RESPONSE TO REVIEWER COMMENTS *Reviewer comments in italics*; author responses to bold

<u>Reviewer #1:</u>

Two size cohorts of hard clams, oysters, scallops, and mussels, were grown with and without macroalga Ulva in two CO2 treatments. The results show higher growth rates of bivalves in presence of Ulva, with a small benefit in the high CO2 treatment. Increased bivalve growth rates in the presence of Ulva was attributed to the increase in saturation state caused by Ulva. The study is an interesting approach to study the potential benefit of Ulva on growth of multiple bivalve species, in the context of aquaculture management with ocean acidification. The strength of this study is that the experiment was conducted on multiple species, two size classes, and there are multiple growth metrics with consistent results. The weakness of this study is the seawater chemistry and the conclusions drawn from the data. The results are intriguing and merit further exploration of why bivalves exhibited enhanced growth in the presence of Ulva. As not all factors were controlled in this experiment (e.g. unknown effect of algae and mussels on seawater chemistry, independently and by treatment), this study provides results to further develop specific hypotheses as to why these trends were observed. In its current form, I am not convinced by the conclusion that Ulva alters seawater chemistry which in turn causes increased bivalve growth under high CO2.

We thank the reviewer for their feedback.

1. The authors attribute what is a substantial biological response by bivalves in the presence of Ulva and high CO2 to a very MINOR increase in saturation state over time (only 0.04!). A lot of emphasis is placed on statistical comparisons of saturation state across treatments, probably because the change is so small but offers an attractive explanation. However, a statistically significant difference in a carbonate chemistry parameter across treatments does not mean that it is biologically relevant. The authors do not discuss if the magnitude of change in growth is realistic for a 0.04 change in saturation state (perhaps some summary plot showing growth metrics of each species by treatment, with aragonite saturation state of each treatment on the x-axis, would provide insight). However, Comment #2 explains why the sampling design is insufficient to characterize seawater chemistry in this experiment in the first place.

We appreciate the reviewer's perspective on this point. First, we note that small changes in saturation state, even when saturated, can be biologically important and significant. In prior studies (Talmage and Gobler, 2010, 2011), the growth of early life stage bivalves used in the present study (*Mercenaria mercenaria* and *Argopecten irradians*) was assessed under three concentrations of CO₂ (pre-industrial, present-day, and elevated CO₂ = 280, 390, and ~780 ppm, respectively), significant increases in growth were observed between 280 and 390 ppm CO₂ which often corresponded to small changes in $\Omega_{aragonite}$ (<0.1 units) within the saturated ranged. We note that we did refer to this example in the discussion of the manuscript. We also note that other studies have ascribed small changes in $\Omega_{aragonite}$ to significant changes in early life stage bivalve survival (Barton et al 2012).

We agree with the reviewer that plots of saturation states against the growth would be of value hence, as suggested by the reviewer, for this revision we will provide plots showing growth metrics of each species by treatment, with aragonite saturation state of each treatment on the x-axis. We will also place the resulting statistics in tables as supplements to the manuscript, with references throughout the manuscript. To summarize these new findings, there was a strong positive and significant (p<0.05) correlation between shell length-based growth and saturation states of aragonite and calcite in all species and size classes, save for *Mytilus edulis*. In at least half of the experiments, there was a strong positive correlation and significant (p<0.05) correlation between tissue and shell weight-based growth and the saturation states of calcite and aragonite, with several additional results approaching significance (p<0.07). We are grateful for this comment by the reviewer, as it assisted in discovering these important trends.

2. The seawater chemistry sampling design and measurements are not sufficient to describe how organisms contributed to seawater chemistry or what they actually experienced.

a. Water was only sampled at the start and end of the experiment, despite multiple water changes during the closed-system experiment. If the changes in saturation state come from cumulative effect of nitrate assimilation by Ulva, this is in fact a change that since the last water change (every 3 days). It means that the bivalves mostly experienced the same saturation state across high CO2 treatments, regardless of the 0.04 change that would have occurred over 3 days.

The conclusion stated here is not supported by the data. *Ulva* is capable of the rapid uptake of nutrients, which were added after every water change. Within 24 hrs of each water change, pH values within containers with *Ulva*, regardless of CO₂ concentration, were higher than in the containers without *Ulva*, meaning bivalves mostly experienced higher saturation states during experiments. We have made new plots of the day-by-day change in pH for each of our seven experiments that demonstrate the strong and significant effect of *Ulva* on pH from the start of each water change in each of the experiments. This will be included in the revision of our manuscript. We are again extremely grateful for this comment by the reviewer, as it assisted in discovering this important and what we find to be highly convincing trend.

b. Seawater chemistry was highly variable. According to the authors, Ulva changes carbonate chemistry via CO2 uptake (decreasing DIC; P9, L11-22) and/or nutrient uptake (increasing TA, estimated at 10-20 umol/kg; P9,L29). During the experiment, the effect of CO2 uptake via primary production by Ulva is presumably removed with continuous bubbling with treatment concentrations of air/CO2 gas mix (P9). However, pCO2 is quite variable across treatments and experiments, indicating that the method used for bubbling did not actually bring the system (treatments + biology) into equilibrium. For example, within one experiment, the standard error in pCO2 reported in Table S1 is up to 200 uatm (based on N=2, start and end samples?). TA also varied substantially, even across treatments without Ulva, and TA did not always increase in the presence of Ulva (Table S1, this is masked by Table 1 which somewhat deceptively summarizes treatments across all experiments). For example, TA was 230 umol/kg less in the CO2 treatment compared to control in the experiment for Mercenaria mercenaria, even without Ulva. The authors do not describe why all their measurements are so variable and inconsistent

in what they define as a well-controlled system. It is unclear if SE refers to a start and end sampling, which again is not a relevant design if the authors think that biological processes contribute to changes in seawater chemistry.

We agree with the reviewer. There was variance in the chemistry driven by the biology and that the productivity of the *Ulva* was capable of overpowering the bubbling. We note experimental that the variability reported is based on replicate vessels with n=4 of the final time point and the daily measurement of pH, not two time points. We will clarify this point in the revision. Importantly, these are true biological replicates representing the cumulative effects of the whole experimental ecosystem: *Ulva*, bivalves, microbial communities, etc on the chemistry. While there is variance generated by these communities, the presence of *Ulva* indeed caused the calcium carbonate saturation states within experimental vessels to be statistically significantly higher than vessels without them and caused a day-by-day rise in pH which we will provide in the revised version of the manuscript, along with the new statistically significant regressions of bivalve growth with saturation state.

We are uncertain as to why the reviewer would suggest we were being 'deceptive' by creating summary supplementary tables. If the tables were available to the reviewer and will be available to all readers, we find this to be full transparent. We note, however, while abiotic systems bubbled with CO₂ generally have consistent alkalinity, alkalinity can be affected by multiple biotic and abiotic processes associated all living organisms within each experimental vessel: Respiration, photosynthesis, shell dissolution, calcification, nitrate uptake, phosphate uptake, ammonium uptake, microbial degradation, etc.

c. Chemistry was calculated using pH that was measured by a Durafet but no information on calibration and quality control was provided. It is unclear how and where the daily pH measurements are used.

The DuraFET III used in the present study were calibrated with a seawater pH standard, as per Dickson (1993), and compared to measurements made spectrophotometrically using *m*-cresol (Dickson et al., 2007). Both methods yielded pH measurements that were identical and never significantly different. We agree with the reviewer's concerns here and will add this information to the revised manuscript. Measurements of pH were used in the calculation of carbonate chemistry, which is stated on P4, L11-12. We will also be providing the day-by-day pH values from experiments to illustrate the effects of nitrate uptake by *Ulva*.

d. For all of the above reasons, I am not convinced that photosynthesis or nitrate assimilation by Ulva increased saturation state which then enhanced growth of bivalves (as claimed on P11, L29-30). Unless the authors can clarify these points, alternative hypotheses should be discussed. For example, could proliferation of algal cells in high CO2 have provided more food to the bivalves and therefore contributed to their growth.

We applaud the reviewer's skepticism as this is a core element of the review process. As the reviewer requested, we have shown that the growth rates of bivalves are significantly correlated with the saturation states of two forms of calcium carbonate and that calcium carbonate saturation states were always significantly higher within the treatments with *Ulva*, two key data sets supporting the hypothesis that improved conditions for calcification was the key factor driving trends observed in this study. The reviewer has provided an alternative hypothesis but one that does not fit the data since if high CO₂ led to the proliferation of algal cells, and thus more food for the bivalves, once would expect their growth to increase but our results showed they actually decreased growth under elevated CO₂. We do, however, agree with the reviewer's point that differences in algal cells within treatments could impact the growth of bivalves. Therefore, for this revision we have enumerated final algal cell densities within experimental vessels for all treatments. To summarize these findings, there were no significant differences in algal cell counts across any treatment within individual experiments. A table with this data will be created for this revision and added to the supplementary materials.

After all, nutrients were added and this would benefit Isochrysis spp. (spelling error on P3,L23) and Chaetoceros spp.

Yes, nutrients were added to all experiments and vessels. This point is specified on P3, L17. Despite the plausibility that the microalgae could have influenced the growth of bivalves, analyses of phytoplankton cell densities within each treatment and experiment rule out this possibility as there were no difference in algal cell counts across treatments within individual experiments. A table has been created and will be added to the supplementary materials.

3. The extensive discussion (e.g. last four paragraphs) on macroalgae/seagrass benefits to bivalves detracts from the discussion of the results of this study, and makes the authors appear biased towards the hypothesis that macroalgae will mitigate ocean acidification (e.g., their interpretation of Unsworth et al 2012 on P11,L17, comments below). The ability for seagrass and macroalgae to chemically buffer ocean acidification (e.g., P12, L1-2) is not a fact, and needs to be considered in the context of the greater coastal environment that the habitat is in (e.g., freshwater inputs, upwelling, water residence time, etc., e.g. see Cyronak at al 2018 "Short-term spatial and temporal carbonate chemistry variability in two contrasting seagrass meadows: implications for pH buffering capacities"). The authors do not discuss the fact that their experiment was conducted in a closed system. It is unrealistic to conclude that a minute impact on alkalinity by Ulva (if verified, see comment 1 & 2) would mitigate ocean acidification in an open system. For these reasons, extrapolating these results to field applications should not take up more than a paragraph, and the authors should only do so if all of the issues with seawater chemistry can be sufficiently resolved.

We do not suggest that macroalgae alone can mitigate ocean acidification, but rather merely that primary productivity and/or nitrate assimilation by macroalgae may provide a small temporal and spatial refuge for bivalves and other calcifying organisms as has been stated and concluded in prior studies. Given the scale, this may be particularly relevant to bivalves in an aquaculture setting with macroalgae purposely grown in copious quantities in close proximity to bivalves potentially providing regional "chemical resilience". As per the reviewer's comments, we will, however, significantly scale back this discussion. Title: based on the issues with seawater chemistry, this title may need to be revised

We believe the title aptly describes the paper given the linear relationships between saturation states and the growth of bivalves and the day-by-day increases in pH provided by *Ulva*.

Abstract: remove p-values

We will remove the p-values from the Abstract.

- Half of this study has to do with large vs. small bivalves but the significance of this is not mentioned in the introduction. Please add the motivation for this in the Introduction.

This was done since vulnerability of bivalves to acidification can be size-dependent. We will add this information to the Introduction.

- P2, L18: specify that pH and saturation state in seagrass meadows provide *temporal* refuge from acidification (as pH also declines below background seawater pH at night or in winter seasons).

We agree with the reviewer and have specified that daytime primary productivity increases pH and saturation states of aragonite, which provides a temporal refuge from acidification.

- Were nutrients added to vessels without Ulva as well? If not, the presence of Ulva is confounded with presence of nutrients which could influence the growth of Isochrysis and Chaetoceros and therefore the food supply by treatment.

Nutrients were added to all experimental vessels, *Ulva* or not, for the reason that the reviewer states. This point is specified on P3, L17.

- P3, L 24: how can 'ad libitum' food supply be exactly 4 x 104 cells mL-1 d-1?

For the bivalves used in the present study, the rate of 4x10⁴ cells mL⁻¹ d⁻¹ of the specified microalgae is an amount that is more than sufficient (*'ad libitum'*) for the growth of the studied bivalves as per Helm MM, Bourne N, Lovatelli A (2004). Hatchery Culture of Bivalves: A Practical Manual. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO), which we will reference in our revision. In addition, we will demonstrate in this revision that there were always excess algal cells at the end of experiments, providing direct evidence that this food supply was indeed, *ad libitum*.

- *Report on assumptions of ANOVA (i.e., do residuals exhibit a normal distribution? was this tested?)*

In this revision we will report on the assumptions of the ANOVA tests. In order to ensure that our data met the assumptions of the ANOVA (normality and equal variance), all data

were log transformed before ANOVA were performed. We will add these details to the Methods section and have update the supplementary materials to reflect these changes.

- P4, L34: add # of circles of algae added to each vessel. Was this scaled by container volume for small (1 L) and large bivalves (8 L)? If Ulva changes seawater chemistry in a consistent way, this data can be used to explore that (e.g., weight to volume and magnitude change in seawater chemistry).

A single disk of *Ulva* was added per container, which we will include in the Methods. In terms of weight, the amount of *Ulva* added to 1 L and 8 L containers was consistent with the benthic coverage of *Ulva* in Shinnecock Bay based on several years of benthic trawl data as well as other estuarine regions (Liu et al., 2015; Sfriso et al., 2001) and thus, yes, it was scaled to the size of the vessels. This point is specified in the Methods on P4, L31-34 and P5, L1-5.

- Tables in supplement: check consistency of * with p < 0.05

We will change the text within the supplementary materials to make consistent use of asterisks for significant results.

- Please report the actual p-values in the text since the tables are in supplemental files.

We will change the text to reflect the actual p-values within the Results section.

- I don't understand how ANOVA results are used to make statements like "When in the presence of Ulva, shell length-based growth was significantly increased by 42% (Two-way ANOVA; p<0.05)" when it is unlikely that the % change is the same in high CO2 and low CO2 treatments. If the authors are reporting the effect of Ulva only at high CO2, then the statistics should come from the Tukey post-hoc comparison. Authors should also report on the interaction of the two-way ANOVA (significant or not).

In this text, we reference Fig. 3 which demonstrates the increased shell length-based growth in the presence of *Ulva* (by 42%). The reference to Table S4 shows the ANOVA results, not the percent difference. The point being illustrated here is that growth increased in the presence of *Ulva* by a certain percent, which, by way of Two-way ANOVA, was found to be statistically significant. We agree that with the reviewer that the 42% increase may not be the same within elevated and ambient CO₂ treatments, and will change the text to separate the percent increase between the two CO₂ treatments for the revised version of the manusctipt.

- I was expecting the Ulva results in the Results section. It's not critical, but a small point of confusion.

We had to not include *Ulva* growth results in the Results section since it is not related to the primary goals of the study and since our previous studies have already reported on

enhanced growth in *Ulva* incubated under elevated CO₂. We will add the mean response of *Ulva* as a supplementary figure for this revision and refer to this at the end of the results.

- P5, L35: report tests of ANOVA assumptions, report p-values that are corrected for multiple comparisons.

For this revision we will specifically report on the use of Shapiro-Wilk test to test for normality, in addition to an equal variance test, both of which are built into SigmaPlot. We performed log transformations of the data to ensure that they passed both tests and will update the supplementary materials to reflect this change. We will also change the text within the manuscript to state what assumptions were made for ANOVA.

- P11,L16-19: this statement is incorrect. Unsworth et al 2012 is a theoretical modeling study. Model results were then applied to coral calcification rates that came from laboratory-based experiments. The authors themselves state that the results from the modeling need to be field tested.

We thank the reviewer for pointing out our error. We will change the text to state that Unsworth et al. (2012) used a theoretical model to determine that coral calcification downstream from seagrass meadows could increase by ~18% should the full extent of a seagrass meadow's ability to increase pH and $\Omega_{aragonite}$ be realized in a natural setting.

- Discussion should include information about the magnitude of the beneficial effect of Ulva under high CO2.

For this revision, we will state the percent increase in growth rate of the bivalves in the discussion.

Table 1: indicate which parameters were measured, and sample size (N).

We agree with including the sample size, and will add an asterisk next to the parameters that were measured but not the ones that were calculated and explain what the asterisk represents in the table legend.

Figures: define error bars and indicate when there are significant differences among groups

We have indicated the definition of the error bars within the figure captions, and have placed significant differences within the figures, specifically the main treatment effects $(CO_2 \text{ and } Ulva)$.