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Vulnerability of seagrass blue carbon to microbial attack following exposure to warming and oxygen

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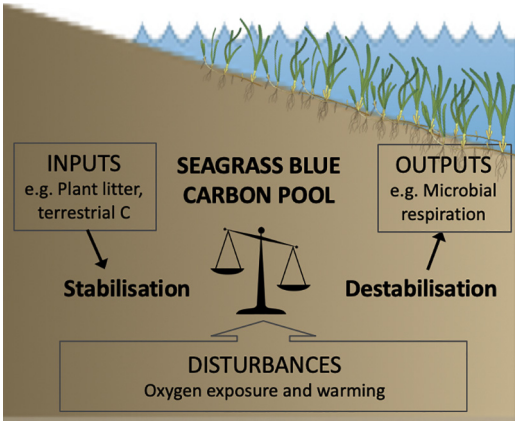
HIGHLIGHTS

- Seagrass ecosystems store globally-significant amounts of blue carbon
- Mechanisms underpinning blue carbon destabilisation are largely unknown
- Exposure of blue carbon to oxygen triggered microbial remineralization
- Sediment carbon turnover increased 34–38-fold following oxygen exposure
- Activities that expose seagrass sediments to oxygen threaten blue carbon stability

GRAPHICAL ABSTRACT



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ABSTRACT

Seagrass meadows store globally-significant quantities of organic 'blue' carbon. These blue carbon stocks are potentially vulnerable to anthropogenic stressors (e.g. coastal development, climate change). Here, we tested the impact of oxygen exposure and warming (major consequences of human disturbance) on rates of microbial carbon break-down in seagrass sediments. Active microbes occurred throughout seagrass sediment profiles, but deep, ancient sediments (~5000 yrs. old) contained only 3% of the abundance of active microbes as young, surface sediments (<2 yrs. old). Metagenomic analysis revealed that microbial community structure and function changed with depth, with a shift from proteobacteria and high levels of genes involved in sulfur cycling in the near surface samples, to a higher proportion of firmicutes and euraracheota and genes involved in methanogenesis at depth. Ancient carbon consisted almost entirely (97%) of carbon considered 'thermally recalcitrant', and therefore presumably inaccessible to microbial attack. Experimental warming had little impact on carbon; however, exposure of ancient sediments to oxygen increased microbial abundance, carbon uptake and

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Blue carbon
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Emissions

sediment carbon turnover (34–38 fold). Overall, this study provides detailed characterization of seagrass blue carbon (chemical stability, age, associated microbes) and suggests that environmental disturbances that expose coastal sediments to oxygen (e.g. dredging) have the capacity to diminish seagrass sediment carbon stocks by facilitating microbial remineralisation.

1. Introduction

'Blue carbon' ecosystems, which include seagrass meadows, tidal marshes and mangrove forests, hold large reservoirs of organic carbon (McLeod et al., 2011). Despite occupying <1% of the seafloor, blue carbon ecosystems contribute half of all organic carbon (OC) sequestered through burial in the oceans (Duarte et al., 2013). Blue carbon ecosystems have the ability to capture CO_2-OC in soil, where it can remain locked away for millennia; thereby acting as natural sinks of carbon, effectively reducing atmospheric CO_2 concentrations that contribute to global warming (McLeod et al., 2011). The disproportionally large influence of blue carbon ecosystems on the global carbon cycle means that altering the stock of carbon in their soils will likely influence global greenhouse gas budgets.

Despite the impact of blue carbon, we know very little about the mechanisms responsible for the retention of carbon in blue carbon environments. For example, when existing blue carbon ecosystems are disturbed (e.g. via coastal development, climate change), little is known to what extent is stored OC broken down and released into the atmosphere, and whether this varies in response to environmental and edaphic conditions, or when management actions result in new lands coming under tidal influence at what rate is carbon sequestered. Knowledge of these mechanisms is critical in understanding the dynamics of blue carbon cycling and informing such conservation management issues as: identifying coasts under threat of blue carbon loss, quantifying the impact/outcomes of blue carbon loss, identifying where enhanced blue carbon sequestration may be possible and developing blue carbon markets. Due to lack of robust data on the fate of blue carbon following disturbance, the scientific community has relied on risk assessment approaches and expert solicitation until this knowledge gap is filled (Lovelock et al., 2017).

A key aspect in the success of blue carbon ecosystems as natural carbon sinks is their ability to accumulate OC that resists or evades microbial attack (Macreadie et al., 2017). Microbes control key biogeochemical pathways, therefore changes in microbial activity and community structure could affect the stability of blue carbon (Kearns et al., 2016). According to the terrestrial soil literature, which is much more advanced in its understanding of the mechanisms underpinning the stabilisation or destabilisation of OC (Sollins et al., 1996), there are four key pathways in which carbon might remain preserved within blue carbon environments and evade breakdown (remineralisation) by microbes: intrinsic chemical stability (Baldock et al., 2004); physical mechanisms of protection (e.g. adsorption or binding to minerals) (Torn et al., 1997); the absence of *in situ* microbial communities with the capacity to produce enzymes for degradation to occur (Arnosti, 2004); and environmental conditions hostile to microbial activity (Bianchi, 2011). Much research effort has focused on intrinsic chemical stability, mostly from the terrestrial soil literature (Kleber, 2010; Kleber et al., 2011), but also more recently from marine environments (Trevathan-Tackett et al., 2015; Trevathan-Tackett et al., 2017a), where OC is often viewed on a spectrum of susceptibility to microbial attack, ranging from 'labile' OC, that has high susceptibility to microbial attack, to 'recalcitrant' OC, which has low susceptibility to microbial attack.

We focus on seagrass meadows, because they are thought to be facing a 'global crisis' (Orth et al., 2006), putting at risk up to 299 million tons OC yr^{-1} (Fourqurean et al., 2012). Warmer waters and oxygen exposure of seagrass sediments have already been implicated as causes of loss of blue carbon from seagrass ecosystems (e.g. Arias-Ortiz et al., 2018; Macreadie et al., 2015), but as yet our understanding of the microbial processes and mechanisms behind the observed responses is very limited and have been identified as an urgent knowledge gap for seagrass ecosystem management (Belshe et al., 2017; Macreadie et al., 2014; Trevathan-Tackett et al., 2017c). Furthermore, there have been conflicting results. For example, while some studies report increased OC remineralization due to warming (Pedersen et al., 2011), others have reported no effect. It is generally agreed that disturbances (e.g. via eutrophication, dredging, coastal development, climate change) to seagrass and other blue carbon ecosystems cause losses of OC (Macreadie et al., 2013), but what's not clear is whether the OC becomes remineralised by microbes and converted into atmospheric CO_2, or whether the OC is simply exported elsewhere and re-buried? The answer to this question is crucial for global seagrass OC budgets.

Our overarching aim of this study was to gain understanding of how blue carbon recalcitrance is achieved and the risk of seagrass blue carbon becoming destabilized in response to biotic and environmental change. More specifically, our objectives were to: 1) Characterise recalcitrant blue carbon from seagrass meadows; 2) Characterise the functional capacity of microbial communities associated with recalcitrant blue carbon from seagrass meadows; and 3) Quantify the importance of environmental controls (oxygen exposure and warming) over seagrass blue carbon recalcitrance.

2. Materials and methods

We sought to understand which of the key pathways in Table 1 may be responsible (whole or in part) for blue carbon preservation in seagrass meadows. To achieve this we performed a series of surveys and experiments to infer and test the potential vulnerability of seagrass OC to disturbance, specifically temperature increases and oxygen exposure. Warming and oxygen were selected because they are among the most common and widespread forms of disturbances to seagrass meadows; warming due to climate change, and oxygen exposure due to human activities in the coastal zone such as wetland drainage and dredging. The specific hypotheses tested and approach used are summarized in Table 1.

2.1. Study site and sample collection

Sediment cores were collected from *Zostera muelleri* seagrass meadows in Fagans Bay (33°26′04.20″ S, 151°19′19.98″ E, Fig. 1), a site situated on the east coast of Australia, characterized by high OC content. *Zostera muelleri* is among the most important Australian seagrass species with regards to OC storage capacity per unit area (Lavery et al., 2013). Full-length cores were collected using push coring techniques, as per Ewers Lewis et al. (2019) for long cores, and as per Brodersen et al. (2019) for short cores.

Full-length cores for geochemical profiling, age dating, thermogravimetric analysis (TGA), ^{13}C nuclear magnetic resonance (NMR) spectroscopy, and chemical fractionation were returned to the lab for sectioning. Cores for microbial analyses were sampled *in situ* from pre-drilled holes along the PVC core to fit 5 cc syringes as per Macreadie et al. (2015), transferred into cryovials in the field, then transported to the laboratory on ice and then stored at −80 °C. Finally, cores undergoing laboratory

Table 1
Proposed pathways and rationale to achieving blue carbon (C) recalcitrance (i.e. preservation in sediments), and hypotheses tested in this study to interrogate the proposed pathways.

Pathway	Rationale	Hypotheses (tested herein)	Approach
Intrinsic chemical stability	Microbes lack enzymes to breakdown intrinsically recalcitrant C, or the energy required to breakdown the C outweighs the energy gained	Blue C that has been preserved long-term will have a high proportion of intrinsically-stable organic C	• Thermogravimetric analyses (TGA) • Solid state-13C Nuclear Magnetic Resonance (NMR)
Physical mechanisms of protection	Binding of blue C to minerals make the C inaccessible to microbial attack	Mineral bound C will have higher amounts of old and chemically-labile organic C than bulk fractions	• Chemical fractionation to isolate C pools bound to minerals
Absence of active microbial taxa	Blue C sediments lack active microbes with capacity to break-down blue C	Microbes will be more active in surface soils (oxic sediments), but will decline with depth (increasing levels of anoxia)	• Analysis of downcore microbial activity via flow cytometry • Metagenomic sequencing to examine microbial diversity and function
Environmental conditions hostile to microbial activity	Anoxic sediments limit activity of key microbes that use oxygen as an electron receptor to metabolise blue C	Exposure of sediments (from deep layers) to oxygen and warming will trigger blue C remineralisation and microbial C turnover	• Geochemical survey of blue C core profiles (incl. C content and stable isotopes) • Experimental exposure of blue C sediments to oxygen and warming • Microsensor analyses • Microbial C uptake via radiolabelling assays

incubation were sectioned *in situ* into 'surface' (0–10 cm) and 'deep' (a 10 cm interval from a depth > 50 cm) samples using a reciprocating saw and immediately capped (within ~1 min) with PVC caps to prevent oxygen exposure.

2.2. Age dating

A chronology for the sediments at Fagans Bay was determined from ^{210}Pb profiles (sediment accumulation rates) for recent (<200 years

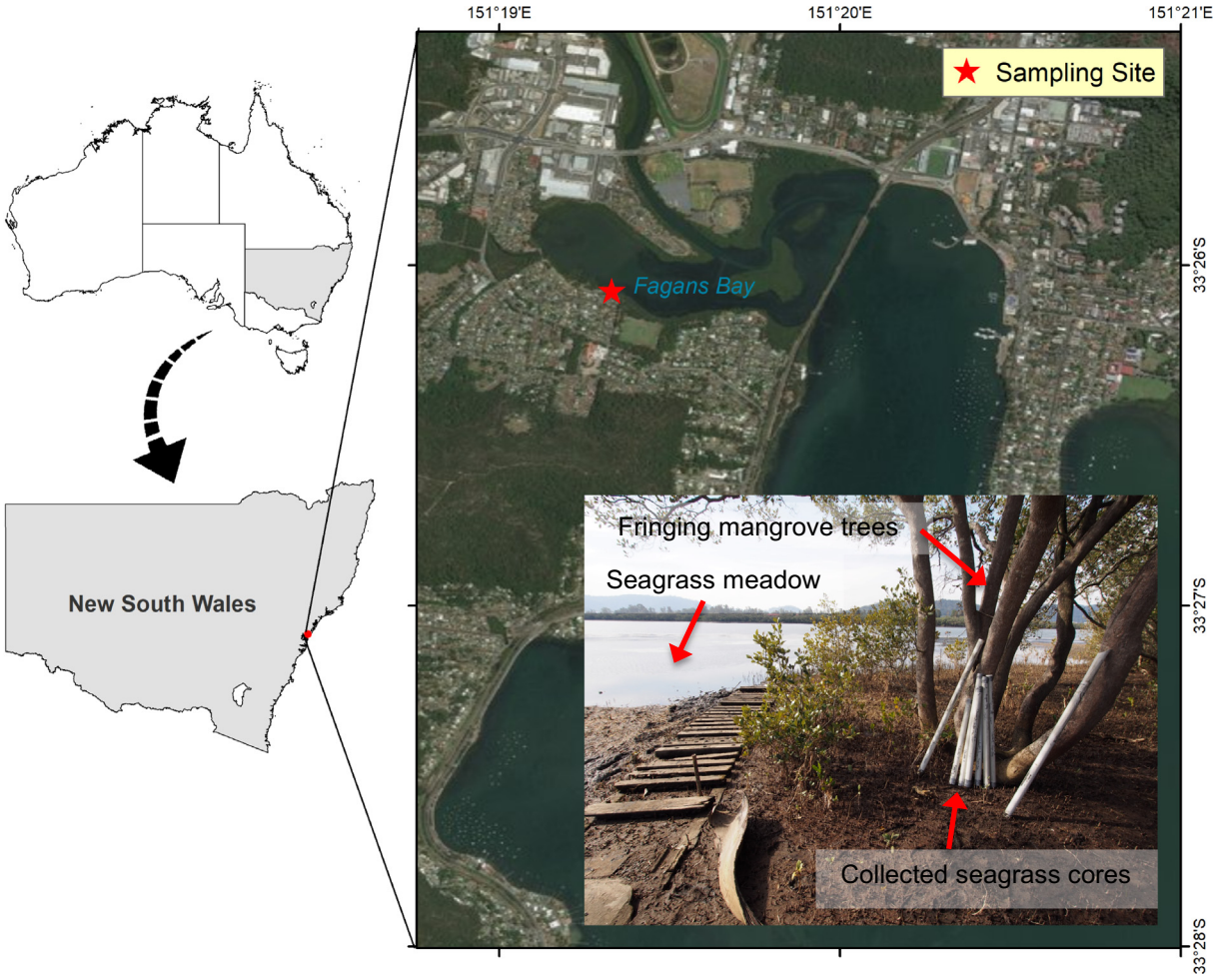

Fig. 1. The Study Site. Sediment cores were collected from a shallow, subtidal *Zostera muelleri* seagrass meadow within Fagans Bay on the east coast of New South Wales, Australia. The bay was surrounded by residential and industry developments, and fringed by an intertidal mangrove forest.

old), and radiocarbon (^{14}C) dating for older sediments (Table S1). ^{210}Pb dating was determined using alpha spectroscopy and radiocarbon (^{14}C) dating was performed using accelerator mass spectrometry via the Australian Nuclear Science and Technology Organisation (ANSTO). Prior to analysis, samples were dried, sieved through a 63 μm sieve to remove sandy and shell material prior, and then crushed. The detection limit for alpha spectrometry was 0.001 Bq/g. For ^{14}C, ages were rounded according to Stuiver and Polach (1977). Ages quoted are radiocarbon ages (not calendar ages), and no reservoir correction has been made. Sediments were aged using ^{210}Pb at eleven intervals over the top 0–20 cm; however, only the top 0–4.5 cm had enough unsupported ^{210}Pb to establish a decay profile. ^{210}Pb activities in sediments below 4.5 cm were close to background levels. For the top 0–4.5 cm, sediment ages were calculated using CIC and CRS ^{210}Pb dating models. For the CIC model, unsupported ^{210}Pb data between 1 and 3.5 cm were used to estimate a mass accumulation rate. Assuming the same mass accumulation rate throughout the upper part of the core, sediment ages between 0 and 4.5 cm were calculated. For the CRS model, all unsupported ^{210}Pb data between 0 and 4.5 cm were used in calculation of CRS model sediment ages and mass accumulation rates.

2.3. Organic carbon content determination

Measurement of OC content of sediments was performed following procedures described by Macreadie et al. (2015). In brief, sediments were extruded from cores, and the top 0–1 cm was sectioned, and then dried at 60 °C to a constant weight. Dry bulk density was calculated as the dry weight divided by the core volume. Dried samples were homogenised using a ball mill. Samples were split into two equal parts. The first set of duplicates was measured for total sediment C ($TC_{sediment}$) using a Costech 410 Elemental Analyzer. The second set of duplicates were ashed in a furnace at 500 °C for 6 h, and the inorganic content of the ash (IC_{ash}) remaining after loss on ignition (LOI) was determined using the above elemental analyzer. The IC_{ash} was scaled back to the original weight of the unashed sample using LOI to calculate the inorganic content of the original sediment ($IC_{sediment}$). We the calculated OC (expressed in units of % dry weight) as: $OC = TC - IC_{sediment}$.

2.4. Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) was used to estimate structural complexity of C within seagrass sediments by apportioning sediment C into labile (200–400 °C), recalcitrant (400–550 °C), and refractory (650–900 °C) C pools according to Capel et al. (2006). This was performed for five sediment depths: 0, 5, 25, 50, and 80 cm. For TGA, a SDT Q600 (TA instruments) with a 0.1 μg balance sensitivity was used for all samples. An aliquot of ground sample (30 mg ±2 mg) was placed in a platinum cup and heated under air (gas flow 50 mL min^{-1}), at 20 °C min^{-1} from ambient temperature up to 1000 °C (Capel et al., 2006). Universal Analysis software (TA Instruments) was used to aid the identification and quantification of specific exotherms.

2.5. Amount of carbon bound to minerals

Chemical fractionation procedures were used to separate C fractions that differ in physiochemical stability. This procedure involves oxidation of samples by disodium peroxodisulfate and mineral dissolution by hydrofluoric acid (HF). The oxidation approach is believed to imitate the natural oxidative processes of microorganisms and therefore isolate C that is completely protected from oxidative degradation (Eusterhues et al., 2003). HF dissolves silicate minerals and thus releases any low molecular weight C that is bound to mineral surfaces. Treated and untreated samples for each depth (0, 5, 25, 50, and 80 cm) underwent analysis for ^{14}C via AMS at ANSTO (as per Macreadie et al., 2015).

2.6. Solid-state nuclear magnetic resonance (NMR) spectroscopy

The chemical composition of OC in seagrass sediment core sections was determined using solid-state ^{13}C NMR spectroscopy following the protocols of Baldock et al. (2013). We used a Bruker Avance system (200 MHz) equipped with a 4.7 T wide-bore magnet with a resonance frequency of 50.33 MHz. Samples for NMR analysis were packed into a 7 mm zirconia rotor, and a standard cross polarization experiment was performed using a pulse of 3.2 μs, 195 W, 90°, a contact time of 1 ms and a recycle delay of 1 s. 20,000 scans were collected on each sample. Total signal intensity was divided into eight major chemical shift regions: 0–45 ppm (Alkyl), 45–60 ppm (N-Alkyl/Methoxyl), 60–95 ppm (O-Alkyl), 95–110 ppm (Di-OAlkyl), 110–145 ppm (Aryl), 145–165 ppm (O-Aryl), 165–190 ppm (Amide/Carboxyl), and 190–215 ppm (Ketone). To aid in the interpretation of the NMR spectra, we used a molecular mixing model (Baldock et al., 2004) that solves for the best combination of six biochemical components (carbohydrates, protein, lignin, lipids, carbonyl and charcoal).

2.7. Microbial cell abundance and activity

Flow cytometry was used for determination of microbial cell abundance and activity within seagrass sediments. 300 μL sub-samples were taken at 1 cm intervals from the sediment surface to a depth of approximately 70–80 cm. The 300 μL sediment samples were immediately mixed with 1 mL of 0.2 μm pre-filtered seawater, collected from the overlaying seawater at the time of sampling. Samples were transferred to cryotubes, fixed with glutaraldehyde (1% final concentration) and quickly frozen in liquid nitrogen. Samples were subsequently stored at −80 °C prior to flow cytometry analysis. Immediately before flow cytometric analysis, samples were quickly thawed in warm water and diluted 1:10 in TE. Samples were vortexed for 1 min, Tween 80 (2.5% final concentration) added to the Eppendorf tubes, vortexed again for 2 min, and incubated for 15 min in the fridge. A third vortex process was done for 3 min and samples were centrifuged at 800 ×*g* for 1 min. Supernatant was filtered onto 20 μm mesh in order to remove larger particles or cells and approximately 500 μL were transferred into FCM tubes (Duhamel and Jacquet, 2006). SYBR Green (at a final dilution of 1:10,000) (Invitrogen Molecular Probes USA) was added to each vial prior to the last 15 min incubation in the dark. Fluorescent microspheres (1 μm, Invitrogen Molecular Probes USA) were added as an internal reference (final concentration 3.6×10^6 microspheres mL^{-1}) before cell counting was performed using a Becton & Dickinson LSR II flow cytometer (BD Biosciences). Side-scatter and SYBR green fluorescence were used to discriminate microbial populations (Marie et al., 2000). Data was analysed using Cyflogic [version 1.2.1] flow cytometry analysis software (http://www.cyflogic.com; Perttu Terho & CyFlo Ltd) and the microbial population was separated into H-DNA (high-nucleic acid containing bacterial) and L-DNA (low-nucleic acid containing bacteria) populations according to the approach described by Lebaron et al. (2001).

2.8. Metagenome sequencing

For analysis of microbial community composition and function, extracted shotgun metagenomes were sequenced from 9 sediment samples (3 replicates each of three sediment depths: 'sub-surface' – 5-6 cm, 'middle' – 25-26 cm, 'deep' – 70-71 cm). DNA was extracted using the MO BIO PowerMax Soil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Genomic DNA concentrations were measured in Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). Metagenomes were sequenced using the Illumina HiSeq 2000 platform (100 bp paired-ends,) at the Australia Genome Research Facility Ltd. (AGRF).

Metagenomic sequences were analysed using the Meta Genome Rapid Annotation using Subsystems Technology (MG-RAST, version

3.5) (Meyer et al., 2008). Quality control was performed using DRISEE (Duplicate Read Inferred Sequencing Error Estimation) (Keegan et al., 2012) to check for Artificial Duplicate Reads (ADRs), or estimating sequence error, KMER and base computations (Gomez-Alvarez et al., 2009). A total of 1.5 to 2.3 Gbp were generated per metagenome (after quality control). Clusters of proteins were based on a 90% identity level (Edgar, 2010; Wilke et al., 2014), with the protein identification/alignment performed using BLAT (Kent, 2002) and OpenMP (Wilke et al., 2014). Metabolic assignments were annotated using the SEED subsystems database (Overbeek et al., 2005), and taxonomic identification was performed using the top BLAST hits to the SEED taxonomy. Matches with E-values of 1×10^{-5} were considered significant with a minimum alignment length of 50 bp.

2.9. Laboratory incubations

Laboratory mesocosm experiments involving cores (see 'Study site and sample collection' section for details of coring procedures, preparation, etc.) were established in 32 L rectangular tanks at the University of Technology Sydney to test two types of disturbance – warming and oxygen exposure. To inform what temperatures to use for warming treatments, we deployed temperature loggers (HOBO Pendant) at the field site where cores were collected. Loggers were deployed at five depths below the sediment surface: 0, 20, 40, 60 and 80 cm. The loggers recorded temperature every hour for 6 months, which covered the duration of the austral spring and summer. Average temperatures of sediments (0–80 cm below the sediment surface, Fig. S1) were 21.4 °C in spring and 25.3 °C in summer. A peak temperature of 31.8 °C for surface sediments occurred on 26 February 2014. Air temperatures over adjacent land that day peaked at 37.2 °C. Sediment temperatures over a depth range of 0–80 cm below the sediment surface varied relatively little; 0.75 °C during spring and 1.12 °C during summer.

Based on logger data gathered from the field site, and IPCC projections (IPCC 2007), the effects of warming were tested at 26 °C (ambient - average summer conditions), 29 °C (ambient 3 °C), 32 °C (ambient 6 °C), 35 °C (ambient 9 °C). Temperature treatments were established with aquarium heaters (accuracy ±0.5 °C) and monitored 3-times per week. Oxygen treatments were established at ambient temperature, with oxygen exposure manipulated by either removing the top PVC cap from cores ('oxic' treatment, 100% oxygen saturated) or leaving caps on ('anoxic' treatment; 0% oxygen saturated). Removing caps exposed surface sediments to oxygenated (100% saturated) seawater within mesocosms. Seawater was replaced daily.

Effects of temperature and oxygen were monitored at four time points: 0, 7, 30, and 90 days. Detection of remineralisation was performed via analysis of a decline in sediment OC content as a percentage. Unique cores were used for each sampling time ($n = 3$) to avoid repeated measures artifacts. In addition, due the destructive nature of some of the sampling techniques, separate cores were used for microbial and geochemical analyses. For geochemical analyses, all samples were dried at 50 °C prior to analysis.

2.10. Microsensor profiling

Electrochemical microsensor profiling (O_2, H_2S and pH) was employed to characterise the chemical environment within the top 0–3 cm of three replicate surface and deep sediment cores. Prior to profiling, each core was submerged in a small (2 L) tank of seawater that was positioned in a water bath at a constant 26 °C. The water in the tank was continuously aerated via an air-stone connected to an air pump and stirring of the water column was achieved with a small water pump attached to the tank wall and adjusted to a height that allowed for sufficient water flow across the sediment surface (clear and continuous movement of organic particles on the sediment surface but no movement of sand particles). Each core was incubated in the water bath for at least 20 min at constant flow before profiling in order to ensure steady state profiles in the sediment.

Oxygen, H_2S and pH profiles were measured in individual cores with Clark type O_2 (Revsbech, 1989) and H_2S (Jeroschewski et al., 1996) microsensors (Ø = 100 μm, 90% response time < 8 and 6 s, respectively, stirring sensitivity <1%) and a pH minisensor (Ø = 500 μm, 90% response time < 10 s) (Unisense A/S, Denmark) with an external standard 2 mm reference electrode (Ionode LLC, Australia). All three sensors were connected to a multimeter (Unisense A/S, Denmark), which in turn was connected to a laptop computer where the acquired signal was logged using dedicated software (SensorTrace Pro v.3.1.1, Unisense A/S, Denmark). Each microsensor was linearly calibrated (O_2: 0% and 100% saturation, H_2S: 0, 50, 100, 250 μM, pH: 4, 7 and 10) at experimental temperature and salinity before profiling commenced (further information on the calibration procedures is provided by the manufacturer: www.unisense.com). For each profile, the tip of the respective microsensor was positioned at the sediment surface (defined as 0 μm depth) with a micromanipulator, guided by observations through a stereo-microscope attached to a reticulating arm. Measurements started at a set distance from the sediment and extended step-wise (100 μm for O_2 profiles and 500 or 1000 μm for H_2S and pH, respectively) towards and into the sediment via an automated micro-profiler stepper motor (Unisense A/S, Denmark) controlled through the logger software. Three replicate profiles were performed with each sensor at random locations in each core while avoiding the edges of the PVC tubing to limit edge effects and areas of complex flow. Finally, the three profiles within each core were averaged to produce one replicate profile for each replicate core per sensor.

Flux of O_2 and total sulfide (S^{-2}_{tot}) was calculated via Fick's first law based on the slope of the linear change in concentration with depth:

$$f = \phi * D_s * \frac{dC}{dT}$$

where f is the flux (nmol cm^{-2} s^{-1}) of the compound, ϕ (arbitrary unit) is the porosity of the medium (water or sediment), D_s (cm^2 s^{-1}) is the temperature and salinity dependent diffusivity of the respective molecule in water and dC (nmol cm^{-3}) is the change in concentration over the distance dT (cm). Specifically, O_2 flux was calculated from the slope of the linear change in concentration in the boundary layer just above the sediment surface (Jørgensen and Revsbech, 1985), with $\phi = 1$ (water) and $D_{s,O2} = 2.29 * 10^{-5}$ cm^2 s^{-1} at 26 °C and a salinity of 35 ppt (value obtained from Unisense gas table; Ramsing and Gundersen). The flux of total sulfide was calculated from the bottom linear segment of the concentration profile in the sediment, with $\phi = 0.95$ in surface cores and 0.6 in deep cores and the diffusivity of H_2S calculated as $D_{s,H2S} = 0.7573 * D_{s,O2} = 1.73 * 10^{-5}$ cm^2 s^{-1} (described in Unisense gas table). As the H_2S microsensor only detects the H_2S component of the total sulfide pool (defined as $S^{-2}_{tot} = [H_2S]$ $[HS^-]$ $[S^{2-}]$), the speciation of which is directly related to pH (Jeroschewski et al., 1996), the total sulfide concentration was calculated for each H_2S measurement as described in Jeroschewski et al. (1996) based on the pH measured in the same depth. Calculations were done based on the averaged profiles ($n = 3$) from each core, and the presented fluxes are the average (±SE, $n = 3$) of the three replicate cores.

2.11. Bacterial production measured using radiolabelling

Bacterial production was measured in the surface and deep layers of sediment exposed to two levels of warming treatments (26 °C and 32 °C), and two treatments in regards to oxygen (oxygen-exposure and non oxygen-exposure). Rates of bacterial production were quantified by incorporation of ^{3}H-leucine into protein fraction, according to Buesing and Gessner (2003). A total volume of 1 mL of

each sample (500 μL of sediment plus 500 μL of local pre-filtered water in 0.2 μm filter) was incubated for 1 h with 50 μL of 1 mM unlabelled leucine and 1.5 μL of L-^{3}H-leucine (Perkin Elmer, 161 Ci mmol^{-1}) of 4.44–7.03 TBq mmol^{-1}, at a final concentration of 50 μM, and a specific activity of 6.1–7.8 GBq mmol^{-1}. Three replicates and one blank were measured per treatment, where blanks and the triplicate tubes received the addition of TCA 5% (final concentration) for protein precipitation. The subsequent protein washing, precipitation, and cleaning steps were conducted as described in Buesing and Gessner (2003), with the number of disintegrations per minute measured using a Tri-Carb Scintillation Counter – Tri-Carb 2810TR scintillation counter (Perkin Elmer). Protein production was converted to bacterial C production (BCP) by using the conversion factor of BCP (kg) = 1.44 × Leucine (Leucine = leucine incorporation in mol) (Buesing and Marxsen, 2005).

2.12. Statistical analyses

Taxonomic and functional reconstructions generated using MG-RAST were imported into the Statistical Analysis of Metagenomic Profiles (STAMP 2.0.2) package to test for statistically significant differences among metagenomes (Parks et al., 2014). We conducted a Welch's two-sided *t*-test to compare the averages of two groups with unequal variance (surface x middle depth; middle x deep depth). Differences between mean proportions (confidence intervals of 95%) were calculated using the Welch's inverted, and p- value corrected for the two categories: taxonomic or functional reconstructions (Rivals et al., 2007). The *p*-values represent corrected values, with only values <0.05 reported in the analysis (Parks and Beiko, 2010). Differences between proportions of two groups were shown within the 95% confidence intervals as positive and negative values for the most different phyla and functions present (Parks and Beiko, 2010). SIMPER and multidimensional scaling (MDS) analyses of bacterial assemblages were conducted in PRIMER.

We used a linear mixed effect model to determine the effects of warming and oxygen exposure on sediment percent organic carbon for samples of varying depth (surface or deep), treatment (oxygen exposure: oxic or anoxic; warming: ambient, 3 °C, 6 °C, 9 °C), and time (0, 7, 30, 90 days). Depth, treatment, and time were fixed effects and tank ID was a random effect in the model. Sediment percent carbon was log transformed prior to analyses to meet test assumptions of normality and equal variance. All analyses were conducted using the statistical programming package R (R Core Team, 2015).

3. Results and discussion

3.1. Characterising a seagrass blue carbon core: Stocks, provenance, and rates of accumulation

The seagrass sediments used in this research spanned a period of 5000 years, according to ^{210}Pb and radiocarbon (^{14}C) dating (Fig. 2, Table S1). Surface sediment ages were 1.7 ± 1.3 years in the top 0–0.5 cm, reaching 84.8 ± 15.1 years in the 4–4.5 cm (Fig. 2, Table S1), with a mass accumulation rate of 0.112 g cm^{-1} yr^{-1} over this depth. The down-core average of the proportion of OC was 1.66 ± 0.77% (Fig. 2), which is lower than the global average for seagrass of 2.5 ± 0.1% (Fourqurean et al., 2012), but higher than the Australian average of 0.64 ± 0.68% (Lavery et al., 2013). Percent OC declined with depth beneath the sediment surface (Fig. 2); however, this pattern changed when taking into account the bulk density of the sediments to calculate OC density because sediments became increasingly compacted with depth. δ^{13}C values were depleted (−16.7 to −21.8‰) compared to typical Zosteraceae seagrass values (−10.5‰), which can likely be explained by a contribution of OC from mangroves or upland terrestrial sources surrounding the site (Hemminga and Mateo, 1996). This 6–10‰ offset between seagrass and sediment δ^{13}C values suggests that at least 50% of the sedimentary OC is of allochthonous origin (Kennedy et al., 2010). Values for nitrogen (N) concentration (0.10 ± 0.34%) and δ^{15}N (3.89 ± 0.02‰) were typical for seagrass (Lepoint et al., 2004), with %N declining with depth but δ^{15}N showing little downcore variability (Fig. 2) but also within the range commonly observed for terrestrial soil organic matter (Kramer et al., 2003). Mud (i.e., silt and clay, particle sizes <63 μm) content is known to be related to OC content for *Zostera* species (Serrano et al., 2016) and was high (84.7%) at the site.

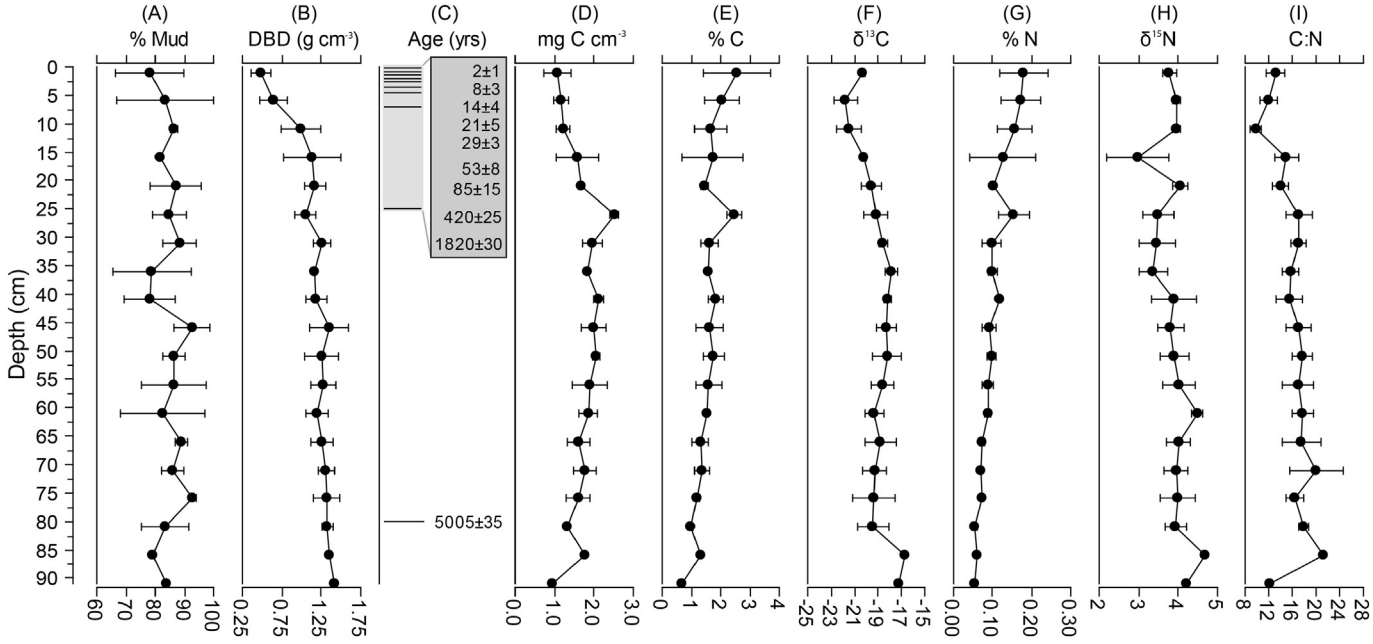

Fig. 2. Nature of seagrass blue carbon. Summary of data from sediment cores taken from seagrass meadows at the study site (Fagans Bay, NSW, Australia), showing changes in key physical, chemical, and biological properties along a depth / age profile. Ages represent calibrated years before present according to ^{210}Pb and ^{14}C dating. DBD = dry bulk density.

3.2. Defense shields: Biochemical recalcitrance and physical protection

Analysis of the thermal stability of OC was used as a proxy estimate of the susceptibility of sediment OC to microbial degradation (Capel et al., 2006). It allowed us to apportion OC to thermally labile, recalcitrant and inert pools, as defined by Capel et al. (2006). The proportion of OC in the labile pool diminishes with depth, declining from 43% in surface sediments to only 3% in deep sediment (80 cm below surface, Fig. 3A), suggesting that this deep 5000 y.o. OC may not be highly susceptible to remineralisation after disturbance. Such downcore decline in the percentage of sediment OC is typical of slow diagenesis that happens with the breakdown of carbohydrates in blue carbon sediments (Benner et al., 1991; Canfield, 1994; Marchand et al., 2005).

To test for the importance of mineral binding in protecting OC from remineralization, sediments were subjected to two chemical treatments. Dissolution of minerals with dilute hydrofluoric acid (HF) led to a release of 10–40% of the total OC (Fig. 3B), demonstrating that a substantial proportion of sediment OC was mineral-bound and therefore likely to have been physically-protected from microbial remineralization (Eusterhues et al., 2003). The wet oxidation treatment, which putatively oxidizes anything that is not strongly chemical bound to mineral surfaces, removed 80–95% of the OC and N from the samples, with less OC being susceptible to loss at depth (Fig. 3C). These results were consistent with the thermal analysis demonstrating an increase in recalcitrant and inert OC with depth (Fig. 3A).

There was no shift in age of the OC (estimated using ^{14}C) after HF treatment despite loss of up to 40% of the OC, suggesting that the OC that was bound by minerals was about the same age as the non-bound OC (Fig. 3D). This could also indicate that the mineral-bound OC is no more stable than the non-mineral-bound fraction. This contrasts with some terrestrial studies, which show that mineral-bound OC is much older than surrounding non-bound OC (Paul et al., 1997). The OC that was resistant to wet oxidation was thousands of years older than the bulk or mineral-associated OC (Fig. 3D) suggesting that this 5–15% of the OC is extremely refractory.

Solid-state NMR spectroscopy suggests that the sediment samples contained a complex mixture of organic material, with more similarities to upland (terrestrial) soil organic matter than the local vegetative (seagrass) material (Fig. S2). We offer two possible explanations for this finding. The first is that the sediment spectra could be interpreted to suggest that very little seagrass derived organic matter is being preserved in these sediments and most of the organic matter accumulating is from allochthonous (terrestrial) sources (Sanderman et al., 2015). A second possibility is that local seagrass is rapidly degraded by microbes and incorporated into microbial biomass, thereby masking the original seagrass signal (Baldock et al., 1997). In such a situation the seagrass spectra may undergo change when it goes through microbial degradation (Trevathan-Tackett et al., 2017a; Trevathan-Tackett et al., 2017b). There is likely to be a contribution from both of these possibilities.

The NMR spectra were also interpreted using a molecular mixing model (Baldock et al., 2004) which solves for the best combination of six biochemical components (i.e., carbohydrates, protein, lignin, lipids, carbonyl and char) that typically dominate organic matter in terrestrial and aquatic environments. There was a large composition difference from fresh litter material to the organic matter preserved in sediments with a relative enhancement in protein, lignin, lipids and char relative to carbohydrates (Fig. S3). Lignin appears to selectively accumulate in these marine sediments in contrast to the typical trend of a decreasing contribution of lignin to organic matter in upland aerobic soils (Baldock et al., 1997). Consistent with the thermogravimetric analysis (TGA) data, with increasing depth the abundance of carbohydrates decreased while lipid-like organic matter increased, suggesting that organic matter is slowly transformed over time.

Both direct interpretation of the NMR spectra (Fig. S2) and results from the molecular mixing model (Fig. S3) suggest that char is a significant component of the organic matter in these sediments. Char is typically underestimated by a factor of two due to the nature of the cross polarization NMR experiments performed (Baldock et al., 2013) meaning that char represents 28–36% of total organic matter. We suggest that this is the minimum terrestrial contribution to the organic matter found

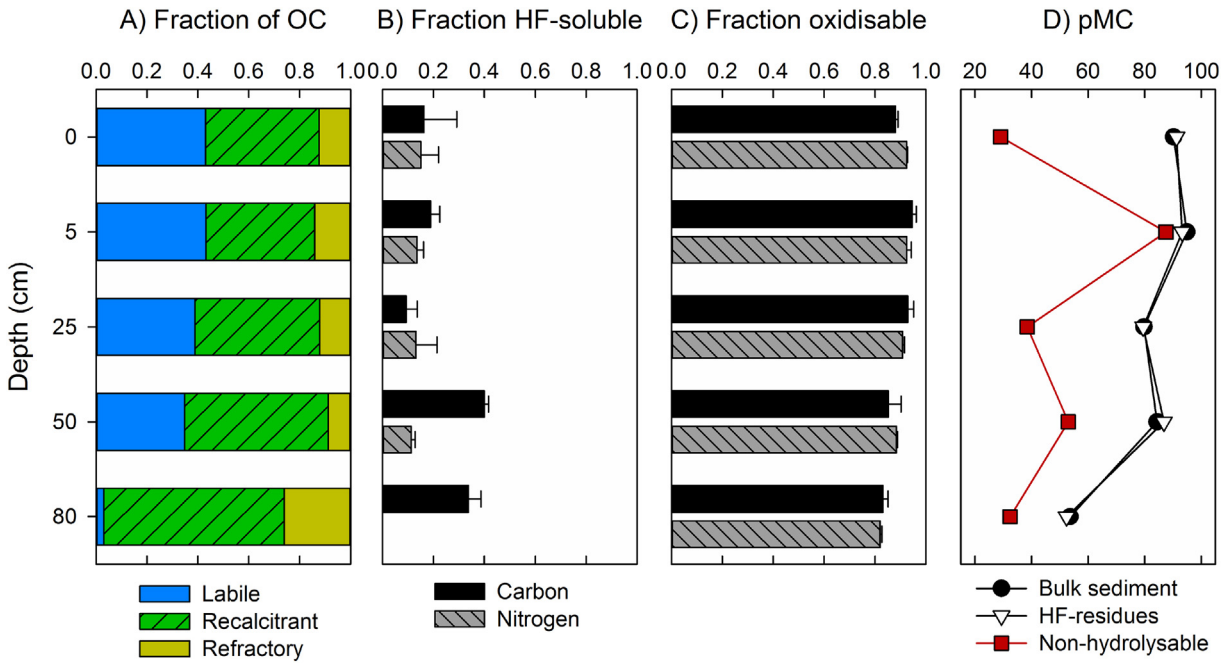

Fig. 3. Defense shields: biogeochemical recalcitrance and physical protection. (A) Proportion (pooled across $n = 3$ cores) of organic carbon of sediment in labile, recalcitrant, and refractory C pools at different depths below the surface, as determined by thermal gravimetric analysis (TGA). Data are normalised to the total amount of organic carbon in the sample. Carbon (C) and nitrogen (N) loss from seagrass organic matter taken from different depths below the sediment surface after (B) demineralization (10% HF) and (C) wet oxidation treatments (mean ± sd). (D) Radiocarbon results presented as percent Modern Carbon (pMC) for sediments before and after HF and wet oxidation treatments.

in these sediments. It is likely that the non-char fraction of the organic matter also has a significant terrestrial component, as it is hard to argue that only char would be delivered to this Bay without associated soil organic matter. The $\delta^{13}C$ values of surface sediment support this hypothesis (Fig. 2).

3.3. Microbial activity, composition, and functional potential varies with soil depth

We saw a marked shift in the proportion of bacterial cells within the flow cytometrically-defined high-DNA (HDNA) category, with sediment depth (Fig. S4). Separation of bacterial assemblages into at least two (and often more) flow cytometrically defined sub-populations according to differences in apparent nucleic acid content is a consistent characteristic of aquatic samples (Gasol and Del Giorgio, 2000; Gasol et al., 1999; Lebaron et al., 2001). These bacterial sub-populations, generally categorized as high and low DNA populations (HDNA and LDNA), exhibit dissimilar spatial and temporal dynamics and have been proposed to represent the active and in-active compartments of bacterial assemblages (Lebaron et al., 2001), with the proportion of HDNA cells within an environment shown to be correlated with bacterial respiration and often applied as a proxy measure for bacterial activity (Gasol et al., 1999; Lebaron et al., 2001; Seymour et al., 2004). The application of these criteria to our data suggests that bacterial activity declines with sediment depth (Fig. S4). While similar, depth-related decreases in bacterial activity have been reported for terrestrial soils (Taylor et al., 2002), this conclusion must be tempered by an alternative interpretation that the HDNA – LDNA separation is a consequence of phylogenetic differences, rather than bacterial metabolism (Zubkov et al., 2002). We directly investigated this possibility that shifts with depth in the proportion of HDNA cells reflect changes in composition of bacterial assemblages using a shotgun metagenomic approach.

Metagenomic analysis indicated that the microbial community in near-surface seagrass sediments differed substantially from communities in deeper sediments, both within the context of microbial taxonomic composition (Fig. 4A) and function (Fig. 4B). Surface sediments had a higher relative contribution of microbes, with an increasing importance of Archaea (Euryarchaeota) occurring with depth (Fig. 4C). In near-surface sediments, the proportion of Proteobacteria was significantly ($p < 0.01$) higher than intermediate and deep samples (Fig. 4C), a trend largely driven by higher relative abundances of Desulfobacteraceae (Fig. 4D), which are indicative of sulphate reducing bacteria (SRB) (Seymour et al., 2004). This pattern is consistent with the widespread observation that this family of SRB dominate the microbial community in the anoxic upper sediments of seagrass meadows (Cucio et al., 2016; Ugarelli et al., 2017). Indeed, our metagenome data showed a significantly higher proportion of genes involved in sulfur metabolism occurred in the upper sediment layer (Fig. 4F). On the other hand, intermediate and deep samples were characterized by a significantly higher ($p < 0.01$) proportion of Firmicutes and Euryarchaeota (Fig. 4C). The increased contribution of Firmicutes was largely explained by a significantly ($p < 0.01$) higher relative abundance of members of the Clostridia (Clostridiaceae, Thermoanaerobacteraceae, Peptococcaceae), which are generally obligate anaerobes widely found within soil environments. The significantly ($p < 0.01$) higher proportion of Euryarchaeota in the deep samples (Fig. 4D) coincided with the peak in genes involved in methanogenesis in the deep samples (Fig. 4E). Sulphate reducing bacteria, such as the Desulfobacteraceae, and methanogenic archaea, such as the Methanosarcinales, play important roles in the anearobic mineralisation of organic material (Raskin et al., 1996), but in many environments, including marine sediments, kinetic and thermodynamic advantages allow SRB to outcompete methanogens for substrate when sulphate is available (Raskin et al., 1996; Ward and Winfrey, 1985; Widdel, 1988). However, while SRB exhibit higher energy and growth yield per mol substrate than methanogens (Roden and Jin, 2011), a recent study has shown that OC turnover rate in anoxic sediment is limited by sediment age and thus OC composition rather than terminal metabolism (Beulig et al., 2018). This suggests that any mixing of the sediment within the anoxic compartment of the sediment column will not change the decomposition rate of the OC available.

3.4. Response of carbon to environmental disturbances

According to *bulk* analyses of seagrass OC, there was no significant effect of warming or oxygen exposure on OC content (Table S2, Fig. S5). Two other studies have performed warming simulations similar to this study. The first was by Pedersen et al. (2011) who simulated warming under anoxic conditions in the laboratory for seven months with *Posidonia oceanica* seagrass mats from the Mediterranean. They found that increasing temperature 10 °C above ambient increased remineralization rates 4.5-fold, indicating that microbial activity was enhanced by increasing temperatures, but above 10 °C bacterial growth declined, suggesting that microbial breakdown of seagrass OC fits a hump-shaped distribution. By contrast, Macreadie and Hardy (2018), who studied warming in the field (*Zostera muelleri*, Australia) using the thermal plume generated from a coal-fired power plant, found no effect of warming on seagrass organic after 6 months of temperatures elevated 4 °C above ambient. We suggest that warming is not a major controller of seagrass sediment microbial activity at this location, or that longer durations of exposure may be required to detect subtle, but cumulative, effects on warming on microbial remineralization of OC.

There was an apparent effect of oxygen on the percent of OC on surface vs deep sediment, as well as a depth by time interaction (Table S2). We further explored this potential effect of oxygen exposure on sediment %OC using microsensor analyses, which provide much higher spatial resolution analysis than bulk measures of OC. In using microsensor approaches, we sought to reduce the variability and noise as seen in Fig. S5 from bulk OC analyses. In addition to providing a more precise analysis of sediment fluxes, we combined microsensor analyses with C radiolabelling in an attempt to confirm whether any changes in OC fluxes were caused by changes in microbial activity.

According to microsensor analysis, which permits fine-scale measurement of diffusive flux across the sediment-water interface, after sediments were exposed to oxygen, oxygen consumption at the sediment/water interface was consistently higher (70–80%) in surface sediment than in deep sediment (Figs. 5A, S6), suggesting a higher overall microbial activity in the young sediment. This finding was consistent throughout the length of the experiment (90 days), although the magnitude of the effect that oxygen exposure had on respiration rates changed with time; oxygen consumption decreased by 25 and 27% in the young and old sediment, respectively, which was most likely caused by a reduction in the pool of readily degradable OC at the sediment surface (Fig. S6).

Sulphate reduction is the dominant biogeochemical process in anoxic, marine sediments (Jorgensen, 1982) and is therefore used as a proxy for overall anaerobic activity and OC turnover (Jorgensen, 1982). Here we calculated the sulphate reduction rates from the upward flux of total sulphide (S^{2-}) in the sealed sediment cores as described by Kuhl and Jorgensen (1992). Estimated sulphate reduction rate and thereby OC turnover rate per area in the anoxic part of the young sediment was more than one order of magnitude higher than in the old sediment (Fig. 5B). This likely resulted from differences in the availability of labile OC (Fontaine et al., 2007; Sollins et al., 1996) and highlights the low rate of OC turnover in deep sediments, corroborated by decreasing sulphate reducing microbes with depth whereas increasing methanogenesis in the old sediments (Fig. 4). Assuming that O_2 consumption at the sediment surface was primarily driven by aerobic respiration, a comparison between the sulphate reduction rate and the O_2 consumption rate in the sediment clearly show how the availability of O_2 can increase overall microbial turnover of OC: Taking into account the O_2 consumed by re-oxidation of sulphide at the sediment

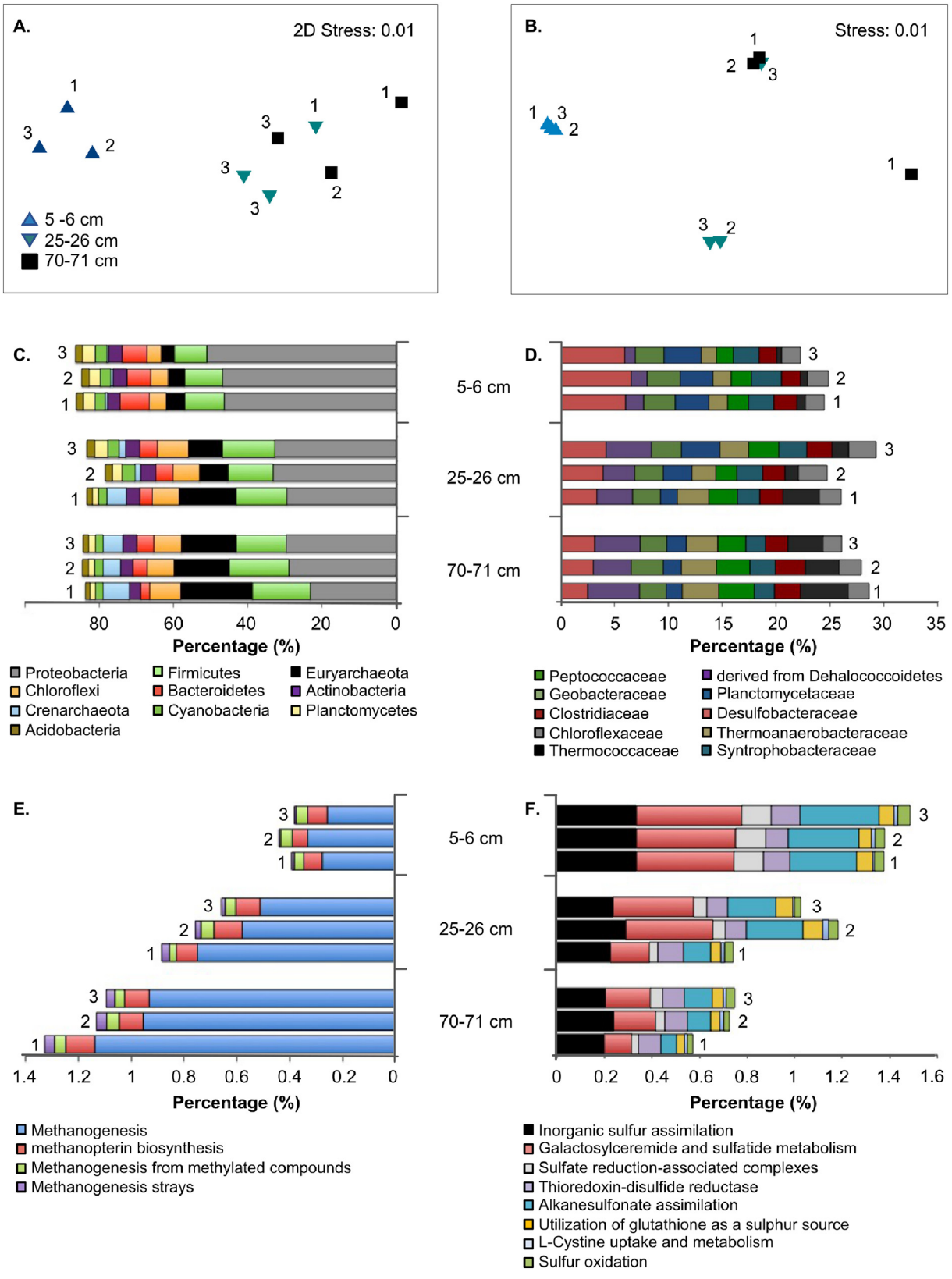

Fig. 4. Microbial activities, composition, and functional potential varies with soil depth. Outputs of metagenomic analysis (using SEED database) of microbes (n per depth = 3) in surface (5–6 cm), middle (25–26 cm), and deep (70–71 cm) seagrass sediments at Fagans Bay. MDS (multidimensional scaling) results of (A) taxonomic (phylum level) and (B) functional diversity (level 2); (C) ten most abundant phyla; and (D) most abundant families, in percentage. (E) Predictive methanogenesis pathways (F) sulfur metabolism (level 3), in percentage, using SEED database. The numbers '1', '2', and '3' refer to the core numbers – i.e. Core1, Core2, Core3.

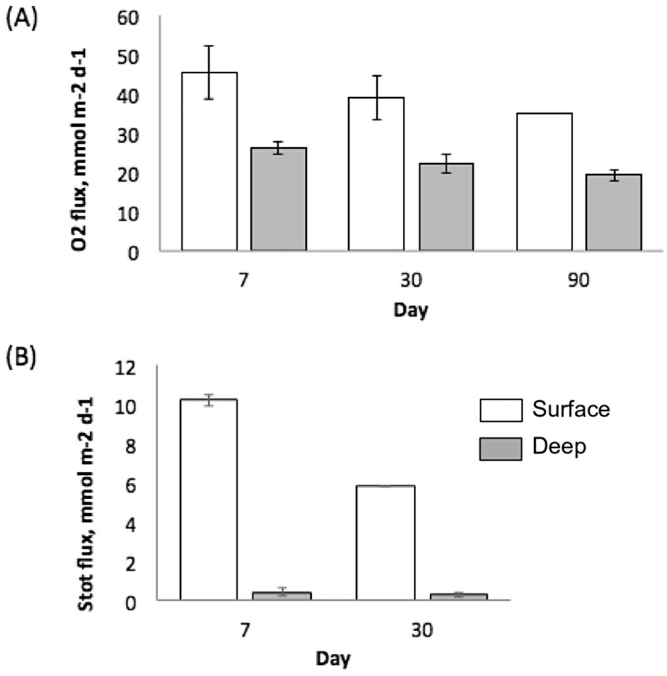

Fig. 5. Oxygen exposure triggers microbial break-down of blue carbon. Summary of microsensor data out puts showing (A) oxygen (O_2) and (B) total sulphide (Stot) flux rates for surface and deep seagrass sediments exposed to oxygen through time (7, 30, and 90 days). These estimates are based on microsensor profiles provided in Fig. S7. Note: there was no replication for surface cores at day 90 due to two replicates having tipped over during the long-term incubation. Data represents mean ± s.e.

surface ($HS^- \ 2O_2 \rightarrow SO_4^{2-} \ H$) (Fenchel et al., 1998), aerobic O_2 consumption ($CH_2O \ O_2 \rightarrow CO_2 \ H_2O$) equated to an estimated production of 19–26 mmol CO_2 m^{-2} d^{-1} in the old sediment in comparison to just 0.5–0.8 mmol CO_2 m^{-2} d^{-1} from anaerobic sulphate reduction ($2CH_2O \ SO_4^{2-} \rightarrow 2CO_2 \ HS^- \ H_20$) (Ivanov et al., 1989), representing an increase in CO_2 production by ~34–38 fold with O_2 exposure. Similarly, in the young sediment calculated aerobic and anaerobic CO_2 production was comparable at 23–25 mmol and 12–20 mmol CO_2 m^{-2} d^{-1}, respectively. Importantly, estimated total aerobic OC turnover in the old and young sediment was similar despite the low amount of labile OC in the old sediment (Fig. 3A, 80 cm). This effect of O_2 exposure on mineralisation of recalcitrant OC has been observed previously in marine sediments (Kristensen et al., 1995) and may result from a more efficient degradation of the recalcitrant OC in the old sediment with the activation of aggressive mono- and dioxygenases in aerobic bacteria (Fuchs et al., 2011).

Building on our microsensor findings, next we used radiolabelling technology to estimate the effects of warming and oxygen exposure on bacterial abundance and bacterial OC production. This allowed us to determine if the changes in OC turnover as measured from microsensor analysis translated into expected change in OC uptake by microbes. We found that warming had no effect on bacterial abundance (Fig. S6A) or bacterial carbon production (Fig. S6B) for either surface or deep sediments, at any time. However, bacterial abundance significantly increased under exposure to oxygen in both surface and deep sediments in the first week of the experiment (Fig. S6B). The same pattern was observed for bacterial carbon production, which was stimulated after one week of oxygen exposure (Fig. S7D), but was not maintained at later times. Overall, the analysis of bacterial abundance and bacterial carbon production support the hypothesis that oxygen exposure can trigger increased microbial remineralisation of sediment OC within seagrass meadows. To explain why the effect of oxygen exposure on bacterial abundance and bacterial carbon production was short-lived, we would recommend further experiments involving phylogenetic analysis of the microbial community during the 12-week incubation to assess how community composition is changing through time in response to oxygen.

4. Summary and conclusions

As the coastal zone faces increasing anthropogenic pressure, it is important to understand whether blue carbon reservoirs are at risk of being remineralised by microbes and released to the atmosphere as CO_2 or other greenhouse gases. Here, we characterized blue carbon sediment profiles from seagrass meadows and then tested the vulnerability of blue carbon to oxygen exposure and warming. Sediment profiles spanned a period from the present day to ~5000 years old. Flow cytometric analysis showed that putatively active microbes were present throughout the entire sediment profile, but that the proportion of these microbes within deeper, ancient (5000 year old) sediments was only 1% of that observed in surface sediments, a pattern consistent with a shift from sulphate reducing bacteria to methanogenic archaea and one carbon metabolism genes with depth. The abundance of active microbes declined most dramatically within the top 25 cm. Percent OC in sediments declined gradually with depth, and thermogravimetric analysis showed a shift in OC stability with depth that was attributable to the depletion of the 'labile' OC pool. The ancient sediment OC pool consisted almost entirely (97%) of 'refractory' and 'recalcitrant' OC. Dissolution of minerals via HF led to release of up to 40% of total OC, indicating that minerals bind a substantial proportion of sediment OC, but ^{14}C dating showed that mineral-bound OC was no older than bulk OC. Stable carbon isotope data suggest a significant contribution from allochthonous (terrestrial) sources at all depths. Solid-state NMR spectroscopy and molecular mixing models suggests that the sediment samples contained a complex mixture of organic materials with microbial processing of OC occurring very slowly. Despite the apparent intrinsic chemical recalcitrance of the seagrass sediment OC, we found that oxygen exposure significantly increased microbial remineralisation and OC turnover. This was evident through microsensor analysis and changes in bacterial carbon production and abundance rather than being detected in bulk OC analyses over the course of a disturbance experiment. Overall, this research suggests that blue carbon buried deep in seagrass sediments is most vulnerable to exposure to oxygen, through altered redox chemistry and microbial activity. Environmental disturbances such as dredging, wetland drainage and major storms that expose coastal sediments to oxygen have the capacity to diminish blue carbon stocks.

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Author contributions

PM conceived the idea. PM, TA, JS, MSF, JS and DA carried out the experiments and analyses. All authors contributed to writing the manuscript. The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.05.462.

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