We would like to thank anonymous referee #1 and #2 for their thorough and constructive review of our manuscript “Ocean acidification changes the structure of an Antarctic coastal protistan community”. We are thankful to the referees for the recognition of the strengths of this paper studying the biological effects of ocean acidification on a natural community in Antarctica, an area that is rarely studied.

We accept and agree with all comments; the larger changes are listed below, followed by a point per point response to each of the referees’ comments.

**Introduction**

1. On reflection, we agree with the referees that the comparisons made to previous studies in both the introduction and discussion could be improved, and these have now been changed to more appropriately reflect the findings of previous studies and how these differ or agree with our findings.

**Methods**

1. The methods section has been expanded to include introductory paragraphs explaining the experimental design, followed by a section addressing the minicosm operation with further details on the light adjustments and intensities.

2. The text describing carbonate chemistry manipulation, sampling, measurements and calculations have been expanded, and an additional section has been included on macronutrient sampling and measurements. The data for both is presented in the supplement.

3. Additional detail has been included in the light microscopy section to address concerns of anonymous referee #1, including the additional steps taken to ensure accurate estimates of cell abundance, particularly for rare and/or species with patchy distributions.

**Results**

1. We have incorporated a discussion of taxa abundances and the community structure at time points prior to day 18 to account for the nutrient depletion towards the end of the experiment.

2. The section on size-related responses in diatoms has been amended to be clearer to the reader, and an additional figure showing responses of large (>20 μm) vs. small (<20 μm) diatoms to fCO2 has been included.

**Discussion**

1. The “lag in growth” discussed in the acclimation section has been removed, which on further investigation was not significant when growth rates were calculated.

2. As outlined above, the paragraphs comparing the responses of previous studies in the “Autotrophic protist taxa specific responses” section was poorly worded or at times incorrect. This has now been amended to accurately reflect the findings of previous papers and how they compare to ours.

3. A specific paragraph has been added into the discussion as part of the “Autotrophic protist taxa specific responses” considering the effect of nutrient depletion on microbial abundances.
Figures
1. Addition of figure showing the response of small vs. large diatoms to fCO₂
2. Change pCO₂ to fCO₂ in figure legends
3. Amended axis to “cells mL⁻¹” with no exponents rather than “cells x exponent L⁻¹”
4. Average longest dimension of the cell (i.e. valve diameter or pervalvar length) added to figure captions for each taxa/functional group (Figures 1-7, now 1-8 with additional figure)

Supplementary material – now included
1. Table S1. Measurements of seawater conditions at time of sampling from Prydz Bay, Antarctica (19th November 2014).
2. Table S2. Mean carbonate chemistry speciation of DIC and pH₇ (measured) and fCO₂ and PA (calculated) for each minicosm tank after acclimation (days 8 to 18).
3. Figure S1. Temporal development of DIC within each minicosm throughout the experimental period.
4. Figure S2. Temporal development of pH₇ (total proton scale) within each minicosm throughout the experimental period.
5. Figure S3. Nitrate/nitrite (NOₓ) concentrations within each minicosm throughout the experimental period.
6. Figure S4. Dissolved reactive phosphorus (P) concentrations within each minicosm throughout the experimental period.
7. Figure S5. Molybdate reactive silica (Si) concentrations within each minicosm throughout the experimental period.

Anonymous Referee #1;

General comments: This study investigates the effect of elevated CO₂ concentrations on protistan community composition of Prydz Bay, East Antarctica. As the quantification of cell abundances at the species level is very labour intensive, often studies tend to neglect this very important aspect that has been considered in detail in the study by Hancock and co-authors. The data presented are interesting and I believe it should be published, but it needs a considerable revision to be acceptable for Biogeosciences.

The authors need elaborate in more detail about the counting procedure, in particular for cells which were present only in low abundance as they often tend not to be evenly distributed in the Utermöhl chamber, causing easily wrong cell abundance estimates.

The section “Methods – Light Microscopy” has been expanded to include more detail on the counting procedure and the steps taken to ensure representative abundance estimates were gained for all taxa/functional groups. The Utermöhl counting method described in Olrick et al. 1998 for protistan abundance estimates was used with a number of additional steps.

In brief, a stratified counting procedure (small cells <20 μm at 400x and large cells >20 μm at 200x) was employed to provide both accurate identifications of small cells that are difficult to identify under 200x magnification, but still
allowing accurate estimates of larger cells that have lower abundances (therefore fewer cells per field of view, FOV) at 400x magnification. To check that abundance estimates were accurate, mean cell counts of each taxon was recorded versus number of FOVs counted. These plots showed that the mean stabilised at 10-15 FOVs for small cells and 15-20 for large cells, therefore 20 randomly chosen FOVs were counted at each magnification. For nanoplanktonic cells (<20 μm), 20 randomly chosen FOVs at 3.66x10^6 um^2 were counted providing on average counts totalling approximately 2,000 cells, ranging from 50 for rare taxa and over 1,000 for abundant species. For microplanktonic cells (>20 μm), 20 randomly chosen FOVs at 2.51x10^5 um^2 were counted, providing on average counts of approximately 1,000 cells, ranging from 5 for rare taxa to over 300 for abundant chain forming taxa (i.e. Chaetoceros). Lastly, rare species with similar ecological function, and similar response to fCO2 treatment, were combined together into functional groups to reduce noise in the multivariate analysis. These rare species with high standard deviation in their abundance estimate were identified in the results, and not discussed further in terms of response to fCO2.

For better readability of the manuscript, information on carbonate chemistry as well as on macronutrient concentrations over the course of the experiments is needed. Details on carbonate chemistry and macronutrient measurements are now provided in a supplement.

In particular, the onset of nutrient limitation on day 16 needs to be accounted for in the discussion of the development of the protistan community, which has been neglected so far. At the moment, the discussion mainly concentrates solely on the CO2 effects, which is fine until day 15, but not after this time point. This aspect needs to be addressed. Throughout the results consideration has been included on the effects of fCO2 on the abundance of taxa/functional groups and community structure prior to day 18 of the experiment, when nitrate/nitrite levels dropped below the level of detection. An additional paragraph within the section “Autotrophic protist taxa specific responses” has been included to address the potential effect of nutrient depletion on the autotrophic responses to fCO2. Also, information has been added to the section “Community-level responses”, I.e. NOx had no statistically significant effect on community structure in the multivariate analysis, as opposed to phosphorus and silicate (which were replete throughout the entire experiment).

For better and faster comparability of the figures of species-specific cell abundances, I recommend to use the unit ‘cells per mL’. Figures have been adjusted to show “cells per mL”, rather than “cells per L” with an exponent.
Furthermore, to strengthen the author's argument that growth of large-sized diatoms is more prone to high CO2 concentrations, a graph showing actually the different trends in total abundance of all small versus all large diatoms, similar to figure 3, is needed. A figure (Figure 2 in the amended manuscript) similar to figure 3 has been added as suggested. This plot clearly shows the different responses of large (>20 μm) and small (<20 μm) diatoms to fCO2 treatment.

Introduction:
P2, L5-6: This statement is not right as there are several studies that were already published on OA effects in various natural assemblages of Southern Ocean microbes (Tortell et al. 2008, Feng et al. 2010, Hoppe et al. 2013, McMinn et al. 2014, Young et al. 2015, Coad et al. 2016, Davidson et al. 2016, Thomson et al. 2016). Please rephrase.
This sentence has been removed.

P2, L19-35: Considering that the authors already cited 8 papers that were published on CO2 effects, it is not really appropriate to write that “there have been relatively few studies”. Please also cite the studies by Hoppe et al. 2013 PLOS One and Young et al. 2015 MEPS, which are currently missing. The latter two studies need also to be taken into account when summarizing the findings on CO2-dependent shifts in community composition in this paragraph.
This sentence has been removed and the whole paragraph re-written to accurately summarise the findings of previous studies including Hoppe et al. 2015 and Young et al. 2015.

P2, L23-25: Please note that Feng et al. (2010) reported a shift from Cylindrotheca to Chaetoceros from 380 to 750 μatm pCO2, and not from Pseudonitzschia. Further Tortell et al. (2008) did not observe a CO2-triggered shift in Phaeocystis antarctica. It was reported that both summer and spring phytoplankton communities were dominated by P. antarctica and within the communities a shift among diatoms was observed.
These sentences have now been re-written to accurately summarise the findings of Feng et al. 2010 and Tortell et al. 2008, and the species covered in those papers.

Methods:
P3, L17: Did the authors assess whether the gravity filtration procedure introduced cell damage and/or physiological fitness of the sampled microbial community? The latter could have affected the evolution of the community structure.
There was no direct assessment of the potential effects of gravity filtration on cellular fitness, however the filling speed was slow to prevent damage due to turbulence (this is now added to the filling description in methods). Furthermore, we would not expect negative impacts on the cells that are small enough to pass through the mesh into the mesocosms.
P3, L25: To me is unclear why during the initial acclimation phase the community was exposed to the extremely low light intensity of ∼1 μmol photons m⁻² s⁻¹. A low light intensity was used during the acclimation period to preclude growth of the phytoplankton community whilst cellular physiology acclimated to the increase in fCO₂ to target levels in each minicosm. This information is now included in the methods section that has been restructured (outlined below).

P3, L28-32: How was the light intensity adjusted? Were the minicosms not exposed to the natural irradiance cycle? Did the authors monitor daily in situ irradiances over the whole experiment? The manipulation of the light intensity remains unclear to me.

The beginning of the methods section has been restructured to include a “Minicosm operation” section.

The minicosms were not exposed to a natural irradiance cycle as the minicosms were contained within a single shipping container with artificial lighting. The light intensity and cycle during the incubation phase of the experiment (days 8 to 18) was saturating but not inhibitory to the phytoplankton.

P4, L2-12: I can understand that carbonate chemistry results are reported in detail in Deppeler et al. (submitted), but also for this manuscript there is the need to give information on the successful CO₂ manipulation of each CO₂ treatment at least in a table. For the interpretation and discussion on the results of the development of the community composition, it would be also helpful to give the information on carbonate chemistry (e.g. pH, fCO₂) at the day of seawater sampling. Carbonate chemistry speciation throughout the experiment and at the time of seawater collection is now provided in supplementary material.

P5, L2-11: The counting of particularly large diatoms can be problematic. To this end, it is recommended to count the whole Utermöhl chamber as species are not distributed evenly. In particular, chain-forming diatom species can be very patchy, making their quantification on the basis of 20 chosen fields of view difficult. How many cells did the authors count per species? How was the patchiness of species distribution within the chamber accounted for? Considering the low cell numbers, it is important to address this issue as otherwise easily wrong cell abundance estimates can be made. The section “Methods – Light Microscopy” has been expanded to include a more detailed description of the counting procedure. A brief description of the extra steps taken to assure accurate estimates is mentioned above.

I miss information on the development of the macronutrient (N, P, Si) concentrations over the duration of the experiment. This info needs to be provided either in a table or a figure. Macronutrient concentration changes over time are now provided in a supplement.
According to Deppeler et al. (submitted to Biogeosciences) N was depleted for most treatments at day 16, this means that in addition to the changes in fCO2 N also potentially acted as stressor at the end of the experiment, potentially influencing community composition at the end of the experiment. The latter information is not obvious when nutrient data are not shown in this manuscript and needs to be accounted for in the interpretation and discussion of the results. Hence, to assess the effect of increasing fCO2 levels on community composition, the authors should rather compare results at day 16 instead of day 18. For instance, the abundance of Fragilariopsis species < 20 μm of the 343, 506, and 634 μatm fCO2 treatments strongly dropped between day 16 and 18, coinciding with nitrate limitation at these specific fCO2 levels. Nitrate/nitrite fell below the level of detection only at day 18 of the experiment (NOx measurements throughout the experiment are now shown in a supplement). A consideration of single-species and community structure responses to fCO2 prior to day 18 has now been included in the results and discussion. Concerning the decrease in nano-sized (<20 μm) Fragilariopsis abundance between day 16 and 18 of the experiment, the low NOx concentrations on day 16 do not correlate with a decline in cellular abundances on day 18 as, for instance, there was hardly any change in abundance between these two days at 953 μatm despite relatively low NOx levels. This is also observed in other taxa i.e. Chaetoceros.

Results:
P6, L21-22: It seems very unlikely that the high variation in protist abundance of the 635 μatm pCO2 treatment, accounting for ~10000 cells per mL, comes from the increase in rare large cell diatom species, which were only present between 5 up to 200 cells per mL (Fig. 2-5).
The observation of anonymous reviewer 1 is valid; therefore this sentence has been removed.

P6, L27-29: It is not really clear which figure underlines this statement. Also, it would be helpful to point out which diatoms were classified as 'large' and 'small'. For this statement, it would be good to have a graph showing actually the different trends in total abundance of all small versus all large diatoms, similar to figure 3.
A new figure has been added to the manuscript showing large (>20 μm) vs. small (<20 μm) diatom abundances.

P6, L30: Do the authors now refer to Fig. 2b-d when they refer to discoid centric diatoms or is Fig. 2a meant, but then it does not make sense to refer to 'unidentified discoid centric'. Does the latter term refer to one single species or does it summarize all counts of unidentified discoid diatom species that were smaller than 2 μm? Another idea would be to add the cell volume of the species next to its name on the graph, making it easier to see the size differences at a glance.
The term “discoid centric diatoms” has been clarified by including a description in what the term is referring to (centric diatoms with a valvar diameter greater than the pervalvar dimension i.e. centric diatoms of the genera *Thalassiosira*, *Landeria* and *Stellarima*).

The wording “unidentified discoid centric” has also been amended to reflect that this is referring to all discoid centric diatoms with a valve diameter <2 μm which could not be identified to genus or species level.

The average longest length dimension has been added to all fCO2 response graph captions (Figures 1 to 5, now 1 to 6 with additional large vs. small figure). We believe that adding it into the name of the graph will clutter the figure and make the graphs difficult to read; therefore added it into the caption. We have used the average longest length dimension as the sizing parameter as this has already been used throughout the manuscript i.e. *Fragilariopsis* below and above 20 μm in length (Figure 3 and P7L10-17).

P7, L14: To what fCO2 treatment does the control refer to? 343 μatm? We removed “control” and replaced it with “ambient (343 μatm)” throughout the manuscript.

P7, L12-14: Looking at figure 3a, small Fragilariopsis cells of the 953 μatm reached highest cell abundances in comparison with all other fCO2 treatments at day 16 and 18. The authors, however, write “Abundances in the AEŠCO2 treatments >953 μatm were lower but less than those in the control treatment. .” How can this be? The wording was adjusted to correctly reflect the response seen in Figure 3a.

P7, L15-17: Why is a tolerance lower when cell abundance is higher? The sentence was amended and wording made clearer.

P7, L20-21: The species name is O. weissflogii and not weissfloggi. Also write ‘pennate’ instead of ‘pennant’. Also, it is Pseudo- nitzschia and not Pseudonitzschia. Also change turgidulodies to turgiduloides. The miss-spelling was amended.

Discussion:
P9, L26-28: As in almost all figures cell abundances did not change between day 1 and 8, considering also that irradiance was very low, I am surprised about the statement that community composition changed.

Additional information has been added to the discussion section “Acclimation to high CO₂” to clear confusion regarding the change in community composition during the acclimation period of the experiment. During the acclimation the light irradiance was very low to preclude phytoplankton growth during physiological acclimation, therefore this change in community composition is hypothesized to be due to death of delicate cells (from sample collection or the minicosm environment i.e. lighting, being sub-optimal and different species having different environmental requirements/sensitivities). This change in community composition was the same across all minicosm tanks/ fCO2 treatments (as shown in the cluster analysis, Figure 9).
Did the authors characterize species composition of the initial community? As the information on the characterization of the initial community is missing this complicates the interpretation on subsequent species changes through the sampling. 

The initial community was characterized on day 1 of the experiment, one day after filling of the tanks and prior to any CO\textsubscript{2} manipulation (outlined in the Methods section). 

P9, L32-33: Taking into account the very low irradiance between day 1 and 8 the cells were exposed to, it is not surprising the community showed a severe delay in growth among all treatments, a finding which is not mentioned here. Apparently, the combination of very low irradiance and high fCO\textsubscript{2} caused even stronger delay. This is worth to be mentioned. On which observation is the statement based that ‘the protists required more than 8 days to acclimate to this high fCO\textsubscript{2}? After careful calculations, there is no “lag in growth” as described in the manuscript. As such we have removed this finding from the discussion and conclusion. 

P10, L1-6: To underline the statement that community growth of the highest fCO\textsubscript{2} treatment was lowest, why do the authors not calculate community growth rate? All data are there and this would strengthen their argumentation. As such we have removed this finding from the manuscript. 

P10, L9-15: I am not yet convinced about the statement that in ‘diatoms the response was mainly size-related’. To underline this, a graph showing actually the different trends in total abundance of all small versus all large diatoms, similar to figure 3, is required. The authors even point out that ‘a couple of species did not follow this trend’. An additional figure has been included with small versus large diatom cells as described above. We have now reworded this section in the discussion to reflect that whilst the majority of the diatom species follow this size-related trend, there is a few that do not (i.e. Proboscia and Chaetoceros). 

P10, L9-19: The fact that nutrients became limiting either on day 16 or 18 needs to be elaborated in more detail. This aspect was fully neglected, only in L14 it is mentioned that ‘Chaetoceros did not show a response to fCO\textsubscript{2}, but instead reflected the nutrient availability’. This aspect needs to be discussed also for the other species. Done. 

P10, L19-20: The low tolerance to high pCO\textsubscript{2} is also found and reported in Tortell et al. (2008) and Hoppe et al. (2013). A comparison to Tortell et al., 2008 and Hoppe et al., 2013 has been added.
P10, L20: ‘Unlike diatom species, . . . Phaeocystis dramatically declined . . . at the three highest fCO2 levels’. It was, however, pointed out before that ‘large diatoms showed . . . a decrease at higher fCO2’. There is no controversy, please modify. The sentence has been amended to reflect that whilst Phaeocystis is a smaller cell, its response to fCO2 is similar to that of larger not smaller diatoms.

P10, L23: Please specify the statement ‘our study only finds this response in diatoms’. To which response is referred to? The increase in diatom abundance under high fCO2? But the opposite response for diatoms was claimed before. Paragraph re-written.

P10, L21: I disagree that there is a ‘common consensus in other ocean acidification studies that pico- and nanoplanckton abundance increases at high CO2 levels’. Like the dataset of the authors, there are several studies reporting the opposite for Southern Ocean communities (Tortell et al. 2008, Feng et al. 2010, Hoppe et al. 2013). Please rephrase more carefully. Sentence removed.


P10, L25-28: In line with the data by Hancock et al., in none of the cited studies Phaeocystis antarctica showed a positive growth response to high CO2, growth rather remained unaffected by CO2. Please also add Trimborn et al. 2017 Physiol. Plant, which is in line with the latter observation. Sentence adjusted and Trimborn et al. 2017 reference added.

P10, L28-30: I disagree. The results from Feng et al. (2010) show no CO2 effect on the colonial Phaeocystis antarctica. Furthermore, it is not clear to me why the presence of the mucus could have any effect on the CO2 sensitivity of Phaeocystis. These sentences have been removed in the re-writing of the discussion section “Autotrophic protist taxa specific responses”.

P11, L1: Please also cite Wu et al. 2014 that reported enhanced growth rates in response to high CO2 in large diatoms. The Wu et al. 2014 citation added.

P11, L3-6: As mentioned before, no CO2-dependent increase in Phaeocystis was reported in Tortell et al. (2008), Feng et al. (2010) or Trimborn et al. (2013). Please correct. Further Xu et al. did also not observe any CO2-dependent increase in Phaeocystis from the current to the 2060 scenario, but a significant decline from 2060 to 2100. Please note that in the latter study next to CO2, also temperature, light and Fe availability was changed, being therefore more difficult to compare with this data set here. Sentences removed.
P11, L13-15: Please also cite Trimborn et al. (2013) who actually investigated the CCM of Southern Ocean phytoplankton species, among them Phaeocystis antarctica.
The findings from Trimborn et al. 2013 have now been included.

P11, L26-35: For better readability, please specify the direction of the observed responses of the different choanoflagellates, just saying 'there were differences' is not enough.
Done.

Discussion Part 4.4: In particular here, the onset in nutrient limitation at day 16 and 18 needs to be accounted for in the discussion of community-level responses as CO2 was not the only driver. The latter statement also applies for the overall discussion.
Additional discussion has been added to “Community-level responses” considering the depletion of nutrients during the experiment on the protistan community structure and succession (in which NO₃ was not significant in the multivariate analysis only phosphorus and silicate which were replete throughout the entire experiment). As described above this has also been included in the “Autotrophic protist taxa specific responses” section of the discussion.

Figures: Fig. 1-7: For better and faster comparability between cell abundances of the different species, I would use the unit ‘cells per mL’ in all figures instead of using ‘cells x 10⁴L⁻¹’ as in Fig. 2-4,’cellsx10⁵L⁻¹’ as in Fig. 7 or ’cellsx10⁷L⁻¹’ as in Fig. 1 and 6. The latter makes it even more complicated as the Y-axis is also changing, hampering a fast comparison between cell numbers between graphs of different figures.
Figures have been adjusted to cells per mL.

In the legends of Fig. 1 to 7, it is referred to the pCO₂ while in the M&M section it is referred to fCO₂, please stick to one of them throughout the manuscript.
pCO₂ adjusted to fCO₂.
Legends have been adjusted within Figures 1-7 to fCO₂ rather than pCO₂.

Anonymous Referee #2,

This manuscript analyses the effects of elevated CO₂ on the protistan community in East Antarctica. Firstly it is great to read another biological ocean acidification study being conducted in the Antarctic as well as being a community response study. Both these areas of research are not common with many questions left unanswered. It is, therefore, particularly interesting that this study by Hancock et al. addresses community level responses in the Antarctic where biota are considered to be the most vulnerable to OA due to the rising solubility of CO₂ in cold-waters. Overall this manuscript is well written, contains plentiful relevant data and attempts to close the gaps in our knowledge of important questions outstanding in the OA field.
Before consideration for publication, there are a few points that need addressing. In particular, more explanation is needed about the carbonate chemistry analysis. The section of the methods addressing carbonate chemistry has been expanded. In addition the carbonate chemistry speciation throughout the experiment and at the time of seawater collection has been included into the supplementary material (Table S1 and S2, and Figure S1 and S2).

DIC and pH_T are the CO2 parameters directly measured so why is fugacity of CO2 used as the CO2 parameter altered? pH (on any scale) or DIC are the usual parameters directly altered in OA studies and therefore using fCO2 limits the continuity between this study and others. I suggest using either the measured pH or DIC measurements instead.

Like many other studies we have manipulated carbonate chemistry by increasing DIC and leaving total alkalinity constant which perfectly mimics ongoing ocean acidification. The data is presented as fCO2 as this is preferred rather than pH or DIC as fCO2 corresponds to a certain time in the future based on emission scenarios. That is the reason why most studies opt to discuss their results with respect to fCO2 rather than DIC or pH.

A table of differences between the carbonate chemistry of each treatment is also necessary rather than quoting Depper et al. (submitted). Tables and figures now added to the supplementary material;

Table S1. Measurements of seawater conditions at time of sampling from Prydz Bay, Antarctica (19th November 2014).
Table S2. Mean carbonate chemistry speciation of DIC and pH_T (measured) and fCO2 and PA (calculated) for each minicosm tank after acclimation (days 8 to 18).
Figure S1. Temporal development of DIC within each minicosm throughout the experimental period.
Figure S2. Temporal development of pH_T (total proton scale) within each minicosm throughout the experimental period.

Many more details are generally needed. For example, how often was DIC measured? What was the variability between measurements in DIC and pH? How often was the probe calibrated?

The section of the methods addressing carbonate chemistry has been expanded. Daily DIC and pH_T measurements throughout the experiment are now included in the supplementary material Figures S1 and S2. The pH probe was only calibrated initially with freshwater buffers as it was simply used as an indicator of how much CO2 enriched seawater had to be added to the minicosm to maintain the CO2 level. Actual carbonate chemistry speciation samples were taken and measured after the addition using more suitable and robust methods (DIC, spectrophotometric pH_T).

A common problem with OA research is replication. I query why this experiment was not replicated given the short duration? In addition OA research is also moving towards long-term studies spanning many months to years. I also query why such a short duration was chosen for this experiment?
The methods section has been expanded to include introductory paragraphs explaining the experimental design followed by a section addressing the minicosm operation.

In brief, due to the limitation of 6 minicosms (tanks) within a shipping container gradient-dose response design was chosen, allowing to assess potential thresholds or tipping points of microbial community responses to CO₂. On day 18 of the experiment the total volume of samples removed from each minicosm was below 20% of the initial minicosm volume. This 20% level was below sub-optimal as it would effect competition and succession of microbes, therefore sampling was conducted to keep the level of tanks above 20% until the completion of the experiment. This reasoning is now included in the introduction section of the methods.

The experiment was only conducted once due to having a short time period available for set-up, running of the experiment and pack-up between transport options to and from Davis Station (ship and flights).

Throughout the manuscript there are several references to look at Deppler et al. (submitted) for information not detailed in this manuscript. I query whether this manuscript is a “stand-alone” story. Whilst this manuscript is complementary to Deppeler et al., it is a “stand-alone” story. Our manuscript reports single species responses, differences between species and changes in protistan community composition. In contrast, Deppeler et al. (submitted) presents the photo-physiological responses of the phytoplankton community. Carbonate chemistry and macronutrient data has now been added to our manuscript and, therefore, the number of references to Deppeler et al. is now minimal.

Technical corrections:
Page 1 line 1: remove ‘of’
‘Of’ was removed.

Page 1 line 8: should be a semicolon instead of a colon.
Colon was changed to semicolon.

Page 1 line 8/9: is it a case of large cells decrease in abundance in high fCO₂?
That would be a better way to report these results, as high fCO₂ is the environmental stress concerned.
Sentence was reworded.

Page 1 line 12/13: This statement needs clarification as it implies this research is not original.
Wording adjusted to; “Despite interannual differences and the time in the season which the experiment was performed, comparisons with previous experiments show that the threshold fCO₂ remains the same for this nearshore site”.
generally OA studies on organisms higher up the foodweb in the Antarctic are few which adds importance to your study and should be mentioned with some key Antarctic papers referenced. Additional discussion in the paragraph has been added describing indirect effects on higher trophic levels due to the phytoplankton community structural change.

Page 2 line 23: insert a comma. ‘With increased CO\textsuperscript{2}, Tortell et al.....’ Paragraph re-written.

Page 4 line 5: remove ‘the’ before adding Paragraph re-written.

Page 6 line 22: remove ‘a’ should be ‘likely due to’ Sentence now removed through editing for comments by Anonymous Referee #1.

Page 7 line 3: change to ‘had increased to’ Sentence now removed.

Results section: removed ‘show’ and other variations using this word as it is unnecessary. It reads better to just say ‘increased’ instead of ‘showed an increase’. The use of "show" removed throughout the results and discussion section of the manuscript.

Page 10 line 11: typo ‘a’ should be ‘at’ ‘A’ changed to ‘at’.

Page 10 line 17-19: why might there be differences between the results in this study and that of Feng et al. (2009)? Section re-written to include comments by Anonymous Referee #1. Feng et al. 2009 citation now removed.

Page 10 line 22: it is difficult to compare the results in this study to others quoted in this statement when different CO2 parameters were altered. This sentence has now been removed.

Page 10 line 23: ‘response’ instead of ‘responses’ This sentence has now been removed.
Ocean acidification changes the structure of an Antarctic coastal protistan community

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Abstract. Antarctic near-shore waters are amongst the most vulnerable in the world to ocean acidification. Microbes occupying these waters are critical drivers of ecosystem productivity, elemental cycling and ocean biogeochemistry, yet little is known about their sensitivity to ocean acidification. An unreplicated, six-level, dose-response experiment was conducted using 650 L incubation tanks (minicosms) adjusted to a gradient in fugacity of carbon dioxide (fCO₂) from 343 to 1641 µatm. The six minicosms were filled with near-shore water from Prydz Bay, East Antarctica and the protistan composition and abundance was determined by microscopy analysis of samples collected during the 18 day days of incubation. No CO₂-related change in the protistan community composition was observed during the initial 8 day acclimation period under low light. Thereafter, the response of both autotrophic and heterotrophic protists to fCO₂ were species-specific for both heterotrophic and autotrophic protists. The response by diatoms was related to cell size, large cells increasing.

Diatoms’ response was mainly cell size-related; microplanktonic diatoms (>20 µm) increased in abundance with low to moderate fCO₂ (634-953 µatm) but decreased at fCO₂ >953 µatm. Similarly, the abundance of Phaeocystis antarctica increased with increasing fCO₂ peaking at a fCO₂ of 634 µatm. Above this threshold the abundances of large micro-sized diatoms and Phaeocystis antarctica fell dramatically, and small diatoms, nanoplanctonic diatoms (<20 µm) dominated, therefore culminating in a significant change in the composition of the protistan community. The threshold change in the protistan community composition. Comparisons of these results with previous experiments conducted at this site show that the fCO₂ level at which the composition changed agreed with that previously measured at this location, indicating it remains consistent among seasons, thresholds are similar, despite seasonal and interannual differences in the physical and biotic environment. This suggests that near-shore microbial communities are likely to change significantly near the end of this century if anthropogenic CO₂ release continues unabated, with profound ramifications for near-shore Antarctic ecosystems.

food-webs and biogeochemical cycling.
1 Introduction

Eukaryotic and prokaryotic microbes are the most abundant organisms in the oceans and comprise the base of all marine foodwebs (Kirchman, 2008; Cooley and Doney, 2009; Doney et al., 2012). Their composition and abundance determines the quality and quantity of food available to higher trophic levels, affecting fishery productivity and the conservation of biological diversity (Cooley and Doney, 2009; Doney et al., 2012). Without understanding the effect of ocean acidification on protistan community structure, the indirect effects on high trophic levels cannot be assessed. In the Southern Ocean, microbes are not only drivers of productivity, elemental cycles, but also play key roles in elemental cycling and ocean biogeochemistry, meaning their response to environmental stressors is a key determinant of Southern Ocean feedbacks to global climate change (Arrigo and Thomas, 2004; Arrigo et al., 2008; Kirchman, 2008). Despite their importance, relatively little is known about the sensitivity of Antarctica marine microbes to ocean acidification and this limits our ability to predict how the Southern Ocean will be impacted in the future, and the feedback this may have on global climate change.

The Southern Ocean is particularly vulnerable to ocean acidification due to its cold temperatures, naturally low calcium carbonate saturation state, extensive upwelling and naturally large seasonal fluctuations in pH. Carbon-Cold waters have a higher solubility of carbon dioxide (CO₂) has a higher solubility at cold temperatures so that more meaning that CO₂ is being absorbed; concentrations are higher in polar waters compared to that in warmer waters. Added to this, warmer waters, and the calcium carbonate saturation is lower. Furthermore, the surface waters of the Southern Ocean are being exposed to increased CO₂ from upwelling bringing of deep CO₂ rich waters to the surface (Orr et al., 2005). This (Orr et al., 2005), a phenomenon which is enhanced in near-shore Antarctica where upwelling is greater (McNeil et al., 2010; IPCC, 2011).

These waters already experience large seasonal fluctuations in pH, upon which the anthropogenic decrease is superimposed (McNeil et al., 2010; IPCC, 2011). Prydz Bay, off Davis Station East Antarctica, shows an has a large fluctuation in the annual cycle of CO₂ levels ranging from as high as 450 µatm during autumn and winter to below 100 µatm in summer concentration (Gibson and Trull, 1999; Roden et al., 2013). During autumn and winter, sea-ice covers the ocean, light is limiting to photosynthesis, and the draw-down of CO₂ by primary production and air-sea gas exchange is negligible, resulting in an increase in CO₂ to as high as 450 µatm. During spring and summer the sea-ice retreats and phytoplankton bloom, due to the increased light and nutrient availability, resulting in a decrease in CO₂ to levels lower than 100 µatm (Gibson and Trull, 1999; Roden et al., 2013). Thus, phytoplankton in these coastal Antarctic waters are exposed to highly variable carbonate chemistry over the conditions over the full annual cycle.

There have been relatively few studies on the response of Studies investigating the effects of ocean acidification on natural Antarctic microbial communities have mainly been conducted in the Ross Sea and Western Antarctica (Antarctic Peninsula and Weddell Sea area). In the Ross Sea, Tortell et al. (2008b) and Feng et al. (2010) found that the microbial communities to increased CO₂ in the Southern Ocean and Antarctic waters, and out of these fewer have been at the community level (Feng et al., 2010; McMinn et al., 2014; Coad et al., 2016; Davidson et al., 2016; Tarling et al., 2016; Thomson et al., 2016; McMinn et al., 2016; McGillicuddy et al., 2016; and Minas et al., 2016).
These studies report a range of microbial responses to community shifted from being dominated by pennate diatoms to larger chain-forming diatoms with increased CO₂. But one common trend was a shift in community composition. With increased CO₂ and Feng et al. (2010) found an increase in the dominance of levels, whereas in the Weddell Sea Hoppe et al. (2013) found a shift from Chaetoceros Pseudo-nitzschia species over smaller, pennate diatoms such as Pseudo-nitzschia subcurvata. They also found an increase in Phaeocystis antarctica abundance at 800 µatm, although Feng et al. (2010) found no effect on the same species. Davidson et al. (2016) and Thomson et al. (2016) ran a miniecosm study at the same location as this study (Davis Station, East Antarctica), and found a to smaller pennate diatom species. However, there are also studies in both regions that have found no significant shift in community composition (Tortell et al. 2008a in the Ross Sea and Young et al. 2015 at Palmer Station, West Antarctic Peninsula). East Antarctica has received comparatively little attention, with only one series of experiments conducted on pelagic coastal communities in Prydz Bay (Davidson et al. 2016 and Thomson et al. 2016). This study reported significant change in species abundance and biomass with increases in increased CO₂ above 643 to 1281 µatm (Davidson et al., 2016; Thomson et al., 2016). Below this threshold the community had a high abundance of large diatoms 643 µatm the community was dominated by micro-sized diatoms species, but above was dominated by this level nanoplankton and picoplankton and Fragilariaopsis curta/cylindrus <10 µm long. This dominated. These results differ from the findings of Tortell et al. (2008b) and Feng et al. (2010) who found a shift towards larger diatoms species, but is consistent with Hoppe et al. (2013) and microbial communities studied elsewhere in the world, particularly in the Arctic, where studies have observed a shift to picoplankton at high CO₂ has been observed (Hare et al., 2007; Brussaard et al., 2013). Schulz et al. (2017) recently reviewed 31 community level studies, finding an increase in picoeukaryotes at high CO₂ in most studies, particularly prasinophytes and chlorophytes. The effects of increased CO₂ on larger marine diatoms was less clear with evidence for both promotion and inhibition. Schulz et al. (2017) concluded that the effects on marine diatoms are likely to be at a species level rather than the community level, and therefore could be more difficult to detect.

Incubations of natural communities, which include the effects of species interactions and competition among species, are essential to predict accurately assess and project the effects of ocean acidification on microbial communities these communities and future elemental cycling (Schulz et al., 2017). This study will address the following questions on with respect to a natural microbial community from near-shore East Antarctic waters.

1. Do individual species have different tolerances to increased CO₂ level?

2. Does the protistan community composition and abundance change with increased levels of CO₂? And what CO₂ level elicits this change?

3. Does an acclimation period allow the protistan community to better tolerate exposure to elevated CO₂?

4. When compared to Davidson et al. (2016) and Thomson et al. (2016), does our experiment indicate that the response by the protistan community at this site is consistent in nature and threshold, irrespective of seasonal and interannual differences in the composition of the community and the availability of nutrients?
2 Methods

An unreplicated, six-level, dose-response experiment was conducted on a natural, near-shore Antarctic microbial community over a range gradient of CO₂ levels (343, 506, 634, 953, 1140 and 1641 µatm). The experiment was conducted using a temperature controlled shipping container housing six, 650 L polythene tanks (minicosms). This allowed temperature, light and mixing to be controlled, and CO₂ levels to be varied between minicosm tanks. Due to the constraint of six minicosms, an unreplicated design with six CO₂ levels was chosen to best identify potential thresholds in protistan response.

The minicosm experiment was conducted between the 19th November and the 7th December 2014. Initially, the microbes in the tanks were given a day at low light to acclimatize to the minicosm conditions. This was followed by a five-day (days 1-5) acclimation period of gradually increasing CO₂ levels at low light, precluding phytoplankton growth while cellular physiology acclimated to the CO₂ increase (see below in minicosm operation). Thereafter, CO₂ was re-adjusted daily to maintain the CO₂ target level in each treatment for the remainder of the experiment (see below in carbonate chemistry manipulation, measurements and calculations). To minimise potential effects of sample removal on the competition and succession of microbes, the experiment was stopped on day 18 before the volume of water in the minicosms dropped below 20% of the initial volume (due to sample collection).

2.1 Minicosm operation

Seawater was collected on the 19th November 2014 approximately 1 km offshore from Davis Station, Antarctica (68°15’S, 77°35’E) from an area of ice-free water amongst broken fast-ice. The seawater was collected using a thoroughly rinsed 720 L Bambi bucket slung beneath a helicopter and transferred into a 7000 L polypropalene polythene reservoir tank. Six 650 L polythene tanks (minicosms), located in a temperature controlled shipping container, were immediately filled via After filling, the contents of the reservoir tank were immediately gravity fed to the minicosms via a teflon lined hose via gravity fitted with an in-line 200 µm Arkal filter to exclude metazooplankton. The minicosms were simultaneously filled to ensure they contained the same starting community. Tanks were filled simultaneously to ensure a uniform starting community, and at a slow flow rate to minimise damage to delicate cells by turbulence. The ambient water temperature at the time of collection was −1.0 ± 0.17 °C and the minicosms were maintained at a temperature of 0 ± 0.5°C (full properties of the seawater at the time of collection are provided in the supplementary online material Table S1). At the centre of each minicosm there was an auger shielded for much of its length by a tube of polythene. This auger was rotated at 15 rpm to gently mix the contents of the tanks. Each minicosm tank was covered with an acrylic air-tight lid to prevent out-gassing of CO₂ off-gassing outside of from the minicosm headspace. For a more detailed description of the minicosm set-up see Davidson et al. (2016).

The minicosm experiment was conducted between the 19th November and the 7th December 2014. Initially, the contents of the tanks were given a day to equilibrate to the minicosms. This was followed by a five day acclimation period to increasing CO₂ at low light (0.8 ± 0.2 µmol m⁻² s⁻¹), allowing cell physiology to acclimated to the CO₂ increase. Each minicosm was illuminated using a 150W metal halide lamp (Osram) on a 19:5 hr light:dark cycle. During the initial acclimation phase of the experiment (days 1-5) -During this period the CO₂ was progressively adjusted over five days to the target level for each tank.
Thereafter CO₂ was adjusted daily to maintain the CO₂ level in each treatment (see carbonate chemistry section below). The light was filtered using one layer of quarter colour temperature (CT) blue filter, two 90% neutral density (ND) filters (Arri) and a light-scattering filter, resulting in a low light intensity (photosynthetic active radiation of 0.9 ± 0.2 µmol m⁻² s⁻¹). Following acclimation to the various CO₂ treatments, light was progressively adjusted to 89 increamentally increased over 24 hrs to 90.52 ± 46-21.45 µmol m⁻² s⁻¹ at via the removal of the two 90% ND filters leaving the CT blue and light-scattering filter. This final light intensity was maintained for the remainder of the experiment (days 8-18) on a 19 h light:5 h light/dark cycle. The community was incubated and allowed to grow for a further 10 days (days 8-18) with CO₂ adjusted back to target each day (see carbonate chemistry section below). For a detailed description of lighting apparatus and climates see Davidson et al. (2016) and Deppeler et al. (submitted). Despite increasing chlorophyll a concentrations, this light intensity was found to be saturating for photosynthesis from day 8 to the end of the experiment (see Deppeler et al. submitted for details).

### 2.2 Carbonate chemistry manipulation, measurements and calculations

Carbonate chemistry was calculated from dissolved inorganic carbon (DIC), pH₇ (total proton scale), salinity and temperature. Minicosm fugacity Fugacity of CO₂ (fCO₂) was adjusted incrementally during the acclimation phase to the target level for each tank, and then for the remainder of the experiment in each minicosm throughout the experiment by the addition of CO₂ was adjusted daily to maintain the target levels within the tanks. Daily enriched natural seawater. During the acclimation phase adjustments were made based on morning and afternoon pH measurements, and were achieved by the adding the required volume incrementally until target levels were reached on day 5, and thereafter re-adjusted on a daily basis. Every morning pH measurements were conducted prior to regular sampling using a portable, NBS-calibrated probe (Mettler Toledo). This measurement was used to estimate the deviation in fCO₂ from target levels. Re-adjustments were then conducted by adding appropriate quantities of 0.2 µm filtered CO₂ saturated seawater. After adjustment, full carbonate chemistry measurements were performed (DIC, pH, salinity and temperature) to check the adjustment, and if required a secondary adjustment was made. Daily pH measurements were conducted using a portable, NBS calibrated probe (Mettler Toledo) and salinity measurements via a conductivity metre WTW197, fed into the minicosms from 1000 mL infusion bags at a rate of 50 mL min⁻¹. Samples were then collected to quantify the carbonate chemistry speciation in each minicosm using measured DIC, pH₇ (total scale), temperature and salinity.

DIC was measured by infra-red absorption on an Apollo SciTech AS-C3 analyzer equipped with a LICOR7000 and pH₇ with was measured following the spectrophotometric approach described in Dickson et al. (2007) using the pH indicator m-cresol purple on a GBC UV-Vis 916 spectrophotometer in a ten centimetre thermostated cuvette and pH indicator dye (m-cresol purple). All samples 10 cm thermostated cuvette. Samples for the carbonate chemistry were collected in 500 speciation measurements were collected without headspace in 250 mL glass bottles with stoppers following the Dickson et al. (2007) guidelines. Full carbonate chemistry measurements and calculations are described in Deppeler et al. (submitted) (Dickson et al., 2007) and daily measurements of certified reference material (batch CRM127) were used for improved accuracy (Dickson, 2010). Salinity measurements were made using a WTW197 conductivity meter. Daily carbonate chemistry speciation (fCO₂ and Omega) were
calculated from measured DIC and pH7 (for further details see Schulz et al. (2017)). The mean carbonate chemistry conditions for each minicosm are presented in the supplementary online material (Table S2, Figure S1 and S2).

2.3 **Microbial community structure**

Macronutrients sampling and measurements

Samples—Macronutrient samples were taken by filtration through sterile 0.45 µm Sartorius filters into 50 mL falcon tubes following the protocol of Davidson et al. (2016). These samples were frozen at -20 °C and transported to the Australian Antarctic Division, Hobart, Australia. On return to Australia the samples were analysed using flow injection at Analytical Services Tasmania and the concentrations of nitrate/nitrite (NO₃⁻), dissolved reactive phosphorus (P) and molybdate reactive silica (Si) were calculated (online supplementary material Figures S3 - S5).

2.4 **Microbial community structure**

A further 1 L of water was taken on days 0, 6, 13 and 18 for analysis by Field Emission Scanning Electron Microscope (FESEM). These samples were concentrated to 5 mL by filtration over a 0.8 µm polycarbonate filter. Cells were resuspended, the concentrate transferred to a glass vial and fixed to a final concentration of 1% EM-grade gluteraldehyde (ProSciTech Pty Ltd).

All samples were stored and transported at 4 °C to the Australian Antarctic Division, Hobart, Australia for analysis.

2.4.1 **Electron microscopy**

Glutaraldehyde-fixed samples were prepared for FESEM imaging using a modified polylysine technique (Marchant and Thomas, 1983). In brief, a few drops of glutaraldehyde-fixed sample were placed on polylysine coated cover slips and post-fixed for 30 min with OsO₄ (4%) vapour for 30 min, allowing cells to settle onto the coverslips. The coverslips were then rinsed in distilled water and dehydrated through a graded ethanol series ending with emersion in 100% dry acetone before being. Finally, the samples were critically point dried in a Tousimis Autosamdrı-815 Critical Point Drier to replace acetone molecules with CO₂. The coverslips were mounted onto 12.5 mm diameter aluminium stubs and sputter-coated with 7 nm of platinum/palladium in-using a Cressington 208HRD coater. Imaging of stubs was conducted by Samples were examined using a JEOL JSM6701F FESEM and protists identified using Scott and Marchant (2005).
2.4.2 Light microscopy

Lugol's-fixed and sedimented samples were analysed by light microscopy between July 2015 and February 2017 within two years of collection. Between 2 to 10 mL (depending on cell-density) of lugols concentrated samples was Lugol’s-concentrated samples were placed into a 10 mL Utermöhl-Utermöhl cylinder (Hydro-Bios, Keil/Kiel) and the cells allowed to settle overnight using the method of Olrik (1998). Due to the large variation in size and taxa, a stratified counting procedure was employed to ensure both accurate identification of small nanoplanktonic cells and representative counts of larger rare, larger microplanktonic cells. All cells greater than 20 µm were identified and counted at 20x-200x magnification; those less than 20 µm at 40x magnification. For larger cells (>20 µm), 20 randomly chosen 400x magnification. To check that abundance estimates were accurate, mean cell counts of each taxon were recorded versus number of fields of view (FOV) at 3.66x10^6 µm^2 were counted to gain an average cells per L. For smaller cells (<FOVs) counted. These plots showed that the mean stabilised at 10-15 FOVs for small cells and 15-20 for large cells. Consequently, we counted 20 FOVs per sample for both nano- and microplanktonic cells to ensure that counts gave truly representative estimates of the mean species abundance. For nanoplanktonic cells (< 20 µm³), each FOV of 2.51x10^5 µm² area, provided on average counts totaling approximately 2,000 cells, ranging from 50 for rare taxa and over 1,000 for abundant species. For microplanktonic cells (>20 randomly chosen FOVs at 2.51x10^5 µm² were counted µm), each FOV of 3.66x10^6 µm² area, provided on average counts of approximately 1,000 cells, ranging from 5 for rare taxa to over 300 for abundant chain forming taxa (i.e. Chaetoceros spp.). Rare taxa with high variance but similar response to fCO2 treatment, were combined into functional groups and abundant taxa with low variance were examined separately (see Table 1 for taxon/functional groups and abbreviations). Counts were conducted on an Olympus IX 81 microscope with Nomarski interference optics. Identifications were determined using and identifications were based on Scott and Marchant (2005) and FESEM images. Autotrophic protists were distinguished from heterotrophs via their taxonomic identity and the presence of chloroplasts and based on their taxonomic identity.

2.5 Statistical analysis

The minicosm experiment was a six dose, unreplicated dose-response experiment based on a repeated measures design. Due to the lack of replication, no formal statistics could be undertaken on the interactions between time and fCO2 treatment. Temporal changes in species abundances between treatment groups were informally assessed by plotting the mean microbial abundance at each time for each treatment. Means and standard errors were calculated from separate FOV counts; as these are sub-samples are from a single treatment, they should be considered pseudo-replicates and are indicative of treatment-level sampling variability. Abundant taxa with low variance were examined separately, but rare taxa with high variance were combined into functional groups (see Table 1 for abbreviations). In plots, abundance estimate variability. To avoid over-plotting in the figures, data points from the different fCO2 treatments were slightly offset on the x-axis at each sample time to avoid over plotting of the data. Cluster analyses and ordinations were performed on Bray-Curtis resemblance matrices matrixes formed from square-root transformed abundance data. This transformation was assessed as appropriate for reducing the influence of abundant species, as judged from a one-to-one relationship between observed dissimilarities and ordination distances (i.e. Shepard diagram, not
shown). The Bray-Curtis metric was used as it is recommended for ecological data due to its treatment of joint absences (i.e., these do not contribute towards similarity), and giving more weight to abundant taxa rather than rare taxa (Bray and Curtis, 1957). The **data days 1–10 of fCO₂ on protistan community composition was assessed separately for the 8 and then days 8 to 18 were analysed separately to distinguish community structure in the day acclimation period and in the exponential growth phase during the incubation period of the experiment 10 days of growth**.

Hierarchical agglomerative cluster analyses **based on the Bray-Curtis resemblance matrix** were performed using group-average linkage and the Bray-Curtis resemblance matrices. Significantly different clusters of samples were determined using SIMPROF (similarity profile permutations method) (Clarke et al., 2008) with an alpha value of 0.05 and based on 1000 permutations. An unconstrained ordination by non-metric multidimensional scaling (nMDS) was performed on the resemblance matrix with using **a primary (‘weak’) treatment of ties (Kruskal, 1964a, b). This was repeated over 50 random starts (Kruskal, 1964a, b)**. The Procrustean superimposition approach advocated by Peres-Neto and Jackson (2001) was used to ensure a globally optimal solution according to Peres-Neto and Jackson (2001). Clusters are displayed in the nMDS using colour. Weighted average of sample scores are shown in the nMDS. In the nMDS plot, clusters are displayed using colour and weighted averages of species sample scores were plotted to show the approximate contribution of each species to each sample. The assumption of a linear trend for predictors within covariates in the ordination was checked for each covariate, and in all instances was found to be justified.

A constrained canonical analysis of principal coordinates (CAP) was conducted according to the Oksanen et al. (2017) protocol using the Bray-Curtis resemblance matrix. This analysis was used to assess the significance of the environmental covariates, or constraints, in determining the microbial community structure. Unlike the nMDS ordination, the CAP analysis uses the resemblance similarity matrix to partition the total variance in the community composition into unconstrained and constrained components, with the latter comprising into two components, a constrained component that comprises only the variation that can be attributed to the constraining variables (fCO₂, Si, P and NOₓ). Random reassignment of sample resemblance was performed over 199 permutations to compute the pseudo-F statistic as a measure of, and an unconstrained component not attributed to the constraining variables. Permutation tests were used to assess the significance of each environmental constraint in the structural change of the microbial community (Legendre and Anderson, 1999). A (Legendre and Anderson, 1999), with a minimum subset determined by a forward selection strategy was used to choose a minimum subset of significant constraints that still account for the (Legendre et al., 2011). This provided a final model that included only the significant predictors while still accounting for a majority of the variation within the microbial community (Legendre et al., 2011) potentially explained by constraining covariates.

All analyses were performed using R v1.0.136 (R Core Team, 2016) and the add-on package vegan v2.4-2 (Oksanen et al., 2017).
3 Results

3.1 Protistan community overview

The starting microbial community was characteristic of a post sea-ice break-out community in the near-shore seawater of Prydz Bay (Waters et al., 2000). It was highly diverse with over 100 species present, ranging from small flagellates (< 2 μm) to large diatoms (>100 μm). The total overall protistan abundance at the beginning of the experiment was quite low (approximately 3x10^5 cells L^{-1} to 1300 cells mL^{-1}), but increased to between 6.4x10^6 and 1.0x10^7 cells L^{-1} to 6,400 and 19,000 cells mL^{-1} towards the end of the experiment, depending on the treatment. Abundances remained low during the acclimation period (days 1 to 8) then increased exponentially from days 10 to 16 in all treatments except the highest fCO₂ treatment (Figure 1). At day 16 there was a decrease in abundance in all treatments except 635 MPA at 634 and 1641 μatm. The high variance in the 635 μatm treatment on day 18 is likely due to the increase in a few rare large diatom species, which were highly variable. The highest abundance at 634 μatm. From day 14 onwards the total cell abundance increased with low to moderate fCO₂ treatment shown a different trajectory to the other tanks, with low abundance maintained until day 12, after which cell numbers increased exponentially; hereafter this is referred to as a lag in growth (506 - 953 μm) but in higher fCO₂ treatments (1140 and 1641 μm) remained either similar to, or lower than that of ambient (343 μm) (Figure 1).

3.2 Species-specific fCO₂ tolerances

3.2.1 Diatoms

Diatoms dominated the microbial community and showed had marked responses to increased fCO₂ levels. The response of diatoms was mainly size related, with small diatoms (nano-planktonic diatoms (< 20 μm) showing little effect of exposure being more tolerant to higher fCO₂ while larger exposure than micro-planktonic diatoms (>20 μm) (Figure 2). Nano-sized diatoms had abundances similar to, or higher than ambient (343 μatm) in all treatments from days 14 to 18. In contrast, micro-sized diatoms increased in abundance at moderate levels fCO₂ levels (≤ 634 μatm) but declined at the three higher fCO₂. This trend was particularly evident in treatments (Figure 2). The abundance of micro-sized diatoms was particularly low in the highest fCO₂ treatment from day 10 of the experiment.

This size-related response was also observed at a species level, with the response of discoid centric diatoms (Thallassiosira antarctica, Landeria and Stellarima), which showed decreased fCO₂ tolerance with increasing size or similar, have been grouped as "discoid centric diatoms". The smallest discoid taxa, an unidentified 1 to 2 μm diameter centric diatom, showed discoid centric diatoms, had no significant response to increasing fCO₂ (Figure 2a3a). Thalassiosira antarctica, which ranged in size from 10 to with an average valve diameter of 20 μm diameter, also showed no effect of also did not respond to increased fCO₂ until except in the highest treatment level of 1641 μatm (Figure 2b). Landeria annulata, a medium sized cell of 30 to 60 μm diameter, had an increase in abundance in low to moderate fCO₂ treatments (343-634 μatm) after...
which abundances decreased but not lower than that of the control treatment (Figure 2c). The two larger dominant 3b). Two larger discoid centric species, Stellarima microtrias (40 to 80 µm diameter-average valve diameter of 55 µm) and Thalassiosira ritscheri (>average valve diameter of 50 µm diameter) showed an increase in abundance with moderate increases in fCO2 had increased abundances in the low to moderate fCO2 treatments (≤ 634 µatm), but they showed a decrease in abundance at the three higher fCO2 levels (Figure 2d-3d and e respectively). Whilst these two larger species These responses emerged between days 12 and 14 of the experiment. One discoid centric diatom which did not follow this size-related trend was Landeria annulata, a larger sized cell with an average valve diameter of 55 µm. Unlike S. microtrias and T. ritscheri, it had a similar response, there were subtle differences that correlate with differences in the average size of the species; Stellarima was slightly more tolerant than T. ritscheri (average sizes of Stellarima is less than T. ritscheri abundance in all fCO2 treatments except the 634 µatm treatment, where its abundance was higher than the other treatments on days 16 and 18 of the experiment (Figure 3c).

A similar size-related response was observed in for Fragilariopsis spp. cells (mostly F. cylindrus but included occasional F. curta and F. kerguelensis). Fragilariopsis spp., which was the dominant diatom, comprising between 15 to 50% of the total phytoplankton abundance and ranged in length from 2 µm to >50 µm toward the end of the incubation (days 16 and 18) and comprised between 15 to 50% of the total phytoplankton abundance. From day 14, the abundance of small-nano-sized Fragilariopsis cells (<20 µm) was higher in similar or higher in all treatments exposed to moderately-enhanced fCO2 (506-953 µatm) compared to that of the control. Abundances in the fCO2 treatments >953 ambient (343 µatm) were lower but less than those in the control treatment (Figure 3a) (Figure 4a). In contrast, larger micro-sized cells of Fragilariopsis spp. (>20 µm) showed a lower tolerance with abundances highest had higher abundance in the three lower fCO2 treatments and considerably lower abundances in the two highest fCO2 treatments on day 18. The abundance of large-micro-sized cells in the 953 µm treatment fell between these two extremes (Figure 3b-4b). This trend was not evident until day 18 of the experiment.

Other diatoms showed similar responses with larger species having larger diatoms had a similar response to S. microtrias, T. ritscheri and micro-sized Fragilariopsis spp., Odontella spp. (mainly O. weissflogii but also some O. litigiosa) and the pennate diatoms Pseudo-nitzschia subcurvata and Pseudo-nitzschia turgiduloides (Figure 5a, b and c respectively), all had higher abundances in fCO2 treatments <343, 506 and 634 µatm but lower abundances in the three highest fCO2 treatments (≥953 - 1641 µatm). This included members of the centric diatom genus Odontella (mainly O. weissflogii but also some O. litigiosa) and the pennate diatoms Pseudo-nitzschia subcurvata and Pseudo-nitzschia turgiduloides (Figure 4a, b and c respectively).

The abundance of two diatom taxa Proboscia truncata was unrelated to fCO2 treatment. Chaetoceros and Proboscia truncata both showed no fCO2 related trend in their abundances despite being relatively large diatoms (P. truncata can exceed 100 despite being a larger diatom species (average pervalvar length of 130 µm in length) (Figure 5). This may reflect a lack of precision in the microscope counts due to high variance among replicate fields of views. The response of 6a). At day 14, Chaetoceros (mainly spp. (mainly C. castracanei but C. tortissimus and C. bulbosus were also present) is difficult to interpret. At day 14 their abundance was lower had lower abundances in the three higher fCO2 treatments. By but by day 18 no fCO2 related trend was evident in Chaetoceros abundance (Figure 5b(Figure 6b).
3.2.2 Flagellates

Colonial life stage. The **colonial life stage** of *Phaeocystis antarctica* occurred in much higher abundances in the three lower fCO$_2$ treatments. It was the most abundant flagellate in this study, reaching concentrations from $\sim 1.0 \times 10^5$ to $1.26 \times 10^7$ cells L$^{-1}$ at ranging from $\sim 100$ cells mL$^{-1}$ at the start of the experiment to 12,600 cells mL$^{-1}$ on day 18 in fCO$_2$ levels treatments $\leq$634 µatm (Figure 67). This starkly contrasted with abundance abundances at fCO$_2$ levels $\geq$953 µatm in which they which did not exceed $1.6 \times 10^6$ cells L$^{-1}$ - 1,600 cells mL$^{-1}$. This strong difference in abundances between the three lower and three higher fCO$_2$ treatments emerged early in the experiment, with a discernible difference by day 12.

Other abundant flagellate taxa in our study showed had a variety of responses. The choanoflagellate *Bicosta antennigera* showed a fCO$_2$-related response similar to that of responded similarly to *P. antarctica* with higher abundances in fCO$_2$ treatments $\leq$506 µatm and lower abundances at higher fCO$_2$ levels (Figure 7a8a). Other choanoflagellates (mainly *Diaphanoeca multiannulata*) showed had no consistent trend in response to fCO$_2$ level (Figure 7b8b).

The abundances of other nanoflagellates and other heterotrophic protists were low and seemingly unrelated to fCO$_2$ treatment. All other nanoflagellates were low in abundance and had high variance between field of view counts, therefore these were grouped together. This group, termed "Other Flagellates", including species includes species *P. antarctica* (gamete and flagellate forms), *Telonema antarctica*, *Leucocryptos* sp., *Polytoma* sp., *Pyramimonas gelidicola*, *Geminigera* sp., *Mantoniella* sp., *Bodo* sp., *Triparma laevis* subsp *T. ramisping*, *T. laevis* subsp. *pinnatilobata* and an unidentified haptophyte. Similarly microheterotrophs comprised only of $\sim$1% of all cells and was dominated by, of which an unidentified euglenoid ($\sim$0.8% was the most abundant (making up 80% of the total microheterotrophic abundance). Dinoflagellates were grouped into autotrophic dinoflagellates (mainly *Gymnodinium* and *Heterocapsa*) and heterotrophic dinoflagellates (predominantly *Gyrodinium* spp., *G. glaciale*, *G. lachryma* and *Protoperidinum* cf. *antarcticum*). Ciliates were grouped together but were mostly comprised of *Strombidium* spp. While none of these functional groups showed a trend in abundance had a response correlated with fCO$_2$ treatment, this may be due to the abundance of these taxa being poorly resolved uncertainties related to overall low counts.

3.3 Community-level responses

[Analysis Analyses of the community-level responses has have been separated into the 8 day acclimation and 10 day growth periods. During acclimation, the growth of the cells was limited by low light. SIMPROF analysis of the microbial community over the acclimation period identified three significant groups (p <0.05) (Figure 89). Group 1 is comprised of all the treatments at day + group 1. Group 3 contains of all the treatments over days 3, 5 and 8 except for the lowest fCO$_2$ treatment on day 3 (D3T1, group 2).

Cluster analysis and SIMPROF based on the composition of the protistan community, identified ten significantly different groups of samples (p-value <0.05) during the growth period (days 8 to 18) (Figure 9a10a). On days 8 and 10 the communities did not differ among treatments, except for the highest fCO$_2$ treatment on day 10 (D10T6), which was clustered with the day 8 samples (clusters 8 and 9, Figure 9a10a). Day 12 treatments are scattered across the cluster groups but day 14 samples are again grouped together (all treatments except 634 µatm together in cluster 6). On day 16 the fCO$_2$ treatments were
clustered together except at the highest fCO₂ level. By day 18 the fCO₂ treatments had separated into two distinctly different groups; one with the three lowest fCO₂ treatments and the second with the three highest fCO₂ treatments. Interestingly, these three highest fCO₂ treatments fall into the cluster with the day 16 samples (or nearby cluster 2). This shows means that at day 18 the higher fCO₂ treatments (≥ 953 µatm) contained a protistan community more similar to that of day 16, and were significantly different to that of the day 18 lower fCO₂ treatments (Figure 9a-10a).

A nMDS in two dimensions proved a reasonable approximation to the full multivariate structure (stress = 0.05), and shows depicts the similarity among clusters along with the relative contribution of each specific taxon/functional groups to each cluster (the more closely species are located to a sample in the nMDS, the more abundant it is in that sample) (Figure 9b-10b). The community at day 8 was dominated by discoid centric diatoms below unidentified discoid centric diatoms with a valve diameter smaller than 40 µm, flagellates, ciliates, P. subcurvata and dinoflagellates (heterotrophic dinoflagellates are located off to the left of the plot in Figure 9b-10b). By day 18 the community had shifted to be dominated by Fragilariopsis spp., T. antarctica, T. ritscheri, Odontella spp., and P. antarctica (Figure 9b). Between the start and the end of the experiment other taxa emerged between days 10 and 14, including an unidentified euglenoid, L. annulata, B. antennigera and other centric diatoms. Other taxa increased in abundance between days 14 and 16, including other choanoflagellates, large micro-sized centric diatoms (i.e., P. truncata and S. microtrias), and pennate diatoms (i.e., P. turgidulodies turgiduloides) (Figure 9b-10b). At the end of the experiment (day 18) large micro-sized diatoms, T. ritscheri, Odontella spp., and Fragilariopsis spp. (>20 µm) as well as P. antarctica are located close to the lower fCO₂ treatments for day 18 (cluster 1), and resistant nano-sized Fragilariopsis (<20 µm) is found near the high fCO₂ treatments (cluster 4). Interestingly, T. antarctica is also located close to the lower fCO₂ treatments despite being quite resistant to increased fCO₂ when analysed as a single species (Figure 9b and Figure 2b-10b and Figure 3b).

From the nMDS the lag in community growth and succession in the highest fCO₂ level (tank Tank 6, 1641 µatm) can be seen. At day 10, the community is grouped with that in this treatment is grouped among all tanks sampled on day 8, likewise at day 12, 14, 16 and 18 it is consistently closer to samples from the previous time point (Figure 9b). These results are consistent with the changes in total cell abundance (Figure 1). The highest fCO₂ level inhibits growth and succession in the protistan community such that it is consistently a time point behind the other fCO₂ treatments.

CAP analysis showed differences Differences in the trajectory of the protistan community succession over time in the different fCO₂ treatments (Figure 10) in represented in the CAP analysis (Figure 11). This analysis, using covariates fCO₂, NO₃, Si and P, provided a model which explained 71.61% of the variation in similarity among samples (F₄,₃₁ = 19.544, p <0.001 based on 999 permutations). However, NO₃ was not significant (F₁,₃₂ = 1.3714, p >0.200 based on 999 permutations) and was therefore dropped from the model. In this the reduced CAP model, CAP1 and CAP2 were both significant (p <0.05) (Table 2), and the remaining terms fCO₂, Si and P together accounted for 70.35% of the total variance (F₃,₃₁ = 25.308, p <0.001 based on 999 permutations). Considering each term marginal to all others (i.e., the contribution of a term after first accounting for all other terms), fCO₂ accounted for 2.92%, P 22.68% and Si 5.21% of the variance (Table 3a). The remaining terms present in the reduced model were all significant when sequentially added, but in the marginal effects showed that only P and Si were significant, not fCO₂ (p >0.100) (Table 3a and 3b respectively). The CAP analysis shows there is a separation
of the protistan community between low (≤ 506 µatm), medium (634 to 1140 µatm) and high (1641 µatm) fCO₂ treatments (Figure 4011). At day 18 there are two distinct treatment groups, those namely: those exposed to low and moderate fCO₂ (343, 506 and 634 µatm) and those exposed to high fCO₂ (953, 1140 and 1641 µatm).

4 Discussion

4.1 Acclimation to high fCO₂

Changes in the response of the protistan composition and abundance during the acclimation period of the experiment (days 1 to 8) were likely due to the transfer and establishment of the natural communities in the minicosm tanks rather than exposure to increasing fCO₂. There was a significant shift in species composition between day one and all other samples sampling times during acclimation. The collection of seawater (via Bambi bucket under a helicopter) and subsequent filling of the minicosms is likely to have damaged delicate protists (Estrada and Berdalet, 1997), and the minicosm conditions may have been sub-optimal for some species (Kim et al., 2008). Therefore it is likely this change in community composition reflected the decline in delicate species reflects the change in environmental conditions between the natural environment and the minicosms (i.e. light, temperature, turbulence etc.).

The total abundance of the protistan community showed an initial lag in growth. Throughout the experiment the community structure of the highest fCO₂ level. Our results indicate that the protists required more than (1641 µatm) was more closely associated with that of other treatments at the previous time point, rather than those on the same day. For example, the community structure at 1641 µatm on day 10 is associated with the community structure of day 8 days to acclimate to this high, rather than the other treatments on day 10. This suggests that the highest fCO₂ and therefore growth was delayed compared to other treatments inhibits growth and succession in the protistan community. Deppeler et al. (submitted) showed that during acclimation there was a decrease in the photosynthetic health of the community, but whilst all treatments had recovered by day 12, the highest fCO₂ treatment had a greater decline in photosynthetic health and took longer to recover. This lag was also seen in accumulation of the increase in chlorophyll a, rate of productivity and nutrient concentrations where the slower growth meant this was the only treatment in which nutrients were not limiting by the end of the experiment (Deppeler et al., submitted). After this lag depletion of macronutrients (Deppeler et al., submitted). Together with the findings reported herein, this data suggests that there is a "lag" in the development of the rate of growth in the protistan community at the highest fCO₂ treatment is similar to other treatments. To our knowledge this is the first study to report such an acclimation response in a short-term experiment.

4.2 Autotrophic protist taxa-specific responses

The microbial community within this study was highly diverse, and the detailed taxonomic classification employed allowed the range of responses by the individual taxa to be resolved. In diatoms the response was mainly size-related. Small diatoms showed a strong resistance to high levels of Nano-sized diatom abundance was unaffected by fCO₂ but large diatoms showed
a non-linear response with an increase in abundance at moderate fCO2 levels (634-953 µatm) but a decrease in higher fCO2 treatments. This trend is even evident within a single species as Fragilariopsis spp., cells >20 µm which showed a similar response to other large micro-sized centric diatoms, but those ≤20 µm showed a higher resistance similar to other smaller diatoms no effect of fCO2. Whilst the response for most diatom taxa was related to cell size, a couple of species did not follow this trend. Proboscia truncata is a large cell but did not show a and Landeria annulata are both larger diatom species but had no fCO2 response. Likewise, Chaetoceros did not show a response to fCO2 but instead reflected the nutrient availability (see Deppeler et al. submitted for nutrient data). A number of Antarctic studies have shown an enhancement of larger diatom species with increases in CO2 (i.e. Engel et al. 2008, 2 and Feng et al. 2010). There are also other previous studies to which our results do not align. Feng et al. (2009) found an increase in abundance of Pseudonitzschia subcurvata at high fCO2. In this study were Pseudonitzschia had a very low tolerance to fCO2 levels above the control, different to that of Feng et al. (2009).

Unlike diatom species, Interestingly, this size-related trend was not present in non-diatom taxa. Unlike nano-sized diatoms, the dominant autotrophic nanoplankton nano-sized autotrophic flagellate, Phaeocystis antarctica, dramatically declined in abundance at the three highest fCO2 levels. There has been a common consensus in other ocean acidification studies that pico- and nanoplankton abundance increases at high CO2 levels (i.e. Hare et al. 2007, Brussaard et al. 2013, Davidson et al. 2016, Thomson et al. 2016 and a recent review by Schulz et al. 2017), but our study only finds this responses in diatoms. P. antarctica had an increase in abundance with moderate fCO2 levels but had a strong threshold level between 634 and 963 µatm, above which abundances were very low. This contrasts with the findings of many might indicate that the size-related effect of fCO2 is restricted to diatoms.

For some diatom taxa, such as P. truncata and L. annulata, the response to fCO2 was not size-related and other factors effected their abundances. On day 18 of the experiment, NO3 levels fell below detection in all fCO2 treatments and on day 16 treatments of 343, 634 and 953 µatm of fCO2 had lower NO3 concentrations compared to the other three treatments (Figures S3). The abundances of total cells, Fragilariopsis spp. ≤20 µm and Chaetoceros spp. all decreased between days 16 and 18. It was thought that this may have been due to nutrient depletion rather than fCO2, however the decrease in abundances often did not occur in those tanks with the lowest nutrient levels on day 16 (343, 634 and 953 µatm). The greatest rate of decline in the total cell abundance between days 16 and 18 was seen at 1140 µatm, not 343, 634 or 953 µatm that had the lowest NO3 concentration. Similarly, the decline in abundance of nano-sized Fragilariopsis spp. was greatest at 506 µatm, and Chaetoceros spp. had an increase in abundance at 343 µm despite NO3 being lowest at day 16 in these treatments. Thus, while differences in the concentration of nutrients among tanks may have influenced the abundance of protists on day 18 of the experiment, the poor correlation of changes in abundance with nutrient depletion suggest that other factors, such as inter-specific competition, exerted a greater effect on the community composition.

A number of Antarctic studies may have found the ratio of larger sized diatoms to smaller diatoms taxa increased with increasing CO2 (Feng et al., 2010; Tortell et al., 2008b; Hoppe et al., 2013; Trimborn et al., 2013). This study is consistent with that of Tortell et al. (2008b) where Pseudo-nitzschia subcurvata decreased in abundance with increased CO2, unlike Tortell et al. (2008b) there was no increase in larger chain-forming diatoms, but rather a shift towards smaller pennate species similar to Hoppe et al. (2013).
Unlike Tortell et al. (2008b), no effect of CO$_2$ on *Chaetoceros* spp. abundance was observed in this study, but the species of *Chaetoceros* spp. in the two studies differ: mainly *C. castracanei* and *C. tortissimus* here compared to the comparatively larger *C. debilis* in the study by Tortell et al. (2008b). This study also contrasts with other Antarctic ocean acidification studies that found that *P. antarctica* abundance was either not affected or increased by elevated CO$_2$ at the experimental either increased (Trimborn et al., 2017) or was not affected at CO$_2$ levels used in their studies (Feng et al., 2010; Trimborn et al., 2013) above that of the current ambient level (Tortell et al., 2008b; Feng et al., 2010; Trimborn et al., 2013; Young et al., 2015). It is noted that these studies were conducted in the Ross Sea, which is a very different ecosystem to other coastal Antarctic areas (Smith et al., 2014; Deppeler and Davidson, 2017). The difference in response of *Pantarctica* compared to other studies showing an increase in pico- and nanoplankton (Schulz et al., 2017), may be because of the peculiar physiology of this alga in the colonial life stage (which dominated in our study). *Pantarctica*, when in colonial form, protect their cells from intimate contact with the surrounding seawater by a thin tough skin and behave in a similar fashion to a large diatom (Davidson and Marchant, 1992; Hamm et al., 1999).

This study’s In addition to these regional differences, the fCO$_2$ treatments used in this study extend well past the range of other studies, where commonly in which the highest level was typically between 750 and 1000 µatm (compared to 1641 µatm in this study here). When the results of this study are compared in light of these with comparable CO$_2$ levels the conclusions aligned used in the other studies, the conclusions are mostly in agreement. For example, the increase in large diatoms found by Engel et al. (2008), and Feng et al. (2010) was at larger diatoms CO$_2$ levels at between 700 and 800 µatm, CO$_2$ (Tortell et al. (2008b), Feng et al. (2010), and others around the world i.e. Wu et al. 2014), agreeing with the increase in large micro-sized diatoms in this study at fCO$_2$ between 343-634 µatm. Again Trimborn et al. (2017) saw an increase in *P. antarctica* reported by ?, Feng et al. (2010) and Trimborn et al. (2013) was to CO$_2$ levels between 750 and at 800 µatm, again CO$_2$ agreeing with the increase in *P. antarctica* abundance in this study. Xu et al. (2014) observed a non-linear response by *Pantarctica*, with an increase in abundance at 600 µatm here to CO$_2$ but a decrease at 800 < 634 µatm, similar to the finding of this study. This non-linear response could explain the variation in other microbial ocean acidification studies. Previous studies have shown a range of responses to. Thus, the different findings between this study and previous studies are likely due to the choice of experimental CO$_2$; however it is possible that their highest CO$_2$ level is sitting either just below or above the tipping point for their study levels relative to the tolerance threshold of the species or community being studied.

It has been hypothesized that phytoplankton will generally benefit from increased CO$_2$ due to RuBisCo’s low affinity the low affinity of RuBisCO’s for CO$_2$ (the carbon fixation enzyme in photosynthesis) (Reinfelder, 2011). The half saturation constant of RuBisCo RuBisCO for CO$_2$ is substantially higher than occurs in the ocean. The concentration of CO$_2$ in ambient seawater, and it has been proposed that the anthropogenic rise in oceanic CO$_2$ may enhance the rates of phytoplankton photosynthesis (Rost et al., 2008). Most phytoplankton species have highly regulated carbon concentrating mechanisms (CCMs) which enhances the CO$_2$ available for photosynthesis by increasing the CO$_2$ supply so that it is less rate-limiting (Reinfelder, 2011). Yet the – (Reinfelder, 2011; Trimborn et al., 2013). This was observed by Tortell et al. (2008a) who found that natural phytoplankton assemblages up-regulated their CCMs to compensate for low CO$_2$ compared to phytoplankton in higher CO$_2$ conditions (Trimborn et al., 2013). Yet these beneficial effects of enhanced CO$_2$ availability are offset against the
coincident increase of H⁺ ion concentration. It is thought that the energy saved by decreased CCM activity will be outweighed by off-set against the energy required to increase intracellular processes that mitigate this H⁺ increase within the cell (i.e. the proton pump) (Taylor et al., 2012). Our findings—results reported here suggest a combination of these two effects. The increase in abundance of many species with moderate increases in fCO₂ evident for many species could be due to the increased availability of CO₂ and down-regulation of CCMs, therefore allowing energy saved from CCM activity to be used in other cellular processes. However there is a limit to this energy saving; a further decrease in pH, and increase in H⁺ ions, results in an increase in energy used to maintain the homeostasis of the cell intracellular pH. Deppeler et al. (submitted) reports the effects of fCO₂ on photosynthetic physiology during our study, showing that the same study, where CCM activity has been down-regulated in the highest fCO₂ treatment of the experiment but not in at the lowest fCO₂. This supports the theory that whilst the CCM activity might be down-regulated, the community is still being inhibited at high eventually inhibited at higher fCO₂ level by the metabolic cost of fCO₂ tolerance mechanisms such as proton pumps levels by metabolic costs of maintaining H⁺ homeostasis.

4.3 Response of heterotrophic protists

Intriguingly, we found different CO₂-induced responses by different responses in the two dominant choanoflagellate taxa; /groups of this study; Bicosta antennigera and abundance declined above 506 μatm, while other choanoflagellates (>90% consisted of being Diaphanoeca multiannulata). To our knowledge this were unaffected by fCO₂. This is the first study to show report differing responses of choanoflagellate species—abundances due to ocean acidification. Previous studies have reported no effect (Moustaka-Gouni et al., 2016a, b) or a decrease (Davidson et al., 2016) of choanoflagellates due to increased CO₂, but this is the first to show a find different responses amongst taxa. The reasons for choanoflagellate taxa responding differently to elevated CO₂ are unclear. Gong et al. (2010) showed that loria formation in choanoflagellates can be affected by pH changes but SEM preparations in this study found no evidence of this. Some studies of sperm flagella on the flagella of sperm from reef invertebrates and oysters have suggested that increased CO₂ can slow metabolic rates or interrupt flagella function (Havenhand and Schlegel, 2009; Morita et al., 2009). Thus This difference in sensitivity to CO₂ may reflect the differences in loria complexity, cellular morphology or physiology among taxa. If the species-specific response of choanoflagellates is indicative of the behaviour of the broader microheterotrophic community, this finding raises then this finding highlights a previously unseen level of complexity into the effect of ocean acidification on microbial communities. Previous studies have observed no direct CO₂ related response on microheterotrophic protozoa effect on microheterotrophic protozoan community composition (Suffrian et al., 2008; Aberle et al., 2013). Unfortunately, the abundance of protistan heterotrophs other than choanoflagellates in this study was generally low and with high variability, making it difficult to detect any CO₂ response. If the species-specific responses of choanoflagellates seen in this study observed in choanoflagellates are indicative of CO₂-induced responses by other microheterotrophic grazers (e.g. autotrophic heterotrophic dinoflagellates and ciliates), the implications for top-down control of protists and prokaryotes could be quite profound significant.
4.4 Community-level responses

This study shows that in this study there is a significant shift in the protistan community structure with increasing $fCO_2$. The community response is not linear with an increase in abundance a general increase in abundances between 343 to 634 $\mu$atm favoring larger micro-sized centric diatoms and *Phaeocystis antarctica*; however there is a threshold, and above. Above a threshold of 634 $\mu$atm there is in $fCO_2$ there is a decrease in abundance and shift towards smaller nano-sized *Fragilariopsis* spp. While the nMDS and CAP showed that the primary driving factor behind community change was time (sample day or nutrient concentrations as a proxy sampling day (which covaried with nutrient concentration), a significant $fCO_2$ induced response was observed. This non-linear response has been previously observed in individual taxa response to increased CO$_2$ (Trimborn et al., 2013; Xu et al., 2014). Concentrations of NO$_x$ were at a minimum in treatments 343, 634 and 953 $\mu$atm on day 16, and dropped below detection in all treatments between days 16 and 18. Despite this depletion of NO$_x$, it is not a significant driver in the shifts of the protistan community structure. NO$_x$ was dropped in the reduced CAP model but P and Si were significant despite these macronutrients remaining above detectable levels throughout the entire experiment. Community level studies have reported a shift shifts in community composition with increased $fCO_2$ (Schulz et al. 2017 and refs therein), but what has not been reported before is the non-linear response at the community level such that has observed in our study.

Comparisons with Davidson et al. (2016) and Thomson et al. (2016) show that there is a consistent threshold curved response of a natural Antarctic protistan community to a CO$_2$ above which protistan communities at this site alter their structure. A similar series of minicosm experiments was conducted in the same location, Prydz Bay East Antarctica, in the austral summer of 2008-09 (Davidson et al., 2016; Thomson et al., 2016). That study showed gradient (with enhanced abundance at intermediate levels and a decrease at higher).

Davidson et al. (2016) and Thomson et al. (2016) found a significant shift in the microbial community above a structure of the protistan community at CO$_2$ levels between 750 to 1281 $\mu$atm during a similar series of minicosm experiments at Prydz Bay in 2008-2009. As here, once that threshold had been reached above the threshold there was a shift in the community structure towards smaller cells and a decrease in structure of the protistan community towards a picoplankton dominated community, and an overall decrease in total protistan abundance. Thomson et al. (2016) reports results from three different starting communities and nutrients levels, but despite these differences the threshold remained the same. Our study differs from that of Davidson et al. (2016) and Thomson et al. (2016) in that it has a narrower $fCO_2$ range in the treatments, and it also included an acclimation period. Despite these differences, the threshold level found in our study falls within that of Davidson et al. (2016) and Thomson et al. (2016). Unlike those previous studies, we saw an increase in large protists below this threshold level microplankton abundance increased at moderate CO$_2$ enrichment (506 to 634 $fCO_2$). This could be due to having six treatments across a smaller $fCO_2$ range, therefore allowing a higher resolution in the response of the community prior to the threshold. It could also be due to the inclusion of an acclimation period, giving cells time to adjust to the $fCO_2$ level prior to the beginning of growth and therefore allowing them to capitalise on the benefits of moderately elevated CO$_2$. The results from these studies show that in summary, there is a consistent $fCO_2$ threshold that elicits changes in the structure of microbial communities in near-shore waters of Prydz Bay, East Antarctica, both within a season and among
seasons. Furthermore, irrespective of including an acclimation phase, the nature of the change in the protistan communities at high fCO\(_2\) remains similar, though the magnitude can change greatly.

The flow on effect of decreased abundance and a structural shift in the protistan community to smaller cells could have a nano- and picoplankton dominated community could be far-reaching impacts through effects on the rest of the, altering the near-shore Antarctic food-web and biogeochemical cycles. Many studies have shown found that a shift in protistan community composition can affect the palatability, nutritional quality and availability of phytoplankton cells available to grazers and the higher trophic levels (Rossoll et al., 2012; Caron and Hutchins, 2013; Bermúdez et al., 2016; Davidson et al., 2016). Antarctic microbes are also a vital component of many elemental cycles, ocean biogeochemistry and provide an play important roles in the feedback of the Southern Ocean to global climate change (Arrigo and Thomas, 2004; Arrigo et al., 2008; Kirchman, 2008). The results of this study show suggest that the abundance of Phaeocystis antarctica could significantly change with future increases in fCO\(_2\). This species is particularly important in a number of near-shore Antarctic nutrient cycles through their substantial production of dimethyl sulfide, which acts as a cloud condensation nuclei when released into the atmosphere (Liss et al., 1994). P. antarctica also plays a vital role in the carbon flux when in colonial form. Davidson and Marchant (1992) show found that the majority of Phaeocystis biomass remains unutilized and therefore enters the carbon flux as dissolved organic carbon cycle in a dissolved organic form. Likewise, larger micro-sized diatom species are also important in the sequestration of carbon to the deep ocean through their role in the vertical carbon flux (Passow and Carlson, 2012; Caron and Hutchins, 2013). The effect of a protistan community dominated by smaller cells nano- and picoplankton on this vertical flux is uncertain, but any decline in this flux would have a positive feedback on atmospheric CO\(_2\) levels, as instead of being sequestrated to the deep ocean it would be respired in the near-surface waters and released into the atmosphere.

5 Conclusions

Returning to the aims of this study, there are four main conclusions;

1. The responses to increased fCO\(_2\) was taxon-specific in both autotrophic and heterotrophic protists, with different taxa showing having different tolerance thresholds for fCO\(_2\). In diatoms this response is was mainly driven by cell size, with smaller cells showing nanoplankton having a high tolerance to increased fCO\(_2\) and larger cells a lower tolerance while microplanktonic diatoms were less tolerant. This trend is consistent even within a taxon as demonstrated by the large size ranging nano- to micro-sized Fragilariopsis spp. cells in this experiment. Whilst size-related responses have previously been observed for nano- and pico plankton vs larger cells (see Schulz et al. 2017 for a review), our study shows that this trend continues in diatoms greater than 20 µm.

2. An increase in fCO\(_2\) significantly changes the composition and abundance of protists in this coastal East Antarctic community. The threshold for this response is with a threshold for change estimated to be between 634 and 953 µatm. Below this threshold there is an increase in community protistan abundance and the community was characterised by large centric diatoms and Phaeocystis antarctica. Above the threshold there was a decrease in total abundance and the
community was dominated by smaller diatoms predominantly nano-sized diatoms, mainly *Fragilariopsis* spp. <20 μm.

3. During the eight day acclimation period there were no differences in community structure between *fCO₂* treatments, however a lag in protistan growth until day 12 of the experiment was observed at 1641 μatm. This suggests that the acclimation period was not sufficient for the community to adjust its composition and/or cellular physiology to cope with this high *fCO₂* level.

4. Comparisons with Davidson et al. (2016) and Thomson et al. (2016) show that this threshold level is not only consistent across a season but also between years for protistan communities at Prydz Bay, East Antarctica.

The results of this study suggest that there is a strong consistent threshold level above which the structure of this near-shore Antarctic microbial community significantly changes, and this threshold is around the CO₂ level predicted for the end of this century (IPCC, 2014). This change could have significant flow-on effects to the coastal Antarctic ecosystem as it would threaten the many ecosystem services that marine microbes provide, and result in cascading effects through the Antarctic food-web, elemental cycles, ocean biogeochemistry and elemental cycling in general, with feedbacks on global climate change.

**Code and data availability.** Abundance data and environmental covariate data used in the statistical analyses is available via the Australian Antarctic Division Data Centre: Hancock, A.M., Davidson, A.T., McKinlay, J., McMinn, A., Schulz, K., van den Enden, D. (2017, updated 2017) Ocean acidification changes the structure of an Antarctic coastal protistan community Australian Antarctic Data Centre - doi:10.4225/15/592b83a5c7506.


**Author contributions.** A. Davidson designed the research and led the minicosm experiment at Davis Station, Antarctica and all carbonate chemistry measurements, calculations and manipulations were performed by K. Schulz. A. Davidson collected the samples during the experiment and A. Hancock performed all light and electron microscopy work with assistance from A. Davidson and R. van den Enden. A. Hancock conducted the data and statistics analysis using R code and statistical approach developed by J. McKinlay. A. Davidson and J. McKinlay provided assistance and guidance with data and statistical analysis and interpretation. A. Hancock prepared the manuscript with contributions from all co-authors.
Competing interests. The authors declare no competing interests.

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Figure 1. Microscope-derived abundance counts of total protists over an 18 day incubation of a natural protistan community in tanks maintained at different $fCO_2$ levels. Error bars are standard errors derived from pseudo-replicates undertaken at each time point for each treatment.
Figure 2. Microscope-derived abundance counts of protists. Abundances of (a) Unidentified diatom centric <2 nano-sized diatoms (longest length ≤ 20 µm) (b) *Thalassiosira antarctica*, micro-sized diatoms (longest length >20 µm) *Landeria annulata*, (d) *Stellarima microtrias*, (e) *Thalassiosira ritscheri* over days 1 to 18 of the incubation of a natural protistan community in tanks maintained at different $fCO_2$ levels. Error bars are standard errors derived from pseudo-replicates undertaken at each time point for each treatment.
Figure 3. Microscope-derived abundance counts of protists. Abundances of (a) *Fragilariopsis* ≤20-µm unidentified discoid centric diatoms (valve diameter ≤ 2 µm), (b) *Fragilariopsis > Thalassiosira antarctica* (valve diameter ≈ 20 µm), (c) *Landeria annulata* (valve diameter ≈ 55 µm), (d) *Stellarima microtrias* (valve diameter ≈ 55 µm), (e) *Thalassiosira ritscheri* (valve diameter ≈ 50 µm) over days 1 to 18 of the incubation of a natural protistan community in tanks maintained at different $f$CO₂ levels. Error bars are standard errors derived from pseudo-replicates undertaken at each time point for each treatment.
Figure 4. Microscope-derived abundance counts of protists. Abundances of (a) *Odontella Fragilariopsis* spp. (≤ 20 µm in length), (b) *Pseudonitzschia subcurvata*-*Fragilariopsis* spp. (>20 µm in length) *Pseudonitzschia turgidulodies* over days 1 to 18 of the incubation of a natural protistan community in tanks maintained at different fCO₂ levels. Error bars are standard errors derived from pseudo-replicates undertaken at each time point for each treatment.
Figure 5. Microscope-derived abundance counts of protists. Abundances of (a) Proboscia truncata Odontella spp. (pervalve length ≈ 70 µm), (b) Chaetoceros Pseudo-nitzschia subcurvata (≈ 55 µm in length), (c) Pseudo-nitzschia turgiduloides (≈ 85 µm in length) over days 1 to 18 of the incubation of a natural protistan community in tanks maintained at different fCO₂ levels. Error bars are standard errors derived from pseudo-replicates undertaken at each time point for each treatment.
Figure 6. Abundances of (a) *Proboscia truncata* (pervalvar length \(\approx 130 \mu m\)), (b) *Chaetoceros* spp. (pervalvar length \(\approx 10 \mu m\)) over days 1 to 18 of the incubation of a natural protistan community in tanks maintained at different \(f\text{CO}_2\) levels. Error bars are standard errors derived from pseudo-replicates undertaken at each time point for each treatment.
Figure 7. Microscope-derived abundance counts of Phaeocystis antarctica (colonial form) over days 1 to 18 of the incubation of a natural protistan community in tanks maintained at different $f$CO$_2$ levels. Error bars are standard errors derived from pseudo-replicates undertaken at each time point for each treatment.
Figure 8. Microscope-derived abundance counts of protists. Abundances of (a) *Bicosta antennigera*, (b) Choanoflagellates (except *Bicosta*) over days 1 to 18 of the incubation of a natural protistan community in tanks maintained at different $fCO_2$ levels. Error bars are standard errors derived from pseudo-replicates undertaken at each time point for each treatment.
Figure 9. Cluster analysis based on similarity in protistan community structure among $fCO_2$ treatments and times during the acclimation period (days 1 to 8). The analysis shows three significantly different groups obtained by SIMPROF (denoted by grey boxes around clusters and coloured lines beneath sample labels). Samples are abbreviated according to days of incubation (D1-8) and level of $fCO_2$ treatment (T1-6 representing 343, 506, 634, 953, 1140, 1641 µatm, respectively).
Figure 10. Cluster analysis and nMDS based on similarity in protistan community structure among fCO₂ treatments and times over days 8 to 18 of the incubation. (a) The cluster analysis shows ten significantly different groups obtained by SIMPROF (denoted by grey boxes around clusters and coloured lines beneath sample labels). (b) nMDS plot structure showing the unconstrained ordination of dissimilarities in protistan community structure with time and fCO₂ treatment in 2 dimensions, overlaid with weighted-averages of the day-treatment scores for each protistan taxa/functional group (see Table 1 for abbreviations). Samples are abbreviated according to days of incubation (D8-18) and level of fCO₂ treatment (T1-6 representing 343, 506, 634, 953, 1140, 1641 µatm, respectively).
Figure 11. Canonical analysis of principal co-ordinates (CAP) based on the similarity in protistan community structure among $f$CO$_2$ treatments and times over days 8 to 18 of the incubation, showing the trajectory of change in the protistan community for each $f$CO$_2$ level (coloured arrows) based on the abundance of the component taxa/functional groups. Arrow starting points are day 8 and all arrows end on day 18 of the experiment. Linear projections of significant constraints CO$_2$, Si and P appear as blue linear arrows.
Table 1. Protistan group abbreviations.

<table>
<thead>
<tr>
<th>Taxon/Functional Group</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>Autotrophic Dinoflagellates</td>
<td>AD</td>
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<tr>
<td><em>Bicosta antennigera</em></td>
<td>Ba</td>
</tr>
<tr>
<td><em>Chaetoceros</em> spp.</td>
<td>Cha</td>
</tr>
<tr>
<td>Choanoflagellates (except <em>Bicosta</em>)</td>
<td>Cho</td>
</tr>
<tr>
<td>Ciliates</td>
<td>Cil</td>
</tr>
<tr>
<td><strong>Unidentified</strong> Discoid Centric Diatoms $&gt; 40 \mu m$</td>
<td>DC.l</td>
</tr>
<tr>
<td><strong>Unidentified</strong> Discoid Centric Diatoms 20 to 40 $\mu m$</td>
<td>DC.m</td>
</tr>
<tr>
<td><strong>Unidentified</strong> Discoid Centric Diatoms $&lt; 20 \mu m$</td>
<td>DC.s</td>
</tr>
<tr>
<td>Euglenoid</td>
<td>Eu</td>
</tr>
<tr>
<td><em>Fragilariopsis</em> spp. $&gt; 20 \mu m$</td>
<td>F.l</td>
</tr>
<tr>
<td><em>Fragilariopsis</em> spp. $&lt; 20 \mu m$</td>
<td>F.s</td>
</tr>
<tr>
<td>Heterotrophic Dinoflagellates</td>
<td>HD</td>
</tr>
<tr>
<td><em>Landeria annulata</em></td>
<td>La</td>
</tr>
<tr>
<td>Other Centric Diatoms</td>
<td>OC</td>
</tr>
<tr>
<td><em>Odontella</em> spp.</td>
<td>Od</td>
</tr>
<tr>
<td>Other Flagellates</td>
<td>OF</td>
</tr>
<tr>
<td>Other Pennate Diatoms</td>
<td>OP</td>
</tr>
<tr>
<td><em>Phaeocystis antarctica</em></td>
<td>Pa</td>
</tr>
<tr>
<td><em>Proboscia truncata</em></td>
<td>Pro</td>
</tr>
<tr>
<td><em>Pseudonitzschia</em> <em>Pseudo-nitzschia subcurvata</em></td>
<td>Ps</td>
</tr>
<tr>
<td><em>Pseudonitzschia turgiduloides</em> <em>Pseudo-nitzschia turgiduloides</em></td>
<td>Pt</td>
</tr>
<tr>
<td><em>Stellarima microtrias</em></td>
<td>Sm</td>
</tr>
<tr>
<td><em>Thalassiosira antarctica</em></td>
<td>Ta</td>
</tr>
<tr>
<td><em>Thalassiosira ritscheri</em></td>
<td>Tr</td>
</tr>
</tbody>
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Table 2. Canonical analysis of principal coordinates (CAP) axis significance against covariates. Permutation tests assessing the significance of each constrained axis in CAP using the covariates $f\text{CO}_2$, P and Si as constraints upon community structure.

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>Variance</th>
<th>$F$</th>
<th>No. Perm</th>
<th>Pr ($&gt;F$)</th>
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<tbody>
<tr>
<td>CAP1</td>
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<td>0.105</td>
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<tr>
<td>Residual</td>
<td>32</td>
<td>1.00992</td>
<td></td>
<td>999</td>
<td>0.105</td>
</tr>
</tbody>
</table>
Table 3. Permutation tests assessing the significance of each environmental covariate (constraint) in determining protistan community structure using principal co-ordinates (CAP), showing with significance of each term when (a) sequential added (b) marginal effects for fCO$_2$, P and Si.

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>Variance</th>
<th>$F$</th>
<th>No. Perm</th>
<th>Pr ($&gt;F$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>$f$CO$_2$</td>
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<td>0.25848</td>
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<td>0.003</td>
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<tr>
<td>P</td>
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<td>1.75541</td>
<td>62.7309</td>
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</tr>
<tr>
<td>Si</td>
<td>1</td>
<td>0.11072</td>
<td>3.9566</td>
<td>999</td>
<td>0.03</td>
</tr>
<tr>
<td>Residual</td>
<td>32</td>
<td>0.89546</td>
<td>3.9566</td>
<td>999</td>
<td>0.027</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$f$CO$_2$</td>
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<td>0.06197</td>
<td>2.2144</td>
<td>999</td>
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<tr>
<td>P</td>
<td>1</td>
<td>0.48178</td>
<td>17.2167</td>
<td>999</td>
<td>0.001</td>
</tr>
<tr>
<td>Si</td>
<td>1</td>
<td>0.11072</td>
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<td>999</td>
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<tr>
<td>Residual</td>
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<td>0.89546</td>
<td></td>
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