Can seagrass modify the effects of ocean acidification on oysters?

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COMPETING INTERESTS

The authors declare no competing interests.

ABSTRACT (150 words max)

Solutions are being sought to ameliorate the impacts of anthropogenic climate change. Seagrass may be a solution to provide refugia from climate change for marine organisms. This study aimed to determine if the seagrass *Zostera muelleri* sub spp. *capricorni* benefits the Sydney rock oyster *Saccostrea glomerata*, and if these benefits can modify any anticipated negative impacts of ocean acidification. Future and ambient ocean acidification conditions were simulated in 52 L mesocosms at control (381 µatm) and elevated (848 µatm) CO_2 with and without *Z. muelleri*. Oyster growth, physiology and microbiomes of oysters and seagrass were measured. Seagrass was beneficial to oyster growth at ambient pCO_2, but did not positively modify the impacts of ocean acidification on oysters at elevated pCO_2. Oyster microbiomes were altered by the presence of seagrass but not by elevated pCO_2. Our results indicate seagrasses may not be a panacea for the impacts of climate change.

INTRODUCTION

Global climate change is causing the oceans to warm and acidify with consequences for the habitat, distribution and abundance of marine organisms and flow-on effects to fisheries and aquaculture (Hoegh-Guldberg et al., 2018). Solutions to halt the losses of fisheries and aquaculture are being sought across the globe. Some habitat forming species may have the potential to provide refuge to other species from the most adverse effects of climate change (Falkenberg et al., 2021).

Excess CO_2 in the Earth's atmosphere is acidifying oceans and coastal environments at a rate faster than any period in Earth's history (Lee et al., 2021). Ocean acidification is predicted to reduce the growth and reproduction of calcifying species such as corals, coralline algae, and shell-forming molluscs (Kroeker et al., 2010; Parker et al., 2013; Ross et al., 2011) although effects on non-calcifiers are also recognised (e.g. noncalcifying macroalgae, sponges, plankton, invertebrates, fish; Falkenberg et al., 2013; Heuer & Grosell, 2014; Nagelkerken &

Connell, 2015). The effects of ocean acidification are greatest in the surface layer of the ocean and exacerbated along coasts and in estuaries that are home to habitat forming organisms (Scanes et al., 2020).

Habitat-forming organisms can facilitate the occurrence and persistence of other organisms by creating favourable environmental conditions (Bulleri et al., 2018; Cole et al. 2021). Such facilitation influences the distributions and abundance of species, allowing some organisms to occur in otherwise unsuitable areas (Bertness & Callaway, 1994; He et al., 2013). In the ocean, large stands of macrophytes can buffer the surrounding water to reverse acidification on a local scale (Fernández, et al., 2019). Such localised habitat refugia are thought to play a vital role in influencing the effects of ocean acidification for organisms and aquaculture (Falkenberg et al., 2012; Fernández et al., 2019; Falkenberg et al., 2021). Although estuaries and coastal environments are acidifying rapidly, diel refugia zones exist as a consequence of the buffering effects of phototrophic organisms (Scanes et al., 2020; Falkenberg et al., 2021).

The co-culture of phototrophs with calcifying organisms may ameliorate the negative effects of ocean acidification. For example, under simulated ocean acidification conditions, there is evidence that some marine macrophytes increase mean seawater pH by 0.3 units, leading to positive effects on the calcification of the blue mussel (*Mytilus edulis*; Wahl et al., 2018). Four species of bivalves also performed better under low pH in the presence of the phototroph *Ulva* (Young & Gobler, 2018). There are, however, some caveats and unknowns, as at night the process of respiration leads to drops in pH (Falkenberg et al., 2016; Cyronak et al., 2018). The potential for a positive effect on calcifying organisms will only be present if the diurnal benefit of photosynthesis outweighs the potential stress of respiration (Falkenberg et al., 2021; Kroeker et al., 2021).

Seagrasses have the potential to benefit and ameliorate the impacts of climate change on calcifying organisms. Seagrasses are present on all continents (except Antarctica) and are

70 foundation species that provide valuable ecosystem services such as carbon sequestration,
71 coastal protection, water purification and habitat for commercially important fish species
72 (Nordlund et al., 2016). Seagrasses are also known for their capacity to modify the
73 carbonate chemistry and pH of their environment (Pacella et al., 2018). The pH of Australian
74 estuaries has been shown to be greater when seagrass abundance is high (Scanes et al.,
75 2020), while research has shown that seagrasses may be able to buffer future ocean
76 acidification (Pacella et al., 2018). The presence of seagrass has also been shown to have
77 positive effects on the growth of calcifying organisms (Ricart et al., 2021).

78 Calcifying organisms, such as molluscs, are particularly vulnerable to ocean acidification
79 because of the decrease in carbonate ions in seawater (Ross et al. 2011; Parker et al., 2013),
80 and their relatively low capacity to remove excess CO_2 from their internal fluids (Melzner et
81 al., 2009). Adult bivalves have been found to grow thinner shells and have a greater
82 mortality rate when exposed to pH levels 0.4 units lower than ambient conditions (Cooley et
83 al., 2012; Gazeau et al., 2013; Wright et al. 2014). Ocean acidification has also been found
84 to change the shell matrix (Fitzer et al., 2018), increase metabolic rate, decrease extracellular
85 pH (Scanes et al., 2017; Pereira et al., 2019) and cause a shift in sex ratios (Parker et al.,
86 2018). Larval stages of bivalves are known to be more vulnerable than adult stages (Ross et
87 al. 2011; Parker et al., 2013). Reductions in fertilisation, increased abnormality, delayed
88 development and decreased settlement have been identified in a range of economically and
89 ecologically important bivalve species including *Mercenaria mercenaria*, *Crassostrea virginica*,
90 *Argopecten irradians* (Talmage & Gobler, 2009), *Crassostrea gigas* (Wright et al., 2014), and
91 *Saccostrea glomerata* (Parker et al., 2010; Parker et al., 2013). It is well established that
92 ocean acidification can cause significant and negative effects on marine molluscs, however the
93 mechanisms behind these effects are not as clear.

94 Recent evidence suggests that ocean acidification can also alter the microbial communities
95 associated with oysters (Scanes et al., 2021a; et al. 2021b). Such shifts are of significance

given that microbial communities associated with marine organisms (i.e. microbiome) play a vital role in the survival and function of their host (Apprill, 2017) and any alteration caused by ocean acidification may have consequences for oyster disease dynamics and health (Dupont et al., 2020; Scanes et al., 2021a). Already, ocean acidification and global temperature rise have been found to alter the haemolymph microbiome of *S. glomerata* with these shifts dependent on genotype (Scanes et al., 2021b). Seagrass also possess a diverse and unique microbiome (Hurtado-McCormick et al., 2019). There is some evidence that ocean acidification can alter the microbiome of macrophytes including kelps leading to blistered leaves and plant death caused by suspected bacterial pathogens (Qui et al., 2019), however, the effects of acidification on the microbiome of seagrasses remains understudied. Understanding the effects of environmental stressors on oyster and seagrass microbial assemblages may assist in predicting and preventing diseases.

The Sydney rock oyster, *S. glomerata*, is native to Australia and New Zealand and farmed in estuaries on the east coast of Australia (Schrobback et al., 2018). *S. glomerata* was used extensively by Australian's first nations people for tens of thousands of years (Nell, 2001). *S. glomerata* is an iconic Australian seafood that supports one of Australia's largest aquaculture industries. Between the 2018-2019 period, *S. glomerata* accounted for $59 million AUD of the New South Wales aquaculture industry (DPI, 2020). In addition to the economic importance of *S. glomerata*, this habitat forming mollusc also increases local biodiversity, provides shelter and nutrients to many species, affords shoreline protection, and filters water (Grabowski et al., 2012). The seagrass *Zostera muelleri* subsp. *capricorni* also grows in estuaries where *S. glomerata* are often farmed (Nell, 2001), potentially providing a positive influence on oysters.

There are concerns that by affecting the growth, physiology and microbiome of oysters, ocean acidification will lead to a reduction in the population of *S. glomerata,* with flow on effects for aquaculture and food security and trigger bottom-up shifts in ecology (Parker et al., 2013; Parker et al., 2018). This study aimed to determine if the seagrass *Z. muelleri* subsp. *capricorni*

would have positive effects on the growth, physiology and microbiome of *S. glomerata*, and if these positive effects would be maintained under ocean acidification. This study also aimed to determine any effects of elevated pCO_2 on the microbiome of seagrass.

METHODS

Experimental Treatments

This experiment used flow-through mesocosms to culture 'Oysters', 'Seagrass' and 'Oysters & Seagrass' under both ambient and elevated pCO_2 (Figure 1). The experimental system was housed in a temperature-controlled room, in the aquarium facility at the Sydney Institute of Marine Science (SIMS) and consisted of 18, 52 L flow-through mesocosms and six 52 L header tanks. All tanks were made from blue food grade plastic (Relfex Co. Wetherill Park, NSW, Australia) with internal dimensions of L x W x H mm: 597 x 362 x 266. Seawater was pumped in directly from the adjacent Chowder Bay, Sydney Harbour (33°50'21.4"S 151°15'10.3"E) and filtered through a size of 25 µm before entering our mesocosms. Incoming seawater was warmed to 22 $\pm$ 0.5 °C via a thermocouple solenoid feedback system that mixed warm (25°C) and ambient temperature seawater in a 2 L mixing chamber prior to flowing into header tanks. Seawater was then maintained at 22°C in the experimental tanks. Seawater from each of six header tanks flowed to three experimental tanks at a rate of 22 L h^{-1}.

To determine the impact of ocean acidification and seagrass on oyster growth and physiology a fully orthogonal experimental design was used. Experimental treatments included 'Oysters', 'Seagrass' or 'Oysters & Seagrass' at ambient and elevated pCO_2, with each combination replicated across three header tanks (Figure 1). 'Oyster' tanks contained eight oysters (*S. glomerata*) and sediment collected from Chowder Bay. 'Seagrass' tanks contained seven shoots of seagrass and sediment collected from Chowder Bay. 'Seagrass & Oysters' tanks contained seven shoots of seagrass and sediment collected from Chowder Bay, as well as eight oysters

147 (*S. glomerata*). To reach the experimental levels of CO_2, seawater in three of the six header
148 tanks were bubbled with premixed CO_2 and air to achieve the desired pCO_2 concentrations
149 (elevated pCO_2), the remaining three header tanks were bubbled with ambient air (ambient
150 treatments). CO_2 was delivered via a controller valve (Parker Hannifin, OH, United States)
151 and a derivative controller injecting food-grade CO_2 (BOC, Australia) into ambient air that
152 had been scrubbed of CO_2. The CO_2-air mix was then bubbled continuously into header tanks
153 using diffusers (Harianto et al., 2021). Experimental pCO_2 levels were selected based upon
154 the SSP3-7 IPCC projection (Lee et al., 2021). Tanks that contained oysters were fed a mix of
155 phytoplankton 50% *Chaetoceros muelleri*, and 50 % *Tisochrysis lutea* at a rate of 1 x 10^9
156 cells/oyster/day on three days a week.

157 *Z. muelleri* subsp. *capricorni* (Jacobs et al., 2006) was collected from Chowder Bay, New South
158 Wales, Australia (33°50'21.4"S 151°15'10.3"E) where it was found in polyculture beds of
159 *Zostera sp.* and *Halophila sp.* at a depth of 1 m below Indian Spring Low Water. Permission to
160 collect was obtained from the New South Wales Department of Primary Industries (NSW DPI)
161 permit number (P03/0029-5.1). The seagrass samples were carefully uprooted in sods with
162 rhizomes and sediments attached, and macrofauna was removed and released on-site at
163 Chowder Bay. The seagrass was then transplanted into tanks at the SIMS within 15 minutes of
164 collection. Sandy sediment was also collected from the swash adjacent to stands of seagrass in
165 Chowder Bay and evenly distributed among all replicates to create a depth of 30mm
166 (approx. 6L of sediment) in the bottom of tanks, including 'Oyster' tanks, carefully covering
167 any exposed rhizomes of *Z. muelleri* in 'Seagrass' and 'Seagrass & Oysters' tanks.

168 *S. glomerata* were sourced from the NSW DPI in Port Stephens, NSW, Australia (32°70' 93"S,
169 152°05' 28"E). Oysters were acclimated to Sydney Harbour conditions for eight weeks in a
170 basket suspended from Chowder Bay wharf. Once cleaned of epibiota, each oyster was
171 measured and assigned a unique identification number using glue-on shellfish tags (Hallprint
172 Co. South Australia).

Once experimental organisms were in tanks, they were left for one week to acclimatise before pCO_2 was added. Over a period of 25 days, pCO_2 was gradually raised in the elevated pCO_2 treatments until the desired pH (7.8). Once the desired pH was reached the experimental period began and ran for a further 20 weeks.

To simulate daylight, artificial LED lighting (Aqua One Strip Glo) was used with 14 hours on (6 am-8 pm), and 10 hours off (8 pm-6 am). The light intensity (µmol) of each tank was measured using a light meter (Li-Cor, 250A) and the average light intensity per condition was calculated (Table 1).

Water chemistry

The pH, temperature and salinity of each experimental tank were measured and recorded twice every week for the entire duration of the experiment (20 weeks). All pH, temperature and salinity measurements were taken using a calibrated handheld multimeter (WTW 3420) with salinity and pH probes attached. The temperature and salinity of incoming seawater was also monitored continuously throughout the experiment. Each week, the alkalinity of incoming seawater was determined using an automatic titrator (Metrohm titrando 902) using triplicate Gran-titrations (Gran 1952), and a CO_2 system calculation program (CO_2 SYS; Lewis et al., 1998), using the dissociation constants of Mehrbach et al., (1973). To determine the pH of tanks over a 24 h dark-light cycle, pH in every tank was measured from 6 pm to 6 am every three hours using a calibrated multimeter (WTW 3420). This period covered the time that lights were turned off nightly (Figure 2).

Growth of Oysters

The surface area (mm^2) of each oyster (n=8 per tank) was measured by taking high-resolution photographs of the upper valve, including a ruler to calibrate scale (mm). The surface area was calculated using image J software. Photographs and surface area measurements were

197 taken after 127 days of experimental exposure. The difference in surface area was
198 calculated by the difference between the initial and final surface area.

199 The wet weight of the oysters (n=8 per tank) was measured by patting the oysters dry and
200 weighing on an electric scale (±0.01g) at the beginning of the experiment, and after 127
201 days of experimental exposure, and the difference calculated.

202 Metabolic Rate

203 After 127 days, metabolic rate of oysters (MR) was measured (n=3 per tank). Prior to MR
204 measurements, feeding was ceased for 24 hours to remove any variability associated with
205 digestive metabolism. Three oysters from each tank were then placed in three individual 75
206 mL airtight chambers, filled with sea water from their respective tanks at the correct pCO_2
207 level. Optical oxygen dipping probes connected to an oximeter (Pre Sens, Oxy 10) were
208 inserted into each chamber. Oxygen measurements (O_2 % air saturation) were taken every 15
209 seconds in each chamber until oysters had reduced the oxygen levels from 100% to 80%
210 (between 1 and 2 hours). The oxygen concentrations were also measured in a blank chamber
211 containing no oyster to determine any levels of background respiration.

212 Following the measurements, oysters were removed from the chambers. Oyster dry shell and
213 dry tissue mass were measured by removing tissue from shell and drying both in an oven at
214 80°C for 24 hours. MR was calculated for each oyster and adjusted for dry tissue weight.
215 Metabolic rate normalised for dry tissue weight (MR; mg O_2 L^{-1} h^{-1} g^{-1}) was calculated using
216 the Resp R package (Harianto et al., 2019) in R software, and Equation 1 (Harianto et al.,
217 2021).

218 $$MR = \frac{\Delta O_2 V}{W}$$

219 Equation 1. Where ΔO_2 is the linear regression over time (mg L^{-1} h^{-1}), V is the volume of the
220 chamber (L) and W is the dry weight of oyster tissue (g).

Extracellular pH (pH_e)

To determine pH_e at the completion of the experiment at least three oysters (but up to five in some cases) from each tank were immediately opened without rupturing the pericardial cavity (n=3-5 per tank). Haemolymph samples were drawn from the interstitial fluid filling the pericardial cavity chamber of the opened oysters using a sealed 1 mL needled syringe. A 0.2 mL sample was drawn carefully to avoid aeration of the haemolymph. The sample was then immediately transferred to an Eppendorf tube where pH_e of the sample was measured at 22 °C using a micro pH probe (Metrohm 827 biotrode).

Microbiome Analysis

After 127 days, the bacterial component of the microbiome of both oysters and seagrass, as well as the surrounding seawater, was measured. To characterise the bacterial communities associated with *S. glomerata* and *Z. muelleri*, 16S rRNA amplicon sequencing was used. DNA was extracted from the gills of three oysters per tank (36 individuals). To extract DNA, oysters were opened using a sterile shucking knife (autoclaved) and gills were removed using sterile scissors (autoclaved), placed into DNA/RNA free tubes and immediately frozen at -80 °C (n=3 per tank). To extract DNA from seagrasses, 30 mm sections of *Z. muelleri* leaf blade were cut from the second leaf of three separate *Z. muelleri* shoots per tank (36 sections of leaf) and placed into DNA/RNA free tubes and immediately frozen at -80 °C (n=3 per tank). A 1L sample of seawater was collected in an autoclaved glass bottle from the centre of each tank. Each seawater sample was then filtered on a 0.22µm filter paper (Millipore S-Pak), filter papers were placed into DNA/RNA free tubes and immediately frozen at -80 °C (n=1 per tank).

DNA from oyster gill and seagrass leaf samples were extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Australia, Chadstone, VIC), according to the manufacturer's instructions and a 12-hour incubation at the lysis stage. DNA from seawater filter papers was

extracted using the Qiagen DNeasy PowerWater Kit (Qiagen Australia, Chadstone, VIC), according to the manufacturer's instructions.

Extracted DNA from all samples was amplified using the 27F (AGAGTTTGATCMTGGCTCAG) (Lane et al., 1999) and 519R (GWATTACCGCGGCKGCTG) (Tumer et al., 1999) primer pair targeting the V1-V3 variable regions of the 16S rRNA gene with the following PCR cycling conditions: 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Amplicons were then sequenced on the Illumina Miseq platform (2x300bp) following the manufacturer's guidelines at the Ramaciotti Centre for Genomics, University of New South Wales. Raw FASTQ data files for the 16S rRNA were deposited in the NCBI Sequence Read Archive (SRA) under Bioproject number PRJNA783421.

Sequence analysis

Raw demultiplexed data was processed using the Quantitative Insights into Microbial Ecology (QIIME 2 version 2019.1.0) pipeline. Briefly, paired-end sequences were imported (QIIME tools import), trimmed and denoised using DADA2 (version 2019.1.0), which also removes chimeras (Callahan et al., 2016). Sequences were identified at the single nucleotide threshold (Amplicon Sequence Variants; ASV) and taxonomy was assigned using the classify-sklearn QIIME 2 feature classifier against the Silva v138 database (Quast et al., 2012). The dataset was further cleaned by removing ASVs with less than 50 reads (0.005 %) and those identified as chloroplasts or mitochondria. Cleaned data was then rarefied at 2990. Data from QIIME 2 was then exported to R v.4.0.1 (R Core team) for analysis and statistical testing using the packages, "Phyloseq" (McMurdie & Holmes, 2013), "DESeq2" (Anders & Huber, 2010) and "Vegan" (Dixon, 2003).

Data analysis

Water chemistry

To validate our novel experimental method of using seagrass to buffer the pH effects of elevated pCO_2 (n=24 per tank), a two-way ANOVA was used. Measurements of tank pH were compared with the first factor of CO_2 (ambient or elevated) and the second factor of Seagrass with three levels (Oysters, Seagrass and Oysters & Seagrass). The software R v.4.0.1 (R Core team), with the packages "lme4" (Bates et al., 2015) and "emmeans" (Russel, 2021) were used for all ANOVA analyses. Data were checked for normality using the Shapiro-Wilk normality test prior to all ANOVAs.

Oyster Growth and Physiology

To determine differences among treatments for oyster surface area (n=8), wet weight (n=8), pH_e (n=3), and metabolic rate (n=3) ANOVA was used. A three-way ANOVA was used to test the effects of the two fixed factors CO_2 (ambient or elevated) and Seagrass ('Oysters Only' or 'Oysters & Seagrass'); the third factor "Tank" (Replicate tanks [n=3]) was included as a random factor nested in "Seagrass". For all analyses, "Tank" was confirmed to not be significant ($\alpha > 0.1$) and was consequently removed from the analysis following the procedure of Underwood (1995). ANOVA were then recalculated using 'CO_2' and 'Seagrass' as the fixed factors. Extracellular pH data did not meet the assumption of normality and normality could not be achieved with transformation. pH_e data was subsequently analysed using an Aligned Rank Transformed ANOVA (Wobbrock et al., 2011) using the "ARTool" package in R software (Kay and Wobbrock, 2016). Post-hoc tests were used for ANOVA to determine the source of variation for significant interactions using the Tukey method.

Microbiome

To determine the effects of treatments on microbial communities, both univariate (alpha diversity) and multivariate (beta diversity) indices were used. Initial comparisons were made among the three sample types that were sampled in each tank; seawater, oyster gills and seagrass leaves. A one-way PERMANOVA was used to compare the bacterial community composition among micro-environments, with sample type (Seawater, oyster gills and seagrass leaves) as the factor. Correlations among samples types and the relative abundance of

297 specific bacterial ASVs were identified with MICtools (Albanese et al., 2018), using
298 Spearman's rank correlation with a Benjamini-Hochberg adjusted threshold of $\alpha < 0.05$.
299 Correlative networks were then graphed using the software Giphy. Data were then divided
300 and analysed separately for each sample type to test for the effects of treatments.
301 Shannon's index of diversity was calculated from bacterial abundance data (n=3 per tank)
302 and analysed using a two-way ANOVA with factors CO_2 (ambient or elevated) and either
303 Oysters ('Seagrass Only' or 'Oysters & Seagrass') or Seagrass ('Oysters Only' or 'Oysters &
304 Seagrass'). Post-hoc tests were used to determine the source of variation for significant
305 interactions using the Tukey method. Shannon's index of diversity data from seagrass leaves
306 were square-root transformed to meet the assumption of normality.
307 To determine differences among the treatments in bacterial community composition, Unifrac
308 and Weighted Unifrac distance matrices (Lozupone & Knight, 2005) were created and
309 analysed using PERMANOVA with the factors CO_2 (ambient or elevated) and either Oysters
310 ('Seagrass Only' or 'Oysters & Seagrass') or Seagrass ('Oysters Only' or 'Oysters &
311 Seagrass'). In the case of seawater analysis, all three tanks were included, so the second
312 factor contained three levels ('Oysters', 'Seagrass' or 'Oysters & Seagrass'). To explore
313 significant PERMANOVA results, Non-Metric Dimensional Scaling (NMDS) and Canonical
314 Analysis of Principal Coordinates (CAP, using Selected line, CO_2 or Temperature as
315 constraining variables) plots were created for distance matrices to allow for comparisons of
316 constrained and unconstrained ordination (Anderson and Willis, 2003). Homogeneity of
317 variances was confirmed for all PERMANOVA using the "Betadisp" function in the "Vegan"
318 package.
319 To determine significant differences in the abundance of ASVs driving significant differences
320 between levels of CO_2 and seagrass/oyster treatments, the "DESeq2" package was used with
321 a Benjamini-Hochberg adjusted P value (Anders & Huber, 2010). The abundances of ASVs
322 were compared between levels identified as significant by PERMANOVA. All microbiome

analyses were done using the "Vegan" and "Phyloseq" packages in R v.4.0.1 (R Core team) software.

RESULTS

Water Chemistry

Both CO_2 and the presence of seagrass significantly affected the pH of seawater in tanks and there was no interaction between these treatments. The pH of tanks that contained seagrass (i.e. 'Seagrass' and 'Seagrass & Oysters') was significantly greater (0.6 ± 0.01 pH units) compared to tanks without seagrass (i.e. 'Oysters') (ANOVA Seagrass; $F_{4,494} = 9.71$, $P > 0.001$). Elevated pCO_2 significantly decreased the pH of tanks (ANOVA CO_2; $F_{4,494} = 143.2$, $P > 0.001$). There was a trend for the buffering effects of seagrass to be less pronounced at elevated pCO_2, where the effect of seagrass on pH was less pronounced (Figure 2). In tanks with seagrass, there was a mean (± SE) decrease in pH between night and day-time of -0.19 pH units (± 0.01) at ambient pCO_2 and a difference of -0.31 pH units (± 0.01) at elevated pCO_2. The lowest pH reading in tanks with seagrass was recorded at night, at 03:00 (Figure 2).

Oyster Growth and Physiology

The change in shell surface area of *S. glomerata* was significantly greater with seagrass at ambient pCO_2 (75.69 ± 16.62 mm^2) than without (39.29 ± 13.22 mm^2) and significantly less with seagrass at elevated pCO_2 (38.36 ± 12.05 mm^2), but the change with seagrass at ambient pCO_2 (75.69 ± 16.62 mm^2) was similar to that observed without seagrass at elevated pCO_2 (77.08 ± 14.45 mm^2) as shown by the Seagrass x CO_2 interaction (Figure 3; Table 2). Seagrass influenced the wet weight of oysters (Figure 4). The wet weight of *S. glomerata* was significantly greater with seagrass at ambient pCO_2 (0.22 ± 0.06 g). There was a non-significant trend for the change in weight wet to be slightly greater with seagrass at elevated pCO_2 (0.05 ± 0.03 g) compared to without seagrass at elevated pCO_2 (0.01 ±

348 0.04 g). There was a loss of weight of oysters without seagrass at ambient CO_2 (-0.04 ± 0.02
349 g) as shown by the significant Seagrass x CO_2 interaction (Figure 4; Table 2).

350 The pH of extracellular fluid (pH_e) of oysters was influenced by seagrass at ambient pCO_2
351 (Figure 5). The mean (±SE) pH_e of *S. glomerata* was greater at ambient pCO_2 with seagrass
352 (7.54 ± 0.03) compared to without seagrass (7.36 ± 0.06) and at elevated pCO_2 with and
353 without seagrass (7.38 ± 0.04 compared to 7.37 + 0.05 respectively; Figure 5; Table 2).
354 There was no difference in the metabolic rate of oysters with or without seagrass or at
355 ambient and elevated CO_2 (Figure 6; Table 2).

356 **Oyster and seagrass-associated bacterial communities**

357 Within each tank, we characterised the bacterial community associated with seagrass leaves,
358 oyster gills and the seawater. Bacterial community composition differed significantly between
359 each sample type (Wunifrac PERMANOVA; $F_{1,80}$=21.93, P<0.01; Supplementary Figure 1).
360 Network analysis identified 2129 significant correlations between sample types and ASVs,
361 with seagrass and oysters sharing the most correlations with ASVs (Supplementary Figure 2).
362 The presence of oysters or seagrass did not have significant effects on bacterial diversity
363 (Table 3) or community composition (Table 4) within the tank seawater.

364 The bacterial communities on oyster gills were dominated by ASVs from the Orders
365 Rhodobacterales, Cyanobacteriales, Rhizobiales, and Ostreoida which comprised 27%, 16%,
366 14% and 5% of relative abundance respectively (Supplementary Figure 3). Shannon's index
367 of bacterial diversity was not significantly affected by experimental treatments (Table 3;
368 Figure 7A). There were significant differences in the bacterial community composition of *S.*
369 *glomerata* gills between tanks that contained seagrass and those that did not (Table 4).
370 Constrained ordination (Figure 8A) plots showed clear separation among groups with and
371 without seagrass with no clear separation between ambient and elevated pCO_2 groups.

There were 175 ASVs from oyster gills that were significantly affected by the presence of seagrass (DESeq analysis, Padj < 0.01; Supplementary Data S1). The four ASVs that experienced the greatest increases in abundance when seagrass was present were from the Family Acaryochloridaceae. ASVs from the Families Ostreoida and Xenococcaceae experienced the greatest reductions in abundance when seagrass was in the tanks.

Bacterial communities on seagrass leaves were dominated by bacteria from the orders Rhodobacterales, Cyanobacteriales and Microtrichales, which comprised 43%, 9% and 8% of relative abundance respectively (Supplementary Figure 4). The bacterial diversity on seagrass leaves (Shannon's index) was dependent on the presence of oysters and pCO_2. Shannon's index of bacterial diversity was significantly lower in tanks held at ambient pCO_2 with oysters than those tanks that just had seagrass while at elevated pCO_2 the presence or absence of oysters did not have a significant effect (Table 3; Figure 7B).

Bacterial community compositions on seagrass leaves were significantly different between tanks that contained oysters and those that did not, however, this pattern was only significant at ambient pCO_2. Constrained ordination plots showed clear separation among groups at ambient, but not elevated pCO_2 (Figure 8B).

There were 137 ASVs from seagrass leaves significantly affected by elevated CO_2 (DESeq analysis, Padj < 0.01; Supplementary Data S1). ASVs from the Family Rhodobacteraceae experienced both the greatest increases and decreases in abundance, with 42 Rhodobacteraceae ASVs significantly increased in abundance, while 23 ASVs significantly decreased in abundance. The presence of oysters significantly altered the abundance of 176 seagrass-associated ASVs with 69 of these ASVs also from the Family Rhodobacteraceae (DESeq analysis, Padj < 0.01; Supplementary Data S1). Three ASVs from the Family Hyphomonadaceae experienced the largest increases in abundance when oysters were present.

DISCUSSION

This study explored whether seagrass will modify the effects of ocean acidification on oysters. Variables measured included the growth, physiology and the microbiome of oysters and seagrass. We found a positive effect on oyster growth when they were cultured with seagrass at ambient pCO_2 however, against expectations, this effect was not present at elevated pCO_2 conditions. These results suggest that the positive effects of seagrass were limited to ambient pCO_2 conditions. The microbiome of both oysters and seagrass was altered by the presence of either seagrass or oysters, respectively, at ambient pCO_2.

Oyster growth

Oyster growth (shell area and wet weight) was significantly greater in the presence of seagrass at ambient pCO_2, however, oysters exposed to elevated pCO_2 had significantly less growth (shell area) and no difference in wet weight in the presence of seagrass. Numerous studies have found that seagrass and other phototrophs can alter the carbonate chemistry of surrounding seawater, making carbonate ions available for calcification, leading to positive effects on bivalve growth (Fernández, Leal & Henríquez, 2019; Thomsen et al., 2015; Unsworth et al., 2012; Wahl et al., 2018; Young & Gobler, 2018). For example, Ricart et al., (2021) found the surface area of C. *gigas* was significantly greater when cultured with the seagrass *Zostera marina* at ambient pCO_2. It was reasoned that the growth of oysters was greater with *Z. marina* because the seagrass changed the seawater carbonate chemistry. This supports our result at ambient pCO_2, however, Ricart et al., (2021) did not test the effects of seagrass on oyster growth at elevated pCO_2 .

In contrast to these findings of enhanced growth, other studies have found negative or neutral effects of seagrass on oyster growth at ambient pCO_2. For example, Lowe et al., (2019) found no effect of the seagrass *Z. marina* on oyster growth in estuarine sites. Overall, the authors argued site-specific trophic interactions were central in oyster growth and survival,

422 rather than the presence of seagrass (Lowe et al., 2019). The limiting factors for oyster growth
423 included food availability in the upper reaches of the estuary and predation in the lower
424 parts of the estuary (Lowe et al., 2019). Groner et al., (2018) also found no significant effects
425 of seagrass on oyster mass at ambient pCO_2 (655 µatm), however, oyster mass was
426 significantly lower at elevated pCO_2 (1157µatm), even in the presence of seagrass. It was
427 suggested that the decreased night-time pH, caused by seagrass respiration, may have
428 counteracted the positive effects of higher day-time pH on oyster growth (Groner et al.,
429 2018). We found that without seagrass at elevated pCO_2, oyster shells were larger
430 compared to ambient pCO_2. Variation in responses in shell size of *S. glomerata* exposed to
431 elevated pCO_2 have been previously observed (Wright et al., 2014). Wright et al., (2014)
432 found that differences in shell size were dependent on family lines of oysters. In this study
433 treatments in which shell surface area increased did not have a concomitant increase in wet
434 weight. This difference may be because oysters can produce shell as a thin layer at the
435 margin called "frill" which is deposited before any concomitant increase in tissue mass (Brown
436 and Hartwick 1988).

437 In another study, where elevated pCO_2 was considered, the macroalgae *Ulva* ameliorated the
438 negative effects of elevated pCO_2 on four bivalve species which had significantly increased
439 shell length and tissue weight (Young & Gobler 2018). Young & Gobler (2018) found this was
440 likely because photosynthesis of *Ulva* changed the pH and availability of carbonate ions in the
441 seawater. Similarly, Wahl et al., (2018), also found *M. edulis* maintained calcification rates in
442 the presence of the algae *Fucus vesiculous* at elevated pCO_2. Young & Gobler (2018)
443 suggested that macroalgae altered carbonate chemistry via the uptake of nitrogenous
444 nutrients. This benefited bivalves because it increased total alkalinity, and therefore a higher
445 pCO_2 is required to reduce pH (Young & Gobler 2018).

446 Before beginning the experiment, we hypothesised that the presence of seagrass would aid
447 oyster growth by increasing seawater pH. There was a significant increase in the pH of tanks

448 with seagrass by approximately 0.06 pH units, compared to tanks without seagrass at
449 ambient and elevated pCO_2. These results support the field observations of greater pH in
450 systems containing seagrass (Scanes et al., 2020), and align with those of Koweek et al.,
451 (2018) which found the pH of seagrass meadows to be on average 0.04 pH units greater
452 than adjacent areas without seagrass. Furthermore, Koweek et al., (2018) found this
453 difference could increase to 0.2 pH units during peak summer photosynthesis. Other mesocosm
454 experiments with macroalgae have also shown the capacity for phototrophs to increase pH
455 under elevated pCO_2 (Falkenberg et al., 2016; Wahl et al., 2018; Young & Gobler, 2018).
456 An increase of 0.06 pH units is in the range of pH change that could benefit oysters (Parker et
457 al., 2009), however, the positive effects that seagrass and the accompanying increase in pH
458 may have on calcifying organisms is more complex than a simple additive interaction
459 (Falkenberg et al., 2021). The capacity for seagrasses to ameliorate the impacts of ocean
460 acidification has been recently questioned by meta-analysies that have revealed the evidence
461 for ocean acidification amelioration by seagrass in natural systems is weak (Kroeker et al.,
462 2021; Van Dam et al., 2021).

463 In addition to the increase in pH during the day, we also predicted that at elevated pCO_2 the
464 overall decline in pH would be lessened by the presence of seagrass. However, the results
465 from this present study suggest that seagrass appeared to cause a decline in night-time pH
466 when respiring, particularly at elevated pCO_2. Thus, as suggested by Falkenberg et al.,
467 (2021), the potential positive effect of seagrass on calcifying organisms was not effective at
468 elevated pCO_2. Recent studies from Western Australia have also found, against expectations,
469 that the seagrass *Amphibolis antarctica* had a significantly lower photosynthetic rate and
470 primary productivity when exposed to elevated pCO_2, a pattern caused by a reduction in the
471 electron transport rate (Grove, 2021). Consequently, Grove (2021) suggested that seagrass
472 may be less effective at buffering pH at elevated pCO_2.

473 **Extracellular pH and metabolic rate**

The extracellular pH of oysters significantly increased in the presence of *Z. muelleri* at ambient pCO_2. The increased extracellular pH in the presence of *Z. muelleri* reflects the buffering of pH by seagrasses, since pH_e is heavily influenced by the external environment of an oyster (Parker et al., 2012; Scanes et al., 2017). Reductions in extracellular pH can negatively affect $CaCO_3$ formation and the subsequent shell growth (Zhao et al., 2017). This negative effect of reduced extracellular pH on $CaCO_3$ formation shell growth was previously observed in the bivalve *Tegillarca granosa* (Zhao et al., 2017). We found lower growth in shell area at elevated pCO_2, possibly due to low extracellular pH which may have caused the dissolution of $CaCO_3$ within shells to buffer against further reductions in extracellular pH.

The capacity of marine organisms and oysters to calcify when exposed to elevated pCO_2 is dependent on the regulation of their acid-base status which is an energy intensive process, requiring metabolic energy production to meet energy needs (Pörtner, 2008). Consequently, oysters with a greater metabolic rate often have an increased extracellular pH (Parker et al., 2012; Scanes et al., 2017). We found that the metabolic rate of *S. glomerata* was not significantly different between ambient and elevated pCO_2 with or without seagrass. Many marine invertebrates, however, experience metabolic depression at elevated pCO_2 which may be a strategy to conserve energy in stressful situations (Guppy & Withers, 1999; Zhao et al., 2017). For example, the blood clam (*Tegillarca granosa*) and the hard-shell mussel (*Mytilus coruscus*), reduced their metabolic rate in response to elevated pCO_2 (Wang et al., 2015; Zhao et al., 2017). While this strategy may be effective in the short term, if metabolic depression is prolonged then it can become lethal to the organism (Pörtner et al., 2004). There are also studies which have found increased metabolic rate at elevated pCO_2. For example, Parker et al., (2012) found oysters increased their metabolic rates after 5 weeks of exposure to elevated pCO_2 (856 µatm) and explained that high metabolic rate may be a consequence of higher energy allocation to homeostasis at elevated pCO_2. Later studies, however,

499 suggested that metabolic depression may be a response to short-term exposure to pCO_2
500 (Parker et al., 2013).

501 **Impact on oyster and seagrass associated bacterial communities**

502 Overall, the bacterial diversity of oyster gill microbiomes increased, and diversity of seagrass
503 leaf microbiomes decreased, when oysters were cultured with seagrass. This relationship was
504 modified by elevated pCO_2, in only the seagrass samples. The order Cyanobacteriales was
505 the second most abundant in both oysters and seagrass samples, and members of this group
506 are well known as the dominant members of seagrass epiphytic microbiomes (Hamisi et al.,
507 2013). Network analysis also found a large proportion of ASVs were shared between oyster
508 and seagrass samples. While the oyster microbiome was unchanged by elevated pCO_2, recent
509 work suggests the microbiome may be driven by changes in oyster metabolism at elevated
510 pCO_2 (Scanes et al., 2021c). This study, however, found no significant changes in oyster
511 metabolic rates. While the oyster microbiome was unchanged by elevated pCO_2, this study
512 shows that the presence of seagrass can significantly alter the oyster microbiome. Given the
513 importance of the microbiome to oyster health (King et al., 2019), seagrasses may play an
514 important role in shaping the oyster microbiome that has not been previously identified.

515 Both the bacterial diversity and community composition of the microbiome of seagrass leaves
516 was affected by pCO_2. When oysters were present, the microbiome of seagrass leaves was
517 less diverse at elevated compared to ambient pCO_2 conditions. This study is among the first to
518 investigate the effects of elevated pCO_2 on seagrass microbiomes. Previous studies on kelp
519 have shown that elevated pCO_2 can significantly shift microbiomes, and may lead to bacterial
520 blisters and plant death (Qiu et al., 2019). Qui et al., (2019) suggested that some bacteria
521 and other microorganisms may not be able to survive under elevated pCO_2 and so the
522 microbial community will change. We found significant changes in ASVs from the Family
523 Rhodobacteraceae, which were among the most common bacterial family in seagrass samples
524 and have previously been found to be commonly associated with *Z. muelleri* from Australia's

east coast (Hurtado-McCormick et al., 2019). The Rhodobacteraceae have also been suggested to possess anti-bacterial compounds that may affect other bacterial members (Dang et al., 2008) and have been implicated in macrophyte disease (Hurtado-McCormick et al., 2019). Previous studies have identified increased Rhodobacteraceae abundance on diseased algae (Fernandes et al., 2012), including diseased algae occurring under elevated pCO_2 (Qiu et al., 2019).

The reduced microbial diversity on seagrass leaves in the presence of oysters may be due to the filter feeding nature of the oysters. As the oysters were ingesting seawater, they may be altering the abundance of bacteria available to settle on seagrass leaves, however, we found no significant effects of oysters on the Shannon's Index of diversity of bacteria in seawater. For example, Groner et al., (2018) found that *Z. marina* was less affected by pathogens causing eelgrass wasting disease when cultured alongside oysters. Groner et al., (2018) hypothesised that this is because the oysters filtered pathogens from the water.

Co-culturing native seagrasses alongside oysters may ensure the continuation of aquaculture in the face of ocean acidification and promote increased abundance of seagrasses. Molluscs including oysters are vulnerable to intensifying climate change and ocean acidification. This study has found that *S. glomerata* oyster aquaculture could benefit from the presence of seagrass such as *Z. muelleri*. The results from this study suggest that elevated pCO_2 should not be assumed to have negligible negative effects on seagrasses, and the impacts of elevated pCO_2 on seagrass communities, including the microbiome, may have a net negative effect on the capacity for seagrass to buffer pH in a future ocean. The results of this study support a novel and sustainable approach to culturing seagrass with oyster aquaculture, however, this approach is unlikely to reverse any negative effects of ocean acidification on oyster aquaculture.

Conclusion

In summary, solutions are being sought to ensure the continuation of aquaculture – particularly of vulnerable calcifying organisms such as oysters – under the challenges of climate change and ocean acidification. One such approach is the co-culture of seagrasses alongside the oysters. The results of this study show that the presence of the seagrass *Z. muelleri* positively modified the growth, physiology and altered the microbiome of Sydney rock oysters, *S. glomerata* at ambient pCO_2. This suggests culturing seagrass with oyster aquaculture has potential economic benefits of increased oyster production. Many of the benefits of seagrass, however, were lost at elevated pCO_2, a finding in agreement with recent research highlighting the complexity of phototroph – calcifier interactions at elevated pCO_2 (Falkenberg et al., 2021; Kroeker et al., 2021). Co-culturing with seagrass is thus unlikely to reverse all the effects of ocean acidification on oyster aquaculture. Further exploration is required to more comprehensively consider the capacity of seagrasses to modify the impacts of ocean acidification.

564 FIGURE LEGENDS

565 **Figure 1.** Simplified schematic of the experimental design. The three treatments (Seagrass,
566 Oysters, Oysters & Seagrass) were replicated three times, under both ambient and elevated
567 pCO_2 conditions. There was a total of six header tanks and 18 treatment mesocosms.

568 **Figure 2.** Mean pH (green and blue lines) measured in all tanks over 24 hours (dark and light
569 conditions) where seagrass was present (n=6) and absent (n=3) at both elevated and ambient
570 pCO_2. To simplify this figure, tanks containing seagrass only and seagrass & oysters have
571 been pooled together. Shading around lines indicates 95% confidence intervals. Yellow
572 shading indicates when lights were on, and grey shading indicates when lights were off.

573 **Figure 3.** Mean change in *S. glomerata* surface area (mm^2) (±SE) after 127 days in
574 experimental treatments of ambient and elevated pCO_2, in tanks of oysters only (Oysters)
575 and oysters with seagrass (Oysters & Seagrass).

576 **Figure 4.** Mean change in *S. glomerata* wet weight (g) (±SE) after 127 days in experimental
577 treatments of ambient and elevated pCO_2, in tanks of oysters only (Oysters) and oysters with
578 seagrass (Oysters & Seagrass).

579 **Figure 5.** Mean *S. glomerata* extracellular pH (pHe) (±SE) after 127 days in experimental
580 treatments of ambient and elevated pCO_2, in tanks of oysters only (Oysters) and oysters with
581 seagrass (Oysters & Seagrass).

582 **Figure 6.** Mean *S. glomerata* metabolic rate (MR mg O2 g^{-1} h^{-1}) after 127 days in
583 experimental treatments of ambient and elevated pCO_2, in tanks of oysters only (Oysters)
584 and oysters with seagrass (Oysters & Seagrass).

585 **Figure 7.** Shannon's index of bacterial diversity in A) *S. glomerata* gills in tanks with (Oysters
586 & Seagrass) and without seagrass (Oysters) and B) Z. *muelleri* leaves in tanks with (Oysters &
587 Seagrass) and without oysters (Seagrass), at ambient and elevated pCO_2 following 127 days
588 in experimental treatments of ambient and elevated pCO_2, in tanks without A) oysters and B)
589 seagrass. The upper and lower "hinges" correspond to the first and third quartiles (the 25th
590 and 75th percentiles) while whiskers extend from the hinge to the highest and lowest value
591 that is within 1.5 × IQR of the hinge.

592 **Figure 8.** Constrained Analysis of Principal Coordinates (CAP) on Weighted Unifrac distances
593 from samples of A) *S. glomerata* gills in tanks with (Oysters & Seagrass) and without seagrass
594 (Oysters) and B) Z. *muelleri* leaves in tanks with (Oysters & Seagrass) and without oysters
595 (Seagrass), at ambient and elevated pCO_2 following 127 days in experimental treatments of

ambient and elevated pCO_2, in tanks with and without A) oysters and B) seagrass. Circles indicate ambient pCO_2 treatments and triangles indicate elevated pCO_2 treatments. In Panel A, green shapes with dashed lines indicate tanks with seagrass and oysters and grey shapes with solid lines indicate tanks without seagrass. In Panel B, green shapes with dashed lines indicate tanks with seagrass blue shapes with solid lines indicate tanks with seagrass and oysters. Group centroids are defined by the mean dissimilarities for each group.

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