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Organic carbon dynamics and microbial community response to oyster reef restoration

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Abstract

Oyster reefs are often touted for their contribution to water quality improvement, shoreline protection, and habitat creation, but few studies highlight another significant ecosystem service: organic carbon (OC) storage and cycling. Although oyster reefs may not fall within the definition of "blue carbon" as a vegetated coastal ecosystem, they may store as much carbon (C) as other well-studied coastal C sinks (e.g., mangroves, salt marshes, and sea grasses), providing an additional justification for restoration activities. Leveraging ongoing *Crassostrea virginica* reef restoration in Mosquito Lagoon, Florida (USA), this study employed a Before-After-Control-Impact sampling design to evaluate short-term (1 yr) changes in sediment OC storage and flux, microbial activity, and microbial community composition on degraded, natural, and restored oyster reefs. Restored reefs exhibited the greatest change in OC cycling within the study year, with increases in dissolved OC (31%), particulate OC (97%), CO_2 flux (707%), and β-glucosidase enzyme activity (467%), resulting in the highest total C and OC concentrations of the three reef types. Results demonstrate that sediment microbial community abundances are resilient and easily reinvigorated through reef restoration, while enzyme assays suggest sediment microbial activity on restored reefs may be limited by labile C availability. Overall, this study suggests restoration and oyster presence can rapidly enhance OC storage and can help offset the C flux from high calcification rates on young oyster reefs, providing an additional ecosystem service to support the importance of restoration activities.

Coastal ecosystems sequester and retain a significant amount of carbon (C), with an estimate of up to over 30,000 Tg of C stored worldwide (Macreadie 2021). Carbon storage in coastal and oceanic ecosystems, such as mangrove forests, seagrasses, and tidal marshes, is commonly referred to as "blue carbon" (Nellemann et al. 2009). These coastal vegetated habitats are well suited to C storage, retaining C via both internal (e.g., above and below ground biomass) and external (e.g., sedimentation) mechanisms. With coastal ecosystems accounting for approximately 50% of all C stored in oceanic sediment worldwide, there is increasing interest in utilizing blue C to mitigate climate change through restoration (Duarte et al. 2005; Macreadie 2021). Previous research found long-term C burial rates in coastal ecosystems were estimated to be 18–1713 g C m^{-2} yr^{-1}, a significantly greater rate than those found in upland environments (Mcleod et al. 2011). Given the vast C storage capacity in coastal environments and increasing CO_2 emissions, the need to enhance C storage is ever present, providing an opportunity to explore a rarely considered coastal C storage resource: oyster reefs.

Oyster reefs provide invaluable ecological resources and ecosystem services, often being referred to as both a "keystone species" and "ecosystem engineer" (Coen et al. 2007). Oysters and their three-dimensional reefs offer a variety of benefits, such as a habitat and food source for wildlife, water quality improvement, wave attenuation, and shoreline stabilization, among many others (Coen et al. 2007; Grabowski et al. 2012). However, a crucial, but often overlooked, service of oyster reefs is their potential role in C storage and cycling. Although oyster reefs do not directly sequester "blue carbon" through primary production, their role in the C cycle may still be significant (Fodrie et al. 2017; Chambers et al. 2018). Recent research suggests some eastern oyster (*Crassostrea virginica*) reefs can capture 0.3–2.7 Mg organic C ha^{-1} yr^{-1} through the accumulation of water column particulate organic matter (OM), though these burial rates are highly dependent upon the landscape position and age of the reef (Fodrie et al. 2017). Considering the widespread interest and investment in oyster reef restoration (Hernández et al. 2018), a better

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understanding of the importance of restored oyster reefs in the global C cycle, and how it may change as a reef develops, is needed. If restoring oyster reefs could serve to mitigate climate change by creating a C sink (a net removal of greenhouse gases from the atmosphere) (Peterson and Lipcius 2003; Grabowski and Peterson 2007), it could serve as an additional incentive for restoration.

Possibly the most recognizable role of oyster reefs in the global C cycle is the transformation of inorganic carbon (IC) through shell synthesis (calcification) and dissolution of calcium carbonate. The process of biogenic calcification ($Ca^{2+} + HCO_3 \leftrightarrow CaCO_3 + H^+$) *releases* CO_2 through the liberation of protons, suggesting young (recently restored) or rapidly accreting oyster reefs may serve as a C source. In fact, Fodrie et al. (2017) found that the reefs that accreted most rapidly in their study were the largest source of CO_2. Assuming the IC cycle of a healthy natural oyster reef is dominated by calcification (serving as a C source) and shell burial (being C neutral), the major mechanism to promote total carbon (TC) storage and climate mitigation on an oyster reef is the accumulation of organic carbon (OC) within the reef. If OC accumulation in the sediment exceeds IC loss during calcification, then even young reefs could prove to be a net TC sink. This study investigates the C sink potential of oyster reef restoration by focusing exclusively on the processes regulating OC storage on recently restored reefs.

The benthic-pelagic coupling of oyster reefs contributes to the accumulation and burial of OC through both biological and physical processes. High rates of filter feeding by oysters remove phytoplankton and other particulate OM from the water column to synthesize new biomass or be released at the sediment surface as biodeposits (a compilation of both feces and pseudofeces; Newell and Jordan 1983). Oyster biodeposits are rich in labile OC, a portion of which can be stored long term (Haven and Morales-Alamo 1966; Hoellein et al. 2015; Locher et al. 2021). Moreover, the three-dimensional structure of an oyster reef slows water velocities and traps particulate OC and IC (Newell and Jordan 1983; Dame et al. 1989; Fodrie et al. 2017). The surface roughness created by a live oyster reef has been shown to increase turbulent kinetic energy 1 cm above the bed by an average of 29%, with an accompanying 29% decline in water velocities, significantly increasing sediment entrapment on live oyster reefs relative to degraded reefs (Kitsikoudis et al. 2020). The combination of these physical and biological processes promotes the development of a "biogeochemical hotspot" in the underlying reef sediment, where higher concentrations of both C and nutrients exist compared to surrounding sediment (Newell et al. 2005; Chambers et al. 2018). The first-year post-restoration can represent a critical period for quantifying OC dynamics to elucidate the role of restoration in the global C cycle (Chambers et al. 2018). A previous research study suggests TC concentrations in the sediments within restored oyster reefs can increase rapidly, as much as 236% in only 1-yr post-restoration on an intertidal *C. virginica* reef in central Florida, USA, with comparable increases in dissolved OC (DOC) and nitrogen (N) pools (Chambers et al. 2018). Althiugh total OC was not quantified in the previous study by Chambers et al. (2018), the observed changes in sediment nutrient content following restoration suggest OC accumulation in reef sediment and the creation of an ideal habitat for microbially mediated biogeochemical transformations.

Sediment microbial communities ultimately regulate the delicate balance between OC burial (sink) and decomposition (source); studying their composition and activity can provide valuable information on restoration success and trajectories as they relate to supporting OC cycling. Moreover, while physical factors such as reef height and oyster density can takes several months to display significant changes, microbes, and their community abundance and diversity can respond quickly to changes in their environment (Chambers et al. 2016). Microbial community response to restoration can be quantified by investigating changes in the community composition itself (e.g., the relative abundance of key functional groups), or the activity of microbes. During the early stages of decomposition, microbial communities catalyze OC break-down by synthesizing and releasing extracellular enzymes. Because enzymes are either constitutive (released in relative proportion to associated nutrient metabolism) or inducible (released in proportion to nutrient need) (Dick 2015), their activities are tightly coupled with OC turnover (Sparling 1992). During the last stages of decomposition, CO_2 and CH_4 production rates are a direct indication of OC mineralization and losses from the system.

The goal of this study is to quantify OC storage and cycling on restored oyster reefs during the first-year post-restoration, relative to degraded and natural reefs, to measure the impacts of reef restoration. Specifically, this study seeks to (1) quantify oyster reef sediment OC pools (particulate and dissolved), transformations (enzyme activities and mineralization), and microbial community composition (functional group gene abundance); (2) determine the timescale of OC cycling and soil microbial community change over the first-year post-restoration; and (3) identify the length of time it takes for each parameter or process to mimic those measured in natural reefs, indicating restoration success. A previous space for time substitution study among similar reefs was conducted in the Indian River Lagoon in 2016 (Chambers et al. 2018). During this study, degraded, restored (1-, 4-, and 7-yr-old), and natural reference reefs were evaluated to determine how biogeochemical properties change following restoration, and how much time is needed for restored reefs to be biogeochemically equivalent to natural reefs. The Chambers et al. (2018) study revealed the first-year post-restoration was significant for reef biogeochemical development, with extractable DOC and NH_4^+, TC, TN, and the activity of three major groups of extracellular enzymes exceeding that of degraded reefs by 1-yr post-restoration, with little additional change as the restored reefs continued to age. Based on this finding, we sought a more

detailed evaluation of the changes and drivers of OC cycling in the first-year post-restoration.

We hypothesized the first year of restoration will be a crucial time for OC cycling, but the timescale and degree of importance remain uncertain (Chambers et al. 2018). Because this study focuses solely on OC cycling and storage within oyster reefs sediments (calcification and dissolution processes were not quantified) a determination of the TC source/sink potential of the study reefs cannot be made. However, this work represents an important step toward understanding the potential for oyster reef restoration to serve as a C sink, similar to the "blue carbon" habitats they juxtapose.

Materials and methods

Study site

This study took place in the Indian River Lagoon, which spans 250 km of central Florida's Atlantic coastline and is considered a highly diverse estuary (Provancha et al. 1992; Fig. 1). At the northernmost region of the Indian River Lagoon is Mosquito Lagoon, a shallow (average of 1.7 m), microtidal estuary with average salinities ranging from 28 to 45 ppt (Hynds and Starkey 2019).

Currents in the lagoon are primarily wind driven, with low water turnover rates (1 yr) and high water retention times. Throughout the Indian River Lagoon, oyster reefs of *C. virginica* have proven to be an especially important resource, valued at over 4.3 million annually when combined with other shellfish resources (Indian River Lagoon Economic Valuation Update 2016). Although oyster reef degradation is often attributed to overharvesting, disease, predation, or invasive species, the primary culprit of reef loss in Mosquito Lagoon (where study reefs are located) is disturbance from boat wakes (Wall et al. 2005; Walters et al. 2021; Zu Ermgassen et al. 2012). Excessive and frequent boat wakes weaken oyster cluster attachment to reef platforms, breaking-off live oysters and depositing them above the intertidal zone where a lack of inundation and food supply eventually leads to death (Walters et al. 2021). Over time, these reefs become primarily composed of degraded, disarticulated oyster shells stacked high above the intertidal zone.

Within Mosquito Lagoon, 24% of reefs have been degraded, leading to extensive efforts to restore/rehabilitate *C. virginica* oyster reefs over the last 13 years (Garvis et al. 2015). As of 2021, over 90 reefs (> 3.5 acres) have been restored through large volunteer efforts associated with the University of Central Florida's Coastal and Estuarine Ecology Lab and various local partners (L.J. Walters pers. comm.). Restoration employs a technique that both encourages oyster spat settlement and makes oysters resistant to displacement. Specifically, loose shells and sediment on degraded reefs are manually raked down to intertidal height and 0.25 m^2 mesh mats

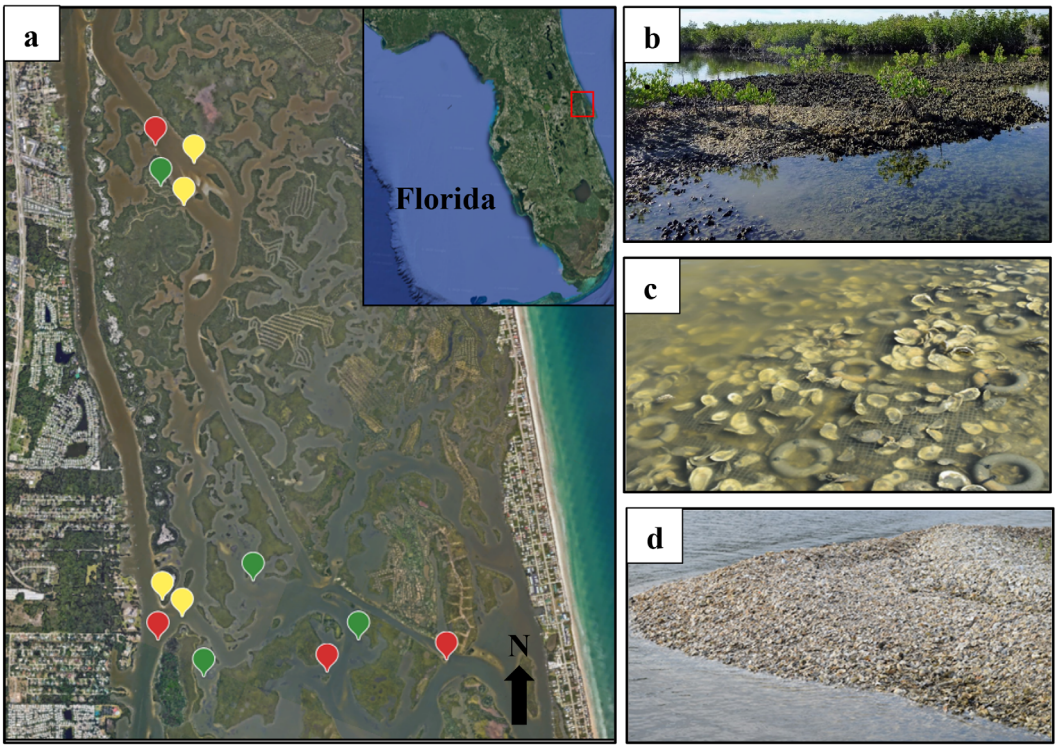

Fig. 1. (**a**) Restored (yellow), natural (green), and degraded (red) oyster reef sampling locations in Mosquito Lagoon, Florida. (**b**) Natural, (**c**) restored, and (**d**) degraded reefs in Mosquito Lagoon, Florida.

with 36 individual oyster shells zip-tied to each mat are placed on the leveled reefs and held down with concrete donut weights at each corner (Walters et al. 2021; Fig. 1c).

Sampling design

A Before-After-Control-Impact (BACI) design was implemented to evaluate restoration impact while accounting for the influence of spatial and temporal variability and disturbances, such as temperature, water levels, salinity, and natural disasters (Underwood 1992). Consequently, both reference (natural) and degraded reefs were also studied to serve as positive and negative controls, respectively. Within Mosquito Lagoon, four intertidal reefs of each type: restored, natural, and degraded, were evaluated over 1 year resulting in a total of 12 oyster reef sites (Fig. 1a–d). Degraded reefs are composed of bleached, disarticulated shells that are continuously exposed during the tidal cycle, with no live oysters/clusters partially buried in the sediment (Walters et al. 2021). Aerial imagery was utilized to identify the degraded sites, which are bright white in color, making them reflective in aerial images. All selected degraded reefs for this project were classified as dead for a minimum of 15 years prior based on aerial photography and ground truthing. Care was taken to choose reefs with similar geophysical and geographical properties. Previous research within the Mosquito Lagoon has noted an impact of distance from the ocean inlet on reef properties (Chambers et al. 2018; Locher et al. 2021). Thus, live and degraded reefs adjacent to restored sites were selected to help better account for the spatial variability in reef locations.

Sediment and water samples were collected from each reef during low tide prior to restoration (May 2017) and at 1 (June 2017), 6 (December 2017), 9 (March 2017), and 12 (June 2018) months of post-restoration (a 3-month sampling was not possible due to high water levels). Within each reef, four sampling locations were haphazardly selected within the intertidal zone (between the low tide elevation and the reef crest). Sediment cores were collected in each location (a total of four cores per reef) to a depth of 5 cm (3.5 cm diameter) using the push-core method. Samples were stored in sterile whirl-pak bags, placed on dry ice, and transported back to the University of Central Florida. Oyster density was measured by counting the number of live oysters within a 0.25 m^2 quadrant at five haphazard locations on each reef. Water immediately adjacent to each reef was measured for in situ temperature, dissolved oxygen, salinity, and pH using a ProDSS YSI sonde (Yellow Springs, Ohio, USA) and for chlorophyll *a* using a Manta plus probe (Eureka Water Probes).

Soil physicochemical properties

Sediment samples from each reef were evaluated for OM, TC, IC, OC, and DOC. Upon return to the lab, shell fragments larger than 2 cm were removed from each sample and samples were thoroughly homogenized by hand. Dried soil subsamples were combusted at 550°C for 4 h to measure OM content using the loss on ignition (LOI) method. TC and IC were measured on dried and resultant LOI samples, respectively, by oxygen combustion analysis at 950°C using a Vario Micro Cube (Elementar). OC was determined by the difference between TC and IC. DOC in each sample was extracted with a 0.5 M K_2SO_4 solution and subsequently analyzed on a TOC-L Analyzer (Shimadzu).

Potential carbon mineralization

Potential CO_2 respiration and methane (CH_4) production rates were measured to estimate rates of C mineralization from reef sediments. Production rates were measured using anaerobic bottle incubations in which 7 g of homogenized, field-moist sediment and 15 mL of filtered, *N*-purged site water were added to 180-mL glass serum bottles. The serum bottles were capped with rubber septa and aluminum crimps, purged with 99.99% N_2 gas to ensure anaerobic conditions, and placed in a shaking incubator at 25°C and 150 rpm. Duplicates from each reef type (restored, natural, and degraded) were included to capture intrasite variance. The gaseous headspace of each bottle was sampled four times over 7 days (Days 1, 2, 4, and 7) with a gas-tight glass syringe to measure CO_2 and CH_4 production and analyzed on a GC-2014 Shimadzu gas chromatograph (Kyoto, Japan). Respiration rates were calculated using the Ideal Gas Law and Henry's Law to account for temperature, pressure, and pH in the bottles, followed by linear regression analysis of the change in CO_2 and CH_4 concentrations over time.

Microbial enzyme activity

β-Glucosidase (β-gluc), alkaline phosphatase (Phos), and *N*-acetyl-glucosaminidase (NAG) enzyme activities were measured using fluorometric analysis to evaluate microbial activity and nutrient availability. β-Gluc enzymes catalyze the breakdown of cellulose into β-D-glucose and indicates the availability of labile C substrates; Phos is an inducible enzyme that hydrolyzes organic P, providing free phosphates (PO_4^{3-}) for microbial uptake; and NAG is a constitutive enzyme that degrades chitin and supports the release of available N (Dick 2015). Enzyme activity was measured using 4-methylumbelliferone (MUF, a fluorescent substrate) for standardization and its associated fluorescent labeled MUF-specific substrates. All enzyme activities were measured within 48 h of field collection, following the methods of Steinmuller et al. (2019, 2020). Briefly, 0.5 g of field-moist sediment from each sample was shaken with 39 mL of DI water for 1 h, creating a 1 : 80 soil slurry. The subsequent sediment slurry was pipetted into 96-well plates along with separate β-gluc, Phos, and NAG MUF-labeled substrates. Plates were read fluorometrically using a BioTek Synergy HTX (BioTek Instruments, Inc.) microplate reader immediately after substrate addition and then again 24 h later. Linear regression analysis was used to determine rate of enzyme activity.

DNA extraction

Sediment samples were homogenized by hand and a subsample (~ 1 g) was wet sieved through a 2-mm sieve into a 2-mL microcentrifuge tube, centrifuged to decant water, and stored within 24 h of field collection at − 80°C until extraction. DNA was subsequently extracted from 0.25 g of sediment following DNeasy Powersoil kit (Qiagen) protocol and stored at −80°C until qualitative polymerase chain reaction (qPCR) analysis. DNA purity and concentration were quantified by absorbance using a BioTek Synergy HTX (BioTek Instruments, Inc.) microplate reader.

qPCR analysis

qPCR was used to determine changes in abundances of genes related to all bacteria (16S), archaea (16S), and sulfate reduction (*dsrA*) (Table 1). All assays had a total reaction volume of 20 μL, with primer concentrations of 0.5 μM, and contained 3 ng of DNA per reaction. Triplicate reactions were run for each sample and assays were created using Power Up Sybr Green Master Mix (Applied Biosystems) on a Bio-Rad CFX96 Real-Time System. Thermal cycling conditions for all genes were: 2 min at 50°C, 2 min at 95°C, and 40 cycles of 95°C for 15 s and 60°C for 1 min (Applied Biosystems, doc. no. 100031508, Rev. C). Results are reported as number of gene copies per g of sediment. The 9-month sampling was excluded from community analysis due to time constraints, resulting in four sampling points for microbial communities.

Statistical analysis

Statistical analyses were performed in R (R Foundation for Statistical Computing) in R Studio (Rstudio, Inc. version 3.6.3). Data for each parameter (i.e., oyster density, sediment bulk density (BD), DOC, OM, TC, IC, OC, potential CO_2 and CH_4 production, enzyme activities, and gene abundance) were evaluated for normality and homogeneity of variance using the Shapiro–Wilk test and the Levene test, respectively. Non-normal data were transformed using a logarithmic transformation. Data were analyzed via a linear model (package 'lme4') to determine the effects of the interaction between treatment and time, as well as the individual reef effect. Least-square mean post hoc tests (package 'emmeans') were used to determine differences among treatment groups (live, degraded, and restored) and time since restoration. Due to the number of model iterations performed, a Bonferroni correction was applied to the initial alpha value of 0.05, thus reducing the likelihood of Type I error and simultaneously lowering the p value to reject the null hypothesis to 0.003 (Bonferroni 1936). Correlation matrices were generated using Pearson product–moment correlations with a critical value of 0.164 and an alpha value of 0.05. Data are presented as an average ± 1 standard error (SE).

Results

Reef biophysical properties

Moisture content (MC) of reef sediment significant differed by treatment ($p < 0.001$; see Supporting Information Table S1 for full statistical results); MC was highest in natural reefs and lowest in degraded reefs, but restored reefs changed the most over time with a 32% increase. BD also significantly differed over time and treatment ($p < 0.001$) and demonstrated the opposite trend of MC. The interaction between time and treatment was significant with oyster density, which was greatest in natural sites at all times but increased the most in restored sites over the first year until it reached natural reef levels by 12 months; oyster density on degraded reefs did not change (Fig. 2a).

Sediment carbon pools

TC was significantly different between treatments, with restored reefs containing the greatest average TC content for the study year ($p < 0.001$; Fig. 2b). TC was well correlated to OM, DOC, OC, IC, CH_4, β-gluc, Phos, MC, archaea, and DNA concentration (Table 2). IC differed significantly with the interaction between treatment and time ($p < 0.001$) and was greatest in restored sites and lowest in natural sites (Fig. 2c), though restored sites experienced the most change throughout the year, with a 27% decrease. IC was positively correlated to TC, β-gluc, and Phos, but negatively related to OC and dsrA. OC was also significant with treatment and time ($p < 0.001$ and $p < 0.001$), though the interaction between the two was not significantly different ($p = 0.357$). OC was highest in natural sites and lowest in degraded sites, but again, restored sites changed the most over

Table 1. Forward and reverse primers for each functional and taxonomic gene measured, including annealing temperatures and standard bacterium used in qPCR analysis.

Functional/ taxonomic group	Gene	Amplicon length (bp)	Forward/ reverse primer	Annealing temperature (°C)	Standard bacterium	References
Archaea	16S RNA	140	Arch967F/Arch-1060R	60	*Methanococcus voltae* (ATCC BAA-1334D-5)	Karlsson et al. (2012), Morrissey et al. (2014)
Bacteria	16S RNA	200	Eub338/Eub518	60	*Bacillus subtillus* (Carolina Biological Supply 154921)	Fierer et al. (2005)
Sulfate reduction	dsrA	370	dsrA_290F/ dsra_660R	60	*Desulfobacterium autotrophicum* (ATCC 43914D-5)	Pereyra et al. (2010)

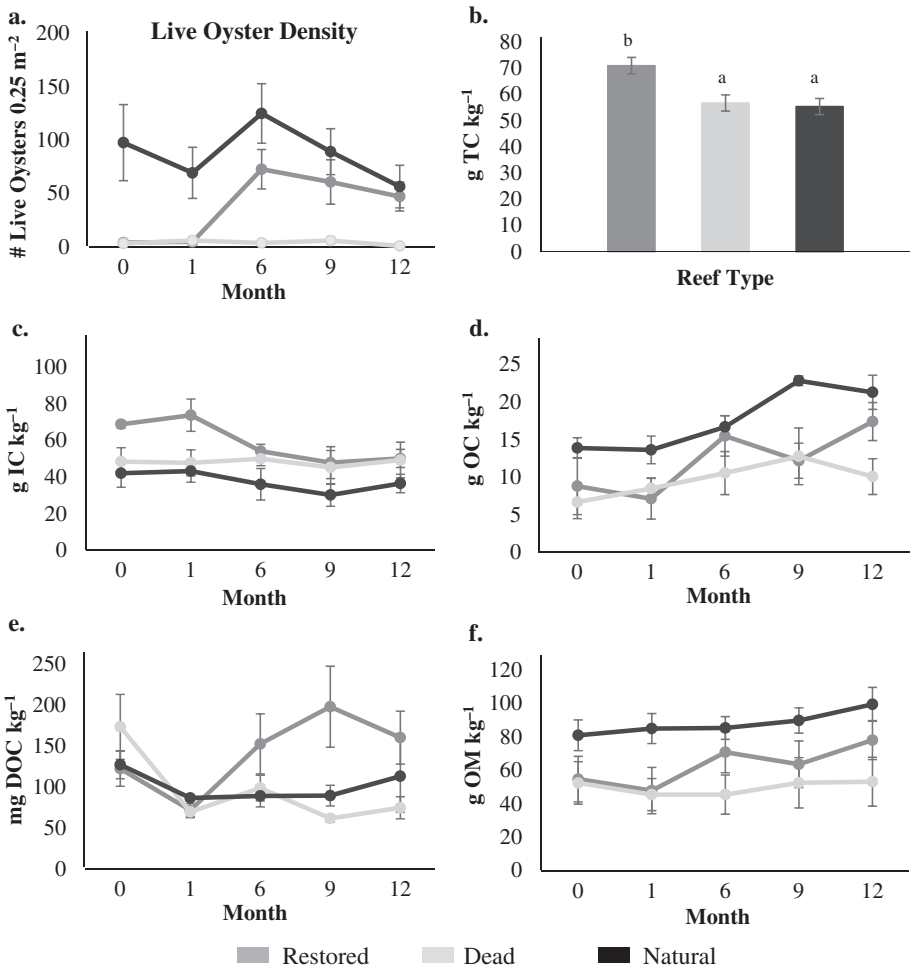

Fig. 2. (**a**) Oyster live density, (**b**) TC, (**c**) IC, (**d**) OC, (**e**) DOC, and (**f**) OM. Data are means and standard error. Bars represent ± 1 SE. Different letters in (**b**) indicate statistically significant differences between sites.

time with an average 97% increase in OC by 12 months (Fig. 2d). OC was positively correlated to MC, OM, TC, DOC, CH_4, oyster density, all microbial gene abundances, and DNA concentration, but negatively correlated with IC. DOC concentrations varied, both over time and between treatments, but restored site DOC exceeded concentrations measured in natural and degraded sites by Month 9 ($p < 0.001$; Fig. 2e). DOC differed with the interaction between treatment and time ($p < 0.001$), and was positively related to OM, MC, TC, OC, CH_4, oyster density, Phos, archaea, and DNA concentration. OM was significantly different between treatments ($p < 0.001$) was greatest in natural sites and lowest in degraded sites, but increased the most throughout the year in restored sites (43%; Fig. 2f). OM was well correlated to every other variable excluding CO_2, NAG, and IC.

Arch., archaea; Bac., bacteria; Oys. Den., oyster density.

Microbial community characterization

Archaea concentrations in reef sediments did not significantly change throughout the study year when applying a Bonferroni corrected alpha-value ($p = 0.063$), but differed by treatment ($p < 0.001$); natural sites had greater Archaea concentrations than degraded sites while restored reefs were of intermediate concentration (Fig. 3a–d). Bacteria concentrations were significant with both time and treatment ($p < 0.001$ and $p < 0.001$), with highest concentrations within the live sites. Concentrations of DNA, archaea, and bacteria were all positively correlated with 9 or 10 of other parameters measured, but not CO_2 production (Table 2). Natural sites had the greatest abundances of dsrA compared to both restored and degraded sites for the study year ($p < 0.001$; Fig. 3d), though restored sites increased to near natural levels around 6 months.

Carbon cycling

The CO_2 production rate differed significantly with the interaction between treatment and time ($p = 0.002$). Restored sites exhibited the greatest increase in CO_2 production throughout the year (707%; Fig. 4a). CO_2 production was

Table 2. Correlation matrix for each measured parameter (*r* values). Bold values indicate a positive correlation, italicized values indicate a negative correlation. Only significant *r* values are shown ($n = 144$, df = 142, alpha = 0.05, critical value = 0.164).

	CO_2	CH_4	β-gluc	NAG	Phos	BD	MC	TC	DOC	OC	IC	Oys.Den.	Bac.	Arch.	dsrA	DNA
CH_4	—															
β-gluc	**0.173**	—														
NAG	—	**0.466**	—													
Phos	—	**0.218**	**0.197**	**0.357**												
BD	—	*−0.255*	*−0.165*	*−0.222*	*−0.361*											
MC	—	**0.209**	**0.179**	**0.192**	**0.309**	*−0.762*										
TC	—	**0.170**	**0.199**	—	**0.436**	*−0.391*	**0.196**									
DOC	—	**0.295**	—	—	**0.212**	*−0.259*	**0.304**	**0.208**								
OC	—	**0.189**	—	—	—	*−0.437*	**0.591**	**0.184**	**0.218**							
IC	—	—	**0.176**	—	**0.464**	*−0.204*	—	**0.780**	—	*−0.276*						
Oys. Den.	—	—	—	—	—	*−0.369*	**0.503**	—	**0.234**	**0.354**	—					
Bac.	—	**0.230**	**0.164**	—	—	*−0.427*	**0.507**	—	—	**0.367**	—	**0.414**				
Arch.	*−0.173*	—	—	—	**0.190**	*−0.422*	**0.576**	**0.169**	**0.185**	**0.355**	—	**0.446**	**0.422**			
dsrA	—	—	—	—	—	*−0.398*	**0.551**	—	—	**0.409**	*−0.234*	**0.537**	**0.732**	**0.596**		
DNA	—	**0.312**	—	**0.257**	**0.293**	*−0.496*	**0.543**	**0.168**	**0.179**	**0.328**	—	**0.375**	**0.751**	**0.501**	**0.643**	
OM	—	**0.300**	**0.191**	—	**0.324**	*−0.661*	**0.844**	**0.275**	**0.316**	**0.522**	—	**0.524**	**0.488**	**0.620**	**0.479**	**0.531**

positively correlated to β-gluc and negatively correlated with archaea (Table 2). Methane production significantly differed with both treatment and time individually, ($p < 0.001$ and $p < 0.001$) and was greatest in restored sites and lowest in natural sites (Fig. 4b). Methane was positively correlated to all other parameters excluding IC, oyster density, archaea, dsrA, and CO_2, where there were no correlations (Table 2).

β-Gluc enzyme activity was widely variable over time and between treatments, but by Month 12, restored sites had the greatest activity and it was well correlated with several other parameters ($p < 0.001$; Fig. 4c; Table 2). NAG activity was significantly different with treatment ($p < 0.001$), time ($p < 0.001$), and the interaction between the two ($p < 0.001$, Fig. 4d). Phosphatase enzyme activity also differed between times ($p < 0.001$), reefs ($p < 0.001$), and the interaction between treatment and time ($p = 0.002$, Fig. 4e).

Discussion

Sediment carbon as an indicator of reef development

The influence of restoration on reef C dynamics was most evident from changes in sediment OM, OC, IC, and CO_2 production. Although increases in OM were expected in all reefs from oyster biodeposition and sedimentation, OM increased by an average of 43% on restored reefs, nearly double the increase observed on natural reefs during the same time (Fig. 2f). The large increase in OM in restored reefs mirrors a rapid increase in oyster density. Although TC content did not significantly change over time in any reef, the relative contributions of the OC and IC changed, most notably in restored reefs. Specifically, IC decreased by 27% while OC increased by 97% on restored reefs, changing the OC:IC ratio from 7.8 (at Month 0) to 2.9 (at Month 12). This represents a substantial change in nature of the sediment C pool on recently restored reefs, brings the OC : IC ratio down to a value comparable to that of natural reefs (mean OC : IC = 2.3 for natural reefs) by the end of the first year (Fig. 2c,d). DOC concentrations in restored sites also increased significantly over time, with higher amounts than either degraded or natural sites by 9 months (Fig. 2e). Young oysters (< 12 months) have been shown to produce biodeposits with a greater concentration of DOC than those from mature oysters (Locher et al. 2021), which is believed to enhance OC cycling in restored reefs. The positive relationship of OM, OC, and DOC with oyster density supports the critical role of oyster biodeposition on reef OC content. In addition, OC was positively related to all microbial gene abundances and DNA concentration, implying OC, and not TC alone, is a significant indicator of reef development.

Restored sites had the greatest increase in CO_2 production during the first year and, despite having the lowest initial rates, had the highest average rates of production by the end of the year. Previous studies demonstrate a strong relationship between soil OC content and CO_2 production (Woodwell 1984; Ho et al. 2018; Steinmuller et al. 2019), but

that trend was not observed in this study. Though not significantly, restored reefs exhibited more CO_2 respiration than natural reefs, which was unexpected. However, it could be the result of labile C input from young oyster biodeposition, as supported by a concomitant increase in β-gluc activity (Fig. 4c). Although there was an increased availability of OC in restored reefs, there was no notable difference in CO_2 production between the reef treatments at any time, presumably due to the high degree of variability within each treatment.

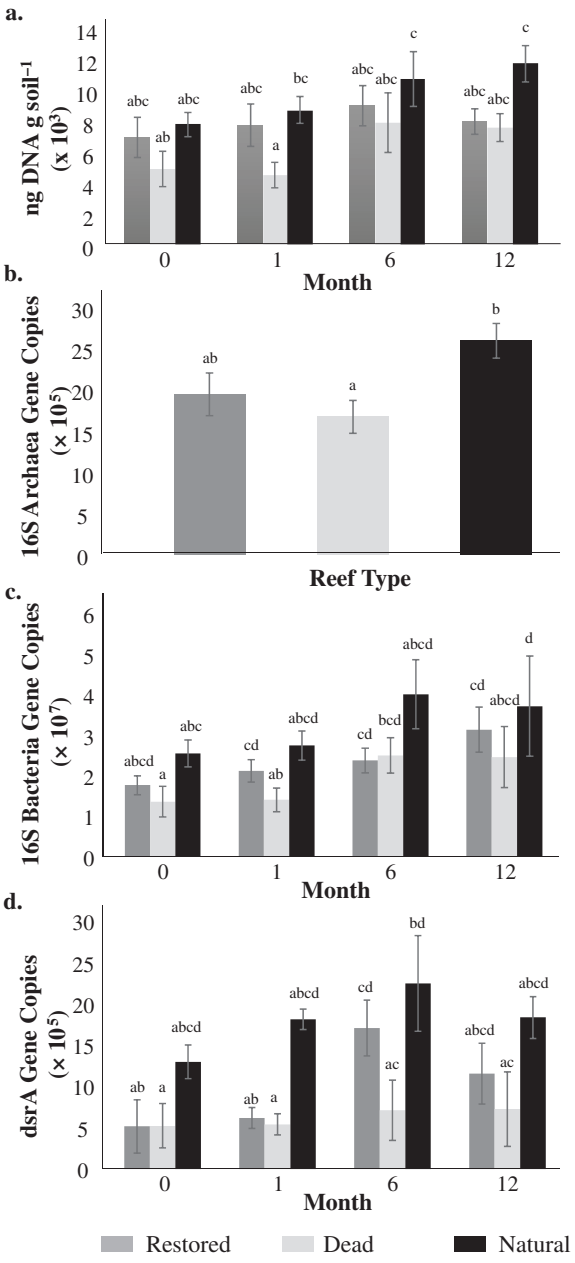

Fig. 3. (**a**) Sediment DNA concentration and (**b**–**d**) gene copy numbers. Data are means and standard errors, different letters represent significant differences based on least-square mean post hoc tests.

This study attempted to capture the immediate impacts of restoration, so only the top 5 cm of reef sediment was measured. However, relatively greater proportions of OC may be located below 10 cm in reef sediment (Fodrie et al. 2017). Other intertidal reefs evaluated elsewhere served as net C sources due to rapid reef accretion that inhibited shell dissolution (Fodrie et al. 2017). The reef depths used in this study, while appropriate for studying restoration effects, cannot effectively capture C source vs. sink as it would omit OC stores buried at depth (Fodrie et al. 2017). However, previous studies suggest long-term increases in TC pools as restored reefs mature, indicating immediate increases in OC could eventually translate into long-term storages of TC (Chambers et al. 2018). Utilizing OC concentrations, BD from Locher et al. (2021), and the 5 cm increment of sample collected at the soil surface, restored, natural, and degraded sites contained 870, 850, and 600 kg OC ha^{-1}, respectively, at the end of the 12-month sampling. As restored reefs held the most OC in surface sediments by the end of the study year, results suggest restoration and oyster presence can rapidly enhance OC storage and can help offset C flux from high calcification rates on young oyster reefs. Future studies can leverage results from this study to understand the impact of increased OC burial on net C stores on oyster reefs.

Microbial community as an indicator of reef development

Enzyme activity was measured to understand nutrient supply and demand for the microbial community in oyster reef sediments. β-Gluc activity was tightly coupled with the other enzymes measured and CO_2 (Table 2). In fact, β-gluc was the only parameter with a positive correlation to CO_2, suggesting labile C availability may regulate potential sediment respiration on these reefs. A notable increase in β-gluc (a constitutive enzyme) activity in restored sites by 12 months, in addition to an increase in CO_2 production, suggests reefs are experiencing increased rates of mineralization and microbial activity (Hoellein and Zarnoch 2014; Fig. 4c; Table 2). The high rates of β-gluc activity observed at 12 months in this study may persist in the long-term, as Chambers et al. (2018) also found significant increases in β-gluc activity in 1- to 7-yr-old reefs compared to degraded reefs.

NAG enzyme activity in restored sites remained relatively constant over time, excluding the large increases in all reefs at Month 9. NAG was well correlated to the other enzymes measured, but not to any extractable nutrients, contradictory to other studies where NAG activity co-occurred with an increase in extractable NH_4^+ (Steinmuller et al. 2020). Locher et al. (2021) reported rapid increases in extractable NH_4^+ in restored reefs within 1 month, but we did not see a concomitant increase in NAG activity. As NAG is a constitutive enzyme, an increase in NH_4^+ would be expected to be accompanied by an increase in NAG activity. Varying and changing N concentrations with no impact on NAG activity could imply the increase in N availability is from direct oyster release, not microbial

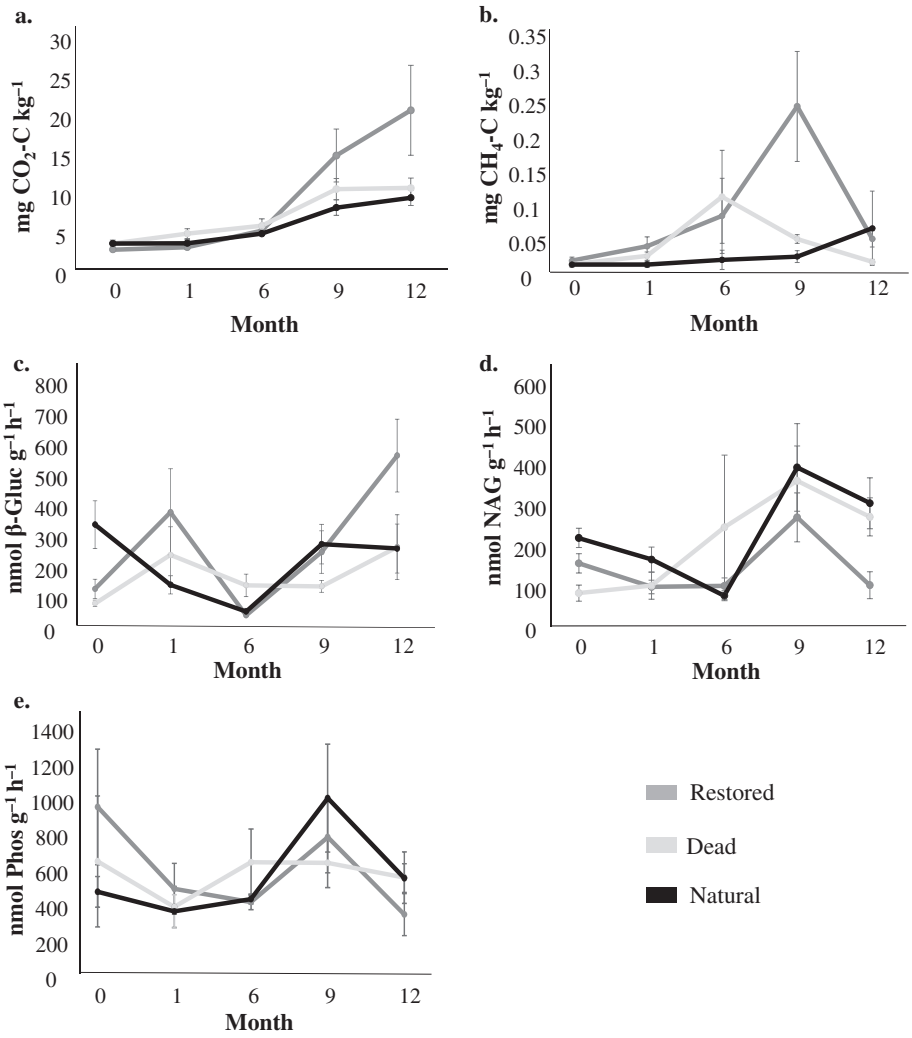

Fig. 4. Sediment (**a**) potential respiration, (**b**) potential methanogenesis, (**c**) β-gluc, (**d**) NAG, (**e**) Phos. Data are means and standard errors.

mineralization. Previous studies show oyster reefs release relatively large amounts of N as NH_4^+ back into the water column, with one study finding that 66% of total N taken up by reefs was eventually released as NH_4^+ (Dame et al. 1985; Dame et al. 1989). The persistent availability of NH_4^+ would result in stable sediment NAG concentrations and indicate N is not a limiting nutrient for sediment microbes in this system.

Phosphatase activity, in contrast to β-gluc, generally decreased in restored sites immediately after restoration and remained low thereafter (Fig. 4e). As an inducible enzyme (Dick 2015), this would suggest a decreasing demand (increasing availability) of phosphate on restored reefs. The raking down of reefs to intertidal height and reworking of reef sediment could have provided initial pulses of P to restored reefs and depressed rates of Phos activity. However, there were no corresponding increases in sediment P as we expected, suggesting lowered rates of Phos activity are a result of P from another source and are not directly attributed to sediment P (Locher et al. 2021).

In terms of the microbial community data, there were generally no differences between restored sites and natural or degraded sites. The exceptions to this rule were at 6 months, when the abundance of dsrA was significantly higher in natural sites than degraded sites, and at 1 month, when bacterial abundances were exclusively different between the restored and degraded site (Fig. 3d). The lack of differences does not necessarily suggest that the microbial function was equivalent between the three treatments, but perhaps may highlight the issue of microbial viability. Given that not all microbial DNA evaluated is viable (i.e., derived from intact cells), relict DNA abundance can potentially mask changes in total microbial community data (Nocker et al. 2007; Carini et al. 2016). Thus, the long-term restoration impacts on microbial communities in these sediments may be obfuscated by the impacts of relict

DNA persistence within degraded reefs. The short-term increase in dsrA gene abundance after 6 months does indicate an increase in microbial respiration via sulfate respiration within the restored reefs, but the impermanence of the trend suggests a high degree of variability, perhaps through seasonal changes. However, oyster density was most strongly correlated with dsrA out of all other measurements (Table 2), inversely correlated to IC, and strongly positively related to OC, all of which suggests its abundance is also indicative of sites with more OC (which is positively related to oyster density; Table 2). Consequently, dsrA, out of three genes within this study, may be the best indicator for sediment microbial community development, provided efforts are taken to minimize variability within the dataset and account for the potential masking effects of relict DNA. The lack of trends observed in microbial community data may also indicate that while these measurements demonstrate the resiliency and ease of restoring microbes within oyster reefs, they struggle to elucidate reef development due to high variability. Changes in OM and C cycling are better indicators of reef development as they change most abundantly following restoration.

Restoration effects on carbon and microbes

An initial hypothesis of this study was that degraded and natural sites would be significantly different from one another for almost all measured parameters. Although degraded sites did have lower oyster density and less abundant microbial communities than natural sites, many biogeochemical properties were similar among treatments. Specifically, degraded sites often maintained similar levels of C concentrations (TC, DOC, CO_2, and CH_4) and enzyme activities to natural sites throughout the study, which was unexpected. Therefore, results from this study did not indicate the microbial (and associated biogeochemical) properties of degraded and natural oyster reef sediment are inherently different. On the contrary, their biogeochemical properties may simply represent a background level for lagoonal sediments, with microbially mediated processes becoming rapidly reinvigorated following restoration. The stimulation of microbial and biogeochemical processes with restoration starts with the physical disturbance of the restoration process (e.g., raking down the elevation), redistributing sediment and allowing reef surface sediment to interact with nutrients from the water column, and is followed by the biodeposition of labile nutrients as oysters begin to recruit on the reef. It is also important to consider the role of the broader coastal food web in influencing reef biogeochemistry. For example, data suggest natural and restored reefs in our study region are often utilized by birds as a food source, while birds are more often observed loafing (or idling) on degraded reefs for long periods of time (Shaffer et al. 2019; Copertino 2021). Observed loafing on degraded reefs could be the result of these reefs having increased elevation and access during high tide events or a lack of foraging capacity, which leaves loafing as the only available option. Regardless of the mechanism, deposition of nutrient-rich avian feces (Portnoy 1990) during bird loafing may help sustain an active microbial pool on degraded reefs, causing their biogeochemical properties to diverge less than expected from live reefs.

Several analyses displayed high levels of variability within treatments (particularly enzyme activity), contributing to some of the lack of significant differences observed between treatment groups. Many microbial indicators are inherently variable over temporal and spatial scales, which is why repeated measurements may often be necessary (Franklin and Mills 2003; Piotrowska-Długosz et al. 2016; Piotrowska-Długosz et al. 2019). Care was taken in this study to choose reefs that offered both spatial representation and treatment replication. However, Locher et al. (2021), which focused on the same reefs as this study, found the unique characteristics of individual degraded and restored reefs caused significant differences within treatments that was greater than the differences observed between treatments. Due to the uniqueness of every reef (e.g., geophysical properties, flow dynamics, site history, etc.) and the variability of microbial data, microbial community analysis may be an approach better suited for understanding how reefs function within a larger ecological context than a metric for measuring restoration success. Alternatively, this study demonstrates that sediment OC content and its associated metrics (OM, DOC, OC, and CO_2) are strong, early indicators of reef development, as all these variables displayed direct increases with reef age after restoration.

Although live oyster density is often considered the driving factor influencing sediment biogeochemistry and microbial communities in oyster reefs, this study indicates microbial pools related to OC cycling and mineralization are resilient and remain on reefs long after they have been degraded. As stated previously, sediment microbial community abundances varied little across sites, indicating these communities are robust, even in degraded reefs. Although natural and degraded sites displayed changes throughout the year as expected, restored sites exhibited the most changes. The sudden and rapid influx of labile C, N, and P to newly restored reefs, primarily from oyster biodeposition, can account for the increased biogeochemical cycling, microbial activity, and C dynamics during the first year following restoration, as has been previously described (Chambers et al. 2018). Oysters appear to demonstrate a top-down control on reef biogeochemical cycling, where oyster presence increases OM accumulation from biodeposition and sedimentation, which increases OC content, and then supports enhanced microbial community abundance, activity, and nutrient cycling. At the end of the study year, restored sites retained the greatest concentrations of total OC in surface sediments, highlighting the rapid recovery of C cycling after restoration. This study suggests that sediment OC content and its associated metrics (OM, DOC, OC, and CO_2) are strong, early indicators of reef development, as all these variables displayed direct increases

with reef age after restoration. Study results demonstrate the contribution of oyster reef restoration to enhancing OC storage in reef sediment and help further the understanding of oyster reef significance in the global C cycle.

Data Availability Statement

Data will be made accessible through UCF's Showcase of Text, Archives, Research and Scholarship (STARS; https://stars.library.ucf.edu/) within 1 year of publication and are also accessible by contacting the corresponding author directly.

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Conflict of Interest

None declared.

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