

Interactive comment on “Picoplankton diversity in the South-East Pacific Ocean from cultures” by F. Le Gall et al.

F. Le Gall et al.

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We have prepared a revised version of the paper taking into account the comments by the referees (see below, referee comments appear in bold and our response is below).

Reply to Anonymous Referee #1

The paper is well organized and presents biological data from an area that is poorly studied because of its remoteness and insights into organisms that are difficult to culture and study by regular microscopy and culturing techniques. I think it should be accepted with minor changes.

The present manuscript is an extensive effort to obtain cultures and subsequently knowledge about oceanic picoplankton. It presents new data on the

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occurrence of cultivable picoplankton in the South-East Pacific Ocean. The culturing efforts of the authors involve no new methods, but an interesting mix of already established methods that proved successful to a certain degree. They were able to exclude very many of the bigger species (nano and particularly microplankton) and quite a great variety of cultures were established. I think this is quite an achievement.

I would like the authors to explain why they filter their samples through 3 and 0.6 μm filters, why did you choose 0.6 and 3 μm ?

Our aim was to isolate picoplankton sized cultures ($<2\text{--}3\ \mu\text{m}$). We chose 3 μm filters to remove larger species such as diatoms that would take over very quickly following nutrient addition and 0.6 μm filters to have more chance to obtain prokaryote cultures such as *Prochlorococcus* or *Synechococcus*. In fact, in our past experience, we have been quite successful at isolating small picoeukaryotes using very small filtration thresholds such as 0.6 μm (see for example Table 4 in Vault, D., Le Gall, F., Marie, D., Guillou, L., and Partensky, F.: The Roscoff Culture Collection (RCC): a collection dedicated to marine picoplankton. *Nova Hedwigia*, 79, 49-70, 2004). This is probably due to the fact that a few 2 μm cells can squeeze through 0.6 μm holes.

I would like to oppose to the stated fact that picoplankton were discovered 30 years ago. At that time their existence had been known for quite a while. I think that their importance and wide distribution was being gradually recognized to a much larger extent from that time (30 years ago).

We agree with the referee and have changed the text accordingly.

At the end of the introduction, p. 2702 l. 27, p. 2703 l. 1-3; it is stated that the cultures established encompass five major phylogenetic divisions, which is true. For the alveolates however, the two species brought into culture were ca 15 μm , which is much larger than picoplankton. I think it should be mentioned already at this time that for the alveolates no picoplankton cultures were established.

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We have added a sentence at the end of the introduction to state that not all the recovered cultures were picoplanktonic in size, in particular for alveolates.

Page 2703 I. 19 remove full stop after photons

It was removed in the revised version.

Page 2706 I 1; 1% glutaraldehyde. Is this final concentration?

1% glutaraldehyde is the final concentration. It was added in the revised version.

On page 2709 in the first paragraph I would like you to add that you isolated many picosized stramenopiles, chlorophyte and haptophyte cultures, but no pico-sized dinoflagellates.

This was added in the in the revised version.

Page 2710, the sentence on I 19-21 does not make sense to me.

The sentence has hopefully been clarified as :

“These strains are phylogenetically related to the recently established genus Picochlorum (Fig. 4) that now regroupes salt-tolerant species previously classified within the genus Nanochlorum (Henley et al., 2004).”

Page 2711 I 20-21, I would use heterokont flagella since one is hairy and the other is smooth.

It was changed in the revised version.

Table 1

I think the legend is incomplete. The term sort should be explained.

The legend was updated in the revised version.

Table 2

The columns CTD and names of the pre-cultures does not, in my opinion add much info to the reader and may be omitted. Do the sized measure length and or diameter? This should be mentioned in the legend.

Former Tables 2 and 3 have been moved to the Supplementary material (now named Tables S1 and S2 respectively). We think that the information available in these tables is important for future cross reference, in particular in the light of other papers published in the BG special issue devoted to the BIOSOPE cruise. However these tables are too bulky to be part of the paper itself and this is why we have moved them to the Supplementary material.

The size corresponds to the cell diameter. It was added in the legend of Tables S1 and S2.

Table 3

For *Minutocellus* I think spherical would be more appropriate than rectangular since it is a centric diatom.

This was changed in Table S2.

Is not *Prorocentrum dentatum* flagellated?

Prorocentrum dentatum is flagellated. It was changed in the Table S2.

I think you should use cf *Prasinoderma* instead of *Prasinoderma*?

This was changed in the Tables S1 and S2.

Also you should consider the use of the terms coccoid and round. Do you mean round cells that have a cell wall when you say coccoid? Is round the same as spherical? May be spherical is a better term?

We replaced everywhere coccoid and round by spherical which is indeed more appropriate.

Figure 3

For the 15 first pictures (counted from the top and from left to right) the legends correspond with the pictures. This is also true for the last two pictures. Something has happened to the Pelagophyceae and Bolidophyceae. RCC 852 and 853 are on the plate. 879, 871 and 852 are mentioned in the legends.

We do not understand what the referee means exactly. The legend and the plate content match exactly. The pictures have been arranged in order to fit the available space.

Pelagophyceae: (RCC 879, and RCC 871). Bolidophyceae (RCC 852). Heterokontophyta: unknown species (RCC 853).

Is the picture labelled 852 a *Bolidomonas*? To me it looks more like a *Pelagomonas*.

The picture labelled RCC 852 is indeed a *Bolidomonas*. We confirmed its morphology by electron microscopy (presence of two flagella, one hairy and the other smooth).

The picture of RCC 1025 does not show the characteristics of *Chaetoceros* cells.

We replaced the picture with new picture showing the characteristics features of the genus *Chaetoceros*.

English is not my native tongue and I will restrain from making improvements to the spelling and grammar of the manuscript.

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Reply to Anonymous Referee #2

This descriptive paper reports on a nice and timely effort to “culture the unculturable”, here the marine photosynthetic eukaryotic picoplankton (PEP). Marine eukaryotic pikoplankton is special in that it is a very heterologous group in which

in the last decades novel classes and even a novel division was detected. As with other groups, these findings partially stem from environmental 18S sequencing and the relevant cultures are missing. PEP is more important in oligotrophic waters, so the focus of this study on oligotrophic ocean areas was a wise one. The paper adds new insights into pikoplankton species abundance and occurrence.

The major aim of this study, the culturing of novel pikoplanktonic groups mostly failed, with one possible exception which unfortunately later died. Was this the fault of the authors? Probably not. They started a large number (1900) of precultures of which roughly 10% yielded clean cultures. This number is in good agreement with other microbiological assemblages for which estimates exist of 90-99% to be unculturable.

Perhaps it would have been better, to use more than just one medium (K) to culture novel PEP, especially from the very oligotrophic areas, but this is only hypothetical.

Indeed this is a very good point. In a subsequent cruise this summer to the North Sea, we have tried other media and indeed success rate seems to depend on the medium used. During the BIOSOPE cruise we made the choice to concentrate on the use of flow cytometry and therefore did not focus on changing the medium since in our hands K is the medium for which we had the best results previously to obtain novel species.

The analysis of pure cultures by LM, EM and partial 18S sequencing seems ok to me. I only wonder why not all cultured were sequenced, given that 200 sequencing reactions no longer cost a fortune and the Roscoff Station has modern sequencing facilities. I assume not all could be amplified? Why?

The problem was not really the sequencing cost but rather the time needed to grow the cultures, to extract the DNA, to perform the PCR and sequencing reactions and then to analyze the sequences. As we tried to meet the deadline of the BG species issue on the BIOSOPE cruise, we decided to select a representative subset of the cultures

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to be able to have the sequences in time to write up the paper.

I also wonder whether it was always easy, to group the unknown sequences safely into an ARB tree if only partial (500 bp?) of 18S sequence were available. In our hands this sometimes is difficult e.g. because of poor bootstrap support.

As detailed in the method sections, both ARB and BLAST have been used jointly to assign sequences to existing phylogenetic groups. Moreover, sequences were grouped together using Fast Group II. Finally a more complete phylogenetic analysis of representative was performed with MEGA. We did not encounter any difficulty to assign any of the sequences obtained in this work.

Minor things: I could not find the names of the oligos Euk328f and Euk329r in the Moon et al 2000 paper.

Indeed, in the Moon et al 2000 paper, these oligos are not named. A later paper (Romari et al. 2004) provides names and sequences for these oligos. The reference was changed in the revised version of our paper.

Were these really the oligos to amplify the total 18S rDNA?

Yes, these oligos amplify the total 18S rRNA gene.

I could not read table 2 and 3 and figure 4, unless I use a magnification lens.

We have moved Tables 2 and 3 to the supplementary material (where they should appear in landscape orientation and therefore be easier to read). Although these tables are hard to read, they provide a lot of information that hopefully should be useful to other researchers. Fig. 4 is phylogenetic tree and as usual with this kind of figure, it needs a full page display to be read more easily. Hopefully, in the final printed version it should appear on a full page and not half a page as in the BG Discussion.

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Reply to Anonymous Referee #3

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This paper presents a huge culturing effort for marine picoeukaryotes. These small protists are ecologically relevant in marine systems, but they are only partly represented in culture collections. Molecular environmental analyses of this assemblage have shown the discrepancy for some groups (alveolates, stramenopiles, picobiliphytes) between in situ populations and culture collections, whereas other groups, like prasinophytes, appear to be well represented by cultures. So, culturing efforts such as the one presented here are required for a better characterization of marine picoeukaryotes. Another remarkable aspect of this study is that cultures were initiated with seawater collected during an oceanographic cruise, including coastal upwelled waters and open sea ultra-oligotrophic waters. After screening 1900 precultures, the authors isolated 212 strains, which were all characterized morphologically and about half of them by sequencing the 18S rDNA. Most of these strains were very small (2-3 μm), so true picoeukaryotes. This represents an interesting contribution of biological material, and strains have been deposited in culture collections and are available for further studies. It will help to mention in the introduction if similar culturing efforts have been done before (when, where, and how many strains). However, as mentioned by the authors, the study failed in the two most interesting and novel aspects, the isolation of picoeukaryotes only known from molecular surveys and an extensive strain isolation from the ultraoligotrophic waters. Sadly, the two more promising strains by their novelty were lost, probably due to the difficulty to keep them into culture. So, this study also points to the necessity to apply novel culturing strategies. Only a few 18S rDNA sequences are shown in Fig. 4, implying that those represent the whole collection of strains isolated. However, it would be interesting to know which is the variability seen (at the 18S rDNA sequenced) by the repeated cultures. Was it always 100%? For instance, only 2 rDNA sequences are shown for the 38 *Pelagomonas* strains. Are the other 36 identical to one or the other?

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Indeed we grouped sequences using FastGroup and selected one or two for each group, so that the phylogenetic tree (Fig. 4) would not be too crowded. We have added some more data on the *Pelagomonas* which offer in fact an interesting case. . . Out of the 37 sequences available, 34 display 100% similarity to each other while 3 have very low level of divergence ($> 99\%$ similarity) which could be due to sequencing errors.

Specific comments

Page 2701, line 5-7. Picoplankton was first proposed in 1978 to include essentially bacteria. A few years later it was seen that also included protists. Rephrase, since it seems that picoplankton was defined in base of the recently discovered small protists.

We have tried to rephrase the first sentences of the introduction in the revised version to make it clearer that picoplankton refers to a cell size range.

Page 2703, line 1-3. “Five phylogenetic divisions” but six groups are listed.

This has been changed in the revised version.

Page 2704, line2. A better reference for the rice-based media is A.J. Cowling 1991. Free-living heterotrophic flagellates: methods of isolation and maintenance, including sources of strains in culture. In: D.J. Patterson and J. Larsen, eds., *The Biology of Free-Living Heterotrophic Flagellates*, Clarendon Press, Oxford, pp. 477-492

This reference has been changed in the revised version.

Page 2704, line 18-19. Change “to target the rarer cells” to “in order that rarer cells form visible populations in the cytogram”

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This has been changed in the revised version.

Page 2705, line 11. Transporting photosynthetic cultures growing at 20_C in an ice box (dark and around 0C) is probably not ideal. There is a better way to do it? Were many cultures lost during the transport?

We rephrased this part. In fact the cultures were transported in the dark at ambient temperature in isothermal boxes (called “ice box”). Cultures were exposed to moderate light when possible during the transit and then rushed to the Roscoff culture room on arrival. This is now made explicit in the revised text.

It is difficult to assess the number of culture lost during transport because it was not possible to check all cultures right upon arrival. Moreover some cultures may have survived the transport but not the new culture conditions in Roscoff.

Page 2707-2708. I don't see why cultures during the initial growth phase are more stable. Is this something general?

We must admit that is more of an intuition than based on real facts. The incriminated sentence has been removed.

Page 2710, lines 23-26. Rephrase: there are 16 Phaeocystis strains, 3 from upwelling regions and 5 for the east of the gyre. What about the remaining 8?

The corresponding sentences have been clarified and all 16 strains are now mentioned.

Page 2711, line 6-8. I don't understand the reasoning, because I believe that the same sample could include 2 (and many more) different taxonomical different Pelagomonas

Indeed you are right. The sentence has been modified to give the two possible hypotheses (different life stages or different species). However the fact that two strains isolated from the same bottle, one flagellated and the other not, share 100% 18S similarity over

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697 base pairs (this detail has been now added in the text) pleads for the former hypothesis, although this would have to be confirmed by sequencing other genes (or the full genome money permitting !).

Table 1. Change “min” and “max” by “surface” and “DCM” respectively

This has not been changed because although the minimum sampled depth often corresponds to the surface layer, the maximum depth does not always correspond to the DCM (deep chlorophyll maximum), eg in the upwelling region.

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