Responses to referees' comments

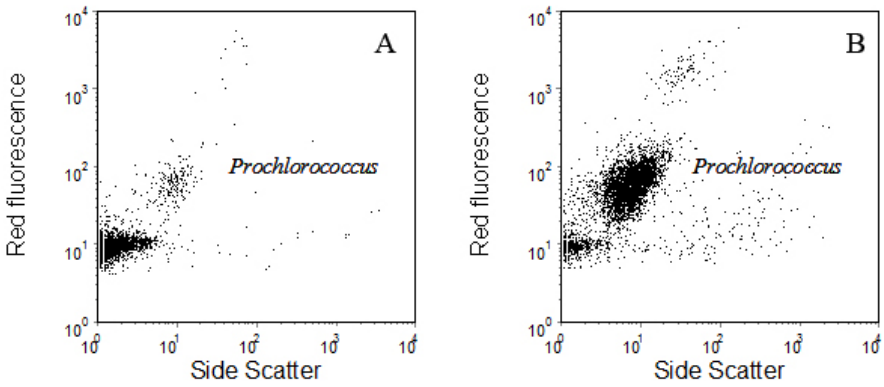
We thank the three anonymous reviewers for their detailed comments and useful suggestions. We have fully considered the comments and revised the manuscript accordingly. Below are our responses to the questions one by one (words in blue color. A few abbreviations: FCM- flow cytometer/cytometry, Pro- *Prochlorococcus,* Syn- *Synechococcus* , Deep Pro- *Prochlorococcus* in the deep aphotic waters).

Anonymous Referee #1

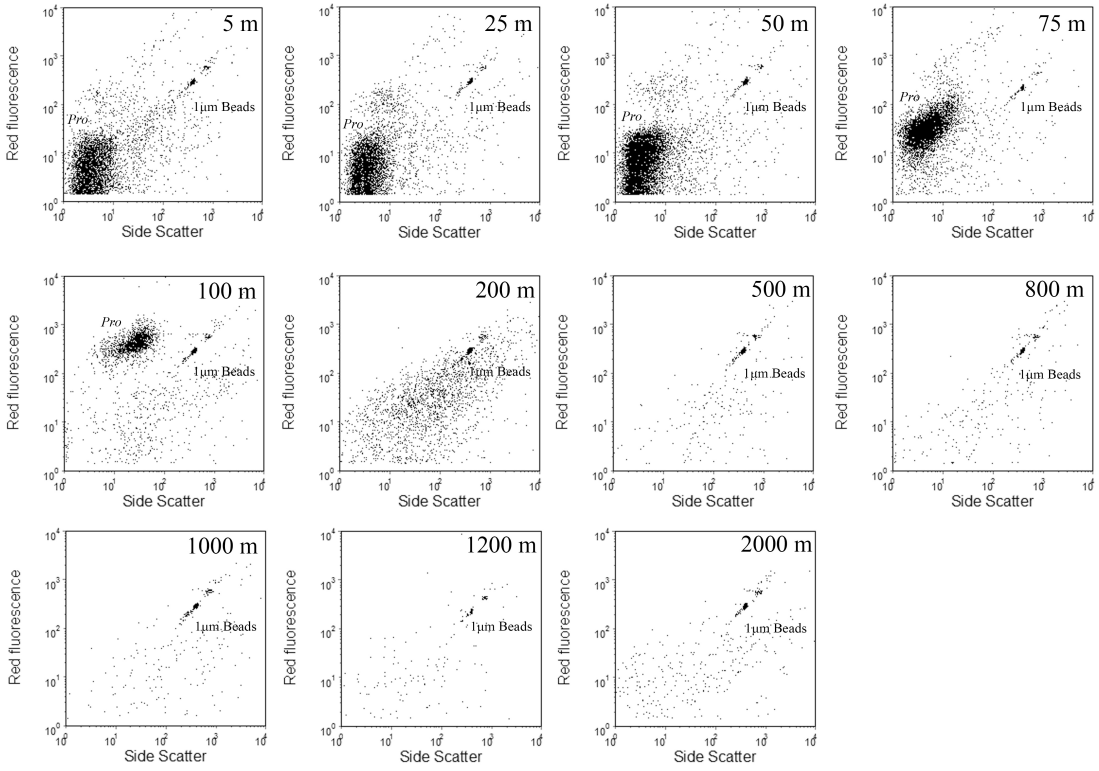
Referee #1 # 1. there are seemingly no negative control, i.e. analysis of 0.2 μm filtered deep sea water, that would demonstrate for sure that these are not just merely cells released from the flow cell, as it may happen with some flow cytomters after analysis of dense *Prochlorococcus* populations (as found in the upper lit layer).

Response: In addition to FCM "backflush" to get rid of tubing memory effects between samples, we did have negative controls such as sheath water, filtered seawater and even condensed deep sea water samples (see FCM plots below: Response Fig#1), not every time though. We also had field-negative-controls: a FCM depth profile at a reference site (N18-5) out of the Luzon Strait on the same cruise, in which no deep Pro were recorded, demonstrating that the deep sea samples were not contaminated by the surface samples during our sampling and FCM analysis.

In fact, we doubted deep Pro at the first time we found it in 2008, and we have been collecting evidences and explanations at all possibilities since then. That's why it took us about 5 years to come to the point we write this paper with confidence. During such a long period we tried all possible means we have on hand to assure what we are looking at are signals rather than noises or false positives. In addition to negative controls, we performed all operations carefully, at each station, we always run the deep water samples first and then move on upward (see FCM plots in Response Fig#2). This working procedure assured the elimination of potential contaminations from epipelagic Pro populations; In addition to the on board analysis, we also conducted re-analysis of the preserved samples in the lab (as indicated in the text).



Response Fig.#1 Comparison between FCM plots of a normal sample (A) and a 50 X condensed sample (B) from 800m of St. N8-12



Response Fig.#2 A depth profile of FCM plots at a reference site (N18-5) out of the Luzon Strait (as a field negative control)

Referee #1 # 2. The abstract claims the presence of abundant and "active" *Prochlorococcus* populations in the aphotic zone, while the fact that these populations is active is supported by ONLY ONE (none replicated!) measurement of rRNA per *Prochlorococcus* HLI cell at 300 m in the Luzon

strait (Fig. 3B). These data are therefore clearly not statistically valid and many more (replicated) data need to be shown to claim that deep *Prochlorococcus* are "active" in the deep ocean, a claim which to my viewpoint is somewhat doubtful since *Prochlorococcus* cells certainly cannot photosynthesize (and therefore grow) in the dark, cold waters of the aphotic zone.

Response:

We agree that the RNA data is limited and not enough for the conclusion independently, it is actually serving as a confirmation of our FCM data which are plenty. The comparable FCM red fluorescence signals of deep Pro cells observed in all samples suggested potentially living populations with quite high cellular pigment contents. As reported that changes in cellular chlorophyll fluorescence are a sign of adaptation to light and depth in the ocean (Veldhuis and Kraay, 2000; Jochem, 2000). It is also reported that the percentage of active cells in total population increases with decreasing light (Agustí, 2004). In addition, previous studies have reported that Pro could be partially heterotrophic (del Carmen Muñoz-Marín et al., 2013). Therefore, although Pro cells cannot photosynthesize and grow in the dark, cold deep waters, they may remain alive there for certain period of time by consuming their stored organic matters. For all that, in order to avoid misunderstanding by the wording "active", we will replace it with "alive/viable" "intact cells" in the revised version.

Referee #1 # 3**.** Concerning the quantification of the abundance of *Prochlorococcus* ecotypes, authors seemingly missed one important recent reference (Malmstrom et al. Temporal dynamics of *Prochlorococcus* ecotypes in the Atlantic and Pacific oceans. ISME J 2010, 4:1252-1264), since they only looked at HLI and LLIV ecotypes, whereas this paper showed the co-occurrence at equal abundances of the low light ecotypes LLI and LLIV populations at depth.

Response: Valid comments. In the revised version, we will cite the work by Malmstrom et al. (2010). Still, based on our ITS sequence analysis, more LLIV and HLII were found in our deep Pro samples, therefore we choose LLIV and HLII for

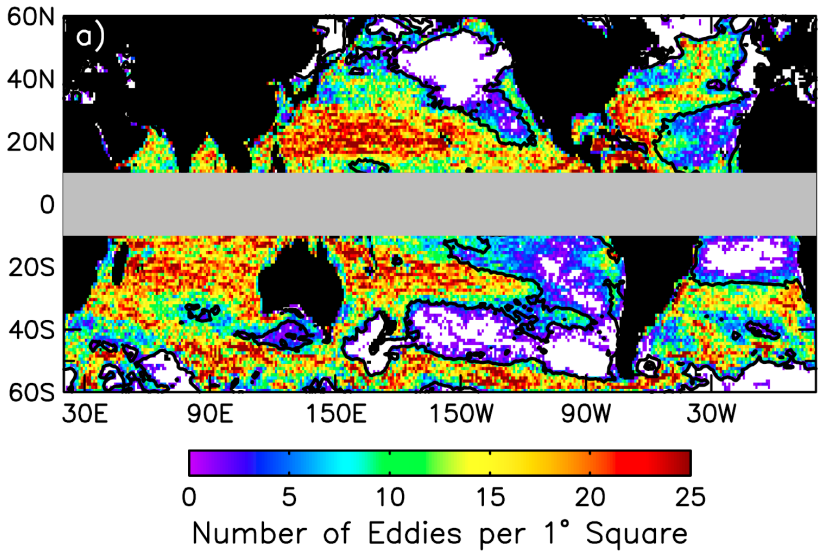
qPCR/RT-qPCR analysis.

Referee #1 # 4. Phylogenetic data shown Figure 4 are also not detailed enough since there is no information on how many environmental sequences were obtained for each clade. A tree clearly showing the novel environmental sequences with a clear indication of their location and depth and how they relate to previously published sequences is absolutely required and solid phylogenetic analyses using different methods and bootstrap support are necessary, not just a mere NJ analysis using MEGA4.

Response: A phylogenetic tree constructed using ML and NJ method, together with more detailed metadata, will be shown in the revised manuscript.

Referee #1 # 5. it is most likely that these deep populations are found only in specific areas with strong vertical mixing and are thus globally not very significant.

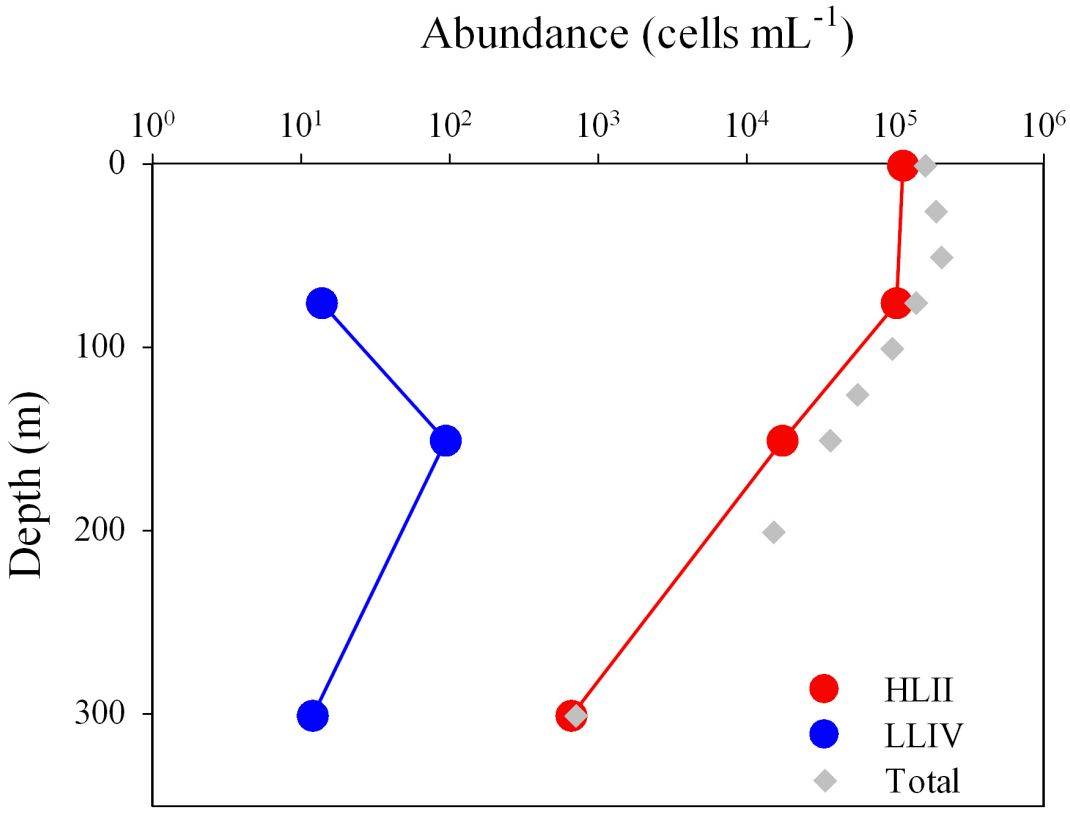
Response: Yes, deep Pro is not everywhere, yet it could be potentially of global significance. Although our study area focused on the western Pacific Ocean and its marginal seas, the significance of the deep Pro is surely present beyond this areas given the fact that physical transportation mechanisms like meso-scale eddies, internal waves and other mixing processes are widely occurring in the global ocean (Chelton et al. 2007) (see Response Fig. 3).



Response Fig. # 3 Distribution of big eddies (with lifetimes >4 weeks) in the global oceans (Chelton et al. 2007)

Referee #1 # 6. Specific comments Fig. 3A: The *Prochlorococcus* abundance as determined by flow cytometry for this specific profile (DC01) should be reminded for easier comparison with molecular data.

Response: Valid suggestion. The figure has been replaced with a revised one including both FCM and molecular data in the revised version. The grey dots show the total Pro abundance as determined by FCM.



Response Fig. # 4 Vertical profiles of the abundance of two Pro ecotypes in the Luzon Strait area (St. DC1).

Anonymous Referee #2

General comments: This article presents evidence that *Prochlorococcus* is present in deep samples (>300 m) at a number of locations in the western Pacific. The authors also discuss some physical mechanisms which can be responsible for the transport of *Prochlorococcus* to deep waters. It is also stated that these deep populations are metabolically active, but no measurements of metabolic activity (e.g. uptake or release of compounds) have been conducted. While some of the observations presented here are useful, I find that the authors greatly exaggerate their biogeochemical implications. The frequent use of the term 'abundant' to refer to the deep *Prochorococcus* is misleading, as the authors use it to convey a sense of importance - which however should be based on carbon biomass, not cell density. My overall recommendation is that this article requires major revision before publication in Biogeosciences.

Response: For "active" issue, please refer to our responses to Reviewer #1 questions #2, and reviewer#2 question #6, #7. For "abundance" issue, refer to our responses to Reviewer #1 questions #5, and Reviewer #2 question #5, #8. In addition, the main purpose of this paper is to bring the phenomena of deep Pro to light and address the potential mechanisms rather than conclude on the quantification of carbon exported to the deep which would be another project. We have revised the manuscript to avoid misleading in this regard.

Referee #2 # 1. The Introduction seems to work on the basis that photoautrophs should be confined to the euphotic layer. However, what is confined to the euphotic layer depth is photoautotrophic growth. In the absence of a perfect physical barrier, it should not come as a surprise that some photoautotrophic cells are present well below the euphotic layer. Downward water movement is bound to result in a downward particle transport, and this will affect all particles of a given size, irrespective of whether they are living or dead particles, or whether they are photoautotrophic, mixotrophic or heterotrophic cells. The question then is to assess how globally important are these events of fast vertical transport.

Response:

While agreed with the specific point of view on "Downward water movement is bound to result in a downward particle transport, and this will affect all particles of a given size, irrespective of whether they are living or dead particles, or whether they are photoautotrophic, mixotrophic or heterotrophic cells" , we have to point out that although theoretically it is " not a surprise that some photoautotrophic cells are present well below the euphotic layer", in fact there has been no report on abundant Pro living cells in the dark water to date. And the known fact is that living photoautotrophs have been found only in the euphotic zone. For global importance, please refer to the response to the Reviewer #1 question #5 with the map of eddy in global ocean for reference.

Referee #2 # 2. The authors refer often (in the Introduction and the Discussion) to the modelling results of Richardson and Jackson (2007) as supporting evidence for the importance of pico-phytoplankton for deep export. However, these modelling results have not been, as far as I can tell, substantiated by direct, sea-true data.

Response: We agree with the reviewer and actually our study provided real measurements supporting the model somewhat. Our study also provided a novel mechanism other than previously known sinking processes through phytodetritus, aggregation, and mesozooplankton grazing. This, at least, shed a light on the explanation of modeling results.

Referee #2 # 3. I concur with reviewer #1 that some methodological precautions should have been taken, such as running blank samples through the flow cytometer to make sure that no contamination from surface samples is contributing to the cell abundances measured in deep samples.

Response: Please refer to our responses to Referee #1 question #1.

Referee #2 # 4. Table 1 indicates that *Prochlorococcus* was present at depths as large as 1500 m but no abundances are shown. Vertical profiles of abundance are given only for the Luzon strait. However, it would be helpful to see a plot showing all

pairwise depth and abundance data, using different symbols to distinguish regions.

Response:

The reason we only listed the depth profiles for the stations in the Luzon strait are 1) that Luzon Strait is the only area where physical processes are extensively studied. To understand such physical processes as solitary waves takes great efforts including time-series observations in the field; 2) that the studied western Pacific area is vast and the controlling mechanisms of the deep Pro in each of the stations may be different. It would be confusing if we list all the profiles in a single figure; 3) that as mentioned out above that it took us about 5 years to conclude the presence of Pro intact cells in the deep water, i.e., we took chances of every affordable ship time rather than design a systematic long lasting cruise to scan the vast ocean for a mapping of the distribution of the deep Pro and a thorough exploration of the controlling mechanisms. After all, this paper serve as a pilot study to call more efforts in this research direction, rather than claim too many things at this stage.

Referee #2 # 5. The results presented in the current manuscript, when converted into carbon biomass data (which is the relevant currency for biogeochemistry, not cell abundance) do not seem to support the view that picophytoplankton are key players in carbon export to the deep ocean. Assuming a mean *Prochlorococcus* carbon biomass of 30 fgC per cell (Heldal et al 2003 L&O 48(5), 1732–1743), a mean deep abundance of, say, 5000 cell/mL (Fig. 2) translates into ca. 0.15 µgC/L of organic carbon. If surface chla in the studied region was, say, 0.2 µg/L, one can have a surface phytoplankton C biomass of around 20 µgC/L. Add to this the non-phytoplanktonic material (detritus, bacteria, heterotrophic protists) and one easily reaches a POC concentration of 40- 50 µgC/L. The authors should examine the biogeochemical relevance of the observed *Prochlorococcus* taking into account that these deep cells may represent, in terms of carbon, <0.5% of surface stocks.

Response: The biomass of deep Pro does only account for a small part of total autotrophs in the surface, however, it is comparable to carbon exported from euphotic zone based on our preliminary calculations, which is not negligible in biogeochemical cycling. In addition, previous work (Lomas et al., 2011), also found that Syn, Pro and nano-plankton derived aggregates contribute respective 2–13% 1–20%, and 6–43% of the total sediment trap POC flux, although it is based on known sinking mechanism.

Nevertheless, we have replaced the word “key” with “important ” in the revised version.

Referee #2 # 6. The authors assert that *Prochloroccous* cells were viable, but no actual study of cell viability has been done. If those cells have recently (e.g. a few days) been transported to deep waters, they may still retain properties of actively growing cells such as possessing pigments and rRNA.

Response: We did have data on cellular pigments as seen by FCM fluorescence, as well as limited RNA data. Please refer to the responses to Referee #1 question #2 for reference.

Referee #2 # 7. These issues could easily be tackled in the laboratory, by taking exponentially growing cells, transferring them to conditions of low temperature and darkness, and then monitoring the evolution of cell abundance, pigment and RNA content and, importantly, ability to fix CO2 or uptake dissolved substrates. Hot temperatures can obviously be very destructive, but there seems to be nothing surprising in a photoautotrophic microbe resisting low temperatures and dark- ness for a few days, and then being able to resume active growth upon transfer to favorable conditions.

Response:

While agreed with the reviewer that isolation and cell viability analysis are a plus, now that “there seems to be nothing surprising in a photoautotrophic microbe resisting low temperatures and darkness for a few days”, we didn’t make every effort on more evidence, in stead we made efforts on the mechanisms for vertical transportation which is novel compared with previously reported sinking processes through phytodetritus, aggregation, and mesozooplankton grazing. Meanwhile, we have been keeping in touch with as many as possible colleagues in this field to keep our study on the right track (see acknowledgements). e.g., we are aware of that Chisholm lab at MIT has been working on Pro cultures in the dark and got some positive results as seen from their posters at ASLO meeting etc, we will cite their work if it is out by the time this paper is ready for publication. For all that we still modified the wording about deep-Pro viability in the revised version. (also refer to the response to the Reviewer #1 question #2)

Referee #2 # 8. When comparing the deep to surface *Prochlorococcus* ratio to general estimates of the f-ratio the authors seem to extrapolate their observations to the global ocean. This, however, would require that the mechanisms of rapid vertical transport discussed here are widespread in the ocean - which seems highly unlikely and has not been demonstrated by the authors.

Response:

Please refer to our response to Referee #1 question #5. Nevertheless we have revised the statement in the text of the new version.

Referee #2 # 9. Related to the above, the mechanisms of downward particle transport should operate also for larger cells. If *Prochlorococcus* is carried to the bottom, so must be other cells - only that, their size being larger and their abundance being smaller, conventional sampling methods will not detect them. My point is that finding picophytoplankton cells at great depths does not necessarily reinforce the importance of the microbial carbon pump - unless these downward water movements selectively transport picoplankton, leaving behind the larger cells.

Response: Yes, all cells no matter big or small would be transported to the deep if they are carried by water movements. However, in our study area, Luzon Strait, the solitary waves were much stronger in the bottom of the the euphotic zone where Pro rather than big diatoms and other phytoplankton are prevailing. These small cells are supposed to be non-sinking and are recycled in the surface layer of the ocean, but are actually demonstrated to be abundantly present in the deep water, contributing to the microbial loop and furthermore to the microbial carbon pump down there. For all that, we still made some modifications to avoid misunderstanding.

Referee #2 # 10. Related to the Figure above, it seems strange that no *Synecochoccus* sequences were found, not even in the euphotic layer. Flow cytometry data (for instance, papers by Zubkov et al. from the AMT cruises) indicate that *Synechococcus* is always present when *Prochlorococus* is present (but not the other way around). Some comments on this should be included - perhaps I am misreading the Figure and Syn sequences were indeed found.

Response: Yes, Syn often presented together with Pro, but at much less abundance so

that they are not visible in the deep water samples by FCM. We did have *Synechococcus* sequences in our clone library. We have added Syn data in the revised manuscript

Referee #2 # 11. Additional basic hydrographic information is needed. At a minimum, temperature and chl a (or fluorescence) profiles should be shown for all studied regions. Estimates of surface suspended POC concentrations would be required, to place in context the significance of deep *Prochlorococcus* in terms of organic carbon.

Response: These are good suggestions for a following project. In addition to our response to Reviewer #1 questions #5, and Reviewer#2 question #5, #8, we should state it clearly that the main purpose of this paper is to bring the phenomena of deep Pro to light and address the potential mechanisms rather than conclude on the quantification of carbon exported to the deep in the context of whole picture of hydrographic information of all the sampled stations (a huge area), which would be another big project. We have revised the manuscript to avoid misleading in this regard.

Anonymous Referee #3

General comments: In this manuscript Jiao *et al* report on the discovery of *Prochlorococcus* populations far below the euphotic zone and discuss possible mechanisms for their transport and implications to C cycling. Overall I think this is an important observation and the oceanographic community needs to hear about it. However, the present form of this paper has shortcomings that need to be addressed. For example, the evidence for metabolic activity or viability of deep *Prochlorococcus* is sparse and not particularly convincing. There was only one set of measurements of rRNA content and they only went down to 300m. It would be interesting if *Prochlorococcus* cells were still active in mesopelagic waters, but without supporting data the discovery of deep *Prochlorococcus* tells use more about physical processes and C transport than ecology and physiology of *Prochlorococcus*. In fact, the transport of *Prochlorococcus* cells seems less important in terms of C than the transport of DOC and other microbes from the bottom of the euphotic zone, which is not dominated by *Prochlorococcus* in terms of biomass. *Prochlorococcus* cells serve as tracers for physical transport, and I believe the paper would be stronger if it

deemphasized issues of *Prochlorococcus* viability and *Prochlorococcus* C, and focused more on estimating total C transport from the bottom of the euphotic zone.

Response:

We admit that the limited RNA data is not enough for a firm conclusion. We wanted to sample again for RNA analysis during the extended revising period but could not have a ship time to make it yet. On the other hand we have plenty of FCM data which can back up, to certain extent, the viability of the deep Pro cells, as it has been reported that changes in cellular chlorophyll fluorescence and changes in cell size are signs of physiological states of the cells (Abalde *et al*., 1995; Cid *et al*., 1997; Veldhuis and Kraay, 2000; Jochem, F.J., 2000). It is also reported that the percentage of active cells in total population increases with decreasing light (Agustí, S., 2004). In addition, previous studies have reported that Pro could be partially heterotrophic (del Carmen Muñoz-Marín et al., 2013). Therefore, they may remain alive there for certain period of time by consuming their stored organic matters. For all that, in order to avoid misunderstanding by the wording "active", we have replaced it with "alive/viable" "intact cells" in the revised version.

Another concern, the biomass of deep Pro does only account for a small part of total autotrophic carbon in the euphotic zone, however, it is comparable to C exported from euphotic zone according to our preliminary calculation. The suggestions on estimating total C transport from the bottom of the euphotic zone is valid, but we do not have data on other phytoplankton. Fortunately, previous work (Lomas *et al*., 2011) reported that Syn, Pro and nano-plankton derived aggregates contribute 2–13%, 1–20%, and 6–43% of the total sediment trap POC flux, respectively. Which provide a reference for our case.

For all that, the main purpose of this paper is to bring the phenomena of deep Pro to light and address the potential mechanisms, we think it worth publishing to call attention from the community to address the other derived importance issues, such as the viability of the deep Pro, why and for how long? As well as how much the impact of the deep-Pro on ecological processes such as microbial loop, microbial carbon pump, and DOC/ POC transport and sequestration.

Referee #3 # 1. Does the model of transport by solitons explain transport to and

from the mesopelagic? The paper mentions transporting a certain fraction of cells back to the euphotic. More background on solitons would be helpful to understand if it is a bidirectional process.

Resonse: Solitary waves are going with water movements up and down (Jackson and Apel, 2002), therefore there must be some cells being brought back from the deep where Pro cells brought to from the euphotic zone. But we were not be able to separate the brought-up cells from the rest at the present. Nevertheless, mentioning this point would be good for a comprehensive understanding of the present story and future study. As for the depth a soliton reaches, it could be case by case. In the case of Luzon strait, it can reach 500m or deeper, according to our record.

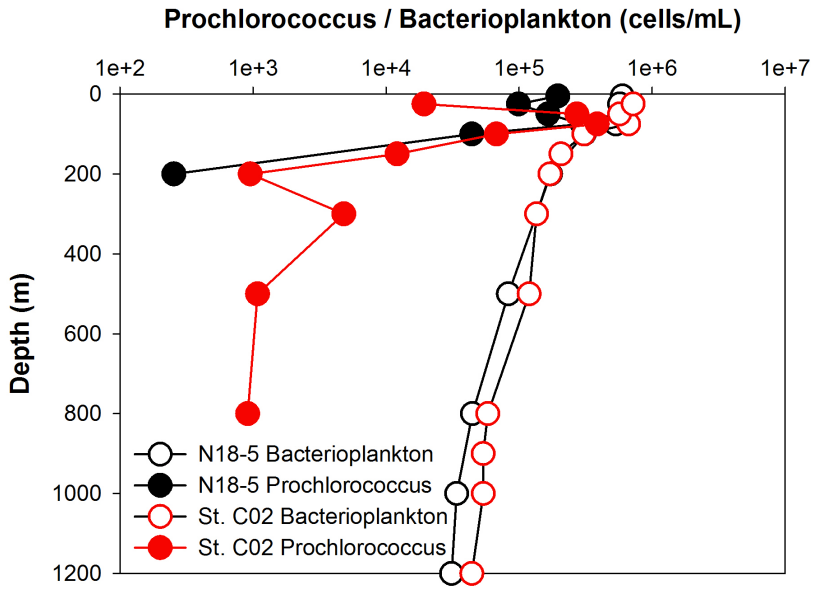
Referee #3 # 2. Why do deep *Prochlorococcus* maintain high pigment concentrations? Pigments are expensive but useless in the mesopelagic. Does high pigment concentration indicate the cells were recently transported?

Response: Light-limited cells (usually near the bottom of euphotic zone) tend to have higher Chl per unit of biomass thus higher efficiency to capture light even though it is expensive. Our data suggested that deep Pro cells were mainly brought from the bottom layer of the euphotic zone where solitons are most active and strong. After brought down to the deep water, their cell sizes were further shrinked, and therefore showing high cellular pigment contents apparently. Yes, the high cellular pigment contents we observed in the present study suggest that the cells be recently transported.

Referee #3 # 3. Are there flow cytometry counts for total bacteria from the bottom of the euphotic zone? What about counts from the mesopelagic? I know *Prochlorococcus* was not "supposed' to be down deep, but were they a larger or small fraction of total deep populations compared to the bottom of the euphotic zone? It's hard to believe that *Prochlorococcus* were preferentially transported, so how many cells might have gone down to the mesopelagic with them?

Response: Yes, we do have bacteria data, the depth profiles below (Response Fig.# 6 showing the difference between station with and without deep Pro. Obviously Pro

were not preferentially transported compared to total bacteria. The fraction of deep Pro in total populatons is smaller than that in the euphotic zone. This does not mean Pro were less proportionally transported but due to 1) that heterotrophic bacteria can grow with and without light while Pro can grow only under light, and part of the Pro cells transported to the dark may become dead; and 2) that heterotrophic bacteria are everywhere and the concentrations of the transported bacteria from the euphotic zone to the deep waters were diluted at proportions lower than that of Pro who were originally present only in the euphotic zone.



Response Fig. # 6 Depth profiles of total bacteria and *Prochlorococcus* at sites in the Luzon Strait and outside in the western Pacific Ocean as a reference.

Referee #3 # 4. Is it really important to emphasize picoplankton-derived C when discussing the importance of the microbial carbon pump? Why would it matter if refractory C was produced from *Prochlorococcus* instead of heterotrophic bacteria?

Response: These arguments are valid, and we have revised the manuscript according to the suggestions.

Referee #3 # 5. I am confused why the authors suggest deep *Prochlorococcus* populations emphasize the importance of the microbial carbon pump in subtropical waters. This observation really supports a greater role for the biological pump since the observation is of POC transported in the deep ocean. There were no measurements

in this study regarding the production or refractory C.

Response: As Pro are most abundant in subtropical and tropical oceans, and Pro cells are so small that the carbon carried by Pro are normally going only through the microbial loop, during which, the microbial processing of organic carbon can generate RDOC , and thus contributing to the MCP.

As autotrophs, Pro are supposed to be only present in the euphotic zone, but now they are found to be present in the deep water where Pro cells will finally become dead or grazed through microbial loop, and thus contributing to the MCP. Still, in order to avoid misunderstanding, we have revised the statement in the new version.

Referee #3 # 6. Can Figure 4 be more quantitative? Perhaps a table with counts of ITS sequences from each ecotype would provide strong support.

Response: We will provide more quantitative information in Figure 4 in the revised manuscript.

Literature cited:

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