All referee comments are in bold and our answers in normal font**.**
Changes in the manuscript text are kept in italics.

Short Comments by
Lucie Munns, Manon Duret, Chris Daniels, Kyle Mayers, Alex Poulton and Rosie Sheward

GENERAL COMMENTS
The manuscript by Müller et al. examines the potential for high external calcium (Ca) to be inhibitory or toxic to a number of phytoplankton grown in laboratory conditions. The outcome is that calcifying organisms (i.e. coccolithophores) show little or no response to high medium Ca concentrations in terms of reduced growth, calcification rates or photosynthetic rates. An interesting side-line from the work is that a non-calcifying 'mutant' strain begins to calcify at extremely high Ca (relative to present day oceanic Ca concentrations). The paper shows that coccolithophores can regulate their intracellular Ca concentration to a greater degree than the other phytoplankton groups tested and avoid Ca poisoning. It should be noted that the Ca concentrations, which are inhibitory to the other phytoplankton groups examined, are in excess of current levels seen in the modern ocean.
The paper is nicely written, however the discussion leaps straight into the bigger picture, without fully discussing the results, which are very interesting in themselves, in sufficient context and depth to support the wider conclusions. The Results section is very short and could be enhanced by more emphasis being placed on the interesting trends seen in the data (e.g., growth rate and Fv/Fm).
We thank the discussion group at the National Oceanography Centre (NOC) for their comments and critical evaluation of our manuscript.
First, we would like to clarify the point that we only present data on under-calcified strains of *E. huxleyi* that increase their cell cover of coccoliths when exposed to elevated seawater Ca2+ concentrations. Please refer here also the answers to referee 1.

We added now additional discussion on the control of intracellular Ca2+ homeostasis and possible effects of Ca2+ poisoning (see also answers to referee 1):

"*Marine phytoplankton presumably operate several mechanisms which contribute to cellular Ca^{2+} regulation such as intra and extra cellular enzymatic binding capacities and/or the influx regulation via selective channels (Gadd, 2010). Over the past decade progress has been made in the discovery of cellular compartments (e.g. endoplasmic reticulum, chloroplast, mitochondria) regulating plant Ca^{2+} homeostasis and signalling (McAinsh & Pittmann, 2009; Webb, 2008; Brownlee and Hetherington, 2011) and on differences in Ca^{2+} channels between eukaryotes and higher plants and mammalian cells (Wheeler and Brownlee, 2008). However, many unknowns remain about phytoplankton intracellular ion regulation and the homeostasis of the major biological active cations like Ca^{2+} and Mg^{2+} and their interaction and possible influence on each other. For example, Ca^{2+} has a higher ion-exchange capacity than Mg^{2+} (Harris, 2010) and when present in high concentrations might interfere with enzymatic reactions where Mg^{2+} acts as a cofactor (Moore et al., 1960; Legong et al., 2001). However, it remains speculative if this is a possible explanation for the observed reduction in growth rate and Fv/Fm of non-calcifying phytoplankton species (Fig. 2).* "

As the authors state in the Discussion (pg 12701: ln 13), it is likely that the ability of the coccolithophores to tightly control internal Ca2+ transport allows them to withstand high Ca2+ concentrations. This point could more clearly be defined as the outcome of the study and the reader is potentially left with the question – did the paper actually address what the title states? Indeed the calcifying types of phytoplankton assessed in this study showed no depression of growth rate at high Ca concentrations, but is it the process of calcification (i.e. combining Ca with bicarbonate, strictly regulating crystal growth and extrusion of the end result from the cell) or the strong control on Ca fluxes into the cell required to regulate the process of calcification that alleviates Ca poisoning? – Simply put, is it the whole process of coccolith formation or just the control of Ca influxes that prevents this group from being influenced by Ca toxicity?
We are not sure that the paper really addresses this and an alternative title could be, for example, 'Superior ability of coccolithophores to maintain their fitness in high Ca2+ concentrations'.
Please refer here also to the answer to J. Young regarding the title of the manuscript. We will change the title to: "*Phytoplankton calcification as an effective mechanism to alleviate cellular calcium poisoning*" and think that this title reflects nicely the main message of the manuscript.
We show that the process of calcification (which includes CaCO3 formation and a tight regulation of cellular Ca entrance and distribution) is responsible for the diverging differences observed between calcifying and non-calcifying phytoplankton species.
It is correct that we cannot distinguish between the responsibilities of the Ca influx control and the CaCO3 formation. We don't think that this is important for the overall message of the manuscript but stated this differentiation in the revised manuscript.

"... exemplifies the level of cellular control involved in coccolithophore calcification. It appears reasonable to assume that this tight cellular control of biogenic calcification (which includes $CaCO_3$ precipitation inside the coccolith vesicle and the regulation of cellular Ca^{2+} entrance and distribution) also allows for the observed tolerance to external Ca^{2+} concentrations."

Clearly, our presented manuscript will lead to further investigations and challenges that need to be addressed in future studies.

Indeed the inclusion of a non-calcifying coccolithophore could indicate that the process of calcification itself is critical – however non-calcifying diploid coccolithophores are 'mutants' not original organisms and the mutation that occurred to prevent them calcifying could, in theory, happen anywhere on the cellular pathways to coccolith production – the fact that the non-calcifying strain did begin to calcify at high Ca after a prolonged period could suggest that the mutation occurred somewhere along it's Ca uptake pathway (i.e. it could only take up Ca successfully and form coccoliths at 3 times the ambient Ca concentration – note that this information is only found in the figure 4 legend, not the paper). Some of this is semantics, but part of it is experimental design and interpretation and generally we remain unconvinced that phytoplankton

calcification is an effective mechanism to prevent cellular calcium poisoning – although the paper does make a convincing case that it is the coccolithophores ability to tightly control internal Ca2+ transport that allows them to withstand high Ca2+ concentrations.
We are happy that the NOC discussion group acknowledge the convincing case we present here that the control of calcium provides the ability to withstand high Ca concentrations. We hope that our clarification of the misunderstanding regarding the under-calcified and non-calcified strains of *E. huxleyi* (see comments above and to referee 1) makes our case stronger that this control is linked to the calcification process.

SPECIFIC COMMENTS -
Methods and quantitative results from the second experiment must be shown (e.g. state how many cells coccolith counts were taken from under the SEM and show the data that the statistics are based on).
During SEM sessions more than 50 cells were visually evaluated and representative pictures were taken.

We will state this in the materials and methods section:

"Photographs were taken with a Hitachi SU-70 field emission scanning electron microscope (SEM) at the Central Science Laboratory of the University of Tasmania. During SEM sessions > 50 cells were visually evaluated and representative pictures taken."

The limitations of using E. huxleyi and G. oceanica should be discussed in the context of the geological record - i.e. both are recently evolved species (0.3 and 1.9 Ma respectively), and are genetically very closely related. These species are often selected for laboratory experiments due to their tolerance to artificial conditions and are perhaps not very representative of all coccolithophores, particularly given the palaeo-ecological emphasis of the introduction and discussion.
We don't see a limitation of using the coccolithophore species *E. huxleyi* and *G. oceanica*. We primarily investigated the process/mechanism of calcification in these two species and our primary findings are supported by additional literature data from *E. huxleyi* and *C. braarudii* (see Fig. 3). These findings give implications for future or past times of high oceanic Ca concentrations of which the Cretaceous is the best known geological era where coccolithophores were present.

It is clear that conducting biological experiments related to past or future events will be always limited because neither do we have the genetically exact same species that were present in the past nor the ones that will be present in the future.

It should also be acknowledged that the high abundance of coccolithophore fossils from sediments of Cretaceous age additionally represents favourable conditions for CaCO3 export and preservation (e.g. extensive warm shallow shelf seas as favourable depositional environments) and not exclusively CaCO3 production or coccolithophore dominance relative to other phytoplankton groups.

Yes, we completely agree and included the warm shallow shelf seas now into our statement regarding the different paleoceanographic conditions during the Cretaceous.

"Paleoceanographic studies have indicated that the oceanic conditions of the Cretaceous were quite different from those in the modern ocean (e.g. see Zeebe, 2001; Hay, 2008). Besides elevated seawater Ca2+ concentrations (Fig. 1), the Cretaceous was marked by a warm greenhouse environment, elevated sea levels, warm shallow shelf seas and altered oceanic circulation."

There is no conclusive evidence that naked strains form a third part of the E. huxleyi natural life cycle (pg. 12701: ln 23 and see comments from other reviewers). Such non-calcifying strains are most widely considered to be the artefacts of mutation in culture, in which any number of 'faults' could be present in the calcium metabolism or coccolith production and exocytosis pathway. It is hard to interpret the results from a strain that is not well understood (e.g. It spontaneously begins calcifying? 1296: ln 17).
We changed this statement (see answer to J. Young) regarding the life cycle of *E. huxleyi*. We agree that the non-calcifying cells of E. huxleyi (N-cells) are not very well understood which is probably the result of difficulties identifying them in natural samples without the use of molecular tools. However, we hope that this study will encourage other laboratories to start investigating N-cells and the transition from one cellular form to the other.
Additionally, we would like to mention here that we recently showed that N-cells can appear, for example, under unfavourable culture conditions (e.g. low pH, see Müller et al. 2015).
The spontaneous re-calcification of our strain SO-6.13 remains elusive to us as does the spontaneous stop in calcification of N-cells.

It would be beneficial to have a clear discussion early in the paper of what calcium poisoning is and how the authors would define it. For example, why would we expect cells to suffer at high Ca2+? What do we know about how single celled organisms avoid this? This introduction to Ca poisoning could then be used to inform the conclusions drawn.
We cited many of the relevant literature and also added now an additional paragraph in the discussion about possible biochemical mechanisms that lead to Ca poisoning. See answer to referee 1.

Results from other studies (e.g. C.braarudii results) shouldn't be included in the results section, but be brought into the discussion section only.
We see it as a legitimate way to present our results together with available data from the literature. However, we see that this should be more clearly stated in the results section:

"To illustrate the diverging physiological response of calcifying coccolithophores and non-calcifying phytoplankton, we normalized growth and POC production rates from the current study and literature data to the species-specific rates exhibited at modern ocean calcium levels (Fig. 3)."

Cellular PIC and POC quotas and growth rates are meaningful independent of each other and show interesting trends in their own right, whereas PIC and POC production can be misleading as they are heavily affected by growth rate – i.e. they are the combination of growth rates (which change in this study) and cellular carbon inventories (which don't). For example, pg 12699, ln 6 - Significantly lower PIC production by E. huxleyi at low Ca2+ is caused entirely by a decrease in growth rate, as cellular PIC quota remains the same at both Ca2+ levels.
We are not sure if we understand this comment correctly. We agree that cellular quota is different from the cellular production rate of POC and PIC but we see the production rate as the better alternative to present and discuss because it is the product of cellular quota and growth rate and therefore accounts for changes in both.

Fig 2: The note about different methodology for C. clost(erium) should be in the methods section instead of the figure caption.
We agree and added this information now in the method section:

"The physiological response of all species (except C. closterium) was examined in terms of growth rate, particulate organic and inorganic carbon cell quota and production rate, and maximum quantum yield of the photosystem II (Fv=Fm). Physiology of C. closterium was only examined in terms of growth rate. Seawater carbonate chemistry was determined from total alkalinity (AT) and dissolved inorganic carbon (CT) samples taken at the start and the end of the experiment."

pg 12703, ln 15: There is little evidence that the extinction events referred to are related to ocean acidification (temperature and nutrients are also strong controls on fitness for example), and furthermore, it is also highly debated in recent literature whether ocean acidification on geologically-relevant timescales (i.e. hundreds to thousands of years) actually prevents intracellular calcification in coccolithophores.
We removed this statement from the manuscript (see also answers to J. Young and T. Tyrrell).

pg 12703, ln 27: Please explain further the sentence 'this let us suggest that these two species in the modern ocean don't rely on cellular Ca2+ detoxification by mineralization'. How does this fit with the title or that current ocean Ca concentrations are not toxic to any of the phytoplankton examined?
This fits very well with the title as Ca-poisoning is only present or induced at elevated Ca concentrations (above double modern oceanic levels) and as seen from the existence of non-calcifying *E. huxleyi* and *G. oceanica* cells (N-and S-cells) at modern Ca levels, it seems that these species don't rely on the mechanism of calcification for internal Ca regulation at Ca levels of 10 mM. This mechanism (calcification as Ca-detoxification) becomes only relevant at elevated Ca levels.