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Interactive comment on "Distribution of ultraphytoplankton in the western part of the North Pacific subtropical gyre during a strong La Niña condition: relations with the hydrological conditions" *by* M. Girault et al.

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Dear editors of the Biogeosciences and Reviewers,

On behalf on my coauthors, I am pleased to submit the response to the reviewers of the manuscript entitled "Distribution of ultraphytoplankton in the western part of the North Pacific subtropical gyre during a strong La Niña condition: relationship with the hydrological conditions". We thank you and the anonymous Reviewers for the very useful comments. Both Reviewers have suggested numerous useful modifications which have

considerably enhanced the quality of this new version. Both the manuscript and figures were modified according to the Reviewers'comments. In this new version we have detailed the different methods used and added information about the volume analysed by the flow cytometer. Statistical analyses have also been highlighted in this version. Additional statistical analyses were performed during the review process (CAP CcorA, RDA, and Correspondence analysis). Finally, according to our data set and the reviewer suggestions, we decided to add the RDA results only in order to better describe the relationships between the organisms and the environment parameters (Fig 15). On the basis of the Reviewers' comments, figure 14 was modified into a 2D graph in order to better display the various clusters. We have also changed the term "nanocyanobacteria" into "nanocyanobacteria-like" and reported this modification in all the figures. We have also improved the English, revised some sentences and discussion was modified in order to clarify the manuscript. We are looking forward to receiving your decision with the hope that it will be positive.

Best regards, For the authors, Mathias GIRAULT

Reply to Reviewers' comments:

Anonymous Referee #1 Received and published: 20 May 2013

The authors presented, for the first time, the distribution patterns of ultraphytoplankton and heterotrophic prokaryotes in the subtropical western North Pacific during a La Niña condition. Although flow cytometry (FCM) has become a conventional analytical technique for determining the abundance of ultraplankton in seawater in the field of Oceanography since 1990s, the combination of their FCM and macronutrient data at nanomolar levels enabled them to examine the relationships between these parameters. Overall, this paper seems to be rather descriptive, but it could be publishable after revision - I have a few major concerns about their analytical procedures used in this study.

Major comments: -In terms of FCM, how many replicate samples did you analyze?

As for most scientific cruises there was no replicate to address replicability of the various analyses. Indeed, real replicates would require to perform several CTD casts each time and that would require too much time, it is impossible during a cruise. If each Niskin bottle was sampled twice or three times, that would address the repeatability and not replicability. That is why, similarly to most of the works (not to say all of them) available in the literature, in Biogeosciences or in any other Scientific Journal, we did collect only one sample per depth and per station. During his Ph.D, Gérald GREGORI (Grégori. 2001. Ultraplancton dans la baie de Marseille: séries temporelles. viabilité bactérienne et mesure de la respiration par cytometrie en flux. Thesis of the Université de la Méditerranée) has made some tests to evaluate the variability in the samples collected from 7 Niskin bottles closed together at the same depth (5m) during the POMME Cruise (Latitude : 44 59.98 N ; Longitude : 017 59.98 W ; maximum depth: 4824 m, September 20th 2000). The results for the various counts at the station 2 are displayed in the figure 1 (table additional note): As one can see, the variability between the Niskin bottles is not negligible at all, very likely due partially to the patchiness in situ but also to additional error due to the sampling method and manipulations of the samples before analyses. This error is higher than the replicability of the measures by the flow cytometer which has been estimated to 2-3% for calibration beads (data not shown).

-Also, according to Campbell (2001), thousands of events for each population should be acquired for accuracy. In this study, the authors found that concentrations of nanocyanobacteria were very low (< 600 cells cm-3) and the detection limit was 5 cells cm-3 (P5712. L22-28). Campbell. L. (2001) Flow cytometric analysis of autotrophic picoplankton. In: Marine Microbiology (Ed.. J. H. Paul). Methods in Microbiology. 30. 317-343. Academic Press. If the authors consider that these data are statistically reliable. Please, indicate both the runtime of your FCM with its approximate flow rate and the method for determining the detection limit in the text.

The Reviewer has raised an important question. As defined by Shapiro in his “Practical

Flow Cytometry” (4th Ed. John Wiley & Sons. Inc. pages 19-20), the error on a count depends on Poisson statistics. When one counts N particles, the error is basically $100/\sqrt{(N)}$ (in %). Thus, if one counts 100 cells the error on the counting is about 10%. If one counts 10.000 cells, the error drops down to 1%, which is much better. Finally if one counts 25 cells only, the error is about 20%. Obviously this does not seem good at first sight. However, that does not make a drastic difference in term of abundance compared to any other more abundant population. Indeed a rare population (such as nanocyanobacteria-like population in our case) does remain a rare population. During the analyses performed in this study, a decent number of particles has been analyzed for each sample: The total number of particles (events) enumerated during the cruise varied from 3.317 (station 9. 175 m) to 43.353 counts (station 11. 95 m). The number of nanocyanobacteria is indeed low but the total number of event is upper than 5.000 counts in each cytogram where the nanocyanobacteria were detected. Consequently, we believe that the nanocyanobacteria concentrations are reliable in relation with the method used and are very low by comparison with the other communities. Obviously, if one wants to be more accurate and be able to address few cells in a liter or a meter cube, a much bigger volume would be needed (liters or more) but that is out of scope of the subject of this manuscript. However, we do believe that increasing the volume analysed to only about 500 μL, like in several other studies, wouldn't impact significantly the final concentration of nanocyanobacteria found in this study in about 150 μL.

As far as the limit of detection is concerned (5 cells.mL-1), we have added in the manuscript the details of the flow rates and running time. The runtime of the flow cytometer was 3 minutes for phytoplankton (about 150 μL analyzed) and 2 minutes for heterotrophic prokaryotes (about 100 μL analyzed) with an approximate flow rate of 50 μL.min-1. The FACScalibur flow cytometer provides three different sample flow rates. In the case of highest (HI) flow rate (about 100 μl.min-1), the number of particles crossing the laser was higher than the maximal limit of the instrument (about 2.000 events.s-1). One possible solution to face this problem would have been to dilute the samples. But instead, we have chosen to work with the medium flow rate of the flow

cytometer (MED) because it was a good compromise between the number of events per second and the time available on the PRECYM flow cytometry platform to run all the samples (phytoplankton and heterotrophic prokaryotes were analyzed separately in about 10 days). Initially the sample analyses were planned in Japan. However, due to the dramatic accident of Fukushima and because a Thesis was engaged, we have modified the plan and took the opportunity offered by the PRECYM platform run the analyses in France. The running time of all the Tokyo Palau cruise samples were integrated in the busy schedule of the PRECYM flow cytometry platform. This is why the best compromise had to be found between the time needed per sample (i.e. the volume analyzed) and the time available on the platform.

-For nanocyanobacteria, how were you able to separate nanocyanobacteria from cryptophytes which could possess phycoerythrin?

We claim we have analyzed nanocyanobacteria because in two previous publications (Kitajima et al.,. 2009; Sato et al., 2010) both named nanocyanobacteria the organisms analysed by flow cytometry with a size larger than 2 μm and a bright orange fluorescence intensity. In our manuscript the flow cytometry protocol to identify the nanocyanobacteria was similar with the method of Sato et al. (2010). In addition, the experts at PRECYM did not notice in the past any cryptophyte population in the very place where the nanocyanobacteria-like population did show up on the various cytograms. At least, one picture of nanocyanobacteria collected during the Tokyo Palau cruise was taken and recently published in J. Plankton Res. (figure 9G): Girault et al. (2013), Phosphorus stress of microphytoplankton community in the western subtropical North Pacific. J. Plankton Res., 35. 146-157. However, in order to take into consideration the Reviewers'comments, we did not perform any molecular biology to genetically identify the nanocyanobacteria, we have modified the term nanocyanobacteria into nanocyanobacteria-like in this new version of the manuscript.

Kitajima. S., Furuya. K., Hashihama. F., Takeda. S., and Kanda. J.: Latitudinal distribution of diazotrophs and their nitrogen fixation in the tropical and subtropical

western North Pacific. Limnol. Oceanogr., 54. 537–547. 2009. Sato. M., Hashihama. F., Kitajima. S., Takeda. S., and Furuya. K.: Distribution of nano-sized cyanobacteria in the western and central Pacific Ocean. Aquat. Microb. Ecol., 56. 273–282. 2010.

-Why did you select the conversion factors of Tuit et al. (2004), Karayanni et al. (2005) and Caron et al. (1995) out of the literature values published previously?

These conversion factors were selected due to their utilizations in subtropical western Pacific or in report on the organisms living in the oligotrophic area (Denis et al., 2010 in Biogeosciences; Tsai et al., 2013). These conversion factors were in the range of several estimations published in the literature. For example, the carbon biomass of heterotrophic prokaryotes was reported to be 12.4 fg.C.cell-1 $\pm$6.3 for the oceanic bacterial assemblages (Fukuda et al., 1998). In some cases, some literature used an higher carbon biomass 20 fg.C.cell-1. However, Fukuda et al., (1998) reported that the higher carbon biomass (30.2 fg.C.cell-1 $\pm$12.3) is associated with coastal bacterial assemblages. In our study, the value of 15 fg.C.cell-1 (Caron et al., 1995) was chosen because our samples were collected in open Ocean.

Denis. M.. Thyssen. M.. Martin. V.. Manca. B.. and Vidussi. F.: Ultraphytoplankotn basin-scale distribution in the eastern Mediteranean sea in winter: link to hydrodynamism and nutrients. Biogeosciences, 7. 2227-2244, 2010. Fukuda. R.. Ogawa. H.. Nagata. T.. and Koike. I.: Direct determination of carbon and nitrogen contents of natural bacterial assemblages in marine environment. Applied in environmental microbiology, 64. 3352-3358, 1998. Tsai A. Y. Gong. G.–C.. and Hung. J.: Seasonal variations of virus- and nanoflagellate-mediated mortality of heterotrophic bacteria in the coastal ecosystem of subtropical western Pacific. Biogeosciences, 103055-3065, 2013.

-In P5707 and L24. SYBR Green II should be replaced by SYBR Green I. because the former is a stain for single stranded DNA and RNA and Marie et al. (1999) did not use SYBR Green II.

We used the SYBR Green II as it is the protocol used in the PRECYM flow cytometry platform where the samples were analysed. This choice has been made based on the following publication: Lebaron. P., Parthuisot. N., and Catala. P.: Comparison of blue nucleic acid dyes for flow cytometric enumeration of bacteria in aquatic systems. Appl. Environ. Microbiol., 64, 1725-1730, 1998.

In this article, SYBR Green II performs better than SYBR Green I in salted samples. SYBR Green II is also used by Grégori et al. (2003) combined to Propidium iodide to discriminate live and membrane compromised bacteria. Grégori. G., Denis. M., Sgorbati. S., and Citterio. S.: Resolution of viable and membrane-compromised free bacteria in aquatic environments by flow cytometry. Current protocol in cytometry, 11, 15.1-15.7. 2003.

-The authors used principal component analysis (PCA), a simple, unconstrained ordination technique with one data matrix. PCA is a good statistical method to grasp the major structures of the whole data with a reduced set of orthogonal axes. However, PCA can hardly explain relationships between the parameters used (i.e.. plankton and environmental variables in this case) - the authors tried to examine these, but the relationships obtained were qualitative. If the authors would like to compare their plankton data with the environmental variables, I believe they should use canonical ordination methods such as redundancy analysis (RDA), a popular statistical technique combining regression and PCA with multiple data matrices, and discuss the outputs. For example, Fehling et al. (2012) and Peng et al. (2012) succeeded in determining the environmental factors controlling the distribution patterns of each phytoplankton group using RDA. Fehling. J. et al. (2012) The relationship between phytoplankton distribution and wa- ter column characteristics in North West European Shelf Sea Waters. PLoS ONE. 7. e34098. doi: 10.1371/journal.pone.0034098. Peng. S. et al. (2012) Distribution and controlling factors of phytoplankton assemblages in a semi-enclosed bay during spring and summer. Marine Pollution Bulletin. 64. 941- 908. doi: 10.1016/j.marpolbul.2012.03.004.

As suggested by the Reviewer, we have included the results of the RDA in the manuscript and performed a partial RDA in order to quantify the effect of the each environmental variable on the ultraphytoplankton composition. Although results were not included in the manuscript, Canonical analysis of principal coordinate (CAP) technique was also calculated to investigate the effects of environmental variables on the phytoplankton community (Anderson and Willis, 2003) (Fig. 2, additional note). For CAP, the data were log10(x+1)-transformed to remove large differences in scale among the original variables. Then, Bray-Curtis dissimilarities were calculated between every pair of observations and an unconstrained ordination was done using metric multidimensional scaling (MDS) on the dissimilarity matrix (Bray and Curtis, 1957). Finally, classical canonical correlation analysis (CCorA) was performed on unscaled orthonormal axes of principal coordinate analysis (PCO) (Anderson and Robinson, 2003). Two CAPs were performed: one for the surface observations, and another one for the vertical profile data (Sta. 5 to 11). Similarly to the RDA, the canonical test statistics for the surface data were not significant (P=0.1. using 999 permutations). The variation partitioning is not yet developed on the current Anderson software. So, we decided to add only the RDA in the manuscript in order to better characterize and quantify the influence of each variable on the phytoplankton and heterotrophic prokaryote distribution.

Anderson. M. J. and Robinson. J.: Generalised discriminant analysis based on distances. Aust. Nz. J. Stat. 45. 301-318, 2003. Anderson. M. J., and Willis. T.: Canonical analysis of principal coordinates a useful method of constrained ordination for ecology. Ecology, 84. 511-525, 2003. Bray. J. R., and Curtis. J. T.: An ordination of the upland forest communities of the southern Wisconsin. Ecol. Monogr., 27. 325-349, 1957.

Minor comments: -P5703. L20-24: I do not agree on your statement that HPLC pigment analysis is im- practical for estimating phytoplankton community structure in oligotrophic waters. It is true that cellular pigment content becomes low in such waters with high irradiance. However, this weakness can be retrievable by increasing the filtration

volume of seawater. Please amend or delete the sentence.

In order to consider this comment, we have amended the sentence. In the first version we just indicated that results from HPLC analyses have a global approach and cannot measure at the single cell level the pigment content. Based on the pigment concentrations it is possible to estimate the percentage of various communities in the sample (Diatom, Synechococcus. etc...). However, the concentration of pigments in each community does vary depending on the environmental conditions such as irradiance and nutrient limitations (Noble et al., 2003 publications therein). Thus, it is difficult to rely the pigment contents and abundance of organisms in oligotrophic condition, particularly where irradiance is particularly high and thus cells have a very low pigment content.

Noble. P. A., Tymowski. R. G., Fletcher. M., Morris. J. T., and Lewitus. A. J.: Contrasting patterns of phytoplankton community pigment composition in two salt Marsh Estuaries in southeastern united states, Appl. Environ. Microbiol., 69. 4129-4143, 2003.

-P5703. L27 and P5724. L19: “a” after chlorophyll should be italicized. Modified as suggested -P5704. L25 and thereafter: Remove the space between number and % (i.e.. 1%). Modified as suggested -P 5706. L14: because Modified as suggested P 5706. L15: was referred to Modified as suggested -P 5706. L 16: were not shown Modified as suggested P5708. L5-19: What statistical software did you use? We used R for the statistical analysis, we add information in the materials and methods -P5709. L14: Please cite a more appropriate literature instead of the paleoceanographic paper of Oba and Murayama (2004). because they did not indicate the hydrographic characteristics of Kuroshio waters. Modified as suggested (Sekine and Miyamoto. 2002) -P5709. L14-15 and P5718. L3: the second group Subtropical gyre (stations 5-8) C2005 Modified as suggested -P5709. L18-19: the last group Transition zone Modified as suggested -P5710. L18: nitrogen availability in diatoms (Fig. 7) Modified as suggested. -P5711. L13: Use station 8. not 22.83âŮęN changed. P5712. L24:

Nanocyanobacteria were mainly Modified as suggested -P5717. L10: modify salinity. especially Modified as suggested -P5719. L16: Figs. 13 and 14 Corrected -P5720. L2: zone was mostly Modified as suggested -P5723. L16: diatoms Modified as suggested -P5726. L12: be mainly controlled Modified as suggested Figs. 12-14: “Prochlorococcus” and “Synechococcus” should be italic Modified as suggested.

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Anonymous Referee #2 Received and published: 20 May 2013

Overall quality/General comments The authors present the distribution patterns of ultraphytoplankton, heterotrophic prokaryotes and hydrographic variables in the subtropical western North Pacific during a period of La Niña. The majority of the manuscript comes across as being very descriptive which is often a consequence of having a significant number of determinants at multiple stations. Although the manuscript focuses on the distribution of ultraphytoplankton, measurements of the microbial community and hydrographic variables are brought together using principle component analysis in an attempt to examine the relationships between these parameters. At present I am unconvinced about the relationships between microbial and hydrographic data (see concerns in Specific Comments below) but the manuscript might be publishable following revision, particularly of the section concerning relationships between parameters and clarification of other concerns and rewriting of the discussion relating to microbes/nutrient interactions. The discussion would also benefit from being shorter and focussing on fewer topics.

Specific Comments -A major part of the manuscript is based on an analysis of wide-ranging measurements using PCA and so the success of the manuscript hinges on figures 13 and 14. These were found to be somewhat unclear, crowded and therefore unconvincing. Whilst it is felt that there is definitely evidence that the microbial community DID change with latitude, the way in which it changed in relation to the hydrographic variables needs to be more convincingly articulated and presented to the

reader.

We have modified Figure 14 in order to make clearer the group of stations. We have performed a redundancy analysis (RDA) and a partial redundancy (partial RDA) to statistically confirm the qualitative information of the PCA. Monte-Carlo simulations were also used to test the canonical axes of the RDA. For more details, please refer to the above reply addressed to the previous Reviewer.

-Flow cytometry - The abundances of microbes measured by flow cytometry are often very low and a detection limit of 5 cells cm-3 (P5712. L22-28) was stated. Therefore, the authors must state how they determined their detection limit and the degree of confidence they have in this detection limit. In addition to this, was there any replication in sample analysis?

There is no replication in the analysis due to the time available on the flow cytometry platform. For more details please, refer to the above reply addressed to the previous Reviewer.

-If not, what confidence do the authors have in the abundance estimates that they have generated and how can they evidence their confidence? As the volume analyzed by the flow cytometer varied roughly from about 150, we have stated that if 1 cell was detected in these 150 μL, it means that the lower concentration we could detect would be about 5 cells.mL-1.

This detection limit took into consideration the dilution factors (volume of bead and formaldehyde solutions added).

-Obviously this is an estimate based on the hypothesis that cell distribution in the sample is homogeneous and is representative of the in situ. Indeed, the samples may settle long enough in the Niskin bottles before sampling to sediment. What is measured, for any parameter, is in fact what is subsampled from the Niskin bottle (which is never ever mixed before sampling). It is this subsample which is homogenized, but

never ever the Niskin bottles. However, as we are dealing with ultraplankton, we may assume that these cells are too small, particularly in oligotrophic areas, to significantly sediment during the time they remain in the Niskin Bottle. We assume therefore that the samples are homogeneous and representative of the in situ condition.

The concentration of microorganisms in the various samples did vary depending on the sampling stations and depth. The number of events during the analyses varied in a range from 3.317 counts (station 9. 175 m) to 43.353 counts (station 11. 95 m). According to the previous response to Reviewer 1 (above), the total events was higher than 5.000 counts, where the low abundant nanocyanobacteria were detected. For example, the station 11 at the depth of 45 m, there are: 284 Synechococcus, 8.386 Prochlorococcus but only 38 picoeukaryotes, 83 nanoeukaryotes and 34 nanocyanobacteria. The error on the counting relies on Poisson Law (and can be estimated as $100/\sqrt{N}$ with N= the number of counted particles). To be sure to deal with the right volume analyzed, (which may vary slightly as it is based on a pressurized system), Trucount calibration beads were used. The number of Trucount bead (typically between 1.500 and 2.000 beads) counted in the samples was used to accurately determine the volume analyzed. In addition, to verify the flow rate of the instrument, and the reproductibility of the FACScalibur a Trucount bead solution in distilled water with NaCl (salinity adjusted at 35) was analyzed every day in the morning, at noon and late afternoon after the analyses. The volume analyzed was also determined by weighing a tube before and after a 3 minute analysis. The volumes determined by weighing the sample or by the Trucount beads are in agreement. We did not observe significant difference in the number of Trucount beads detected by the flow cytometer, i.e. no significant change in the flow rate of the instrument. The position of the beads on several cytograms (depending on fluorescence and scatter intensities) is also monitored on every single sample in order to prevent any drift of the instrument. Following these tests run on a daily basis, we expect that the detection and count of organism using the FACScalibur is reliable. Obviously we considered that the concentrations of nanocyanobacteria are particularly low. Incidentally, it is difficult to obtain 1.000 events

of nanocyanobacteria in order to have at least a 5% of confidence. The volume of seawater needed would be higher than allowed by the crioval tubes (4.8 mL) for all samples with a number of events lower than 32 in 150μL (1.000*150/4.800=31.25).

-Confidence in abundance estimates increases with the number of cells analysed. Typically, a count of 1000 'events' results in an error in abundance estimation of approx., 5%. but this also depends on the volume of sample analysed. Therefore, the authors need to state what volume of sample was analysed for the different components of the microbial community, or at least provide information about the analysis time and flow rate, the latter of which should be possible as Trucount beads were used as an internal standard.

The volume of heterotrophic prokaryotes was 100μL and volume for the phytoplankton analysis was 150μL. The time to perform one analysis is 2 minutes for the heterotrophic prokaryotes and 3 minutes for the phytoplankton community. We added information of the flow rates and the running time for one analysis.

-These beads would also provide a means of testing sample analysis reproducibility. at least in well-mixed waters if there was no real sample analysis replication as there is often very little variation in cell numbers through the water column in mixed waters.

By using the Trucount beads, we routinely tested the reproducibility. Tests were performed at different hours in order to check the modification of the ambient temperature and pressure on the volumes analyzed and the correct detection of the beads. Our results revealed no significant differences between the samples (t-test. $p<0.05$).

-Separation of nano and picophytoplankton – The authors state that they used 2 μm beads to separate the nano and picophytoplankton. I fail to see how this has been done. Size is measured relatively with a flow cytometer using forward scatter. primarily. however the light scattering properties of beads are very different to phytoplankton: the beads scatter far more light relative to phytoplankton of the same size and so would appear much larger in terms of their forward scatter signal. It should be possible to

demonstrate where the 2μm beads were in relation to the phytoplankton as samples were run with 2 μm beads. Fig 3a. shows beads in the plot. However, it is not known whether the beads are 2 μm beads or the larger 4.2μm Trucount beads. I would have thought that both bead types would have been in the plot as that is what is reported in the methods (page 5707. lines 1-6).

The figure 3.a displays the phytoplankton and the Trucount beads only. We added this information in the legend. Sample and 2 μm beads were not show because it can be easily misidentified and counted as phytoplankton. Before running the Tokyo Palau samples, we prepared an artificial seawater sample (distilled water and NaCl; salinity 35). Then, we inoculated a cocktail of 2 μm and Trucount beads. The figure 3.a (Fig 3 additional note) shows one example of the forward scatter versus red fluorescence cytogram. By using the Summit V4.3 software, it's possible to found the center of the gravity of each region defined by beads. The center of the gravity of the 2 μm beads region characterized the threshold value of 2 μm used to help in separating pico- from nanophytoplankton. The positions of the 2 μm axes were also controlled by addition of 2 different types of beads (1 μm and 6 μm). The figures 3.b and 3.c (Fig 3 additional note) showed 2 examples of cytogram using a set of beads. . Calculation of the position by using a set of 1, 2, 6 μm beads were similar to the center of gravity method. As there is no change in the setting of flow cytometer, the Tokyo Palau samples were analyzed according to the protocol listed in the materials and methods part. Obviously we do know that polystyrene beads do not have the same refractive index than cells constituted of organic matter. It means that this 2 μm limit in size, in term of forward angle light scatter intensity is not exactly the same for both types of particles. But as for most publications on similar topics, these beads were still used to help separating pico- from nanophytoplankton. PRECYM platform is now investigating Silica beads which do have a refractive index closer from cells. But these are just preliminary essays.

-Nanocyanobacteria – How were the authors able to discriminate nanocyanobacteria from cryptophytes? Molecular analyses? If there is no actual evidence of the identity of

the high phycoerythrin-containing group. then the uncertainty needs to be stated. If it cannot be confirmed that the group in question is nanocyanobacteria then the section beginning on line 25 of page 5720 is brought into question and should be removed.

As mentioned previously in the reply to the previous Reviewer's comments, we claim we have analyzed nanocyanobacteria because in two previous publications (Kitajima et al.. 2009; Sato et al.. 2010), both named nanocyanobacteria the organisms analysed by flow cytometry with a size larger than 2 μm and a bright orange fluorescence intensity. In our manuscript the flow cytometry protocol to identify the nanocyanobacteria was similar with the method of Sato et al. (2010). In addition, the experts at PRECYM did not notice in the past any cryptophyte population in the very place where the nanocyanobacteria-like population did show up on the various cytograms. At least, one picture of nanocyanobacteria collected during the Tokyo Palau cruise was taken and recently published in J. Plankton. Res. (figure 9G): Girault et al., (2013) Phosphorus stress of microphytoplankton community in the western subtropical North Pacific, J. Plankton. Res., 35. 146-157. However, in order to take into consideration the Reviewers' comments, we have modified the term nanocyanobacteria into nanocyanobacteria-like in this new version of the manuscript.

Kitajima. S., Furuya. K., Hashihama. F., Takeda. S., and Kanda. J.: Latitudinal distribution of diazotrophs and their nitrogen fixation in the tropical and subtropical western North Pacific. Limnol. Oceanogr., 54. 537–547. 2009. Sato. M., Hashihama, F., Kitajima. S., Takeda, S., and Furuya. K.: Distribution of nano-sized cyanobacteria in the western and central Pacific Ocean. Aquat. Microb. Ecol., 56. 273–282. 2010.

-Heterotrophic prokaryotes – Marie et al. (1999) did not use SYBR Green II. One assumes. therefore that Sybr Green I was used. Please confirm. This is important as Sybr Green II stains single stranded DNA and RNA. whereas Sybr Green I targets double stranded DNA.

As replied to the previous Reviewer, we used the SYBR Green II as it is the protocol

used in the PRECYM flow cytometry platform where the samples were analysed. This choice has been made based on the following publication: Lebaron. P., Parthuisot. N., and Catala. P.: Comparison of blue nucleic acid dyes for flow cytometric enumeration of bacteria in aquatic systems. Appl. Environ. Microbiol., 64, 1725-1730, 1998.

In this article, SYBR Green II performs better than SYBR Green I in salted samples. SYBR Green II is also used by Grégori et al., (2003) combined to Propidium iodide to discriminate live and membrane compromised bacteria.

Grégori. G., Denis. M., Sgorbati. S., and Citterio. S.: Resolution of viable and membrane-compromised free bacteria in aquatic environments by flow cytometry. Current protocol in cytometry. 11. 1-15, 2003.

-Introduction – I feel I must disagree with the authors regarding their comments regarding difficulties using HPLC in oligotrophic waters. Yes. it is well known that pigment per cell of phytoplankton is low in surface waters of oligotrophic regions but by simply increasing the sample volume filtered it is still perfectly possible to measure pigment concentrations of even the smallest organisms such as Prochlorococcus.

We have modified the sentence in the text. We do agree that the increase of the volume filtered improves the measurement, especially when the microorganisms have a low concentration of intracellular photosynthetic pigments. However, Schluter et al., (2000) reported that the concentration of pigments in each community can vary depending on the environmental factors such as irradiance and nutrients. The modification of pigment content is usually independent of the group of phytoplankton (for example: Synechococcus, Prochlorococcus). In this case, the increase of the volume filtered would improve the detection of the cell with low pigment content but would not necessary correct for the difference between the groups of phytoplankton. HPLC has numerous advantages in term of cost, method, etc. However, HPLC does not theoretically link the concentration of pigments with the abundance of particles. The Tokyo Palau cruise samples were collected in the oligotrophic subtropical area. In the subtropical area the

possible bias linked to nutrients depletion and strong irradiance are important, leading to small cells with a low pigment content. In this environmental condition, we decided to use the classical flow cytometry in order get information of the microbial community based on data collected at the single cell level.

Schlüter. L., Mohlenberg. F., Havskum. H., and Larsen. S.: The use of phytoplankton pigments for identifying and quantifying phytoplankton groups in coastal areas: testing the influence of light and nutrients on pigment/chlorophyll a ratios. Mar. Ecol. Progr. Ser. 192, 49-63, 2000.

-Discussion – The authors should take a look at the literature pertaining to the Atlantic Ocean to gain a better understanding of the way in which microbes in oligotrophic waters are able to utilise apparently scarce nutrient resources.

Technical corrections – NOTE corrections in italics or explained 5701: Title. line 4 – change to.' relationship with hydrological conditions' done 5702 line 3 –a north-south transect (33.6oN-13.25oN) along the 141.5oE meridian. . . done Line 10 – explain warm pool description of Warm pool was detailed in the discussion part. We didn't add information in the abstract because of samples were not collected in this area. Line 12 – However. - add comma done Line 17 – Explain (35) 35 is the isohaline Line 20 – the distribution done Line 22 – remove (>100 m). add at depths >100 m after (STCC) done 5703: line 13 – add comma after 200 m done Line 14 – change hydrology to hydrography done Line – 27 – change maximum to maxima done 5704: Line 14 – comma after conditions done Line 15 – s after cast done Line 21 – with an in situ... done 5705: line 13 – the MAGIC... done Line 16 – n after show done 5706: line 4 – filtered not filtrated done Line 10 – remove s from otes done Line 13 – using a FACSCalibur flow cytometer done 5707: line 15 – separated from Synechococcus done Line 25 – on the basis. . . done 5708: line 14 – points below... done Line 21 Hydrographic done 5709: line 18 – remove the word 'the' before station 7 and station 8. done 5710: line 1 – remove sides. replace with ends done Line 4 – tions varied from. . . done Line 5 – remove of and replace with in Line 4-6 – Sentence does not make sense we corrected Line 9 –

remove spread and replace with varied done Line 10 – comma after 8. remove s from phosphates done Line 11 – Except for station 3. done Line 12- at the surface done Line 15 – at station 3 (remove the word the) done Line 17 – replace among with for done Line 18 – replace The with A done Line 19 – remove discriminated the with was the. and silica (remove the) done Line 21 – remove the word the before phosphorus done Line 22 – consisting instead of consisted done Line 24 – was instead of were done Line – 26 Its Si:P. comma between 15 and observed done Line 27 – at the surface. add had instead of has done Line 28 – the highest Si:P ratio of the cruise. 5711: line 2 - remove the s from fluorescences done Line 16 – add a space after cells done Line 21 – minimum abundance was found. done Line 22 – replace from with between done Line 23 – had the lowest concentration compared . . . done Line 24 – remove communities and replace with populations. add The before distribution done Line 25 – add the word the between at and surface. replace was with is. remove the word were. change ranging to ranged done Line 26 – remove the word the done Line 27 – remove the s from extremes done 5712: line 4 – between stations done Line 5 – remove group of the done C2032 Line 18 – remove the s from picoeukaryotes done Line 20 – replace is with was done Line 24 – replace are with were done Line 28 – replace under with below done 5713: line 2 – move (MLD) to the other side of layers done Line 3 – add the word the before MLD at the beginning of the sentence done Line 4 – add the word the before MLD done Line 12 – at a depth. . . done Line 14 - remove has been done Line 16 – remove on the first and replace with to done. add the word a after for done Line 17 – replace on with in done. add an n after show done Line 18 – replace both with the done Line 24 – replace biomasses were with biomass was done Line 25 – remove of done. replace spread with varied done Line 27 – was lower at . . .. done 5714: line 1 – decreased done Line 4 – replace is with was done Line 5 – comma after transect. replace at with to done Line 11 remove the done Line 12 – replace are with were done Line 13 – comma after however done Line 20 replace is with was done Line 27 – hydrographic done 5715: line 1 – replace is constituted with consists done Line 2 – replace side with section done Line 5 – add the after in done Line 20 – replace on

with to done Line 23 – move respectively to the end of the sentence done Line 24 – differentiated done 5716: line 2 – characterised done Line 15 – add the after of done Line 23 – remove the comma after 1994 and replace with a full stop done Line 24 – replace at with to done 5717: line 8 – comma after can. remove be and insert after part on the following line done Line 18 – add the before STF done Line 20 – comma after STCC. replace as for instance with including done Line 24 – add the before STCC done 5718: line 1 – add salinity unit after 0.05 Salinity is a dimensionless quantity Line 3 – comma after layer done Line 5 – remove drastic done Line 7 – replace is with was. twice done Line 9 – add being before lower done Line 13 – remove overall done Line 16 – replace revealed with observed done Line 23 – replace apparent with the done Line 24 – add an s at the end of condition done Line 27 – add the before surface done 5719:line 1 – change maxima to maximum and move before was done. remove the of after maxima done Line 3 – add such after organisms done. replace as well as with and done Lines 2-4 – sentence does not make sense we corrected Line 4 - remove the word a done Line 18 – remove overall done Line 27 – move from the surface down to 200m depth and place it before prokaryotes on the following line. add an n after show done 5720: lines 13-18 – 10nM phosphate is not limiting for Prochlorococcus or Synechococcus. Rethink what you want to say. Yes 10nM is not limiting for such kind of organisms. Hashihama et al.. (2009) suggested that the diazotrophs “exhausted” at the nanomolar lever the phosphate in the upper layer. In our study, we did not indicate that 10 nM is limiting, we just observed that the Synechococcus concentrations are higher in the North part of the transect. We suggested that the difference in abundances or biomass displays the various environmental conditions. Utilization of the nutrients as previously reported by Kitajima et al. (2009) could be one factor explaining this spatial distribution. 5721: line 4 – add the after at done Line 8 - remove also done Line 10 – phosphate is not exhausted. merely at a very low concentration. It suits the numerically dominant autotrophs, namely Prochlorococcus and Synechococcus who are capable of turning over the pool of phosphorus at high rates and using phosphorus sources other than phosphate e.g. Hartmann. M.. C. Grob. D.J. Scanlan. A.P. Martin. P.H. Burkill. and

M.V. Zubkov. 2011. Comparison of phosphate uptake rates by the smallest plastidic and aplastidic protists in the North Atlantic subtropical gyre. FEMS Microbiology Ecology 78(2). 327-335. We changed “exhausted” into low concentrations. In the previous version we used the word exhaustion because the phosphate concentrations were in the same order than the Hashihama et al.. (2009) paper. Line 20 - hydrographical done Line 21 – relationship done Line 26 – communities numerically dominated by. . . done Line 27 – replace has with have done 5722: line 2 – anticyclonic eddies lead to done Line 3 – remove sea done Line 28 – add the after at done 5723: line 14 – add the after at done Line 16 – sentence does not make sense we corrected Line 28 – remove the s at the end of despite done 5724: line 12 – add the after at. Similarly done Line 17 – replace is with was done. The references and figure legends have not been checked by the reviewer

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Interactive comment on Biogeosciences Discuss., 10, 5701, 2013.

Replicate	Prochlorococcus $x10^4$ cell.cm^{-3}	Synechococcus $x10^4$ cell.cm^{-3}	Picoeukaryotes $x10^3$ cell.cm^{-3}	Nanoeukaryotes $x10^2$ cell.cm^{-3}
1	5.67	2.33	7.10	3.81
2	5.34	2.00	8.05	3.04
3	4.99	2.19	7.22	2.63
4	5.02	2.19	7.71	3.72
5	5.42	2.15	7.39	4.64
6	4.60	2.05	7.18	2.92
7	4.62	2.13	7.16	3.22
Mean	5.09	2.15	7.40	3.42
Sd	0.40	0.11	0.35	0.68
% variation	7.92	4.92	4.76	19.88

Fig. 1. Variability in the samples collected from 7 Niskin bottles closed together at the same depth (5m) during the POMME Cruise (Latitude 44 59.98 N, Longitude: 017 59.98 W, September 2000)

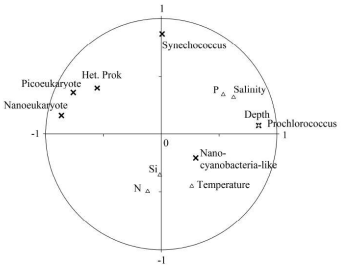

Fig. 2. Correlation plot of the CAP CcorA on the relationship between the environmental variables and ultraplankton composition of the profile data set (first axe (54.8%) and second axe (24.1%) of variation).

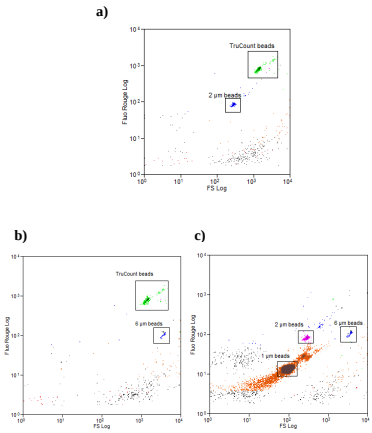

Fig. 3. Examples of cytograms used to discriminate the size and check the reliability of the FACScalibur.

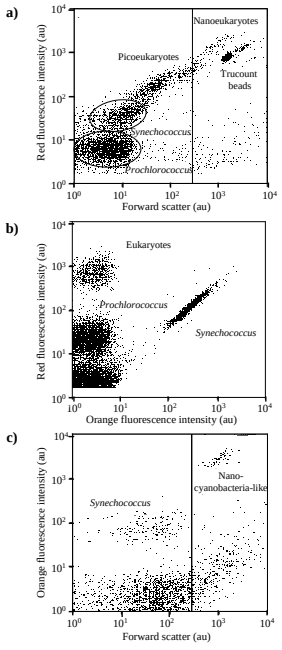

Fig. 4. Modification of the figure 3 (Cluster identification)

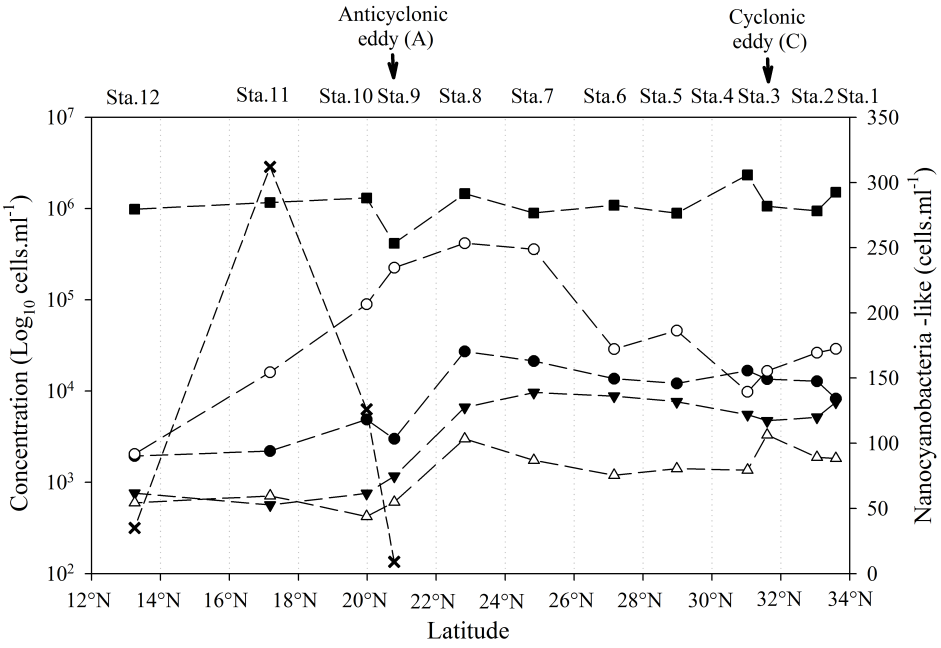

Fig. 5. Modification of the figure 8 (Organisms at surface)

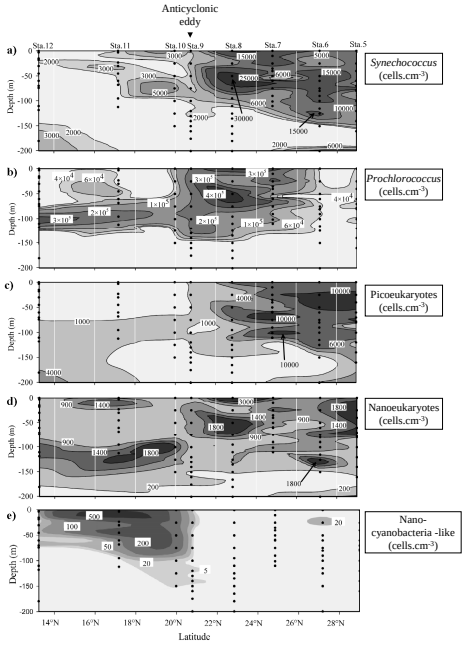

Fig. 6. Modification of the figure 9 (Profile organisms)

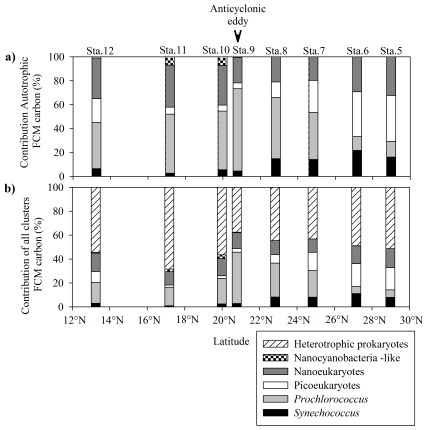

Fig. 7. Modification of the figure 12 (Carbon biomass)

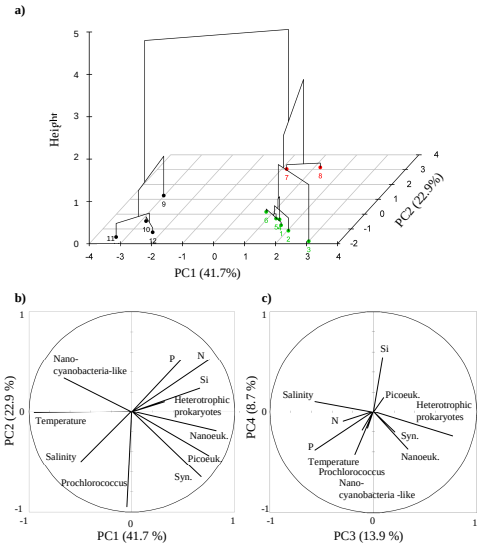

Fig. 8. Modification of the figure 13 (PCA surface)

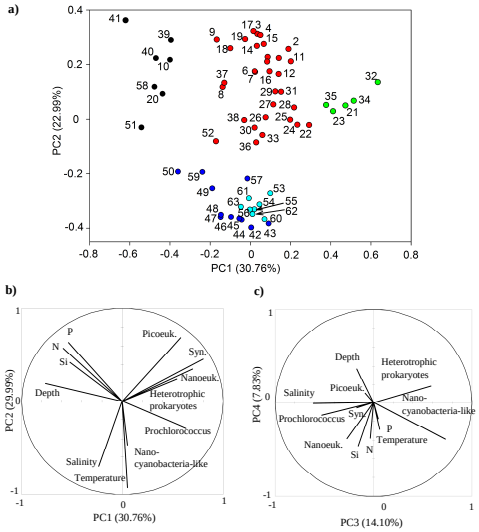

Fig. 9. Modification of the figure 14 (PCA profile)

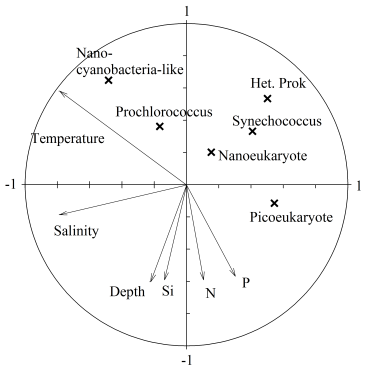

Fig. 10. Modification of the figure 15 (RDA profile)