

# ***Interactive comment on “Diversity of intact polar lipids in the oxygen minimum zone of the Eastern Tropical North Pacific: Biogeochemical implications of non-phosphorus lipids” by Florence Schubotz et al.***

## **Anonymous Referee #3**

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This paper provides a very detailed account of the IPL composition in the waters of the oxygen-deficient zone (ODZ) of the Eastern Tropical North Pacific. It adds to the growing inventory of IPL data. The authors claim that it contributes to our understanding of these systems. I am not entirely convinced. There are also a substantial number of issues related to the analytical methodology that need to be resolved before this paper can be published.

The general aim of the paper is “to evaluate the microbial ecology and ecophysiological adaptations that affect organisms inhabiting the OMZ in the context of biogeochemical

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cycles” (line 30-32). However, when you read the remaining part of the abstract this does not materializes. It ends with a rather vague statement about potential phosphorous limitation, which in the light of the analytical constraints (see below) may be even weaker. I strongly suggest to remove these kinds of claims from the text and just focus on what the paper is about: an inventory of IPLs in the water column of this region.

The authors do report absolute IPL concentrations (Fig. 3b) which show an order of magnitude decrease over the first 100 m of the water column. This is in line what would be expected since this is the zone where primary production is taking place (as is also revealed by the pigment concentrations) and the data would allow to discriminate IPLs produced by phytoplankton in the photic zone from IPLs (produced in much lower concentration) by prokaryotes residing below the photic zone and within the ODZ. However, the focus in the paper is too much on relative abundances of IPLs (e.g. Fig. 5) for unknown reasons. The discussion should be more focused on the zone where IPLs are primarily produced (i.e. the photic zone) vs. the remainder of the water column that may be influenced by IPLs in descending particles (i.e. produced in the upper water column) and additional production by prokaryotes. In this discussion, it should be taken into account that PUFA-containing IPLs may degrade faster than other IPLs. Now, the total inventory of IPLs is too much discussed in terms of relative abundance in relationship with nutrient profiles and other environmental parameters over the whole water column, which is too simplistic.

The paper also suffers from too much detail. It is very hard to follow because every tiny IPL detected is described without a clear environmental implication. The authors should formulate specific research questions (i.e. not understanding the “functioning of OMZs throughout the world ocean” by studying IPLs) and address these. Not every minor IPL detected has to be described!

There are also a number of issue related to the analytical methodology of analyzing the IPLs. Adequate answers should be provided on all issues since this may seriously affect the interpretation of the data.

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1) Filtration. The authors used 0.7 micrometer glass fiber filters for filtration. The limitations of the use of this filter size has extensively been discussed elsewhere and the authors acknowledge the limitations of their approach by admitting that they might be missing smaller cells. However, they should also mention that the pore size will decrease during the filtration process and thus will recover a (variable) part of this material. More importantly, they should stress that this does not only lead to “minimal values” for IPL concentrations but that it may also affect the distribution of IPLs that they report (as percentages). Furthermore, they used a prefiltration device to “remove most eukaryotes” (line 169). It is hard to believe that this will remove most of the algae; if so this would also strongly affect their interpretations.

2) Extraction. The IPLs have been Soxhlet extracted (i.e. boiling DCM/methanol for 8 h). Although this is a common method for extraction of less labile lipids, for IPLs it is rather unusual since these are very labile and the commonly applied method for this type of work is Bligh Dyer extraction at room temperature and controlled pH conditions. The authors should present data to show that their extraction method does not alter the IPL distribution (i.e. their main target of study) due to the fact that some IPLs are more labile than others (e.g. in the ratio of phospho vs. non-phospho IPLs, which plays an important role in the discussion).

3) Analysis. The experimental description indicates that the IPLs have initially been analyzed by HPLC-ESI-IT-MS<sub>n</sub> using the same system as described by Schubotz et al. (2009). In the meanwhile, this group has developed improved methods of analysis of IPLs (e.g. Wormer et al., 2013) and the question arises why these “old” results are still used since the samples were also re-analyzed using these new methods. It does not become clear how IPLs can be quantified with one method and identified by another method (lines 206-207) using an entirely different separation system. One issue that should also be addressed is the timeline of all these analyses. Once extracted, IPLs cannot be stored for a substantial time without significant alteration/degradation. Schubotz et al. (2009) in their very much related work of IPLs in the Black Sea stated

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“Three years after primary qualitative analysis the samples were spiked with C16-PAF (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine) as injection standard and re-run for quantification. Slight changes in the relative distribution of IPLs were observed within the two runs. In particular the differences were identified as a selective loss of the glycolipids Gly-DAG, Gly-Cer, Gly-GDGT and 2Gly-GDGT (data not shown). We interpret this loss as a sign of selective degradation of glycolipids during storage.” So, an important question is how much time evolved between these two analyses and can the results still be compared?

4) Quantitation. It does not become clear from the experimental description if the commercially available standards were run with the HPLC-ESI-IT-MSn system that was used for quantitation. If so, the results (response factors) should be reported. If not, there is a serious problem since response factors should be determined on the same system. The whole procedure of quantitation should be made clear. An “injection standard” is mentioned but it remains unclear what was done exactly. Why didn’t the authors use an internal standard that was added to the extract? Such a standard would at least have been exposed to the same conditions as the IPLs of the samples (e.g. during storage). The authors should also report the analytical errors of their determinations. The data they now report (e.g. Table S2) are extremely accurate (e.g. a relative abundance of 16.68 % SQ-DAG in station 1 at 35 m). I would expect that the analytical error is 10-20%, so the reported data are far too accurate unless the analytical error is extremely low. This also holds for many of the other data: the reported accuracy of absolute pigment concentrations is also (far) too high and so is the data reported in Table S3 (if the SD is larger than the figure itself is odd to report three or four significant numbers).

Additional comments:

Line 40: It is useless to compare relative trends in IPLs with absolute trends of environmental parameters. To this end, absolute concentrations (like you have for pigments) need to be used.

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Lines 68-69: . . . .but not provided in this paper, so remove this sentence.

Lines 117-119: strange sentence

Lines 119-124: I think this overview should be limited to papers describing intact IPLs in the water column. For example, the data presented by Lincoln et al., 2014 are not really solid IPL data. Turich and Freeman, 2001 and Hurley et al., 2016 present only core lipid data.

Line 125: It is not discussed how IPLs can be used as taxonomic indicators. This seems pretty relevant information for this type of study

Line 139: in what way is this approach “complementary”?

Line 146-147: here the authors promise that we should learn a lot (deeper insight into biogeochemical cycling, functioning of OMZs throughout the world ocean) but this is grossly overstated.

Lines 152-152: data on coordinates of sampling stations is incomplete.

Line 166: provide details on sampling volume

Line 186: provide details on pore size of filter

Line 200: storage at -20 degrees C is not sufficient to prevent alteration; IPL extracts should be stored at -80 degrees C and even then, distributions may change. How long were the extracts stored before analysis for IPLs?

Line 210: different column than in other analysis. Why?

Lines 217-219: provide more details on these standards. What are the acyl moieties of these standards? How are the response factors affected by unsaturations in the acyl moieties? What was the time between the arrival of these standards in the lab and their measurement? How were they stored? It is known that these standards degrade over time upon storage. How often were these standards run? Before each batch of

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analyses? How did the response factors vary over time? Answers to these questions are essential for getting a feel for the confidence we can have in the reported relative abundances of the IPLs.

Line 224-224: why would the unknown response factors be in the range of the measured standards? This is not a scientifically acceptable statement in this way. Just say that they are unknown and what you have assumed to be able to calculate a concentration.

Lines 258-262: provide references to indicate that pigments can be used to reveal this information even at the species level (e.g. Rhizosolenia).

Line 267: secondary maxima are not revealed in Fig. 1e.

Lines 268-269: Not evident from Figs 2a-b.

Lines 273-276: So, the whole system is NOT P-limited!

Line 282: How were absolute concentrations obtained?

Line 284: Secondary maxima are not observable in Fig. 3d.

Line 289-291: It would be logical to introduce first all IPL classes observed. Why are absolute concentrations of IPL classes not described and is the manuscript concentrated on relative abundances?

Line 292: “select substitute lipid ratios” is not introduced. It should be introduced in the discussion not in the result since it is an interpretation of the data presented.

Line 293: “total IPLs”? Does this now include archaeal IPLs or not (see line 291)?

Lines 303-317: This section should be moved to the discussion. See also earlier comments on the distinction between the photic zone and remainder of the water column.

Line 319 and Fig. 4: The whole distinction between major and minor IPLs is rather artificial. It becomes especially confusing when minor IPLs are normalized on their

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sum which is a variable part of the IPLs as a whole. It is entirely unclear why this is done other than for “stamp collection” purposes.

Line 332: it would be much more helpful to report absolute concentrations. In that way it can be directly compared with the abundances of archaeal cells. Now, it is normalized to something where it is not at all related to and which varies by more than an order of magnitude (Fig. 3b).

Line 352; formally this statement is incorrect: the chain length was not measured but the number of carbon atoms in the acyl chains. One cannot discriminate between branched and straight chains. The number of double bonds was also not determined since one cannot discriminate between a double bond and a ring. Needs adjustment. Fig. 6 does not really show a lot of useful information since the variation observed is not extensive. I would consider to drop this figure.

Line 366-367: this definition and the one at line 320-321 does not exclude that some groups are both minor and major lipids. Anyhow, this distinction is extremely confusing as mentioned before.

Lines 365-406: only describe IPLs that are useful in the discussion.

Line 383: One cannot make the distinction between an OH group and an epoxy group with the methods applied. Can the authors exclude that these components are formed upon storage of the extract?

Line 408: the authors should make clear why it would be useful to perform statistical correlations between environmental variables and relative abundance of IPLs. This remains entirely unclear. I suggest to skip this entire section.

Line 433: The discussion is extremely lengthy (19 pages of text) and should be focused on the important observations taking into account all the comments made so far. It should be cut by 50% or so. It is, therefore, not useful to provide detailed comments and I will restrict them to more general comments.

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Lines 434-487: General overview which is not connected to IPL dataset at all. Requires substantial shortening.

Lines 490-492: This statement needs to be proven by showing some kind of correlation.

Lines 492-497: First time export of IPLs is mentioned; this should be introduced in the introduction since it is important for the reader to understand that IPLs at depth comprise a mixture of exported and newly produced IPLs.

Lines 497-499: Bold statement that is not (yet?) backed up by any data. Does not belong here.

Lines 502-505: Repetitious.

Lines 508-592: Very lengthy discussion assessing nothing really new: the IPLs in the photic zone derive from algae, cyanobacteria, and heterotrophic bacteria. This was to be expected and has been shown previously.

Lines 595-641: What I miss here is an answer to the intriguing question: how much of the IPLs detected in this zone can be derived from settling from the photic zone.

Lines 631-641: Again, nothing new here. Have the authors evidence for the presence of specific GDGTs derived from Thaumarchaeota (i.e. crenarchaeol)? It would be fair to refer to the original literature for the detection of HPH GDGTs in Thaumarchaeota.

Line 648-653: It is highly unrealistic to suppose that PUFA-containing IPLs would be produced in-situ in the ODZ. It seems the authors agree but the text is really confusing.

Line 655: “due to the increase in bacterial abundance”? I guess bacterial abundance is still highest in the photic zone. The authors seem to forget that they are looking at relative abundances but when they would calculate absolute abundance a completely different picture emerges.

Lines 659: these genes are much more widespread in the bacterial kingdom.

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Line 662: “chain” is incorrect

Line 674: the comparison of the IPL dataset with the FISH dataset is underdeveloped in this manuscript.

Line 693: for PUFAs I would make a clear distinction between C20 and C22 PUFAs and the C18 ones, otherwise the text will become confusing.

Lines 702-703: This strongly depends on the core of the GDGT IPLs detected. Crenarchaeol has only been detected so far in cultures of Thaumarchaeota. Suggestions that it may derive from euryarchaeota or crenarchaeota are only based on very indirect evidence and quoting these references (and not many other literature) in this context is only confusing the issue. In fact, one way to shorten this paper is to take out all the data related to isoprenoidal lipids. Part of this data has been published before (Xie et al., 2014) and the data reported here do not provide any new insight.

Lines 712-825: Extremely confusing title. We just had a very extensive description of how species composition could influence IPL distribution. This section seems to start all over again (lots of repetition). The statistical data treatment is doubtful as explained before. With all the major changes in environmental parameters (light level, oxygen concentration) and its consequences for species composition it is impossible to link changes in nutrient concentrations to differences in IPL distribution. Such studies should be done first with microbial cultures and then, perhaps, these data can be used to interpret environmental datasets like this one.

Figures 1: Explain how (software; kriging method) plots b-e were produced. The figure suggests (but the caption does not explain) that only at stations 1, 2, 5 and 8 full CTD casts have been obtained. Stations 1-8 are almost 3000 km apart. Is it statistically significant to perform interpolation between these stations for the deep (>200 m) waters? One can observe all kind of changes (as shown by colour changes) that are hard to understand from having only 4 data points over 3000 km. Specify the depth scale; it does not seem to be linear but it is not specified in the caption. Most of these

comments also apply to Figs. 2,3, and 5.

Table S3: The authors cannot report the number of double bonds; they determined the DBE number but they cannot discriminate between a ring or a double bond as far as I can tell. The table should be carefully checked; there are instances where the FA combination says 18:4/18:4 but the number of bonds is nine. It is also not clear why sometimes they report the FA combination and sometimes not even for the same type of IPL. They should also specify where the relative abundance is normalized upon. It would be more convenient for the reader when these values are followed by a plus/minus sign and then the SD.

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