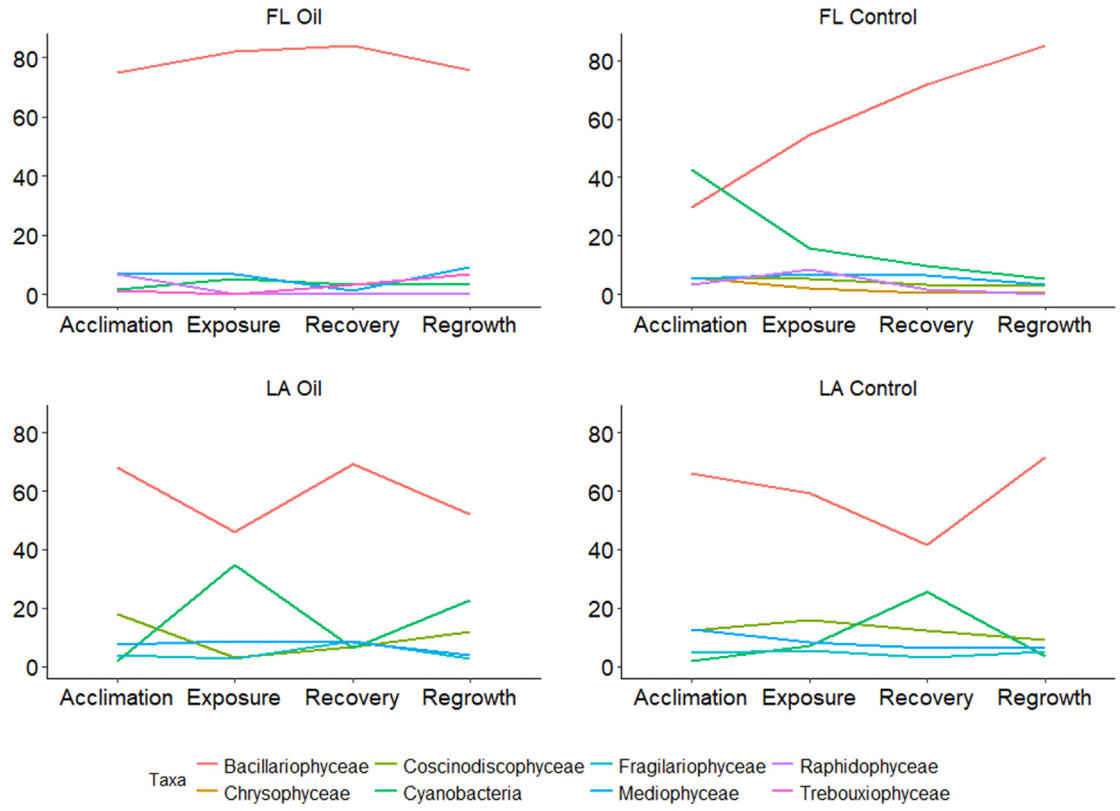

Fig. 4. Time series graph of microphytobenthos major taxa (> 5% of sequence reads) percentage for each locality (FL: Florida and LA: Louisiana) and treatment (oiled and control).

phases, with the trends of each phylotype reversed from those described previously (Fig. 5). There was no clear trend among the phylotypes contributing to the top 80% of dissimilarity between the recovery and regrowth phases.

SIMPER analysis of the Florida oiled sample indicated four phylotypes contributed to roughly 70% of the phylotype composition dissimilarity between the acclimation and exposure phases (Table 3). One Bacillariophyceae phylotype (phylotype-98: *Pseudo-nitzschia* sp.) increased in relative abundance by 47.1%, accounting for the top 38.2% of dissimilarity, and the remaining two Bacillariophyceae (phylotype-85 and 84: *Nitzschia* sp.) and one Raphidophyceae phylotype (phylotype-119: *Heterosigma* sp.) all decreased in relative abundance. There was no clear trend among the phylotypes contributing to the top 80% of dissimilarity between the exposure and recovery phases as they remained similar in phylotype composition (Bray-Curtis dissimilarity: 0.145). The recovery and regrowth phases had three phylotypes contribute to roughly 75% of their dissimilarity. The same Bacillariophyceae phylotype-98 also attributed to the most dissimilarity, accounting for the top 42.8% but this time decreasing in relative abundance by 58.3% as the remaining phylotypes, a Bacillariophyceae (phylotype-83: *Nitzschia* sp.) and Mediophyceae (phylotype-109: *Cerataulina* sp.), increased by 36.3% and 7.4%, respectively.

Of 168 total, 22 major phylotypes were identified, comprising over 5% of sequence reads per sample (Fig. 5). Of these major phylotypes, only one (phylotype-90) was universally found across all mesocosm conditions. Between the Florida and Louisiana sites, 32% of major phylotypes were shared while 32% and 27% of phylotypes overlapped across the treatment and control samples for the Florida and Louisiana samples, respectively. Phylotype-98 in Florida mesocosm, and phylotype-90, 84, and 46 in Louisiana mesocosm were identified as plausibly oil-resistant microphytobenthos phylotypes.

3.6. Microphytobenthic communities identified by microscopy

The mean number of cells counted for each sample was 227 ± 43 (mean ± SE). Pennate diatoms dominated the samples with a mean of 66.4 ± 8.8% (± SE), followed by cyanobacteria (27.6 ± 5.3%). Within pennate diatoms (pennales), Bacillariophyceae occupied a larger fraction (59.7 ± 4.5%). Interestingly, oiled samples displayed a shift from pennate diatom to cyanobacteria dominance for both Louisiana (84.5%) and Florida (71.9%) during the recovery phase but returned to a community composition more similar to the acclimation phase during the regrowth phase (Fig. 1). The control samples displayed a similar shift towards cyanobacteria dominance but during the regrowth phase for Louisiana (71.8%) and Florida (41.2%). The community structure observed in the amplicon sequencing (phylotypes) was generally supported by microscopy with a dominance of Bacillariophyceae diatoms and cyanobacteria across all samples and experimental phases (Fig. 6). The mean difference of abundances for each taxon during each experimental phase was 17.1 ± 2.3% (mean ± SE). The Shannon diversity index ranged between 1.4 and 3.6 by 23S rRNA gene sequencing, and 1.6 and 3.0 by microscopy. There were no clear correlated patterns between these two analyses, except for pennate diatom and cyanobacteria dominance.

4. Discussion

The dominance of pennate diatoms in epiphytic seagrass communities from the Chandeleur Islands, Louisiana, and Estero Bay, Florida upon exposure to high concentrations of oil provides further evidence that many diatom taxa are resilient to oil (Gilde and Pinckney, 2012). Pennate diatoms are known to exhibit enhanced succession in oil-exposed systems under high nutrient conditions most likely attributed to their small surface area-to-volume ratio (Ozhan and Bargu, 2014), as seen in our samples originating from nutrient-rich estuaries (i.e., Estero Bay cores). The high abundances of cyanobacteria in our

Table 3
Summary of similarity terms (SIMPER) analysis. Differences in relative sequence read abundances of microphytobenthos phylotypes contributing to dissimilarities between experimental phases of oiled samples. A cumulative dissimilarity cut-off of 80% was applied.

Phylotype ID	Taxa	Acclimation/exposure			Exposure/recovery			Recovery/regrowth		
		± %[a]	% Contr.[b]	% Cum.[b]	± %	% Contr.	% Cum.	± %	% Contr.	% Cum.
Louisiana										
89	*Fistulifera*	−25.3	18.8	18.8	12.1	11.3	11.3	−9.7	10.6	10.6
48	*Synechococcus*	12.2	9.1	27.9	−11.8	11.0	22.3			
90	*Fistulifera*	9.2	6.9	34.8	−3.5	3.2	51.4			
102	*Rhizosolenia*	−6.6	4.9	39.7						
84	*Nitzschia*	6.6	4.9	44.6	−5.2	4.8	27.1	−2.4	2.6	62.8
103	*Rhizosolenia*	−6.1	4.5	49.0	2.3	2.1	65.1			
46	*Cyanobium*	5.5	4.1	53.1	−5.1	4.7	36.6	2.1	2.3	67.7
85	*Nitzschia*	−4.9	3.6	56.8				3.3	3.7	47.9
49	*Synechococcus*	3.6	2.7	59.4	−3.0	2.8	60.4			
87	*Pseudo-nitzschia*	3.4	2.5	62.0	−3.4	3.2	54.6			
93	*Phaeodactylum*	−3.2	2.4	64.4	4.9	4.6	41.2	−3.7	4.1	36.7
113	*Lithodesmium*	3.0	2.2	66.6	−3.2	3.0	57.6	−0.1	3.0	57.6
86	*Nitzschia*	−2.8	2.1	68.7	4.0	3.7	44.9	−3.0	3.3	54.6
168	*Phaeodactylum*	−2.3	1.7	70.3	0.8	3.3	48.2	−1.4	3.8	40.5
96	*Eunotia*				5.1	4.8	31.9	−4.8	5.2	23.1
102	*Rhizosolenia*				1.4	1.9	67.0			
95	*Eunotia*				2.7	2.6	62.9	3.1	3.4	51.3
107	Mediophyceae				1.9	1.8	68.8	−1.9	2.1	71.9
2	*Cyanobacterium*				−1.8	1.7	70.5	4.5	5.0	28.1
72	Bacillariophyceae							6.6	7.3	17.9
60	Cryptomonas							4.2	4.6	32.7
83	*Nitzschia*							3.3	3.7	44.2
100	*Melosira*							2.4	2.7	60.2
50	*Synechococcus*							2.3	2.5	65.4
55	*Synechococcus*							1.9	2.1	69.8
Florida										
98	*Pseudo-nitzschia*	47.1	38.2	38.2	3.3	11.4	43.6	−58.3	42.8	42.8
85	*Nitzchia*	−19.4	15.7	53.9						
84	*Nitzchia*	−12.5	10.1	64.0	−1.6	5.5	49.1			
119	*Heterosigma*	−6.8	5.5	69.5						
94	*Eunotia*	−5.1	4.1	73.6						
83	*Nitzschia*							36.3	26.7	69.5
109	*Cerataulina*							7.4	5.4	74.9
113	*Lithodesmium*				−6.0	20.6	20.6			
129	*Picochlorum*				3.4	11.5	32.1			
46	*Cyanobium*				−1.4	5.0	54.1			
89	*Fistulifera*				1.2	4.2	58.3			
55	*Synechococcus*				−1.1	3.8	62.1			
61	*Poterioochromonas*				1.1	3.8	65.8			
97	Bacillariophyceae				0.9	3.2	69.1			
103	*Rhizosolenia*				0.9	3.1	72.2			

[a] Percent change between experimental phases.
[b] Contributing percentage.
[c] Cumulative percentage.

oiled samples were also expected, as some species are associated with oil degradation and have been known to dominate oil-exposed environments (Abed et al., 2002; Gilde and Pinckney, 2012). The nutrient-rich origins of the respective cores likely also contributed to the high abundance of cyanobacteria. The overall dominance of diatoms followed by cyanobacteria in amplicon sequencing phylotypes after oil exposure was generally supported by microscopy, but there were observed differences in proportions. This is a noted discrepancy in method comparison studies, which have determined high-throughput sequencing to be a more accurate diversity assessment (Xiao et al., 2014).

Some of the discrepancies between high-throughput sequencing and microscopy community analyses in our study may stem from the nature of the microphytobenthos sampled and biases in our methods (Fig. 6). For example, dominant cyanobacterial taxa (i.e., *Cyanobium* and *Synechococcus*) were picocyanobacteria that are small and difficult to be counted among sediment particles via microscopy. Our microscopic analysis only detected chain-forming and filamentous cyanobacterial species, rather than unicellular picocyanobacterial (due to the use of inverted microscopy at lower magnifications). Additionally, taxa containing a higher number of chloroplasts per cell would lead to a higher representation in sequencing as opposed to microscopy. Alternatively, microalgae such as dinoflagellates are easily detected and enumerated in microscopic analysis, but are less likely to be accurately quantified in 23S rRNA sequencing due to the dramatically reduced gene content of their chloroplasts resulting in a lack of this targeted gene (Howe et al., 2008). We were also unable to collect microscopy samples during the oil exposure phase of the experiment due to the presence of oil WAF.

Even with the exclusion of some dinoflagellates, the selection of the plastid 23S rRNA gene is an advantageous choice when compared to other potential markers (Steven et al., 2012). One of the advantages of our 23S rRNA gene amplicon sequencing approach is a simultaneous detection of both eukaryotic algae and cyanobacteria which cannot be attained by amplicon sequencing of 16S rRNA and 18S rRNA genes; both of which are commonly used in diversity studies of prokaryotes and microeukaryotes (Steven et al., 2012). We successfully identified phylotypes that positively and negatively responded to the oil exposure. These observed succession patterns and shared phylotype sequence data provide a base for future research on microphytobenthic response to oiling. These findings are important and can be easily shared with other researchers through public DNA databases. However, such data sharing could not be easily accomplished by the traditional microscopic

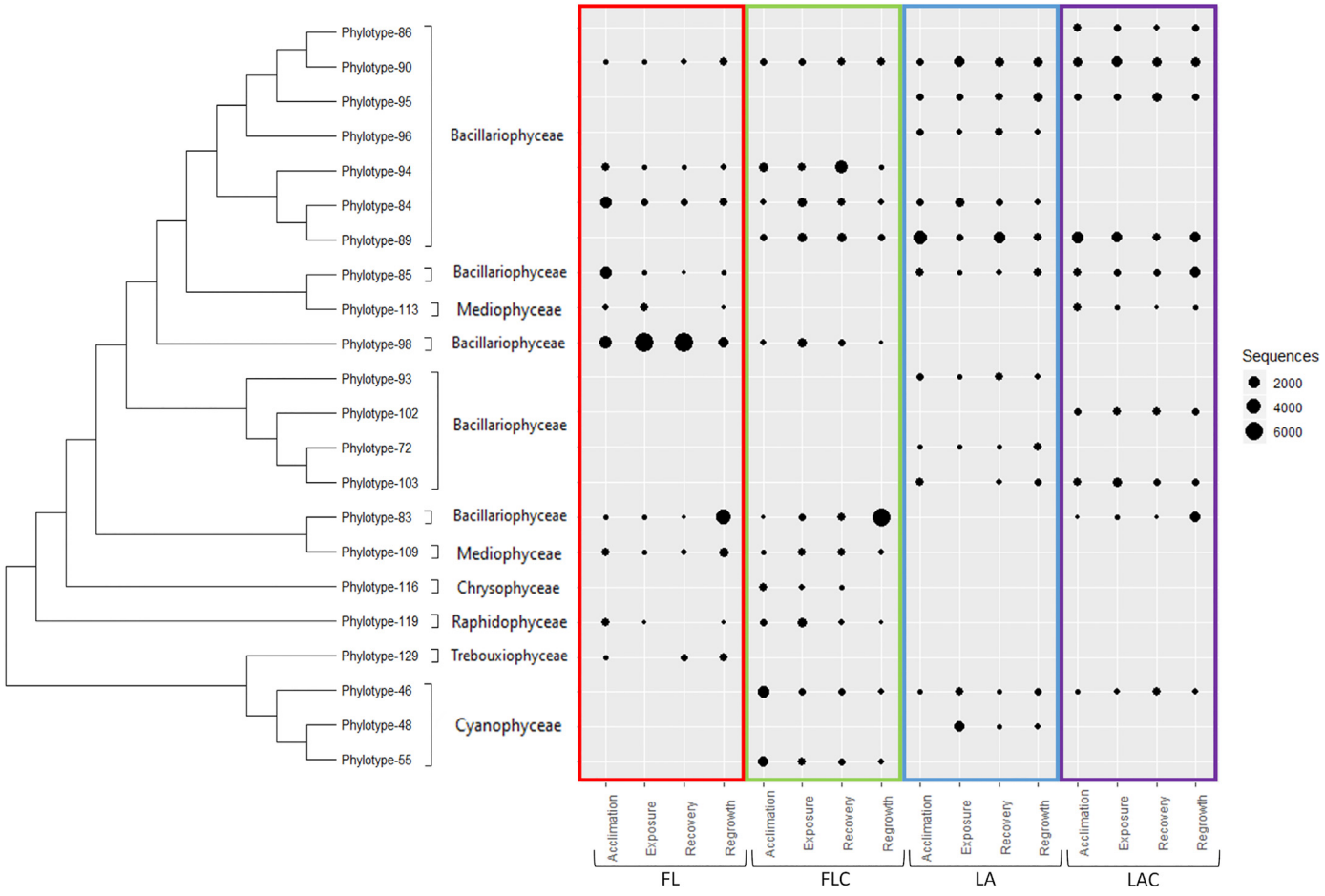

Fig. 5. Bubble chart of major microphytobenthos phylotypes (> 5% of sequence reads) for each sample core location and treatment (FL = Florida oiled, FLC = Florida control, LA = Louisiana oiled, LAC = Louisiana control) with a phylogenetic cladogram based on the neighbor-joining method.

approach which is strongly influenced by the experience and identification skill of researchers and the depth of identification (i.e., species or genus level). Microscopy results are also not comparative with other studies unless the samples are shared by other researchers. Sharing and availability of DNA sequence data might be the strongest point of a molecular-based approach. However, the effectiveness of plastid 23S rRNA gene marker is hindered by limited numbers of available 23S rRNA gene sequences in DNA databases; a universal drawback of molecular community analyses except for microbial 16S rRNA genes, especially concerning tropical microphytobenthic samples (Kermarrec et al., 2014).

Overall, high-throughput sequencing could be a desirable technique for microphytobenthic community analysis. Xiao et al. (2014) concluded that the molecular methods they used (16S and 18S rRNA genes) did not always match with light microscopy and provided a more accurate and uniform diversity assessment lacking the human error and bias of manual cell counting via microscopy as well as being a more economic option. As DNA databases gain more representations and increase in completeness, this method will increase in effectiveness. However, for now more studies are required to systematically assess the nature of these two totally different approaches. For example, species richness detected by microscopy was always higher than that detected by pyrosequencing (Kermarrec et al., 2014). However, Shannon diversity indices obtained from sequencing and microscopy were similar in this study (Fig. 6). Since the nature of these methods is quite different, we cannot anticipate identical results. Therefore, we must identify the potential sources of variability of these two methods.

In our analysis of microphytobenthic taxonomic composition, the Louisiana oiled sample appears to have displayed distinct responses to oil exposure as cyanobacteria increased and Bacillariophyceae diatoms decreased (Fig. 4). The Florida oiled sample appeared to lack any such response as taxonomic composition remained static throughout the experiment and a high dominance of Bacillariophyceae was maintained. Our initial impression was the difference in cyanobacteria trends could be due to differences at higher taxonomic resolution. Upon further examination, the Florida samples had a distinct lack of nitrogen-fixing cyanobacteria such as Oscillatoriales and Nostocales, as identified by 23S rRNA sequencing, but in the Louisiana samples they constituted a miniscule portion of cyanobacteria sequences and do not follow the overall trend of cyanobacteria.

However, our analysis of phylotype composition revealed both the Louisiana and Florida samples displayed distinct responses to oil exposure. This supported our hypothesis that the succession pattern of microphytobenthic communities from a chronically oil-exposed seagrass bed (resilient) and a non-polluted seagrass bed (sensitive) could be differentiated by our experiment. The Louisiana sample contained seven phylotypes that were resilient to oil exposure, belonging to cyanobacteria, Bacillariophyceae, and Mediophyceae. Meanwhile, the Florida sample only had one phylotype resilient to oil exposure, which belonged to the genus *Pseudo-nitzschia* (Bacillariophyceae). Other studies have also reported a similar resilience to oil by *Pseudo-nitzschia* (reviewed by Quigg et al., 2021). Interestingly, all three oil resilient cyanobacteria phylotypes in the Louisiana sample were identified as belonging to the family Synechococcaceae, of which to our knowledge no previous oil resilience has been reported. The Florida oiled sample shared the same oil-resilient phylotypes (except for phylotype-87: *Pseudo-nitzschia* sp.) but did not demonstrate a similar resiliency, most likely due to the nearly absolute dominance of the single resilient Bacillariophyceae phylotype identified.

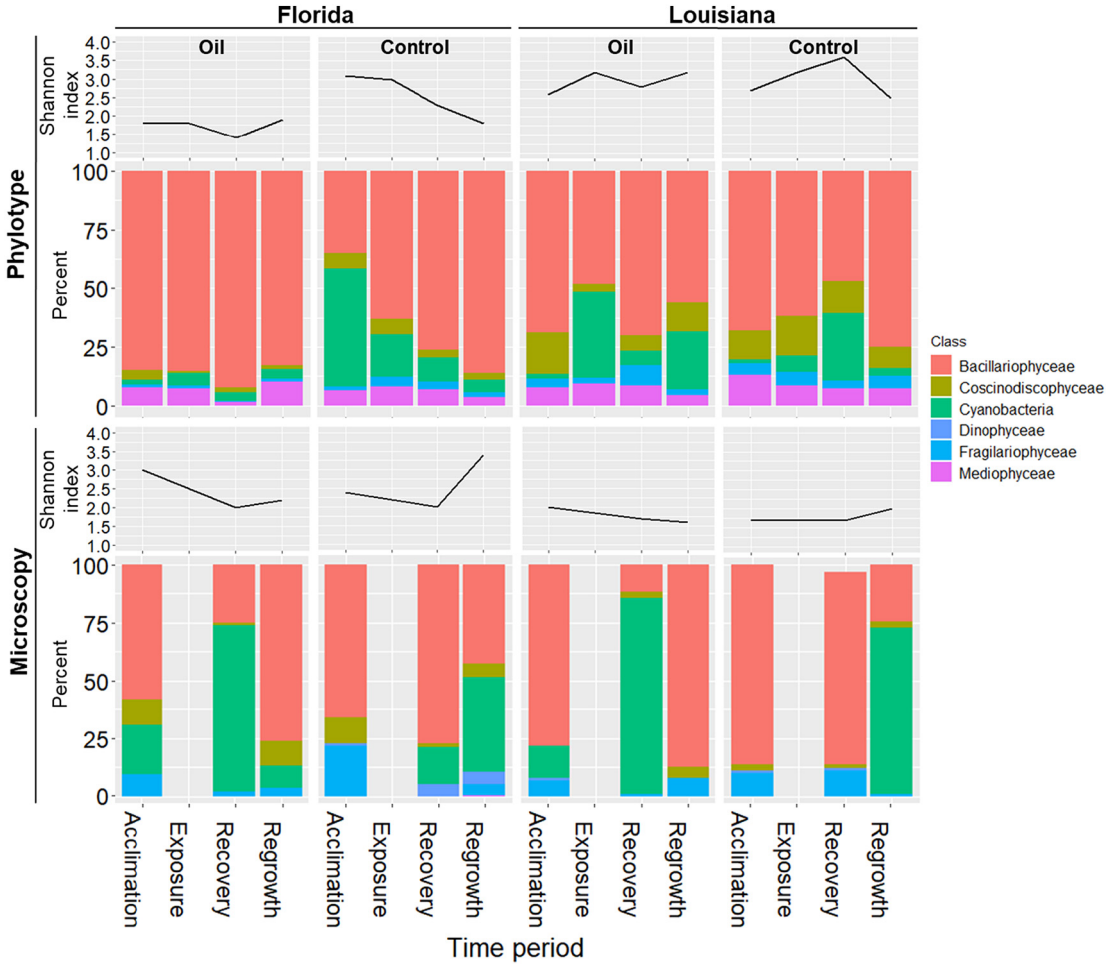

Fig. 6. Molecular and microscopic identification of benthic algal communities. Stacked bar plots of phylotypes (top) and microscopic taxonomic identification (bottom) with line graphs of Shannon biodiversity index values above each respective bar plot.

It should be noted that the Florida sample had fewer overall phylotypes attributing to dissimilarity and a lower phylotype richness in the acclimation and exposure phases than the Louisiana sample. The intermediate oil disturbances that Louisiana has experienced since 1977 have repeatedly introduced otherwise inconsequential or nonexistent selective pressures (Colten et al., 2012), likely resulting in greater phylotype richness and adheres to the Intermediate Disturbance Hypothesis in terms of intraspecific (genetic/phylotype) rather than interspecific competition and succession (Townsend et al., 1997). Additionally, the Louisiana and Florida samples shared some of the same phylotypes, which appeared resilient to oil in one sample but did not exhibit a similar response in the other. These phylotypes were always much lower in abundance in the other samples and highlight how phylotype composition as a whole plays an important role in oil ecological resilience.

As with any mesocosm experiment, our study benefited from a high degree of experimental control but may have been limited by the exclusion of external factors such as water flow, nutrient influx, or species introduction/migration, which have been suggested by the study of nitrifying microorganisms (Urakawa et al., 2019). Additionally, we were unable to account for the differences in our seagrass cores as our Florida treatment and control started with a high degree of compositional differences in terms of phylotypes and taxonomic compositions, although we were able to identify these initial community differences based on the molecular approach.

Further research should incorporate more varied samples collected from diverse climate zones reflecting global oil extraction efforts. Examination of nutrient and oil dynamics, interaction between seagrass and microphytobenthos, and gene expression patterns may help elucidate further mechanisms behind succession patterns described here.

5. Conclusion

Our simulated mesocosm oil spill did not appear to significantly alter microphytobenthic community structure in either chronically exposed or non-polluted seagrass bed cores based on our findings using high-throughput sequencing and microscopic analyses. Rather, taxonomic changes were observed within limited phylotype compositions. These findings support our hypothesis that microphytobenthic communities of chronically oil-exposed sediments from a *Ruppia maritima* seagrass bed will exhibit increased resiliency to stressors introduced by high concentrations of oil as opposed to sediments more naïve to oil (e.g., Estero Bay, Florida). It appears the microphytobenthic communities of the Chandeleur Islands, Louisiana have been selected for resilience to high concentrations of oil alongside *R. maritima* due to chronic exposure. The microphytobenthic communities of Estero Bay, Florida which are naïve to oil have not undergone the same selective pressures, leading to fewer phylotypes across fewer taxa that are resilient to high concentrations of oil. These observed succession patterns and phylotype analysis identifying sentinel benthic microalgal indicators provide a base for future research on benthic microalgae response to ecosystem disturbance.

CRediT authorship contribution statement

Taylor L. Hancock: Investigation, Validation, Formal analysis, Writing – review & editing, Visualization. **Samantha L. Blonder:**

Investigation. **Alison A. Bury:** Investigation. **Rachel A. Smolinski:** Investigation. **Michael L. Parsons:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Alison Robertson:** Project administration, Writing – review & editing, Funding acquisition. **Hidetoshi Urakawa:** Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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