Referee #1

This study aims to determine the intact lipids in suspended particles in the water column using samples collected from ocean oxygen minimum zones from the east/north pacific. OMZs are important marine ecosystems particularly with regard to oceanic N cycles. The comprehensive data presented in this study has significantly advanced our understanding of IPLs in this unique environment. This reviewer has no major concerns. Some specific comments are listed below for further improvement of the manuscript.

We thank Referee #1 for the positive comments.

L93, it would be very useful to specifically discuss previous IPL studies on OMZ samples, e.g ETNP, ETSP, Arabian Sea etc in discussions.

Most of the previous IPL studies performed in other OMZ regions have either focused on very specific IPLs for specific processes (e.g., ladderane lipids and HPH-crenarchaeol for ammonium oxidation in the Arabian Sea) or only discussed surface water IPL distribution of phototrophic organisms (in the ETSP). We therefore consider this study to be the first comprehensive study of IPLs in OMZs. Nevertheless, we made sure to refer to previous IPL studies in the discussion where appropriate, e.g. lines 564-565, lines 667-668.

L101 and throughout the MS, most previous studies have used MGDG, DGDG and SQDG to refer to mono- and di glycosyl- DAG and sulfoquinovosyl DAG, please change to these commonly used acronyms for the sake of consistency in literature.

We are aware that we use different abbreviations for these glycolipids than are often used in the literature. However, since we want to be consistent with our nomenclature, i.e. we are also calling mono- and diglycosyl GDGT 1G-GDGT and 2G-GDGT, we opted to stick to the currently used acronyms 1G-DAG and 2G-DAG. This nomenclature is also relevant when describing head groups with different core lipid structures, i.e. SQ-DAG and SQ-AEG.

We would also like to point out that there is plenty of literature in glycolipid research, particularly bacterial glycolipid research, where besides MGDG and DGDG (which are typically the acronyms for the specific thylakoid lipids monogalactosyl and digalactosyl diacylglycerol), the other sugars are referenced as Glc-DG, GlcGal-DG, depending on sugar type (Glc for glucose, Gal for galactose, etc.). We are therefore not that unique with our chosen nomenclature, which specifically highlights that we do not know the types of sugars (which surely change with the source organisms).

Since we are defining the used acronyms in the text (we also now added a footnote for clarification, page 6), we do not think this to be very problematic.

L104-5 , l307-9; this may be a little misleading since DGTS has been found in a wide range of marine heterotrophic bacteria.

We noted here that DGTS has also been detected in marine heterotrophic bacteria in phosphate-limited environments (line 105-106).

L116, please refer to recent study of Hunter et al., AEM doi: 10.1128/AEM.02034-17 for novel diglycosylceramides found in Thalassiosira.

Done.

L214-215, the authors have referred to previous studies for mass spectral interpretation and IPL assignments. It would be very useful to summarize and synthesise these information in a table (or in the supplementary information) and to detail the criterial for IPL identification. Presumably IPL assignment is based on comparing to retention time of standards (where applicable) and characteristic MS/MS patterns, representative characteristic ions or characteristic neutral loss. How has DGTS but not DGTA been conclusively assigned in this study? Has DGTA been found in any samples?

There are on average between 600 and 800 compounds that are identified and quantified in each sample. Since IPL identification is quite complex, it will be difficult to provide all the necessary information in a comprehensive table that will explain each lipid identification. In spite of this, upon the reviewers' request, we included a table in the Suppl. Material (Suppl. Table 3) that provides examples how mass spectral assignment of lipids was conducted. Furthermore, we clarified in the text how we identified DGTS over DGTA (lines 220-223).

L223, for unknown aminolipids AL1 AL2, do the authors have any hypothesis of their structures based on MSn fragmentation patterns (suppl fig 4)? What are the possible amino head group structures? Have accurate ms of AL1, AL2 been obtained?

Unfortunately, we have not more insights or hypotheses on the headgroup structures of AL-I and AL-II other than the ones provided in the text and figure caption. We have accurate masses of AL-I and AL-II fragments up to the third decimal point, which is why we provide sum formulas for the potential headgroup fragments (see Suppl. Fig. 4), but unfortunately we cannot provide any further insights on their structures.

The authors mentioned CSRD FISH data in supplementary dataset but did not mention how this was done in the materials and methods.

This data is from Podlaska et al. (2012), the methods for the CARD-FISH analyses are also described in this paper. We made a reference to this paper in the respective figure caption.

My general impression for discussion is that it can be shortened significantly.

We revised and shortened the discussion substantially from the originally 19 (Word) pages to 13 pages.

It is a pity that no microbial diversity data were obtained in this study as one would like to see the correlation between specific microbial groups and IPLs, which may provide clues for the origin of these lipids, particularly w.r.t. to AL1 and AL2.

We agree that having microbial diversity data would have greatly improved this manuscript and we will ensure that such data will be available for future IPL studies. We did, however, try to correlate the IPL data with the available CARD-FISH data, but unfortunately did not see significant correlations (see section 4.2.2).

Section 4.1.1 two recent papers (Carini et al, Sebastian et al) have shown marine heterotrophic bacteria are also abundant in MGDG. These need to be discussed here in line with these new evidence.

We now added a sentence stating the potential for heterotrophic bacteria to be sources for these glycolipids (line 482) and then refer to the acyl side chains to further delineate if bacteria are indeed potential sources or not.

Referee N.J. Bale

General comments

This study examines the intact polar lipid (IPL) distribution in suspended particulate matter (SPM) from four stations in the oxygen minimum zone (OMZ) of the Eastern Tropical North Pacific (ETNP). It aims to link the IPL distribution of different water column zones with the microorganisms found there and to examine the ecophysical adaptions to the different zones of the OMZ. This is an extensive data IPL set and authors have theorized which groups of microorganisms are responsible for which IPLs. The strength, as I see it, in this study is the examination of how the IPL distribution changes across the different zones of the water column due to the changes in the bio- geochemical environment. Indeed, due to the generic nature of many of these IPLs it is only possible to put forward tentative assignments of their sources, whereas examining changes in the lipid composition with changing environmental parameters provides more solid information. Overall, I recommend this article for publication with the following edits and with suitable responses to my questions on the analytical methods.

We thank Dr. Bale for these constructive comments.

Specific comments

I have two specific comments.

My first specific comment relates to the extraction and analysis of these samples. The manuscript states that the samples were collected in 2007 and (presumably soon after) that they were extracted using Soxhlet-extraction with DCM:MeOH for 8 hours. From an IPL perspective this seems a "harsh" extraction method that has potential to destroy certain IPLs, resulting in a IPL distribution unrepresentative of that in nature. Could the authors comment on whether this would be their preferred method of extraction for IPLs or whether IPL analysis was not the original reason for the chosen extraction method? Indeed the first author has described utilizing the much gentler modified 'Bligh and Dyer' extraction method in other publications relating to IPLs. The authors also describe two different analytical methods used to quantify and to identify structures. Can you state whether analysis occurred soon after extraction in both cases? If not, how were the extracts stored and for how long? If the two analyses were carried out at different times, did extract storage introduce changes in the lipid distribution? I noted that the reference for the second LC-MS method applied (Wörmer et al., 2013), was published 6 years after the samples were collected. Based on the authors' replies, suitable discussion of these issues should be included in the method section.

We have not performed a direct comparison of the Soxhlet extraction technique with the more common B&D ultrasonication technique using the same sample material, therefore we cannot comment on this with exclusive certainty. Nevertheless, we are not very worried about losing significant proportions of IPLs during Soxhlet extraction due to the following observations: (1) tests using microwave extraction showed optimal IPL yields at 70°C, indicating that IPLs may be more thermally stable than thought, (2) we detect presumably more labile compounds such as HPH-GDGTs in similar abundance using Soxhlet extraction as have been reported from other OMZ zones, indicating that these (presumably) more labile IPLs are not preferentially destroyed during Soxhlet extraction.

With respect to analyzing the samples at different time points: the first analysis using the quantitative data on the LC-ion trap-MS were performed in 2010 and 2011, while the QTOF samples were analyzed 4 years later in 2015. During this time the samples were stored in dry condition at -20°C. Again, we cannot state with absolute certainty that the IPL distribution has not been affected over time, however, based on the following lines of evidence, which were accumulated over more than a

decade worth of experience in IPL analysis we are again not too worried about this: (1) we typically analyze IPL standards every two months (together with our samples) that we store at -20°C over several years. So far these IPL standards have showed no selective degradation of compounds over time, thus indicating that relative IPL abundances will not be affected by storage at -20°C, (2) re-analysis of total lipid extracts that contained an internal standard within a 1.5-year timeframe gave similar absolute concentrations of different IPL species with different headgroups (this is unpublished data). This again indicates that there very likely are no significant selective changes in IPL abundance occurring over year-long storage of IPLs, at least none that would significantly affect the already existing uncertainties in IPL quantification.

Since also reviewer #3 expressed similar concerns, we added some explanatory sentences in the methods section with respect to these issues (see section 2.3).

My second specific comment relates to the length and depth of the discussion. This is a subject I find very interesting and yet I felt rather weighed down in information at certain points. I feel it would aid the reader if this was shortened and made more succinct.

We revised and shortened the discussion substantially from the originally 19 (Word) pages to 13 pages.

Technical corrections

Line 52 - change to "the subsequent"

Done.

Line 70 - change to "(ENTP), situated off the. . ."

Done.

Line 93 - change to "(IPL) are the main building blocks of cellular membrane and may"

Done.

Line 98 - change to "the North Sea"

Done.

Line 100 - you could also include in your reference list here the Western English Channel (White et al., 2015).

"The combined effects of seasonal community succession and adaptive algal physiology on lipid profiles of coastal phytoplankton in the Western English Channel. D.A.White , C.E.Widdicombe, P.J. Somerfield, R.L. Airs, G.A. Tarran, J.L. Maud, A. Atkinson. Marine Chemistry 177 (2015) 638–652)."

Done.

Line 129 - Remove 'here' to read "Notably, replacing. . ."

Done.

Line 141 - change to "extension of that of Xie"

Done.

Line 142 - change to "at two stations described here (station 1 and 8)"

Done.

Line 159 - Should this be "VERTEX I and II" ?

Yes, this has been corrected.

Line 160 The Martin et al. (1987) reference is missing from your reference list.

Is now added.

Line 177 - should define GFF at first use.

Done.

Line 223 - insert “response could not be corrected for”

Done.

Line 350 - The term amino lipid and betaine lipid seem to be used interchangeably throughout the manuscript. Could this be defined at one point?

This should not be the case, because aminolipids refer to the sum of betaine lipids, ornithine lipids and the unknown ALI and ALII (see also legend in Fig. 4) and betaine lipids are just betaine lipids, i.e. in our case DGTS and OH-DGTS. Since ornithine lipids and the unknown AL are often negligibly low in abundance the bulk of aminolipids are indeed dominated by betaine lipids. We checked the manuscript to make sure this distinction is clear and that these terms were not used interchangeably.

Lines 434-439 - This introductory sentence is too long to read and needs to be broken up or shortened.

Done.

Line 436 - replenishment that produces

Done.

Line 454 - “Podlaska et al. (2012)”

Done.

Line 482 - “by Xie et al. (2014)”. I have noticed this citation format error in more places. Please change throughout.

Done.

Line 489 - “coinciding with high Chl-a concentrations, reflecting”

Done.

Line 489 - What do you mean eukaryotic rather than microbial? Eukaryotes can be microbes and microbes can be eukaryotic. Do you mean eukaryotic rather than prokaryotic? But also in your results section, 3.1, you state that Prochlorococcus (not eukaryotes) were an important component of the photoautotrophic community. Hence I think the correct thing would be to say “photoautotrophic rather than heterotrophic”. Is this not what you wanted to say here? Check you have this correct throughout the manuscript.

We agree that term prokaryote should be used here, rather than microbe. We checked for consistency throughout the manuscript.

Line 497 - pluralize “IPLs”

Done.

Lines 540 - 551 - This section is confusing because you contradict each statement. You state that Eukaryotic phytoplankton and cyanos are assumed to be a major source of PG-DAG. Yet you then state that heterotrophic bacteria can also be a dominant source. Maybe using conjoining words “however” and “although” would make this section flow nicely.

We revised this section for clarity.

Line 547 - change to “we therefore suggest that”

Done.

Line 547 - remove heterotrophic bacteria. Cyanobacteria are not heterotrophic bacteria.

Done.

Line 576 - insert “abundance of less”

Done.

Line 586 - change to “fatty acid” or “acyl” rather than “fatty acyl”

Done.

Line 601 - change to “are <20”

Done.

Line 637-638 - remove tab within word “thaumarchaeota”

Done.

Line 648 - insert commas “zone, and that. . ..1993), became”

Done.

Line 667 - replace “microbial” with “bacterial”

Done.

Line 679 - remove “shallower” as it is redundant.

Done.

Lines 694 - 670 - Please make this long sentence shorter or break it into two. You repeat the same words “exported, fossil and signal” twice.

This entire section has been revised and shortened.

Line 767 - insert “waters of the phosphorus-limited”

This entire section has been revised and shortened.

Line 768 - insert “to the phosphorus-replete”

This entire section has been revised and shortened.

Line 770 - insert “observation, the relative abundance”

This entire section has been revised and shortened.

Line 808 - change to “a myriad of bacterial sources”

This entire section has been revised and shortened.

Table 1 - Make the columns wider so that the cell contents all lie on one line. Table 1 caption - should this read “where $p < 0.05$”

The table has been revised accordingly.

Figures 1,2,3 and 5 - Can you indicate the four water column zones on these figures. Perhaps with lines that join the specific depths at which the regions are defined (as was done in figures 4 and 6).

Figures 1, 2, 3 and 5 have been completely revised according to reviewer #3 request and the water column zones are now indicated.

Figure 2 - unnecessary brackets around nitrite in panel b

Figures 2 has been completely revised

Referee #3

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 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.

We thank the reviewer for the constructive comments. We believe that this study contributes to our understanding of the microbial ecology of the system, as (1) a large proportion of the microbial communities in OMZ currently remain grossly uncharacterized (cf. Podlaska et al., 2012) and IPL analysis can at least help evaluate bacterial vs archaeal sources, and (2) we report on a variety of changes in the lipid composition that relate to changes in biogeochemical zones and may represent ecophysiologic stress adaptations. We thus believe that our statements in lines 30-32 are justified. Nevertheless, we slightly modified other instances in the text where our interpretations might have been too bold (e.g., lines 468-470). While we agree that this study may not be able to fully explain the observed IPL distributions, we still provide ideas of what their functional roles or biological sources may be. Thus, this study lays the groundwork for future investigations that may probe the suggested sources and functions of the lipids we report.

In terms of trustworthiness of the presented data due to analytical issues: Even if there may be some issues with respect to providing absolute numbers, the detection of these 'unexpected' lipids in the ODZ cannot be dismissed and remains to be explained. Secondly, we do not believe that the presented data is prone to more bias than is usually the case for any lipid-based quantification. Finally, this is not the first and only study that observes non-phosphorus lipids in anoxic environments, therefore we are encouraged that our data is credible.

While revising the manuscript, we considered to not make bold claims that we cannot substantiate and aimed at making our main conclusions and take home messages clearer. Furthermore, we addressed potential analytical biases (see section 2.3)

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 main focus of the paper lies in observing shifts in community composition by looking at relative changes in IPL composition and we are furthermore putting this into context to the different biogeochemical zones. For this reporting the absolute concentration is only of secondary importance.

It is true that quantitatively IPLs are most abundant in the photic zone and that most of the export of IPL therefore likely occurs from the photic zone (see also Kharbush et al., 2016, OGC 100, 29-41 for more details on this). However, this observation is not very relevant for this paper, because here we focus on the suspended particulate material (SPM, i.e. the in situ living microbiota) and are not looking at exported material as we are filtering out larger particles of marine snow.

Since we see shifts in the relative IPL composition (both in the head group and core lipids) within the different zones we are fairly confident in stating that IPLs are produced throughout the entire water column by different microbial communities and not primarily in the photic zone as stated by reviewer #3.

We would like to highlight here again the importance of microbial processes in ODZ (as introduced in our introduction) and that many important global element cycles, such as the nitrogen cycle are affected by microbes living BELOW the photic zone. Therefore, we see no reason why in this study we should solely focus on the photic zone. This would be completely beside the point we want to make with this study.

In addition, higher absolute abundances do not equal a higher significance: Since small sized prokaryotic picoplankton may not produce as abundant biomass as phytoplankton does, diagnostic IPLs that may be indicative of either changes in the microbial community composition or their adaptations to environmental conditions may only be present in small amounts. For instance, ladderane lipids that are diagnostic for anammox bacteria are only present in very low amounts in the ETNP water column (pg L-1; Rush et al., 2012, OGC 53: 80-87), but this does not mean they are insignificant as these organisms are responsible for large scale denitrification in the global ocean.

In summary, we disagree with reviewer #3 that our approach is too simplistic, instead we would argue that only looking at absolute concentrations and consequently interpreting IPLs in the deeper water column to be mainly derived from exported material out of the photic zone 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!

The manuscript has now been substantially revised, the discussion has been shortened by ca. 6 pages and we made sure to better formulate our research questions (lines 135-143) and the summary of the main results (lines 708 and 731).

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.

We have revised the manuscript accordingly and addresses potential issues with respect to the analytical methodology (see section 2.3).

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.

We added a sentence in the methods section to address variable material recovery due to shrinking filter pore size (lines 165 and 166). We revised the sentence on pre-filtrations and changed 'most eukaryotes' to 'larger eukaryotes'.

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).

We would like to refer here to our comments to the second reviewer Dr. Bale as she raised similar concerns. As stated above (and now also in the manuscript, lines 195-197), Lengger et al. (2012, OGC 47:34-40) compared the typical Bligh and Dyer extraction with Soxhlet and ACE extraction. While overall the IPL yields were best with the Bligh and Dyer extraction, Soxhlet extraction did yield also more presumably labile phospho-IPLs (such as the HPH-GDGT). We are encouraged that we did not greatly discriminate against these allegedly more labile compounds as we abundantly detect HPH-GDGT in our samples. Having said this, our own (unpublished) experiences in comparing various extraction techniques often tell us one thing: extraction efficiencies often predominantly dependent on sample type and even the same extraction techniques may yield different yields in different environments. Extraction efficiencies thus affect any lipid study and are simply part of the known quantification biases that are associated with lipid-based studies (as is also the case for any quantitative gene-based technique for that matter).

We have raised awareness of the 'extraction efficiency issue' in the methods section so that the reader is aware that this problem exists (lines 195-197).

3) Analysis. The experimental description indicates that the IPLs have initially been analyzed by HPLC-ESI-IT-MSn 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 an- other 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

"Three years after primary qualitative analysis the samples were spiked with C16-PAF (1-O-hexadecyl-2-acetoyl-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?

We apologize that we were not very clear in describing the sequence of IPL analysis in this study. We have now substantially revised the methods section 2.3 to clarify when which samples were run and why. Unfortunately, for our second IPL runs in 2015 we are not able to provide absolute quantitative numbers since in between 2010 and 2011 the samples were used for multiple other analyses (published

in Zhu et al., 2016, EM 18: 4324-4336) where overall total lipid extract losses could not be accounted for anymore.

With respect to the selective degradation of IPLs, we would also like to refer to our comments made to reviewer #2 and in addition we would like to state that we did not observe a similar selective loss of glycolipids between the runs that were performed in 2010/2011 and 2015.

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).

Again, we apologize that our quantitative approach was not clearly formulated. We have now revised section 2.3 extensively. We can assure reviewer #3 that the response factors were determined for the same instrument that the samples were run on – anything else would be unacceptable. For quantification of IPLs response factors have to be newly determined every time samples are being run, because not only do they change from instrument to instrument but also over time for the same instrument due to re-tuning after every cleaning. We therefore determined response factors for the 2010 and 2011 runs that were performed on the ion trap system and for the 2015 run on the HPLC-QTOF-MS.

Since response factors are so variable and not only change from instrument to instrument but also within months, we are apprehensive in providing the absolute response factors as this will not be of value to anyone. Instead, we are providing the range of standard variation (line 243) and we have also now provided detailed information on the used standards for response factor correction for the different instruments in a table in the supplementary material (Suppl. Table 2).

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.

As already stated above, this study focused on relative abundances of IPLs in order to track (relative) changes in microbial communities and their ecophysiologic adaptations. Solely looking at changes in absolute abundances would not provide this level of information, therefore we disagree with the reviewer that looking at relative trends is useless.

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

This sentence is a general statement and we don't see the need to remove it just because we cannot provide the answer for this in our study.

Lines 117-119: strange sentence

The sentence was revised.

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.

This section has been revised and references have been removed.

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

We believe that we have extensively introduced the concept of using IPLs as taxonomic markers in the paragraph above this referenced sentence, e.g., glycolipids for phototrophic organisms, betaine lipids for lower plants and algae and specific phospholipids for either phototrophic organisms or bacteria.

Line 139: in what way is this approach "complementary"?

This whole paragraph has been revised (lines 135-143).

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.

This sentence has been revised according to the statements we can actually make (line 142-143).

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

Revised for completeness (lines 148-149).

Line 166: provide details on sampling volume

Pore water volumes are now listed in Supp. Table 1

Line 186: provide details on pore size of filter

Done.

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?

We wonder on which study the reviewer bases this statement. To our knowledge no literature data exists that tested IPL labilities at -20 vs -80°C. We have acquired knowledge on IPL analyses and stabilities for more than 15 years and based on our experience (re-analysis of the same samples and standards, some of which have been stored at -20°C and -80°C), IPLs are not as labile as they were originally assumed to be.

Since both reviewers #2 and #3 have expressed concerns on the storage and analysis of IPLs we addressed these issue in the revised manuscript. (lines 202-204).

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

Because the method has improved (as the reviewer has already noted in comment 3 above regarding separation of compounds, peak shape, etc.) and this is the current state of the art of analysis of IPLs and we wanted to analyze the samples with the best available method. This has been clarified in the revised methods section 2.3.

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 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.

We now included a Table in the Suppl. Material (Suppl. Table 2) that contains all the detailed information on the standards that were used. With respect to the effect of the acyl moieties, yes there are differences in the response according to the chain length, but the effect of acyl chain length does

not affect the response factor as much as the head group does (see also Popendorf et al., 2012, Lipids, 48: 185.195 for details on this). We hereby would also like to point out that it is impossible to have a standard for every single analyte (with different chain lengths and/or unsaturation/ring) that exist in a sample, considering that we are identifying and quantifying 600 to 800 compounds in each sample. Firstly, many of these compounds are not commercially available and secondly it would be utopian to prepare the missing standards in the lab due to the required biomass and necessary time. Due to these mainly practical reasons, it is generally accepted in the IPL community that response factors are merely corrected for the different head group classes.

We cannot stress enough that IPLs are not as labile as reviewers #2 and #3 point out. It is unclear what the reviewer means with "it is known"? We are not aware of a reference supporting this statement. We run standards every time we run samples, firstly to get an understanding of the performance of our system over time and secondly to use them to correct for response factors. The standards change over time as the instrument changes over time, this lies in the nature of every instrument and this is why we run standards to correct for these natural instrumental fluctuations (as should be done for any instrument and method).

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.

The sentence has been revised.

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

Pigment assignments were done according to DiTullio and Geesey, 2002 (the reference is provided in the manuscript) as is usually the case for pigments in POM samples.

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

Between 300 to 400 m there are lighter orange shadings compared to the red colors above and below.
Based on the reviewers comments we exchanged the section plots with XY-plots. Hopefully now the secondary maxima can be better seen.

Lines 268-269: Not evident from Figs 2a-b.
Based on the reviewers comments we exchanged the section plots with XY-plots. Hopefully now the described trends are better visualized.

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

Yes, and no, as this is exactly the point we want to make: From a common stand-point of oceanographers the whole system may not be P-limited (nutrient limitations are based on growth stimulation with nutrient amendment experiments and micromolar phosphate concentrations are concentrations where addition of phosphate may not stimulate additional growth). However, when one looks at bacterial cultures, these are exactly the phosphate concentrations where bacterial cultures replace phospholipids with glycolipids and are indeed growth limited. We have revised the text to clarify this (lines 701-705).

Line 282: How were absolute concentrations obtained?

See methods section 2.3 for this information.

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

Based on the reviewers comments we exchanged the section plots with XY-plots. Hopefully now the secondary maxima can be better seen.

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?

We opt to describe all the IPLs in detail later in the text because we find it logical to first provide a general overview of the grouping of glycolipids, phospholipids and aminolipids with depth. Based on the reviewers' suggestion we now mention the diversity of different IPL classes first and refer to Figure 4. Absolute concentrations of IPLs are described (lines 300-304). The reasons why we then focus more in detail on the relative abundances is explained above.

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.

We already introduced the (by now well-established) concept of substitute lipids in the introduction and we rather view the substitute lipid ratios as result that should be at least mentioned in the results section. We further discuss substitute-lipid rations in the discussion section 4.2.3.

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

We revised the sentence to make it clear that total IPLs excludes archaeal IPLs (line 312 and 316).

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.

This section has been significantly revised and shortened.

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 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.

As explained above we report relative abundances of IPLs as we want to get an understanding of how the IPL composition changes with changing geochemical zones and microbial community composition. For this, minor lipids can be just as important as the major lipids. One example are ladderane lipids, which are highly diagnostic but are only present in very low abundances in the natural environment. If we would have not made the distinction of minor lipids (with an extra plot showing their distribution in Fig. 4) changes in their relative abundance would have not been evident due to their low abundance.

To answer the reviewers question: the distinction between major and minor IPLs was primarily done for visualization purposes in order to identify potential depth-related trends. Furthermore, this distinction also makes it clear which IPLs were used to determine the absolute amounts (as only major IPLs were seen with the ion trap system, while minor IPLs were additionally detected with the QTOF system, see also revised section 2.3).

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).

As explained above the purpose of this study was to get an understanding of how the IPL composition changes with changing geochemical zones and microbial community composition. For this we do not need to examine changes in absolute concentrations but rather in relative concentration. Furthermore, absolute archaeal concentrations are already reported in Xie et al. (2014).

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.

The reviewer is correct with this and we revised our definitions accordingly. Chain length was changed to number of carbon atoms in the hydrophobic chain and double bonds changed to double bond equivalents (DBE).

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.

As described in the text (lines 353-360), we believe there can be significant variation observed in this figure and would therefore like to keep it as one of the main figures.

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.

We agree that at some depths some of the major lipids may be minor (but not the other way around). However, as already mentioned above, this distinction was (a) done for visualization purposes and (b) to distinguish between the ion trap and QTOF runs and we would therefore like to stick to this distinction.

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

The discussion has been substantially streamlined and shortened.

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?

Based on the MS2 data we are quite confident that we can make this distinction. Whenever we are observing the loss of water in the MS2, we see this as an indication for the loss of a OH-group. Even if a ring opening and loss of the oxygen from the epoxy-group would occur in the MS2 (which we doubt because this would require the addition of a nucleotide), all of the observed MS2 fragments could not be explained (due to the missing double bond that is formed during the loss of a OH-group). Whenever we do not have mass spectral evidence for the loss of water we do state that it could also be an epoxy group (line 531).

We do not have evidence that these oxygenated fatty acids are formed during storage: (1) Firstly, we do not see that these lipids appear (or increase in abundance) in other lipid extracts that we have stored over several years at -20°C. (2) Secondly, we know from the literature that OH- and epoxy-lipids are common components of algae and other organisms under stress conditions, therefore it is not surprising that they would be present in these samples. (3) Finally, if they were formed during storage, why would they not be equally present in all samples and why would they only form in some IPLs and not in all fatty acids in similar proportions? Certainly, additional investigations need to be conducted to further identify their structures and potential sources.

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.

We respectfully disagree with the reviewer. This is a concept that has been applied before in many environmental studies, including gene-based studies (e.g., Legendre and Gallagher, 2001, Oecologia 129: 271-280; Ramette 2007, FEMS Microbiol. Ecol. 62:142-160; Rossel et al., 2011 GCA 75:164-184). Reviewer #3 seems to have the misconception that changes in communities can only occur in absolute amounts but not relative to each other. In a simple example: if there is quantitatively more light one can expect a relatively higher abundance of phototrophic organisms (which in turn synthesize phototrophic lipids) compared to low-light areas where there will be relatively lower abundances in phototrophic lipids. This is a simple correlation between relative abundances of IPL (or organisms) to absolute concentrations of environmental parameters that can be statistically evaluated, and we therefore do not see the justification to skip this section as it is an essential part of the paper.

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.

We substantially revised and shortened the discussion by one third from the originally 19 pages to 13 pages.

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

Done.

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

This obvious trend can now be clearly observed in Figure 2.

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.

We actually do not expect the reported IPL signal to represent exported matter from above since we are looking at suspended particulate organic matter and not larger aggregates of exported material from above. Therefore there is no need to introduce this concept in the introduction.

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

We do not think that this is a bold statement since it is indeed backed up by our data: as described in the results section we observe 24 different IPL classes that also exhibit changes in core lipid composition with depth. What else should this IPL diversity reflect? Nevertheless, we toned the statement down (lines 468-470).

Lines 502-505: Repetitious.

This section was revised.

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

The section was revised and shortened.

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.

Not that much, this is why we show the changes in core lipids (carbon atom number and DBE), this is also stated at several instances in the manuscript (lines, 541-545, lines 573-574, lines 588-591).

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.

Yes, as stated in Xie et al. (2014) we detected crenarchaeol and are now also showing the distribution of the core GDGT composition for the individual IP-GDGTs in Supp. Fig. 2. We revised this section according to the reviewers' suggestion (line 562) and are also now citing Schouten et al. (2008).

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.

We do not agree that this is highly unrealistic, PUFAs have been shown to be synthesized by marine bacteria (see references cited in the text, lines 582-586). PUFAs could thus very well be produced in situ. Section 4.1.3 has been substantially revised and hopefully it is less confusing now.

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.

As already explained above, we do not understand the reviewers concern that we cannot use relative abundances of IPL to describe changes in relative community composition and/adaptations to environmental conditions. We disagree that a completely different pictures would emerge when only reporting absolute abundances.

Generally, bacterial abundance is highest in surface waters (with some exceptions at stations 1 and 2, see Fig. 2). However, what the reviewer seems to miss is that relative bacterial abundance is not highest in the photic zone, instead biomass (and IPLs) derived from eukaryotic phytoplankton dominate. In the ODZ, where phytoplankton abundance decreases (or is absent) relative bacterial abundance increases. This is also why we have more IPLs that are derived from bacteria within the ODZ and below.

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

Yes of course, but we are discussing the IPLs here only in light of what is known from the FISH analyses performed at these sites.

Line 662: "chain" is incorrect

The term was replaced.

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

Where possible and applicable we referred to the existing FISH dataset (lines 457, 552, 566, 578 and 637-639).

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.

The distinction we made for this was to call the C20 and C22 PUFAs 'long-chain PUFAs', we tried to be consistent using this distinction.

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.

We disagree with the reviewer that our isoprenoidal data does not provide any new insight as now we also report on the presence of the important thaumarchaeal marker HPH-GDGT, which was not done in the previous Xie et al. (2014) data set. We revised this section of the manuscript and are now also providing supplementary information on the ring distribution of the individual IP-GDGTs (Suppl. Fig. 2).

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.

We substantially revised the discussion, including the headers and the order of the different sections. The purpose of the three sections is to provide an overall synthesis and summary of the above described IPL species. As explained above we do believe our statistical evaluation to be relevant and necessary as it does provide additional insight into the zonation of IPLs into different geochemical

zones. We hope that by shortening and streamlining the discussion our main points we want to make are now expressed in a clearer way.

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.

Based on the reviewers comments we exchanged the section plots with XY-plots.

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 instanced where the FA combination says 18:4/18:4 but the number of bonds in 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.

We changed the terminology and call it now double bond equivalents (DBE) and also revised Suppl. Table 5 according to the reviewers' suggestion.

Diversity of intact polar lipids in the oxygen minimum zone of the Eastern Tropical North Pacific: Biogeochemical implications of non-phosphorus lipids

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Keywords: intact polar lipids, phospholipids, glycolipids, betaine lipids, ether lipids, oxylipins, phospholipid substitution, oxygen minimum zone

Abstract

Intact polar lipids (IPLs) are the main building blocks of cellular membranes and contain chemotaxonomic, ecophysiologic and metabolic information, making them valuable biomarkers in microbial ecology and biogeochemistry. This study investigates IPLs in suspended particulate matter (SPM) in the water column of the Eastern Tropical North Pacific Ocean (ETNP), one of the most extensive open ocean oxygen minimum zones (OMZ) in the world with strong gradients of nutrients, temperature and redox conditions. A wide structural variety in polar lipid head group composition and core structures exists along physical and geochemical gradients within the OMZ. We use this structural diversity in IPLs to evaluate the microbial ecology and ecophysiological adaptations that affect organisms inhabiting the mid-depth OMZ in the context of biogeochemical cycles. Diacylglycerol phospholipids are present at all depths, but exhibit highest relative abundance and compositional variety (including mixed acyl/ether core structures) in the upper and core OMZ where prokaryotic biomass was enriched. Surface ocean SPM is dominated by diacylglycerol glycolipids that are found in photosynthetic membranes. These and other glycolipids with varying core structures composed of ceramides and hydroxylated fatty acids are also detected with varying relative abundances in the OMZ and deep oxycline, signifying additional non-phototrophic bacterial sources for these lipids. Betaine lipids (with zero or multiple hydroxylations in the core structures) that are typically assigned to microalgae are found throughout the water column down to the deep oxycline but do not show a depth-related trend in relative abundance. Archaeal IPLs comprised of glycosidic and mixed glycosidic-phosphatidic glycerol dibiphytanyl glycerol tetraethers (GDGTs) are most abundant in the upper OMZ where nitrate maxima point to ammonium oxidation, but increase in relative abundance in the core OMZ and deep oxycline. Abundant non-phosphorus

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52 "substitute" lipids within the OMZ suggest that the indigenous microbes might be phosphorus limited (P

53 starved) at ambient phosphate concentrations of 1 to 3.5 µM, although specific microbial sources for many

54 of these lipids still remain unknown.

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1. Introduction

Oxygen Minimum Zones (OMZ) are permanently oxygen-deficient regions in the ocean defined by O_2 concentrations <20 µM. They occur in areas where coastal or open ocean upwelling of cold, nutrient-rich waters drive elevated levels of primary production and the subsequent respiration of organic matter exported out of productive surface waters consumes oxygen faster than it is replaced by ventilation or by mid-depth lateral injections of oxygenated water. Low oxygen levels cause habitat compression, whereby species intolerant to low levels of oxygen are restricted to oxygenated surface waters (Keeling et al., 2010; Rush et al., 2012). But even these low levels of oxygen permit vertical migration of some zooplankton taxa into hypoxic waters (e.g., Seibel, 2011; Wishner et al., 2013). Oxygen depletion stimulates diverse microbial life capable of utilizing alternative electron acceptors for respiration under microaerobic conditions (e.g., Ulloa et al., 2012; Tiano et al., 2014; Carolan et al., 2015; Kalvelage et al., 2015; Duret et al., 2015). Important prokaryote-mediated processes within OMZs include denitrification and the anaerobic oxidation of ammonium (anammox), which together may account for 30-50% of the total nitrogen loss from the ocean to the atmosphere (Gruber, 2008; Lam and Kuypers, 2011). Modern day OMZs comprise ~8% of global ocean volume (Karstensen et al., 2008; Paulmier and Ruiz-Pino, 2009; Lam and Kuypers, 2011), but any expansion in the coming decades as a consequence of global warming and increased stratification (Stramma et al., 2008; Keeling et al., 2010) would have profound effects on marine ecology, oceanic productivity, global carbon and nitrogen cycles, the biological pump and sequestration of carbon (Karstensen et al., 2008; Stramma et al., 2010; Wright et al., 2012). A better understanding of the effect of low-O_2 on marine biogeochemistry and microbial ecology is thus warranted.

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The Eastern Tropical North Pacific Ocean (ETNP), situated off the west coast of Mexico and Central America, hosts one of the largest OMZs in the open ocean, extending halfway across the Pacific Ocean and comprising ~41% of global OMZs (Lavín and Fiedler, 2006; Fiedler and Talley, 2006; Paulmier and Ruiz-Pino, 2009). By comparison, OMZs of the Eastern Tropical South Pacific Ocean off Peru and Chile and in the Arabian Sea are ~14% and ~8%, respectively, of global OMZs. In the ETNP, a sharp permanent pycnocline develops where warm, saline surface waters lie on top of a shallow thermocline, producing a highly stratified water column. Moderate primary production, dominated by picoplankton, depends on oceanic upwelling and wind mixing of coastal waters but is generally limited by the lack of micronutrient dissolved iron (Franck et al., 2005; Pennington et al., 2006). Remineralization, ~70% of which is microbially mediated (Cavan et al., 2017), of particulate organic carbon exported out of surface waters consumes oxygen at rates that cannot be balanced by ventilation across the pycnocline and by sluggish lateral circulation, leading to O_2 levels $\leq$2 µM at depths between ~100 and ~800 m. Abundances of micro- (Olson and Daly, 2013) and macro-zooplankton (Wishner et al., 2013; Williams et al., 2014) that are high in surface waters are reduced in the OMZ, and those macrozooplankton that are diel vertical migrators survive in the OMZ with reduced metabolic rates (Maas et al., 2014; Cass and Daly, 2015). Microbial abundances and activities for both heterotrophic and chemoautotrophic metabolisms are high in both surface waters and within the OMZ, but again with reduced metabolic rates in the OMZ (Podlaska et al., 2012). A strong nutricline indicates microbial nitrogen cycling involving co-occurring nitrification, denitrification and anammox (Rush et al., 2012; Podlaska et al., 2012), perhaps contributing up to 45% of the global pelagic denitrification (Codispoti and Richards, 1976). Microbial communities are mainly comprised of proteobacteria, with increasing contributions of archaea in deeper waters. Yet, on

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average ca. 50% of the prokaryotic communities within the OMZ of the ETNP remained uncharacterized (Podlaska et al., 2012).

Intact polar lipids (IPLs) are the main building blocks of cellular membranes and may be used to characterize abundance and physiology of aquatic microorganisms from all three domains of life. IPLs represent a diverse range of molecular structures, including phosphatidyl, glycosidic, phospho-glycosidic, and amino acid polar head groups linked to glyceryl-acyl and glyceryl-*O*-alkyl apolar moieties. IPL distributions have been documented in surface waters of the Eastern Subtropical South Pacific (Van Mooy and Fredricks, 2010), the Western North Atlantic Ocean (Van Mooy et al., 2006; 200; Popendorf et al., 2011a), the South Pacific Ocean (Kharbush et al., 2016), the Mediterranean Sea (Popendorf, et al., 2011b), the North Sea (Brandsma et al., 2012), lakes (Bale et al., 2016), the Western English Channel (White et al., 2015) and throughout the water columns of stratified water bodies (Ertefai et al., 2008; Schubotz et al., 2009; Wakeham et al., 2012; Pitcher et al., 2011; Xie et l., 2014; Basse et al., 2014; Sollai et al., 2015). Surface waters are typically dominated by nine IPL classes. Three diacylglycerol glycolipids, monoglycosyl (1G-), diglycosyl (2G-) and sulfoquinovosyl diacylglycerol (SQ-DAG), are main IPLs found in all thylakoid membranes of phototrophs, including those of cyanobacteria (Siegenthaler et al., 1998)[1]. Three betaine lipids, diacylglyceryl homoserine (DGTS), hydroxymethyl-trimethyl-*β*-alanine (DGTA) and carboxy-*N*-hydroxymethyl-choline (DGCC), are also generally abundant. Betaine lipids are widely distributed in lower plants and green algae (Dembitsky, 1996) and are thus usually assigned to

[1] Elsewhere in the literature 1G-DAG, 2G-DAG, and SQ-DAG are also termed MGDG, DGDG and SQDG. However, we have opted to retain the 1G-DAG, 2-DAG, etc. nomenclature as other IPLs discussed throughout also contain monoglycosyl- and diglycosyl-moieties (e.g., 1G-GDGT and 2G-GDGT). Likewise, we retain the nomenclature PC-DAG, PE-DAG, and PG-DAG for phospholipids elsewhere termed PC, PE, PG.

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eukaryotic algae in the ocean (Popendorf, et al., 2011a), but DGTS was recently also found in bacteria when phosphorus is limited (Yao et al., 2015; Sebastian et al. 2016). Three common detected phospholipids are diacylglycerol phosphatidyl choline (PC-DAG; often simply referred to elsewhere as PC), phosphatidyl ethanolamine (PE-DAG, often PE), and phosphatidyl glycerol (PG-DAG, often PG), all of which have mixed eukaryotic or bacterial sources in the upper water column (Sohlenkamp et al., 2003; Popendorf, et al., 2011a). Microbial source assignments have been broadly confirmed by isotope labeling studies (Popendorf, et al., 2011a). In oxygen-deficient subsurface waters IPL distributions are more diverse and other phospholipids such as diacylglycerol phosphatidyl (*N*)-methylethanolamine (PME-DAG), phosphatidyl (*N,N*)-dimethylethanolamine (PDME-DAG) and diphosphatidyl glycerol (DPG) increase in abundance; these IPLs occur in a number of bacteria that may inhabit low oxygen environments (Schubotz et al., 2009; Wakeham et al., 2012). Dietherglycerol phospholipids and glycosidic ceramides with unidentified sources have also been detected (Schubotz et al., 2009; Wakeham et al., 2012), the latter have been recently observed to be abundant in phosphorus-limited diatoms (Hunter et al., 2018). IPLs that are unique to marine archaea are comprised of glycerol dialkyl glycerol tetraethers (GDGT) core lipids with various glycosidic, diglycosidic and mixed phospho-glyco polar head groups (e.g., Schouten et al., 2008; Pitcher et al., 2011; Zhu et al., 2016; Elling et al., 2017). Abundances of archaeal IP-GDGTs vary considerably with depth, but are typically elevated in zones of water column oxygen depletion, especially where ammonium oxidizing thaumarchaea are abundant (Pitcher et al., 2011; Schouten et al., 2012; Sollai et al., 2015).

IPL can also be indicators of metabolic and physiologic status. Many organisms remodel their IPL composition when faced with environmental stressors such as changes in pH, salinity, temperature or

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availability of nutrients (Zhang and Rock, 2008; Van Mooy et al., 2009; Meador et al., 2014; Carini et al., 2015; Elling et al., 2015). Replacing phospholipids with non-phosphorus containing substitute lipids is an important mechanism when facing nutrient phosphate starvation in oligotrophic surface waters where phosphate concentrations may be as low as nanomolar levels. Cyanobacteria replace PG-DAG with SQ-DAG (Benning et al., 1993; Van Mooy et al., 2006) and microalgae and some bacteria replace PC-DAG with DGTS (Geiger et al., 1999; Van Mooy et al., 2009; Popendorf, et al., 2011b) due to their similar ionic charge at physiological pH. Heterotrophic marine bacteria can replace PE-DAG with either 1G-DAG or DGTS (Carini et al., 2015; Sebastian et al., 2016; Yao et al., 2015). Notably, substitute lipids are also biosynthesized under micromolar concentrations of phosphate (Bosak et al., 2016).

Here, we use IPL distributions in suspended particulate matter (SPM) to characterize eukaryotic, bacterial and archaeal communities inhabiting the OMZ of the ETNP. This study is an extension of that of Xie et al. (2014), which focused on the distribution of core and intact polar archaeal and bacterial tetraether lipids at two stations investigated here (stations 1 and 8). The water column of the ETNP comprises distinct biogeochemical zones based on oxygen concentrations and IPL distributions reflect the localized ecology. Abundant non-phosphorus substitute lipids within the core of the OMZ suggest phosphorus limitation of the source microorganisms even at micromolar concentrations of phosphate. Overall our results provide deeper insight into the broad community composition and the physiologic state of microorganisms inhabiting OMZs.

2. Methods

2.1 Sample collection and CTD data

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Suspended particulate matter (SPM) samples were collected at four stations (distance to shore: 400~600 km; Fig 1) along a northwest-southeast transect (Station 1: 13° 01.87'N, 104° 99.83'W; Station 2: 11° 99.96' N, 101° 22.82' W; Station 5: 10° 68.94' N, 96° 34.12' W; and Station 8: 8° 99.46'N, 90°00.18'W) in the ETNP during the R/V *Seward Johnson* cruise in November 2007 (R/V *Seward Johnson* Cruise Scientists, 2007). Station 1 in the Tehuantepec Bowl is an area of relatively low primary productivity (e.g., 0.05 mg Chl-*a*/m^2; (Fiedler and Talley, 2006; Pennington et al., 2006) whereas Station 8 in the Costa Rica Dome is moderately productive (1 mg Chl-*a*/m^2). All stations are characterized by a strong thermocline/pycnocline/oxycline (at 20-50 m depths depending on location) and a profound and thick OMZ (down to ~2 µM O_2 between ~300-800 m depth). Station 1 is a reoccupation of the Vertical Transport and Exchange II/III site from the early 1980's (Lee and Cronin, 1984; Martin et al., 1987; Wakeham and Canuel, 1988; Wakeham, 1987, 1989).

Seawater was filtered *in-situ* using submersible pumps (McLane Research Laboratories WTS-142 filtration systems) deployed on the conducting cable of the CTD/rosette that measured temperature, conductivity, oxygen, fluorescence/chlorophyll-*a* and transmissivity during pump deployments and during pumping. Filtered water volumes ranged between 130 and 1800 L (Suppl. Table 1). Pumps were fitted with two-tier 142 mm diameter filter holders: a 53 µm mesh Nitex "prefiltration" screen to remove larger eukaryotes and marine snow aggregates and a double-stacked tier of ashed glass fiber filters (142 mm Gelman type A/E, nominal pore size 0.7 µm). POC concentrations we report represent minimum values to reflect potentially inefficient collection of 0.7 µm particles by GFFs. Since pore size of the filters may also decrease during filtration the recovered material may vary dependent on filtration time.

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Deleted: most …arger eukaryotes and marine snow aggregates and a double-stacked tier of ashed glass fiber filters (142 mm Gelman type A/E, nominal pore size 0.7 µm). …It is likely that smaller cells (diameter 0.2-0.7 µm) were not retained on the filter and thus the reported ... [10]

Following pump recovery, GFF filters and Nitex screens were wrapped in pre-combusted foil and stored frozen at -20°C until extraction.

2.2 Elemental, pigment and nutrient analysis

Particulate organic carbon (POC) and total particulate nitrogen (TN) were measured on 14 mm-diameter subsamples of each glass fiber filter (GFF) prior to lipid extraction; therefore, POC and TN concentrations reported here are only for <53 µm material. The plugs were acidified in HCl vapor in a desiccator for 12 hours to remove inorganic carbon. Elemental analysis was performed with a ThermoFinnigan Flash EA Series 1112 interfaced to a ThermoFinnigan Delta V isotope ratio mass spectrometer at the Skidaway Institute Scientific Stable Isotope Laboratory. Organic carbon and nitrogen contents were calibrated against internal laboratory chitin powder standards which in turn had previously been cross-calibrated against USGS 40 and 41 international standards.

Chlorophyll-*a* (Chl-*a*) and pheopigment concentrations were measured on-board the ship (Olson and Daly, 2013). Seawater samples (100 – 500 ml) from CTD casts were filtered onto Whatman GF/F filters (0.7 µm) which were immediately extracted with 90% acetone. Fluorescence was measured with a Turner Designs 10AU fluorometer and Chl-*a* concentrations were determined after Parsons et al (1984). Post-cruise HPLC analysis of pigments in 100 – 500 ml seawater samples filtered onto Whatman GF/F (0.7 µm) filters were conducted at the College of Charleston Grice Marine Laboratory, Charleston, SC on a Hewlett Packard 1050 system (DiTullio and Geesey, 2002).

Seawater samples for nutrient analyses (NO_2^-, NO_3^{-2}, NH_4^+ and PO_4^{3-}) were collected directly from Niskin bottles into acid-washed, 30-mL high-density polyethylene (HDP) bottles. After three rinses,

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bottles were filled to the shoulder, sealed, and frozen (−20°C). All frozen samples were transported to the Oceanic Nutrient Laboratory at USF for analysis using a Technicon Autoanalyzer II.

2.3 Lipid extraction and analysis of intact polar lipids

Lipids associated with the <53 µm SPM on the GFFs were Soxhlet-extracted shortly after the expedition in 2008 using dichloromethane:methanol (DCM:MeOH; 9:1 v/v) for 8 h. Extracted lipids were partitioned into DCM against 5% NaCl solution and dried over Na_2SO_4. Total lipid extracts (TLEs) were stored at -20°C. More recent IPL analyses typically utilize less harsh modified Bligh-Dyer extraction procedures, however, we believe that our finding labile IPLs, such as hexose-phosphate-hexose GDGTs, indicates that our results are not compromised (cf. Lengger et al., 2012).

IPL analyses by high-performance liquid chromatography-mass spectrometry (HPLC-MS) were carried out initially in 2010/2011 and again in 2015 as instrument protocols improved. In between these analyses we did not observe a notable selective loss of IPL compounds, instead we were able to detect a much larger suite of IPL structures due to improved detection and chromatographic separation techniques (Wörmer et al., 2013). The confidence in these results are supported by the analysis of IPL standards (Suppl. Table 2) that are stored at -20 °C over several years (fresh standard mixtures are typically prepared every 2 to 3 years), which do not indicate degradation of any particular IPL over time. The analysis in 2010/2011 focused on absolute concentrations of the major IPLs (for distinction between major and minor IPLs see results section). Aliquots of the TLE were dissolved in DCM/methanol (5:1 v/v) for injection on a ThermoFinnigan Surveyor HPLC system coupled to a ThermoFinnigan LCQ DecaXP Plus ion-trap MS via electrospray interface (HPLC-ESI-IT-MS^n) using conditions described previously (Sturt et al.,

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2004; Xie et al., 2014). Ten µL of a known TLE aliquot spiked with C_{19}-PC as internal standard was injected onto a LiChrosphere Diol-100 column (150 × 2.1 mm, 5 µm, Alltech, Germany) equipped with a guard column of the same packing material. Absolute IPL concentrations were determined in positive ionization mode with automated data-dependent fragmentation of the two most abundant base peak ions. Acyl moieties of glycolipids and aminolipids were identified via HPLC-IT-ESI-MS^2 experiments in positive ionization mode, whereas phospholipid side chain composition was analyzed in negative ionization mode. Details of mass spectral interpretation, and identification of fatty acid moieties are described in Sturt et al. (2004) and Schubotz et al. (2009) and are exemplified in Suppl. Table 3. HPLC-MS analysis is not able to differentiate between double bonds or rings, therefore in the subsequent text we will refer to double bond equivalents (DBE) to include both possibilities, similarly absolute chain length cannot be determined as branched and straight chain alkyl chains cannot be differentiated, therefore we report total carbon atom numbers for the alkyl side chains. Assignment of the betaine lipid DGTS was according to the retention time of the commercially available standard DGTS (Avanti Polar Lipids, USA). The isomer DGTA, which elutes at a different retention time due to its structural difference (e.g., Brandsma et al., 2012) was not observed in the HPLC-MS chromatograms. For all analyses, response factors of individual IPLs relative to the injection standard C_{19}-PC were determined using dilution series of commercially available standards (Suppl. Table 2).

Subsequent analyses in 2015 were used to obtain sum formulas and IPL structures based on exact masses in the MS1 and MS-MS experiments and to additionally provide data on minor lipids, which were below detection limit during the 2010/2011 ion trap analyses (for distinction between major and minor lipids see results section). For these measurements absolute quantities could not be determined since the

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TLE had been used for other experiments and the information on TLE amounts used was unknown; therefore, these analyses are used to describe relative abundances. Analyses were performed on a Bruker maXis Plus ultra-high resolution quadrupole time-of-flight mass spectrometer (Q-TOF) with an ESI source coupled to a Dionex Ultimate 3000RS UHPLC. Separation of IPLs was achieved using a Waters Acquity UPLC BEH Amide column as described in Wörmer et al. (2013), which resulted in better chromatographic separation of compounds and higher sensitivity compared to the 2010/2011 analyses. Relative proportions of compounds were quantified taking the different response factors of IPL classes into account. Peak areas in extracted mass chromatograms were corrected with absolute response factors determined in dilution series of commercially available standards (Suppl. Table 2). Some ions assigned to either PE-AEG and PC-AEG could not be quantified individually due to co-elution of these compounds and were thus quantified as one group using the mean response factor of PE- and PC-DAG. For compound classes for which no standards were available, (e.g., PI-DAG, OL and the unknown aminolipids AL-I and AL-II) the relative responses could not be corrected for. Assuming these compounds may ionize similarly as structurally related IPLs, values may be off by a factor of 0.2 to 1.4, which is the maximum range of response factors observed for the standards.

2.4 Statistical analysis

Nonmetric multidimensional scaling (NMDS) analysis was used to illustrate the relationships among objects hidden in a complex data matrix (Rabinowitz, 1975) and was performed in the free software R (version 3.4.3, www.r-project.org/) with *metaMDS* (vegan library, version 2.4-6) as described by Wakeham et al. (2012). The datasets of relative lipid distribution and variations in carbon number and

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double bond equivalents were standardized by Hellinger transformation using the function *decostand*, while for all other variables (environmental parameters, microbial groups) absolute numbers were used. The compositional dissimilarity was calculated by Euclidean distance measure. The resulting plot shows the distribution of lipids and sampling depths. Microbial groups and geochemical parameters were overlaid by function *envfit*. Lower stress is related to high quality of solution, and stress values ≤ 0.1 indicate results of good quality (Rabinowitz, 1975). Non-parametric Spearman Rank Order Correlation analysis was performed on combined data of environmental variables and IPL ratios and IPL relative abundances of all four stations using SigmaPlot 11.0 (Systat Software Inc., San Jose, USA).

3. Results

3.1 Biogeochemical setting

All along the transect, the thin mixed layer (upper ~20 m) was warm, ~25–28 °C, with oxygen concentrations approaching air saturation at ~200 µM (Fig. 2). The thermocline was abrupt at ~20-50 m, where temperatures dropped to ~15–18 °C and oxygen decreased to ~20 µM. Temperatures stabilized by ~250–300 m depth at ~10–12 °C and oxygen levels were <2 µM; especially at Station 8 there were spatially and temporally variable oxygen intrusions into the upper portion of the OMZ. By ~600–800 m depth, a deep oxycline was observed where oxygen concentrations began to rise again to ~40 µM at temperatures of ~4 °C by 1250 m. For the purposes of this discussion, the water column of the ETNP was partitioned into four horizons based on oxygen content: an oxic epipelagic zone down to the thermocline (0–50 m; 200 µM > O_2 > 20 µM); an upper OMZ (Station 1 and 8: 50–300 m, Station 5: 50 – 350 m, Station 2: 50–200 m; 20 µM > O_2 > 2 µM); the core OMZ (Station 1 and 8: 300–800 m, Station

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5: 350 – 600 m Station 2: 200 – 600 m; $O_2 < 2$ µM); and a deep oxycline (Station 1 and 8 ≥ 800 m, Station 2 and 5 ≥ 600 m; $O_2 > 2$ µM) of rising O_2 levels (Fig. 1a). Note that sampling at stations 1 and 8 reached to 1250 m depth so SPM from >750 m depth best represents the core OMZ and deep oxycline.

Chl-*α* was highest in surface waters with maximum values of 1.8 µg/L at 10 m at station 5, was between 0.2 and 0.7 µg/L at station 1, 2 and 8 and decreased to values close to zero below 100 m at all stations (Fig. 2; see also Fiedler and Talley, 2006, and Pennington et al., 2006, for additional results from previous surveys). HPLC analysis of accessory pigments (Goericke et al., 2000; Ma et al., 2009) showed that picoplankton, primarily *Prochlorococcus* (indicated by divinyl chlorophyll *α*), were an important component of the photoautotrophic community, along with diatoms (fucoxanthin), especially *Rhizosolenia* at the deep fluorescence maximum at stations 1 and 5 but *Chaetoceros* at station 8, and prymnesiophytes (19'hexanoyloxyfucoxanthin and 19'butanoyloxyfucoxanthin; DiTullio and Geesey, 2002; Suppl. Table 4). High phaeopigment abundances (up to 90% of [Chl-*α* + phaeopigments]) attested to algal senescence or grazing by macro- (Wishner et al., 2013; Williams et al. 2014) and micro-zooplankton (Olson and Daly, 2013) above and into the oxycline. Primary maxima in transmissivity corresponded with the peak Chl-*α* concentrations and fluorescence maxima, but secondary transmissivity maxima between 300 and 400 m at stations 1, 5, and 8 indicated elevated particle abundances in the core of the OMZ (Fig. 2).

Nitrite (NO_2^-) maxima in the OMZ at all stations coincided with nitrate (NO_3^{2-}) deficits (Fig. 3). Ammonium (NH_4^+) concentrations changed little through the water column (Fig. 3). Phosphate (PO_4^{3-}; Fig. 3) and total dissolved nitrogen (TDN; not shown) were low (respectively, < 0.5 and < 3 µM) in the upper 20 m of the oxic zone, but increased in the OMZ. High PO_4^{3-} (up to 3.4 µM) and high TDN (up to 44.5 µM) were observed in the deep OMZ at stations 2, 5 and 8 (Fig. 3). N:P ratios were lower than

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the Redfield ratio (16) at all sites and depths (Fig. 3); N:P minima were lowest in surface waters (2.6 to 10 in the upper 20 m) and at ~500 m within the core OMZ and the deep oxycline at station 1 (<9).

POC and TN concentrations (< 53 µm material) were highest in the euphotic zone (POC: 20 – 100 µg/L; TN: 4 – 15 µg/L), rapidly dropping to 5 µg/L and 1 µg/L below the upper OMZ, respectively (Fig. 2; Suppl. Fig. 1). Secondary maxima for POC (~10 µg/L) and TN (~2 µg/L) within the core of the OMZ might reflect elevated microbial biomass there. Concentrations dropped in the deep oxycline to ≤3 µg/L and ≤0.5 µg/L for POC and TN, respectively.

Absolute IPL concentrations were determined by ion trap LCMS and varied between 250 and 1500 ng/L in the oxic zone and abruptly decreased more than 10-fold (to <20 ng/L) in the upper OMZ (Fig. 2). Secondary maxima in IPL concentrations (15–40 ng/L) within the OMZ at all stations roughly coincided with elevated numbers of prokaryotes (Fig. 2). IPL:POC ratios decreased with increasing depth (Fig. 2), tracking trends of POC, TN and IPL concentrations.

3.2 Changes in IPL composition with water column depth in the ETNP

In total, 24 IPL classes were identified in the ETNP (Fig. 4, Suppl. Fig. 2). Eleven major and thirteen minor IPL classes were detected in the QTOF analyses, which were classified according to their relative abundance: if an individual IPL comprised more than 10% of total IPLs at any depth of the four stations it was classified as a major IPL, compounds <10% were minor IPLs. Based on their head group composition IPLs were grouped into glycolipids, phospholipids or aminolipids. Figure 3 shows changes in the relative abundances (as percentages of total IPLs, excluding isoprenoidal archaeal IPLs) of glycolipids, phospholipids and aminolipids as well as several substitute lipid ratios, reflecting preferential

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biosynthesis of non-phosphorus lipids to replace phospholipids under phosphate-limiting growth (cf. Van Mooy et al., 2006; Popendorf, et al., 2011b; Carini et al., 2015; Bosak et al., 2016). Relative abundances of non-isoprenoidal phospholipids were highest in the core OMZ between 400 and 600 m at all sites, where they comprise up to 45–76% at stations 1, 2 and 5 and between 12 and 61% at station 8. Phospholipid abundances were lower within the upper OMZ and oxic zone at all stations (between 4 and 55%) and in the deep oxycline at station 8 (<1%). Aminolipid content was highest in SPM from the upper 55 m at station 5 and 8 (10 to 25%), the core OMZ at station 8 (15 to 34%) and the deep oxycline at station 1 (17%). Lower aminolipid contents (2 to 11%) were observed in the oxic zone and the core OMZ at stations 1 and 2, the upper OMZ at station 5 (0 to 11%) and the deep oxycline at station 8 (<2%). Glycolipid abundance was >9% at all depths, with highest abundance (average 54%, max. 82%) within the upper OMZ and oxic zone at all stations and the deep oxycline at station 8. Values down to 9% were observed within the core OMZ.

3.2.1 Major lipids

The eleven major IPL classes included three IP-GDTs of archaeal origin: (1G-GDGT, 2G-GDGT and HPH-GDGT); and eight IPLs assigned to either a bacterial or eukaryotic origin: three glycolipids (1G-DAG, 2G-DAG, SQ-DAG), four phospholipids (PG-DAG, PE-DAG, PC-DAG, PE+PC-AEG) and one aminolipid (DGTS). All major lipid classes were found at almost all depths at all four stations, but with varying relative abundances (as % of total IPL; Fig. 4, Suppl. Table 1).

Archaeal IP-GDGTs: Relative abundances of archaeal IPL (IP-GDGTs) generally increased with depth from non-detectable in surface waters to >50% of total IPLs at station 8 (bottom of core OMZ and

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deep oxycline). Archaeal IP-GDGT abundances at stations 1 and 2 peaked at 30% (bottom of upper OMZ, core OMZ and deep oxycline) but were generally <10% at station 5 (Fig. 4). At station 1 and 2, 1G-GDGT and 2G-GDGT were most abundant with variable amounts of HPH-GDGTs, whereas 1G-GDGT and HPH-GDGT dominated archaeal IPLs at station 5 and 8 at most depths. Distributions of glycosidic IPL-GDGTs obtained in the present investigation corroborate the absolute values reported by (Xie et al., 2014) for stations 1 and 8: 1G-GDGT was more abundant than 2G-GDGT at station 8 when compared to station 1. The core GDGTs of 1G-GDGTs and HPH-GDGTs are dominated by GDGT-0 and crenarchaeol (Suppl. Fig. 3), whereas 2G-GDGTs are dominated by GDGT-2 and a small amount of crenarchaeol (Zhu et al., 2016)

Diacylglycerol lipids: The oxic zone and the upper OMZ were dominated (~50−80% of IPL) at all sites by the diacylglycerol glycolipids, 1G-DAG, 2G-DAG and SQ-DAG (Fig. 4). In the core OMZ and deep oxycline, relative amounts of 2G-DAG and SQ-DAG decreased to 4% and 12%, respectively. 1G-DAG abundances were lowest in the core OMZ at all stations, but were up to 47% of total IPL in the deep oxycline. Diacylglycerol phospholipids, PE-, PG- and PC-DAG, were the second most abundant IPLs. Abundances of PE- and PG-DAG were highest within the upper and core OMZ, constituting >50% in the core OMZ at station 1, >30% at stations 2 and 5, and 16% at station 8. PC-DAG, with average abundances of 5% at stations 1, 2, 8 and 3% at station 5, did not exhibit depth-related trends. The third most abundant diacylglycerol class was the betaine lipid DGTS, which was present throughout the water column at average abundances of 7% at station 1, 2 and 8, and 5% at station 5.

Major diacylglycerol lipids showed changes in average number of carbon atoms and double bond equivalents (DBE) with depth (Fig. 5, Suppl. Table 5). The glycolipids and PC-DAG decreased in average

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carbon number by up to three carbons and decreased in DBE by up to 2 at the top of the upper OMZ and within the core OMZ compared to the oxic zone and the deep oxycline. Average carbon numbers for PE- and PG-DAG and DGTS showed an inverse trend, both generally increasing up to two carbons between the upper OMZ and the core OMZ. Changes in DBE were not as pronounced for PG-DAG and DGTS, on average 1 to 2 DBE greater in surface waters than in deeper waters, while the number of DBE increased on average with depth for PE-DAG.

Acyl-ether glycerol lipids: Mixed ether-ester glycerol core structures with either PE or PC head groups were observed at all stations and all depths (generally 4-12%) except for the deep oxycline at station 8.

3.2.2 Minor lipids

Thirteen minor IPL classes were identified, five of which were glycolipids, four phospholipids and four aminolipids. All minor lipid classes were detected at each site except for OH-DGTS which was absent at station 1. Some minor lipids were found at all depths, whereas others were restricted to specific depth zones as defined by oxygen content (Fig. 4).

Diacylglycerol lipids: Two minor diacylglycerol glycolipids, 1G-OH-DAG and 3G-DAG, were most abundant within the oxic zone and the upper OMZ, comprising between 2 to 15% of minor lipids on average (0.1 to 0.6% of total IPLs), but were only sporadically found within the core OMZ and deep oxycline. 1G-OH-DAG showed highest relative abundances at station 5, constituting up to 40% of minor lipids. Four additional phospholipids with diacylglycerol core structures with the following head groups were identified: diphosphatidylglycerol (DPG), phosphatidyl-(*N*)-methylethanolamine (PME),

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phosphatidyl-(*N,N*)-dimethylethanolamine (PDME) and phosphatidyl inositol (PI). DPG, PME-DAG and PDME-DAG had highest relative abundances (respectively 65, 56 and 35% of minor IPL) within the upper and core OMZ, but at lower abundances within the oxic zone at all stations and in the deep oxycline at stations 1, 2 and 5. PI-DAG was most abundant in the oxic zone and the upper OMZ (up to 25% of minor IPL), but was also present in the core OMZ and the deep oxycline, except for station 8. Three types of aminolipids were observed as minor lipids. OH-DGTS with up to three hydroxyl-groups attached to the fatty acyl side chains (Suppl. Fig. 4) was observed at most depths at station 8 with an average relative abundance of 23% among the minor lipids; it was also occasionally detected at stations 2 and 5 within the oxic zone and upper OMZ. Two additional aminolipids had an undefined head group that exhibited fragmentation patterns characteristic of betaine lipids, but without established betaine head group fragments (Suppl. Fig. 5b, c). The tentatively assigned sum formula for the head group of the first unknown aminolipid (AL-I) at ca. 6.7 minutes LC retention time was $C_8H_{17}NO_3$ and for the second unknown aminolipid (AL-II) at 10.5 minutes was $C_7H_{15}NO_3$. The head group sum formula for AL-II matches that of DGCC, but the diagnostic head group fragment of m/z 252 was not detected, and furthermore, AL-II did not elute at the expected earlier retention time for DGCC. AL-I and AL-II were detected at most depths at all four stations, with average abundances of 1 to 6% of the minor lipids for AL-I and comparably higher relative abundances ranging from 16 to 36% for AL-II.

Acyl-ether glycerol lipid: One minor compound that eluted slightly earlier than SQ-DAG had a fragmentation pattern similar to SQ-DAG but with exact masses of the parent ion and MS-MS fragments in both positive and negative ion mode that suggested a mixed acyl-ether glycerol core lipid structure (Suppl. Fig. 5d, e). Tentatively assigned as SQ-AEG, this IPL was observed at most depths at all four

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stations with highest relative abundances of 5 to 60% of minor IPLs within the oxic zone.

Sphingolipids: Two types of sphingolipids were identified, monoglycosyl ceramide (1G-CER), and hydroxylated monoglycosyl ceramide (1G-OH-CER) with up to two hydroxyl groups attached to the hydrophobic side chains (Suppl Fig. 3e). Both were observed at all depths at stations 1, 2, and 5 at average relative abundances between 3 and 8% of minor IPLs, but neither was detected in the deeper part of the core OMZ or deep oxycline at station 8.

Ornithine lipids: Trace amounts (<4%) of ornithine lipids were detected in the core OMZ of stations 2 and 5.

3.2.3 Statistical relationships between environmental parameters and lipid distribution

Spearman Rank Order Correlation was used to evaluate relationships between relative lipid abundance of lipid classes and environmental parameters (Table 1). The glycolipids 2G- and SQ-DAG showed highly significant ($p<0.001$) and positive correlations with depth, fluorescence, POC, TN, temperature and Chl-*α*, significant positive correlations were also observed with oxygen. Both also showed highly significant but negative correlations with phosphate and nitrate, and these overall trends were mirrored in the SQ-DAG:PG-DAG ratio. Total glycolipids (GL) and 1G-DAG only showed correlations with a few environmental parameters and total GL were only significantly positively correlated with oxygen. Most aminolipids and phospholipids did not show significant correlations with environmental parameters and any other correlations were neither strongly positive nor negative. Relative abundances of total aminolipids and aminolipid (AL) to phospholipid (PL) ratios correlated positively with ammonium. AL:PL also correlated positively with oxygen. Relative abundance of total

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phospholipids and most individual phospholipids (PG-, PE-, PME-, and PDME-DAG) correlated negatively with oxygen. The only phospholipid that significantly correlated with phosphate was PDME, however, the positive correlation is not strong (r^2<0.4).

NMDS analysis revealed that all samples from the oxic zone had a negative loading on the NMDS2 axis along with environmental variables such as oxygen, fluorescence, TN, POC and Chl-*α*. IPLs with a strong negative loading on the NMDS2 axis (<-0.2) were 1G-OH-DAG, SQ-AEG, 2G-DAG, SQ-DAG, PI-DAG and OH-DGTS. Most samples from the core OMZ and deep oxycline had a positive loading on the NMDS2 axis, together with depth, phosphate and nitrate. IPLs that showed a strong positive loading on the NMDS2 axis (>0.2) were PDME-DAG, 2G-GDGT, DPG, PME-DAG and HPH-GDGT. Almost all environmental variables had low *p*-values (<0.001), indicating highly significant fitted vectors with the exception of temperature, salinity, ammonium and nitrate. Highest goodness of fit statistic was observed with oxygen (r^2=0.54), followed by phosphate (r^2=0.48) and then fluorescence (r^2=0.46).

4. Discussion

The moderate primary productivity in surface waters of the ETNP, intense microbial degradation of particulate organic matter exported to the thermocline, and restricted midwater oxygen replenishment produce the strong, shallow (~20 m deep) oxycline and a ~500 m thick OMZ with dissolved oxygen concentrations of <2 µM, not unlike other oceanic OMZs (e.g., Ulloa et al., 2012). The ETNP is dominated by picoplankton, and micro-grazers reported consuming most phytoplankton production (Landry et al., 2011; Olsen and Daly, 2013). Peak macrozooplankton biomass was located at the thermocline, near the upper boundary of the OMZ, but a secondary biomass peak of a different

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zooplankton assemblage was present at the deep oxycline once O_2 concentrations rose to ~2 µM (Wishner et al., 2013). Shallow-water, plankton-derived particulate organic carbon is the primary food source for zooplankton in the mixed layer, upper oxycline and core OMZ, whereas deep POC, some of which might have been produced by microbes in the OMZ, is important for deep oxycline zooplankton (Williams et al., 2014). Microbial community structure and activities are typical of other OMZs (Taylor et al., 2001; Lin et al., 2006; Woebken et al., 2007; Wakeham et al., 2007; 2012). Cell numbers of total prokaryotes were highest in the euphotic layer and decreased with depth at the thermocline but rose again within the core OMZ (Podlaska et al., 2012). Elevated rates of chemoautotrophy, measured by dark dissolved inorganic carbon (DIC) assimilation, were observed at several depths in the OMZ and in the lower oxycline. Transfer of chemoautotrophically-fixed carbon into zooplankton food webs is also evident (Williams et al., 2014). Bacteria dominate the prokaryotic community at all stations. Nitrifying bacteria constituted 3-7% of total DAPI-positive prokaryotes in surface waters; sulfate-reducing bacteria (17 and 34% of total prokaryotes), planctomycetes (up to 24% of total prokaryotes), and anammox bacteria (<1% of prokaryotes) in the upper OMZ and deep oxycline might be associated with anoxic microzones within particle aggregates even at low dissolved oxygen concentrations (Woebken et al., 2007; Carolan et al., 2015). Archaeal cell abundances peaked at the start of the upper OMZ at all stations (up to 37% of total prokaryotes at station 2), within the core OMZ at station 2 (up to 54% of total detected cells) and within the deep oxycline at station 5 and 8 (around 25%; Fig. 2e). Crenarchaeota/thaumarchaeota represented ~20% of prokaryotes throughout the water column, generally being highest in the lower OMZ and deep oxycline, and at stations 2 and 5 just above the secondary Chl-*a* maxima at ~75 m. Euryarchaeota were 16-20% of total prokaryotes, especially in waters above the OMZ.

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Total IPL concentrations that were over 50 times higher in the surface waters than at deeper depths coincided with high Chl-*α* concentrations, reflecting the importance of phototrophic sources to the IPL pool above the thermocline. Below the thermocline, IPL concentrations generally track trends in microbial cell abundances, and elevated IPL concentrations in the upper and core OMZ coincide with elevated nitrite concentrations. The rapid decrease in IPL concentrations below ~100 m probably results from a combination of a dearth of potential source organisms and the decomposition of sinking detrital lipids (Harvey et al., 1986; Matos and Pham-Thi, 2009). IPL concentration decreases below the euphotic zone are well established (Van Mooy et al., 2006; Schubotz et al., 2009; Van Mooy and Fredricks, 2010; Popendorf et al., 2011b; Wakeham et al., 2012). We believe that the diverse molecular compositions and shifts in relative abundances of IPLs with changing geochemistry reflect a complex biological community structure and their ecophysiologic adaptation throughout the water column.

4.1 Provenance of IPLs in the ETNP

Variations in IPL distributions and head group and core lipid compositions reflect the oxygen-driven biogeochemical stratification of the water column. Below we discuss potential sources of and possible physiological roles for IPLs in the different zones.

4.1.1 Oxic zone

The glycosyldiacylglycerides that dominate the IPL composition in oxic surface waters, 1G-DAG, 2G-DAG and SQ-DAG, are major constituents of photosynthetic thylakoid and chloroplast membranes (Wada and Murata, 1998; Siegenthaler, 1998) and are therefore generally assigned to photosynthetic algae

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or cyanobacteria (Van Mooy et al., 2006; Popendorf et al., 2011b). These are also the likely predominant sources in our study, however, notably 1G-DAG may also be synthesized by heterotrophic bacteria (Popendorf et al., 2011a; Carini et al., 2015; Sebastian et al., 2016). In the oxic zone, 1G- and 2G-DAG are predominantly comprised of C_{16} and C_{18} fatty acids with zero to 5 double bond equivalents, polyunsaturated acid (PUFA) combinations such as $C_{16:4}/C_{18:3}$, $C_{16:4}/C_{18:4}$, $C_{18:3}/C_{16:2}$, $C_{18:4}/C_{14:0}$ and $C_{18:5}/C_{14:0}$ (Suppl. Table 5, Fig. 5). These are characteristic of eukaryotic algae (Brett and Müller-Navarra, 1997; Okuyama et al., 1993), such as diatoms and prymnesiophytes that are the major eukaryotic phytoplankton in the ETNP. SQ-DAG biosynthesized by cyanobacteria do not contain PUFA, but instead predominantly contain combinations of $C_{14:0}$, $C_{16:0}$, and $C_{16:1}$ fatty acids (e.g., Siegenthaler, 1998) yielding shorter chain lengths and a lower average number of double bonds (0.5 to 1) than the other glycolipids as observed at the ETNP (Fig. 5). Betaine lipids (DGTS) in surface waters of the ETNP are comprised of C_{14}, C_{16}, C_{18} and C_{20} with multiple unsaturations or rings (on average 1.5 to 3 double bond equivalents) and are also likely phytoplankton derived (Dembitsky, 1996; Popendorf et al., 2011a).

PC-DAG with fatty acyl combinations of $C_{22:6}$ and $C_{20:5}$ long-chain PUFA and $C_{16:0}$ fatty acids (Suppl. Table 5) in surface waters also point to primarily eukaryotic algal sources. PG-DAG is the only phospholipid in cyanobacteria and thylakoid membranes of eukaryotic phototrophs (Wada and Murata, 1998). Heterotrophic bacteria are an additional source for PG-DAG since it can be a major phospholipid in bacterial membranes (Goldfine, 1984). PE-DAG is a minor phospholipid in eukaryotic algae (e.g., Dembitsky et al., 1996) but is common in membranes of bacteria (Oliver and Colwell, 1973; Goldfine, 1984) and is biosynthesized by heterotrophic marine bacteria (Popendorf et al., 2011a). Lower average number of double bond equivalents in PG- and PE-DAG (<2) in the upper water column of the ETNP are

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consistent with a bacterial origin (Fig. 5).

Oxic ETNP waters contain PE- and PC-based phospholipids with mixed acyl and ether core lipids (AEG), which are often referred to as 1-*O*-monoalkyl glycerol ethers (MAGE) if detected as core lipids. PE-AEG have been described in some sulfate-reducing bacteria (Rütters et al., 2001), which in the oxic zone or OMZ of the ETNP would require anoxic microzones in fecal pellets or aggregates (e.g., Bianchi et al., 1992; Shanks and Reeder, 1993). In the ETNP, MAGE-based phospholipids were 1 to 30% of total IPLs. MAGE, detected as core lipids in surface waters of the Southern Ocean and eastern South Atlantic are thought to be breakdown products of IP-AEGs of aerobic bacterial origin (Hernandez-Sanchez et al., 2014), but culturing experiments have yet to confirm this conclusion. Similarly, aerobic bacteria (possibly cyanobacteria) are likely sources for SQ-AEG, since sulfoquinovosyl is a diagnostic headgroup found in cyanobacteria, although, again, these lipids have not been reported in cultured cyanobacteria. Other minor phospholipids in the euphotic zone include PI-DAG and DPG. They are minor components in several marine algae (Dembitsky, 1996) and bacteria (Morita et al., 2010; Diervo et al., 1975; Mileykovskaya and Dowhan, 2009). Bacteria may also be the source of the low detected levels of *N*-methylated phospholipids PME-DAG and PDME-DAG (Goldfine and Ellis, 1964). 3G-DAG comprised of C_{14}, C_{16} and C_{18} fatty acids with up to six double bond equivalents is another minor IPL detected in the euphotic zone at all stations except for station 5. It has been found in some plants (Hölzl and Dörmann, 2007) and some anaerobic gram-positive bacteria (Exterkate and Veerkamp, 1969), which could both be probable sources in the oxic euphotic zone of the ETNP.

The sphingolipid, 1G-CER, consists of a sphingosine backbone linked to a fatty acid via an amide bond and was a minor component in the oxic zone (<5% of IPL) at all stations (Fig. 4). Glycosidic

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ceramides occur in eukaryotic algae such as the coccolithophore *Emiliania huxleyi* (Vardi et al., 2009). We also detected 1G-OH-CER with up to 2 hydroxylations in the core lipid structure (Suppl. Fig. 4). Multiple-hydroxylated sphingoid bases are potential markers of viral infection and cell death in at least some marine phytoplankton, notably *E. huxleyi* (Vardi et al., 2009). We did not, however, find mass spectral evidence for the presence of viral polyhydroxylated 1G-CER, as described by Vardi et al. (2009) and therefore rather suggest that eukaryotic algal cells are potential sources for the 1G-CER (Lynch and Dunn et al., 2004) in surface waters of the ETNP. We also detected hydroxylated glycolipids (1G-OH-DAG) and aminolipids (OH-DGTS) with up to two hydroxyl-groups or one hydroxyl group combined with an epoxy or keto function attached to the acyl groups (Suppl. Fig. 4). The addition of hydroxyl groups or general oxidation of fatty acids in plants, algae and yeast is a defense mechanism and response to oxidative stress (Kato et al., 1984; Andreou et al., 2009). Hydroxy fatty acids, for example, are intermediates in oxidative degradation of fatty acids (Lehninger, 1970), and since they are constituents of structural biopolymers of many microorganisms (Ratledge and Wilkinson, 1988), they are present in marine particulate matter (e.g., Wakeham, 1999), likely derived from membrane constituents of Gram-negative bacteria, the most abundant bacteria in seawater (Rappé and Giovannoni, 2000).

4.1.2 Upper OMZ

Glycolipid abundance varied between 15 to 80% of total IPL within the upper OMZ below the thermocline/oxycline. SQ-DAG and 2G-DAG exhibited strong decreases in relative and absolute abundance below 125 m at all stations consistent with the decrease in their phototrophic biomass. Number of carbon atoms in the core lipid chains and number of double bond equivalents of glycolipids

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showed considerable variations within the upper OMZ (Fig. 5), indicating a different assemblage of source organisms compared to the oxic zone. Likewise, decreasing carbon numbers and double bond equivalents for PC-DAG and DGTS combined with a dominance by C_{14}, C_{16} and C_{18} saturated and monounsaturated fatty acids (Suppl. Table 5) supports a shift from eukaryotic to bacterial sources. This suggests the diverse proteobacteria in the upper OMZ may biosynthesize non-phosphorus substitute IPLs. 1G-DAG or DGTS are known to replace phospholipids, primarily PE-DAG and PC-DAG under phosphorus limited growth (Geske et al., 2012; Carini et al., 2015; Sebastian et al., 2016; Yao et al., 2015), including at the phosphate concentrations of 2 to 2.5 µM in the upper OMZ. Sulfate-reducing proteobacteria, which comprise up to 10% of the total bacteria in the ETNP (Podlaska et al., 2012) may be candidate organisms for this phospholipid to glycolipid replacement (Bosak et al., 2016). Structures of minor IPLs, AL-I and AL-II were not fully elucidated (see Suppl. Fig. 5) and their origins remain uncertain. PME- and PDME-DAG, DPG, 1G-CER and 1G-OH-CER within the upper OMZ are consistent with previous reports of their production by (unidentified) bacteria near redox boundaries in other stratified water bodies (Schubotz et al., 2009; Wakeham et al., 2012).

Archaeal IPLs with glycosidic headgroups and tetraether core structures (1G- and 2G-GDGT) comprised a greater proportion of the overall IPL pool within the upper OMZ than in surface waters. Analysis of these same samples by Xie et al. (2014) first reported that concentrations of glycosidic GDGTs peak in the ETNP roughly at depths where nitrite maxima are observed. IP-GDGTs with the hexose-phosphate-hexose (HPH) headgroups and the core GDGT crenarchaeol (Suppl. Fig. 3) of thaumarchaeota (Schouten et al., 2008; Elling et al., 2017) were most abundant at depths of nitrate maxima at all ETNP stations, as they are in other oxygen-deficient water columns (e.g., Pitcher et al., 2011; Lengger et al.,

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2012; Schouten et al., 2012; Sollai et al., 2015), although they were present at greater depths in the ENTP as well. The microbial enumerations by Podlaska et al. (2012) had shown previously that thaumarchaeota (referred to as crenarchaeota) and euryarchaeota constitute almost equal amounts to <10% of total cell number in the upper OMZ of the ETNP. Therefore, we conclude that uncultured marine Group II euryarchaeota, are also potential sources for glycosidic GDGTs as has been suggested previously (Lincoln et al., 2014; Zhu et al., 2016).

4.1.3 Core OMZ and deep oxycline

IPL distributions in the core OMZ and at the deep oxycline of the ETNP that were notably different from the oxic zone and the upper OMZ are consistent with *in-situ* microbial origins. Phospholipid abundance at all stations generally increased to over 50% (except for station 8) at the expense of glycolipids. PE and PG-DAG are the most abundant phospholipids in the core OMZ, along with PC-DAG and PE- and PC-AEG, DPG. PME and PDME-DAG are all common lipids in α-, γ- and some β-proteobacteria (Oliver and Colwell, 1973; Goldfine, 1984) that are present in the OMZ (Podlaska et al., 2012). Changes in phospholipids chain length and number of double bond equivalents further support *in-situ* IPL production (Fig. 5). Fatty acid combinations for phospholipids were dominated by saturated $C_{14:0}$, $C_{15:0}$ and $C_{16:0}$ and monounsaturated $C_{16:0}$ C_{17} and $C_{18:0}$ (Suppl. Table 5); PUFA were generally of reduced abundance, and odd-numbered fatty acids increased in proportion. In the case of PUFA, even though they may be biosynthesized by piezophilic aerobic deep-sea bacteria (DeLong and Yayanos,1986, Fang et al. 2003; Valentine and Valentine, 2004), either the microaerophilic bacteria in the deep OMZ of the ETNP do not produce PUFA or these labile fatty acids are rapidly degraded *in-situ* (DeBaar et al., 1983;

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Prahl et al., 1984; Neal et al., 1986).

Among glycolipids, 1G-DAG was most abundant at the deep OMZ/oxycline at stations 1 and 8; here 1G-DAG abundance actually increases over that of shallower depths. Carbon number and number of double bond equivalents for glycolipids are again distinct from the surface waters, with on average 1 to 2 carbon atoms shorter chain lengths and 1 to 3 fewer double bonds (Fig. 5), supporting the notion that at least some of these glycolipids are biosynthesized *in-situ* and not simply exported from the surface waters. In particular, SQ-DAG in the core OMZ/oxycline contained odd-carbon numbered fatty acids (e.g., $C_{15:0}$/$C_{16:0}$ and $C_{14:0}$/$C_{15:0}$) different from the cyanobacterial SQ-DAG in surface waters (Suppl. Table 5). Some Gram-positive bacillus and firmicutes biosynthesize 1G, 2G- and SQ-DAG (Hölzl and Dörmann, 2007) and 1G-, 2G- and SQ-DAG in deeply buried Wadden Sea sediments are attributed to anaerobic bacteria (Seidel et al., 2012). However, Gram-positive bacteria are generally not abundant in seawater.

The core OMZ/deep oxycline are particularly enriched in archaeal GDGT, notably 1G-GDGT and HPH-GDGT, with predominantly GDGT-0 and crenarchaeol as core lipids (Suppl. Fig. 3). At stations 1 and 8 where sampling penetrated below ~800 m depth, 1G-GDGT and HPH-GDGT constitute up to ~60% and ~22%, respectively, of total IPL. Significantly, the elevated abundances of 1G-GDGT and HPH-GDGT at the bottoms of the sampling depth profiles in the deep oxycline of stations 1 and 8 correspond to depths at which ammonium concentrations are higher than shallower in the core OMZ (Fig. 2). Remineralization at the deep-oxycline might provide additional ammonium to drive thaumarchaeotal ammonium oxidation and production of archaeal IPLs.

4.2 Factors influencing IPL distribution in the ENTP

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4.2.1 Factors affecting structural diversity of the core lipid composition

IPL in the ETNP display considerable diversity not only in the headgroup but also core lipid types, from diacylglycerol lipids with varying number of carbon atoms (likely chain lengths) and zero to multiple double bond equivalents (likely reflecting the number of unsaturations), with or without hydroxylations to mixed ether/ester glycerolipids, sphingolipids and ornithine lipids. Statistical analysis provides aids in illuminating influences of environmental factors and microbial community structure on the lipid composition in the water column of the ETNP. Changes in core alkyl lipid chain length and degree of unsaturation are often associated with temperature (Neidleman, 1987), even at the range of temperatures of the ETNP water column. However, NMDS analysis did not yield any strong correlations between temperature and number of carbon atoms in the side chains or double bond equivalents of the major IPL classes ($r^2 < 0.02$, Suppl. Table 6), nor with other environmental parameters (r2 < 0.3, Suppl. Table 6). Instead, changing biological sources may play a decisive role in determining number of carbon atoms and double bond equivalents in the ETNP. For instance, long-chain PUFAs in surface waters are mainly synthesized by phytoplankton, while in deeper waters some bacteria may biosynthesize these PUFAs. The degree of hydroxylation in the acyl side chains also did not show any clear link to specific environmental factors, although, both 1G-OH-CER and OH-DGTS had negative loadings on the NMDS-2 axis indicating a higher abundance of these compounds in oxic samples. It is possible that hydroxylated IPLs play a role during oxidative stress and/or are involved in other defense mechanisms (Kato et al., 1984; Andreou et al., 2009).

Mixed ether-acyl lipids have been reported in various oceanic settings (Hernandez-Sanchez et al., 2014). In our study, there was no noticeable correlation between PE- and PC-AEG and depth or oxygen

concentrations (Fig. 6). Ornithine lipids were strongly negatively loaded on the NMDS-1 axis, but none of the measured environmental parameters could account for this negative loading (Fig. 6). Therefore, it remains unclear what factor(s) ultimately determine their distribution. Likewise, there were no significant correlations between the sphingolipid 1G-CER and any environmental parameter. Since ether-acyl lipids, ornithine lipids and sphingolipids play many functional roles in biological systems, their variable distribution within the water column reflect most likely the diversity of microbes inhabiting the dynamic oxygen regime of the ETNP.

4.2.2 Factors influencing head group composition

NMDS analysis of normalized IPL composition and quantitative microbial data (abundance of α, β, γ, ε-proteobacteria, sulfate-reducing bacteria δ-proteobacteria, planctomycetes, crenarchaeota including thaumarchaeota and euryarchaeota) did not yield any high goodness of fit statistic ($r^2 < 0.3$; Suppl. Table 6) that would clearly delineate specific prokaryotic sources for the various IPL. This absence of statistical correlation would result if neither the IPL compositions of SPM nor the structure and lipid composition of the prokaryotic community were sufficiently unique to strongly distinguish the biogeochemical zones. Indeed, although there are depth-related differences in IPL composition of SPM and prokaryotic community, there is considerable overlap. Therefore, instead of trying to elucidate specific IPL sources, we here query the affect environmental factors such as temperature, nutrient or oxygen concentrations may have on the IPL compositions in the ENTP, and by analogy to natural marine settings in general. Most the major and minor glycolipids were loaded negatively on the NMDS2 axis, as were oxygen, fluorescence, Chl-*a*, POC and TN (Fig. 6). A notable exception was 1G-DAG which

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had only a slightly negative loading on the NMDS-2 axis. These relationships (loadings) roughly reflect the vertical distribution of IPLs in the water column of the ETNP. Glycolipids, particularly 2G-DAG and SQ-DAG, were most abundant in the oxic zone characterized by high oxygen concentration and moderate primary productivity, dominated by phytoplankton, primarily cyanobacteria (high POC, TN and elevated Chl-α and fluorescence). Spearman Rank Order Correlations confirm these observations, including the lack of significant correlations between 1G-DAG and depth or any other environmental parameter. One explanation is that 1G-DAG originates from assorted sources throughout the water column independent of any single environmental variable. Similarly, PC-DAG, PG-DAG, and DGTS did not correlate with any of the tested environmental variables, because their compositions are relatively homogeneous across all biogeochemical zones. PE-, PME- and PDME-DAG, and DPG, on the other hand, that became more prevalent within the core OMZ, and at deeper depths where oxygen concentrations decrease and nutrient (NO_3^- and PO_4^{3-}) concentrations were elevated due to organic matter remineralization, gave positive loadings with these environmental parameters on the NDMS2 axis. Archaeal IPLs showed positive loadings on the NMDS2 axis, consistent with the increasing importance of archaeal abundance with depth and at reduced oxygen concentrations.

4.2.3 Links between substitute lipid ratios and nutrient concentrations

SQ-DAG and PC-DAG are often the most abundant respective glycolipids and phospholipids in the surface ocean (Popendorf et al., 2011a,b), including the Eastern Tropical South Pacific (Van Mooy and Fredricks, 2010). The abundance of SQ-DAG in the surface waters of the ETNP (18-50% of total IPL) is thus not unusual. In the ETNP, however, PC-DAG was comparably minor (3-13% of total IPL).

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Instead, DGTS was abundant at some stations, up to ~20% of major IPL at station 5. SQ-DAG and DGTS serve similar biochemical functions as the phospholipids PG-DAG and PC-DAG, respectively, due to similar ionic charges at physiological pH. The former may be preferentially biosynthesized by phytoplankton and some bacteria as substitute lipids for PG-DAG and PC-DAG when phosphate starved (Benning, 1993; Van Mooy et al., 2006, 2009). Likewise, 1G-DAG, glycuronic acid diacylglycerol (GADG) and ornithine lipids may substitute for PE-DAG in marine bacteria (e.g., chemoheterotrophic α-proteobacteria of the SAR11 clade of *Pelagibacter* sp.: Carini et al., 2015; the sulfate reducing bacterium, *Desulfovibrio alaskensis*: Bosak et al., 2016). In oligotrophic surface waters of the Sargasso Sea (PO_4^{3-} <10 nM) ratios of SQ-DAG:PG-DAG and DGTS:PC-DAG are high (4 to 13) compared to the same ratios (3) in the phosphate replete South Pacific (PO_4^{3-} >100 nM), consistent with cyanobacteria synthesizing phosphorus-free substitute lipids to maintain growth in response to phosphorus deprivation (Van Mooy et al., 2009). At the ETNP, SQ-DAG:PG-DAG ratios ranged between 1 and 10 within the upper 100-200 m along the transect and were <1 deeper into the OMZ (Fig. 3). DGTS:PC-DAG ratios in the ETNP were quite variable, ranging between 0.4 and 2.4 at most depths, but with notable spikes (>30) within the oxic zone at station 5, within the upper core OMZ at station 2 and 8 and in the lower portion of the core OMZ at station 8. 1G-DAG:PE-DAG ratios where highly variable (0.2 to 945) and were highest within the upper OMZ at station 2, 5 and 8 and within the deep oxycline at station 8, where 1G-DAG:PE ratios range between 290 and 945 (Fig. 3). To test the substitute lipid hypothesis for the ETNP, we performed a Spearman Rank Order Correlation analysis of known substitute lipid ratios as well as total aminolipid (AL) to phospholipid (PL) and total glycolipid (GL) to PL ratios with nutrient concentrations and other environmental parameters. Only SQ-DAG:PG-DAG was significantly correlated with phosphate (-0.56,

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p<0.001) but also correlated with other parameters, such as depth (-0.76, p<0.001) and oxygen concentration (0.58, p<0.001). These correlations reflect the elevated SQ-DAG:PG-DAG ratios (2-8) in the surface waters and upper OMZ (Fig. 3) and support the notion that SQ-DAG might serve as a substitute lipid in both surface waters and the OMZ when phosphate concentrations are in the low micromolar range (~0.1-0.4 µM in surface waters; ~2-3.5 µM in the OMZ). Other proposed substitute lipid ratios, DGTS:PC-DAG (Van Mooy et al., 2009) and 1G-DAG:PE-DAG (Carini et al., 2015), did not correlate with nutrient concentrations in the water column of the ETNP but rather showed highly variable distributions. Similarly, AL:PL ratios did not exhibit strong relationships with any environmental parameter, and GL:PL ratios showed similar but less pronounced trends as SQ-DAG:PG-DAG ratios. Overall, we observed no correlation between these substitute lipid ratios and phosphate concentration in the ETNP. We propose that non-phosphorus IPL within the OMZ of the ETNP originate from bacteria growing under low micromolar concentrations of phosphate. Indeed, the culture experiments of Bosak et al. (2016) demonstrated that the sulfate reducer, *Desulfovibrio alaskensis*, begins to replace most of its membrane phospholipids with 1G-DAG, glycuronic acid diacylglycerol and ornithine lipids even at phosphate concentrations as high as 20 µM.

5. Conclusions

The water column of the ETNP is characterized by a diverse suite of intact polar lipids. IPL distributions reflect the dynamic nature of the biological community in the ETNP, with oxygen as a primary determinant, from fully oxygenated surface waters to a strong oxygen minimum zone at mid-depth. Highest concentrations of IPLs (250 – 1500 ng/L) in oxygenated surface waters zone results from

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abundant phototrophic eukaryotic and cyanobacterial sources above the OMZ. , Secondary peaks in IPL concentration (12 – 56 ng/L) within the core of the OMZ mirror elevated abundances of heterotrophic and chemoautotrophic bacteria and archaea under low oxygen conditions. Glycolipids derived from photoautotrophs generally accounted for more than 50% of total IPLs in the euphotic zone (< 200 m, oxic and upper OMZ zones), but bacterial phospholipids were more abundant (avg. 40%) in the OMZ and deep oxycline layers. Archaeal GDGTs were abundant within the OMZ and deep oxycline, consistent with elevated archaeal abundances there. Variations in major fatty acid constituents within IPL classes with acyl core moieties show that biological source(s) for the different IPL were distinct in each depth/oxygen-content horizon. Nevertheless, microbial sources for many of the detected lipids remain unclear and therefore potentially unique ecophysiological adaptations these lipids may represent remain to be explored.

The presence of the glycolipid, monoglycosyl diacylglycerol (1G-DAG), and the betaine lipid, diacylglyceryl homoserine (DGTS), both with varying fatty acid compositions, within all biogeochemical zones, and especially in the OMZ, indicates that these canonical phototrophic markers are not only biosynthesized in surface waters, but may indeed be produced in the aphotic water column and by a much larger host of organisms than previously thought. Since 1G-DAG and DGTS can be biosynthesized by various bacteria to replace phospholipids under phosphorus limited growth, we suggest that they serve as non-phosphorus substitute lipids for some microorganisms in the OMZ. The presence of these substitute lipids at micromolar concentrations of phosphate of the ETNP suggests that the paradigm of substitute lipid biosynthesis being restricted to the PO_4^{3-}-depleted oligotrophic surface ocean may need to be re-evaluated.

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Author contribution

SGW collected the samples. SGW, FS and KUH designed the study. SX and FS measured and processed the data. JSL and FS performed statistical analyses. FS and SGW wrote the paper with input from SX, KUH and JSL.

Competing interests

The authors declare that they have no conflict of interest.

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Tables

Table 1. Spearman Rank Order Correlation coefficients (r) for data combined from all four stations. Only significant correlations, where $p < 0.05$ (highly significant $p < 0.001$, in bold), are presented.

	Glycolipids						Aminolipids				Phospholipids					
	% GL	% 1G	% 2G	% SQ	GL:PL	SQ:PG	% AL	% DGTS	AL:PL	DGTS:PC	% PL	% PC	% PG	% PE	% PME	% PDME
Depth	-0.32		**-0.7**	**-0.67**	-0.41	**-0.76**										
Fluorescence			**0.63**	**0.67**		**0.65**										
POC			**0.61**	**0.6**		**0.6**										
TN			**0.66**	**0.62**		**0.63**										
Oxygen	**0.57**	0.3	**0.48**	0.35	**0.55**	**0.58**			0.36		**-0.49**		-0.38	-0.33	-0.46	-0.52
Temperature	0.3		**0.52**	**0.63**	0.39	**0.69**										
Chl a	0.35		**0.72**	**0.71**	0.42	0.78										-0.33
Phosphate			**-0.62**	**-0.53**	-0.4	**-0.56**										0.36
Nitrate			**-0.53**	**-0.49**		-0.38										
Nitrite		-0.33													0.3	
Ammonium							0.41	0.42	0.35	0.4						
N:P			-0.3	-0.32									-0.36			

Abbreviations: GL – glycolipids, 1G – monoglycosyl, 2G – diglycosyl, SQ – sulfoquinovosyl, PL – phospholipids, AL – aminolipids, DGTS – diacylglyceryl trimethyl homoserine, PC – phosphatidyl choline, PG – phosphatidyl glycerol, PE – phosphatidyl ethanolamine, PME – phosphatidyl methyl-ethanolamine, PDME – phosphatidyl dimethyl-ethanolamine

Figures

Figure 1. a) Map of ETNP with R/V *Seward Johnson* (November 2007) cruise sampling stations investigated in this study.

Figure 2. Depth profiles of (a) oxygen and temperature, (b) chlorophyll-*α* and transmissivity, (c) particulate organic matter (POC) and C:N, (d) intact polar lipid (IPL) to POC ratio and IPL concentration, and (e) absolute cell abundance and relative proportions of archaeal cells (data from Podlaska et al. (2012)). C:N (SPM) is total carbon over total nitrogen of the solid phase collected by water filtration. Note that C:N, POC and IPL/POC are only analyzed for <53 µm particle fraction. Also depicted are the different geochemical zones in the water column.

Figure 3. Depth profiles of (a) nitrate, nitrite, and ammonium, (b) phosphate and N:P, (c) total non-archaeal (non-isoprenoidal) phospholipids, glycolipids and (d) aminolipids shown as percent of total intact polar lipids and ratios of non-phospholipids to phospholipids for DGTS to PC-DAG (e) SQ-DAG to PG-DAG, (e), and 1G-DAG to PE-DAG. Also depicted are the different geochemical zones in the water column.

Figure 4. Relative abundance of (a) major and (b) minor IPLs at sampled depths of stations 1, 2, 5, and 8 in the ETNP. Major IPLs are defined as those comprising more than 10% of total IPLs (minor compounds comprised less than 10%) at more than one depth horizon at the four stations. Also depicted are the different geochemical zones in the water column.

Moved down [11]: Depth profiles of (b) oxygen, (c) temperature, (d) chlorophyll-*α* and (e) transmissivity along a northwest-southeast transect of the study area. Numbers across the top panels denote station, black dots are individual samples.

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Figure 5. Changes in average carbon atoms (CA) and number of double bond equivalents (DB) of the alkyl side chains of major IPLs detected at stations 1, 2, 5 and 8 in the ETNP.

Figure 6. Nonmetric multidimensional scaling (NMDS) ordination plot assessing the relationship between IPL biomarkers, sampling depths and geochemical parameters in the ETNP (stress=0.125). Squares represent the water depth of each sample and are color-coded according to the defined geochemical zonation. Filled circles stand for lipid distribution of major IPLs and open circles for minor IPLs on the ordination. Vector lines of geochemical parameters are weighted by their p-values with each NMDS axis.

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The phospholipid substitute ratio SQ-DAG to PG is based on the observation that

cyanobacteria [FS1]biosynthesize SQ-DAG preferentially over PG when phosphorus limited (Benning, 1993; Van Mooy et al., 2006, 2009) and is here for the purpose of this discussion extended to other bacteria and eukarya that are probable sources of IPLs in subsurface waters. At the ETNP this ratio ranged between 1 and 10 within the upper 100-200 m along the transect and is <1 deeper into the OMZ. The ratio of DGTS to PC is reflective of the algal response to phosphorus limitation since it was observed that microalgae and some bacteria substitute PC-DAG with DGTS when phosphate concentrations are low (Van Mooy et al., 2009; Zavaleta-Pastor et al., 2010). At the ETNP this ratio did not show consistent trends and ranges between 0.4 and 2.4 at most depths, but with notable spikes (>30) within the upper core OMZ at station 2 and 8, in the oxic zone at station 5 and in the lower portion of the core OMZ at station 8. Similarly, the ratio of 1G-DAG to PE, which has been recently proposed to reflect the response of heterotrophic bacteria to phosphorus limitation (Carini et al., 2015) did not show any consistent trend but generally ranges between 0.2 and 6 at most depths except for highly elevated values within the upper OMZ at station 2, 5 and 8 with ratios up to 800 and within the deep oxycline at station 8, where 1G-DAG:PE ratios range between 650 and 950 (Fig. 5).

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Major lipids are defined here as those IPL compound classes that comprised more than 10% of total IPLs at more than one depth at the four stations.

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A previous study on archaeal lipid distributions in these same ETNP samples (Xie et al., 2014) reported on only the two glycosidic archaeal IPLs (1G-GDGT and 2G-GDGT). In that study the TLE had been separated into fractions and only the glycosidic fractions had been analyzed for GDGTs. Subsequent re-examination of the original LCMS data indicate that HPH-GDGT were indeed present in an unanalyzed fraction (fraction 3 in Xie et al., 2014). The remaining eight major IPLs were assigned to either a bacterial or eukaryotic origin and were three glycolipids (1G-DAG, 2G-DAG, SQ-DAG), four phospholipids (PG-DAG, PE-DAG, PC-

DAG, PE+PC-AEG) and one aminolipid (DGTS).

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Minor lipids are defined as those IPL compound classes that comprised less than 10% of total IPLs at more than one depth of the four stations. In total 13

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Minor lipids are defined as those IPL compound classes that comprised less than 10% of total IPLs at more than one depth of the four stations. In total 13

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Carbon and nitrogen stable isotopes in ETNP SPM (splits of the same SPM filters used for IPL analyses) and zooplankton suggest that

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our expedition were investigated via CARD-FISH by Podlaska et al (2012).

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these are observations typical for other oxygen-deficient regions such as the upwelling area off the coast of Namibia (Woebken et al., 2007) and anoxic basins, e.g., the Black Sea and Cariaco Basin (Taylor et al., 2001; Lin et al., 2006; Wakeham et al., 2007, 2012). Bacteria dominate the prokaryotic community at all stations, but archaeal abundance can be as high as 50% and 26% at the bottom of the OMZ at stations 2 and 5, respectively. Heterotrophic activity, measured by uptake of leucine, was prevalent in and above the thermocline/upper oxycline where reactive organic matter is most available.

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Dark DIC assimilation correlated with total prokaryote abundances. Mid-water microbial chemoautotrophy was further indicated by stable carbon isotope values for POC in the upper and core OMZ that are depleted by 2 to 6‰ at the upper oxycline and within the OMZ compared to $\delta^{13}C$ values of -24 to -21‰ for surface water plankton (Podlaska et al., 2009).

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peaks where nitrate was not detectable and in the upper OMZ where ammonium was depleted but nitrate and nitrate were high.

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Similarly, anoxic microzones may be responsible for observed abundances of Planctomycetes (Pla46-positive cells; up to 24% to total prokaryotes), and anammox bacteria (Amx368-positive

cells; <1% of prokaryote numbers; Podlaska et al., 2012) and ladderane lipids in the OMZ correspond with secondary nitrite maxima. Nitrate deficits point to nitrate reduction as a source for nitrite in the OMZ.

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These peaks in archaeal cells are further corroborated by maxima in 1G- and 2G-GDGT abundances at 120 m and 725 m at station 1 and at 200 m and 550 m at station 8 reported by (Xie et al., 2014).

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, and generally correlating with ammonium concentrations and heterotrophic potential

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This is consistent with SQ-DAG being primarily derived from marine cyanobacteria that mainly have saturated and monounsaturated C_{14} and C_{16} fatty acids (e.g., Siegenthaler, 1998). Cyanobacteria are therefore likely the primary source organisms for all three glycolipids in the euphotic zone and upper OMZ of the ETNP as they were abundant from the surface waters into the upper OMZ (as indicated by divinyl chlorophyll *α*, a diagnostic pigment for *Prochlorococcus* cyanobacteria, Suppl. Table 1; see also Goericke et al., 2000; Ma et al., 2009), notably at the secondary fluorescence maxima that were observed just below the thermocline, especially at stations 1 and 8. The PUFA fatty acids in 1G-DAG and 2G-DAG additionally indicate mixtures of eukaryotic algae as source for these lipids. The presence of eukaryotic algae, such as diatoms (characteristic pigment: 19'hexanoyloxyfucoxanthin) and Prymnesiophytes (characteristic pigment: 19'butanoyloxyfucoxanthin; Suppl. Table 1) albeit not as abundant as cyanobacteria, is also indicated by the detection of

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Betaine lipids such as DGTS, which are also diagnostic eukaryotic algal markers (Dembitsky, 1996; Popendorf et al., 2011a), are present in surface waters of the ETNP in abundances similar to those of PC. Major acyl moieties of betaine lipids were C_{14}, C_{16}, C_{18} and C_{20} with multiple unsaturations (on average 1.5 to 3 double bonds).

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minor components in several types of marine algae (Dembitsky, 1996) and bacteria (Morita et al., 2010; Diervo et al., 1975; Mileykovskaya and Dowhan, 2009), mixed inputs are likely for these IPLs. Likewise, bacteria in the oxic euphotic zone may be the origin of the low detected

levels of *N*-methylated phospholipids (Goldfine and Ellis, 1964).

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Core lipid chain length and number of double bonds of all three glycolipids showed considerable variations within the upper OMZ (Fig. 6), indicating a change in source organisms compared to the oxic zone.

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are other non-phosphorus-containing IPL that remain abundant within the minor IPLs in the upper OMZ. Since we could not elucidate the structure of theses lipids (Supp. Fig. 4) potential bacterial source(s) remain unclear. Phospholipids

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G-CER and 1G-OH-CER were detected in the upper OMZ in similar relative amounts as in the oxic zone but microbial sources for these IPLs in suboxic environments remain unclear. Their abundant presence in the anoxic zones of the stratified Black Sea (Schubotz et al., 2009) and Cariaco Basin (Wakeham et al., 2012) has been previously assigned to as yet unidentified anaerobic bacteria. The oxygenated glycolipids and betaine lipids that were observed in the oxic zone are also present within the upper OMZ at most of the stations and thus underline a

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, all of which were abundant within the core OMZ and deep oxycline (20 to 40% of the total bacterial population, Podlaska et al., 2012). C

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were distinctly different within the core OMZ compared to the overlying water column

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For instance, the PUFA that were observed in the euphotic zone and that are widespread in marine phytoplankton (Brett and Müller-Navarra, 1997; Okuyama et al., 1993) became less abundant at the deeper depths (Suppl. Table 3), indicating either the decline of sources or rapid degradation of these labile, highly unsaturated compounds (De Baar et al., 1983; Prahl et al., 1984, Neal et al., 1986). Degradation is the more likely scenario since marine bacteria, including deep-sea species, are capable of biosynthesizing PUFAs (DeLong and Yayanos,1986, Fang et al. 2003; Valentine and Valentine, 2004). PE and PG-DAG were the most abundant phospholipids in the core OMZ, followed by PC-DAG and PE- and PC-AEG. We interpret the increase in phospholipid abundance as due to the increase in bacterial abundance within the OMZ. This is also reinforced by the increase of DPG, PME and PDME-DAG among the minor lipids (Fig. 4). Multiple bacterial sources are possible since PE, PG and DPG are common phospholipids in membranes of most proteobacteria (Oliver and Colwell, 1973; Goldfine, 1984), and genes for the synthesis of PME, PDME and PC are widespread among

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deep-sea species, are capable of biosynthesizing PUFAs (DeLong and Yayanos,1986, Fang et al. 2003; Valentine and Valentine, 2004).

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α-, γ- and some β-proteobacteria, all of which were abundant within the core OMZ and deep

oxycline (20 to 40% of the total bacterial population, Podlaska et al., 2012).

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further underpins a bacterial origin for these phospholipids.

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further support a bacterial source for this IPL, despite its widespread attribution as cyanobacterial marker in environmental studies. Indeed, SQ-, 1G- and 2G-DAG have been reported in s

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were not specifically targeted in previous phylogenetic characterizations of the OMZ of the ETNP (Podlaska et al., 2012). However, in the OMZ of the eastern tropical South Pacific Gram-positive bacteria such as Actinobacteria accounted only for a negligible amount of total prokaryotic community (Stevens and Ulloa, 2008) and are thus likely not contributing significantly to the glycolipids in the core OMZ.

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Aminolipids, DGTS and betaine lipid like AL-II, were observed in similar relative abundances in the core OMZ and deep oxycline as in the overlying shallower water column. The presence of DGTS has so far only been reported in a few aerobic proteobacteria, and then only when grown under phosphorus limitation (Benning et al., 1993; Geiger et al., 1999; Sebastian et al., 2016). Consequently, potential bacterial sources for the aminolipids in the core OMZ remain elusive, particularly since these regions are not considered to be phosphorus limited with phosphate concentrations exceeding several micromolar (Fig. 2).

Bacterial sources for 1G-CER and 1G-OH-CER within the core OMZ and deep oxycline remain similarly unresolved, but as suggested for anoxic water columns (Schubotz et al., 2009; Wakeham et al., 2012), uncultured anaerobic bacteria are potential source organisms. Ornithine lipids, also non-phosphorus containing lipids but which are not known to play important roles in lipid substitutions (cf. Geiger et al., 2010), were present in minor to trace

amounts within the core OMZ and can be assigned to Gram-negative bacteria.

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For several of the major IPLs, such as 2G-DAG, PC-DAG and DGTS, the average chain length and number of double bonds increased again to levels observed in surface waters within the deep oxycline layer (Fig. 6). PC-DAG and DGTS both contained long-chain PUFA, specifically in the case of DGTS and 2G-DAG, $C_{16:4}$ and $C_{18:3}$. [FS2]

Head group variations of GDGTs in t

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depth region were similar to their distributions in the upper OMZ, with a notable increase of

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s with depth at stations 2 and 5. Similar to the upper OMZ, archaeal sources for the detected IPL-GDGTs could be either euryarchaeota, crenarchaeota or thaumarchaeota (Lincoln et al., 2014; Elling et al., 2017) as these phyla were detected within the core OMZ and deep oxycline of the ETNP (Podlaska et al., 2012). Relative archaeal IPL abundances within the core OMZ and deep oxycline vary between stations, but were highest at station 8 where they reach over 50% of the total microbial IPLs. Elevated abundances of archaea had been enumerated by quantification via CARD-FISH (Podlaska et al., 2012), with highest abundances (25 to 50% of total DAPI-stained cells) at stations 2 and 5. It should be noted again, howeve

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r, that >50% of the microbial populations that were DAPI-positive cells remained uncharacterized by the CARD-FISH approach used (Suppl. Fig. 2).

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Relative abundances of IPLs in the oxic zone of the ETNP were distinct from IPL distributions in surface waters of other oceanic ocean regions where SQ-DAG and PC-DAG were typically the most abundant compounds within the glycolipids and phospholipids, respectively (Van Mooy and Fredricks, 2010; Popendorf et al., 2011a,b). Whereas SQ-DAG

was among the most abundant IPL in the surface waters of the ETNP (18-50%), PC-DAG was comparably minor (3-13%). This difference might result from the highly compressed mixed layer of the ETNP compared to other locales, with consequent differences in plankton ecology. Alternatively, there could be differences in physiologic adaptations and hence membrane lipid modifications between the ETNP and other regions, (e.g., Van Mooy et al., 2009). In general, our study confirms the dominance of glycolipids as a common feature of surface ocean waters in which IPL distributions have been determined, in particular the Black Sea (Schubotz et al., 2009), the Eastern Subtropical South Pacific (Van Mooy and Fredricks, 2010), the Western North Atlantic (Popendorf et al., 2011a), the Mediterranean Sea (Popendorf et al., 2011b) and the Cariaco Basin (Wakeham et al., 2012). In addition, the present study highlights the potential importance of glycolipids and other non-phosphorus lipids for bacteria in low oxygen environments as will be discussed in detail below.

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The NMDS analyses and Spearman Rank Order Correlations provide a better understanding of the influence of environmental factors and the microbial community structure on the IPL composition in the water column of the ETNP.

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There are several potential reasons for this, the most likely being that

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derive from eukaryotes in the oxic zone and secondly because many of the proteobacteria in fact also biosynthesize similar IPL assemblages. Changes in bacterial

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structure might not necessarily result in significant variations in

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thus compare any environmental impact with what has been observed in culture studies and

other

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At the ETNP this ratio ranged between 1 and 10 within the upper 100-200 m along the transect and is <1 deeper into the OMZ. The ratio of DGTS to PC is reflective of the algal response to phosphorus limitation since it was observed that microalgae and some bacteria substitute PC-DAG with DGTS when phosphate concentrations are low (Van Mooy et al., 2009; Zavaleta-Pastor et al., 2010). At the ETNP this ratio did not show consistent trends and ranges between 0.4 and 2.4 at most depths, but with notable spikes (>30) within the upper core OMZ at station 2 and 8, in the oxic zone at station 5 and in the lower portion of the core OMZ at station 8. Similarly, the ratio of 1G-DAG to PE, which has been recently proposed to reflect the response of heterotrophic bacteria to phosphorus limitation (Carini et al., 2015) did not show any consistent trend but generally ranges between 0.2 and 6 at most depths except for highly elevated values within the upper OMZ at station 2, 5 and 8 with ratios up to 800 and within the deep oxycline at station 8, where 1G-DAG:PE ratios range between 650 and 950 (Fig. 5).

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Because they have similar biochemical functions and the same ionic charge at physiological pH, SQ-DAG and DGTS are known as substitute lipids for PG-DAG and PC-DAG, respectively, when phosphorus is limiting (Benning et al., 1993; Van Mooy et al., 2009; Popendorf et al., 2011b).

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The elevated ratios of SQ-DAG:PG-DAG and DGTS:PC-DAG in the

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Underlining this observation, relative abundance of phospholipids was positively correlated with phosphate concentration across the Mediterranean Sea (Popendorf et al., 2011b). Microcosm incubations of seawater from the Mediterranean Sea supplemented with phosphate and ammonium confirmed that changes in substitute lipid ratios were partly caused by a

physiological response to nutrients (Popendorf et al., 2011b). However, neither of these substitute lipid ratios was significantly correlated with abundance of phosphate in surface waters of the Eastern Subtropical South Pacific (Van Mooy and Fredericks, 2010), leading the authors to conclude that not only phosphate limitation but also algal community structure may impact these ratios.

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To further explore the possibility that SQ-DAG and aminolipids (DGTS) in the OMZ of the ETNP might serve as substitute lipids,

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Diverse intact polar lipids, including four classes of diacylglycerol glycolipids (with monoglycosyl, diglycosyl, triglycosyl and sulfoquinovosyl head groups), seven diacylglycerol phospholipids (with phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl (*N*)-methylethanolamine, phosphatidyl (*N,N*)-dimethylethanolamine, diphosphatidyl glycerol and phosphatidyl inositol head groups) and three diacylglycerol aminolipids (with homoserine and two unidentified head groups) are present in the water column of the ETNP. Mixed ester-ether glycerol lipids with phosphatidyl ethanolamine, phosphatidyl choline and sulfoquinovosyl head groups as well as glycosidic ceramides and ornithine lipids were detected throughout the water column. A wide range of archaeal GDGTs were most abundant within the OMZ. This diversity in IPL compositions reflects the dynamic nature of the biological community that inhabits the range of environments

fig01

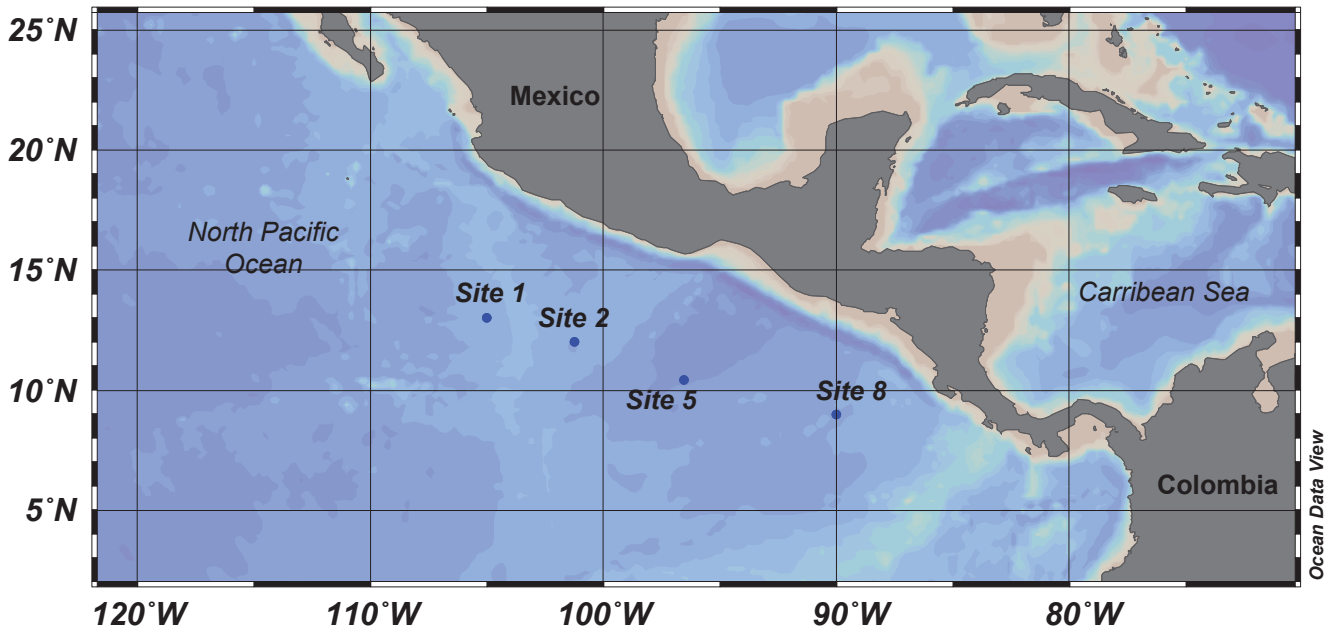

25˚N
20˚N
15˚N
10˚N
5˚N
120˚W
110˚W
100˚W
90˚W
80˚W
Mexico
North Pacific Ocean
Carribean Sea
Colombia
Site 1
Site 2
Site 5
Site 8
Ocean Data View

fig02

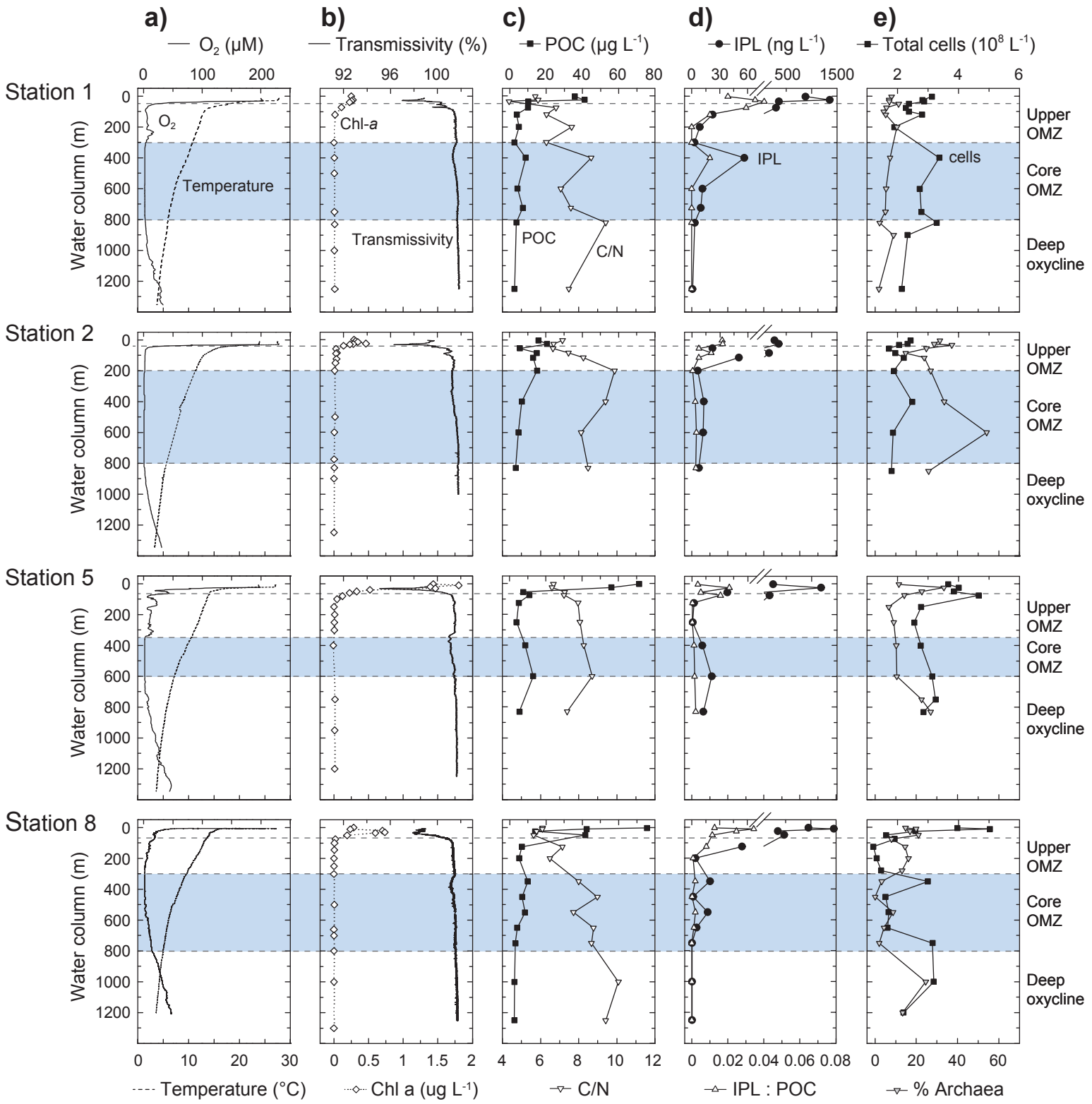

a)
b)
c)
d)
e)
O2 (µM)
Transmissivity (%)
POC (µg L-1)
IPL (ng L-1)
Total cells (10^8 L-1)
Station 1
Station 2
Station 5
Station 8
Water column (m)
O2
Temperature
Chl-a
Transmissivity
POC
C/N
IPL
cells
Upper OMZ
Core OMZ
Deep oxycline
Temperature (°C)
Chl a (ug L-1)
C/N
IPL : POC
% Archaea

fig03

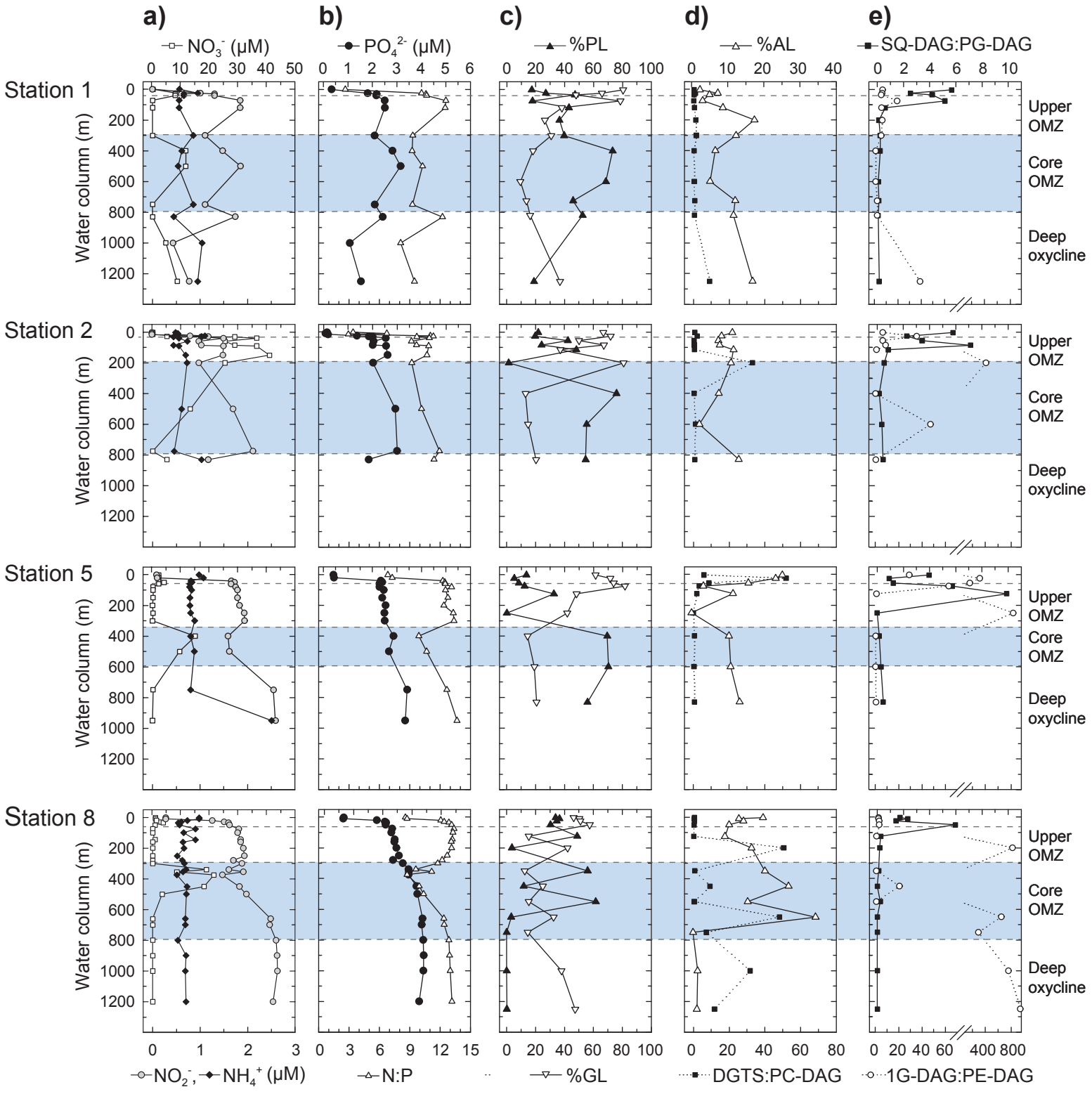

a)
b)
c)
d)
e)
NO3- (µM)
PO4 2- (µM)
%PL
%AL
SQ-DAG:PG-DAG
Station 1
Station 2
Station 5
Station 8
Water column (m)
Upper OMZ
Core OMZ
Deep oxycline
NO2-, NH4+ (µM)
N:P
%GL
DGTS:PC-DAG
1G-DAG:PE-DAG

fig04

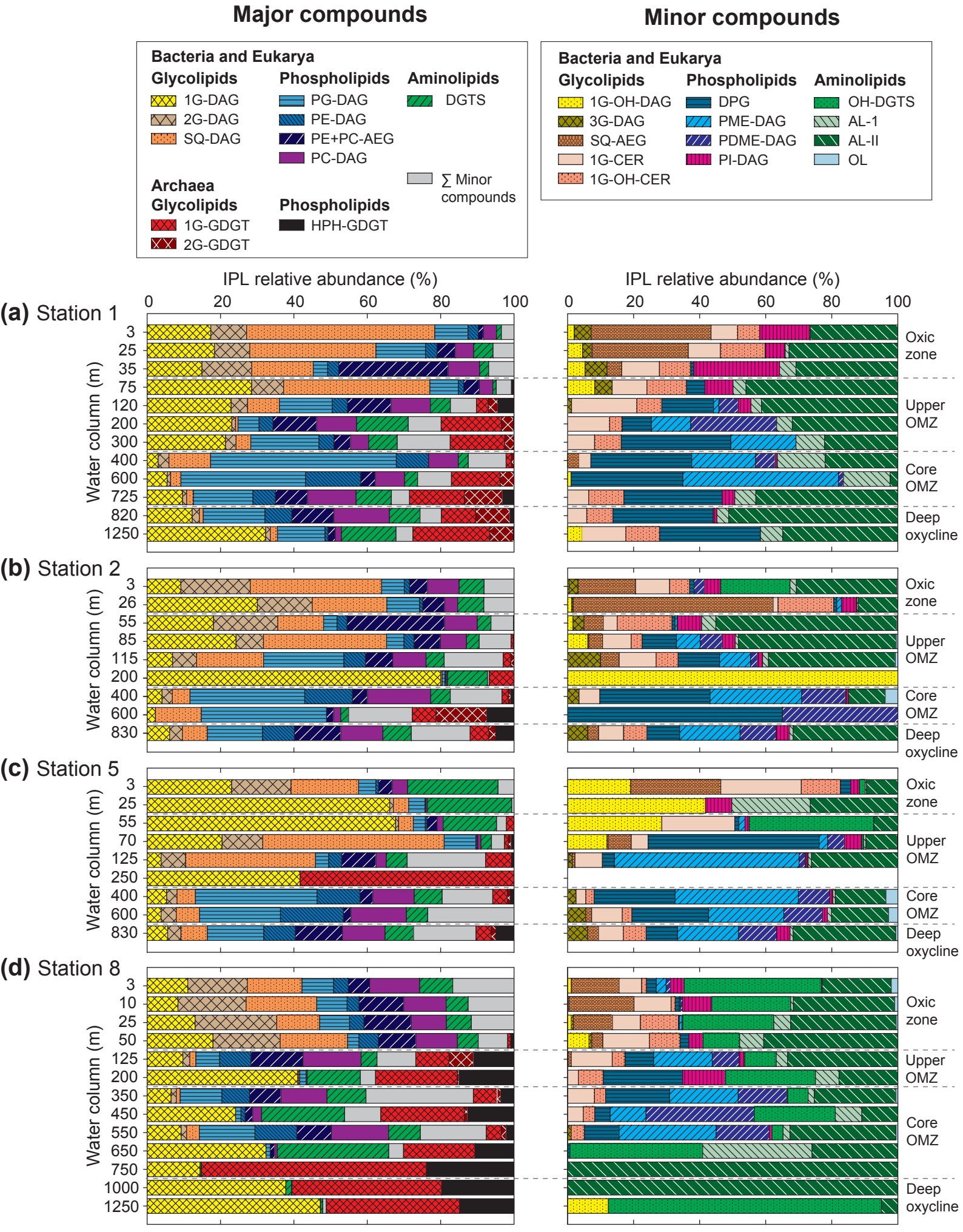

Major compounds
Minor compounds
Bacteria and Eukarya
Glycolipids
1G-DAG
2G-DAG
SQ-DAG
Phospholipids
PG-DAG
PE-DAG
PE+PC-AEG
PC-DAG
Aminolipids
DGTS
∑ Minor compounds
Archaea
Glycolipids
1G-GDGT
2G-GDGT
Phospholipids
HPH-GDGT
Bacteria and Eukarya
Glycolipids
1G-OH-DAG
3G-DAG
SQ-AEG
1G-CER
1G-OH-CER
Phospholipids
DPG
PME-DAG
PDME-DAG
PI-DAG
Aminolipids
OH-DGTS
AL-1
AL-II
OL
IPL relative abundance (%)
0
20
40
60
80
100
(a) Station 1
Water column (m)
3
25
35
75
120
200
300
400
600
725
820
1250
(b) Station 2
3
26
55
85
115
200
400
600
830
(c) Station 5
3
25
55
70
125
250
400
600
830
(d) Station 8
3
10
25
50
125
200
350
450
550
650
750
1000
1250
Oxic zone
Upper OMZ
Core OMZ
Deep oxycline

fig05

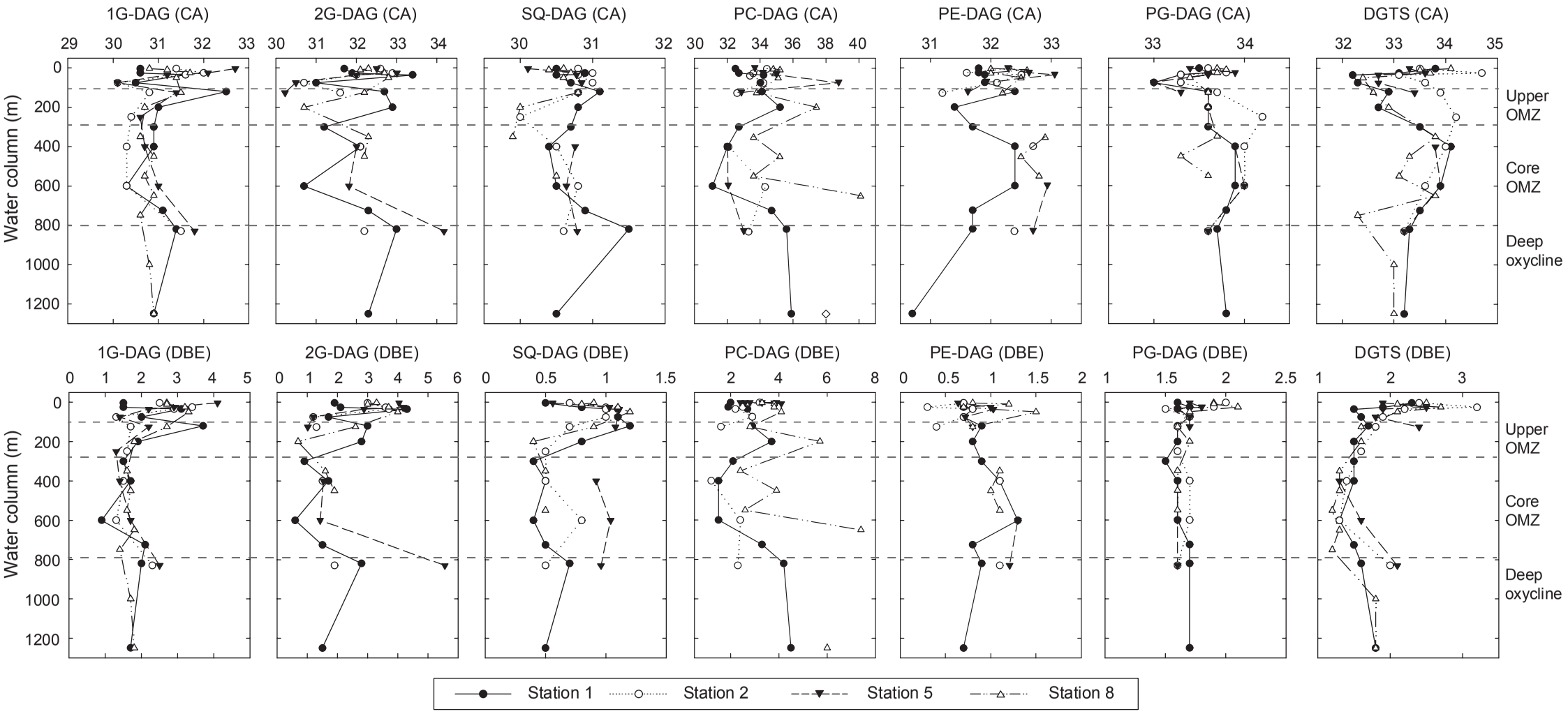
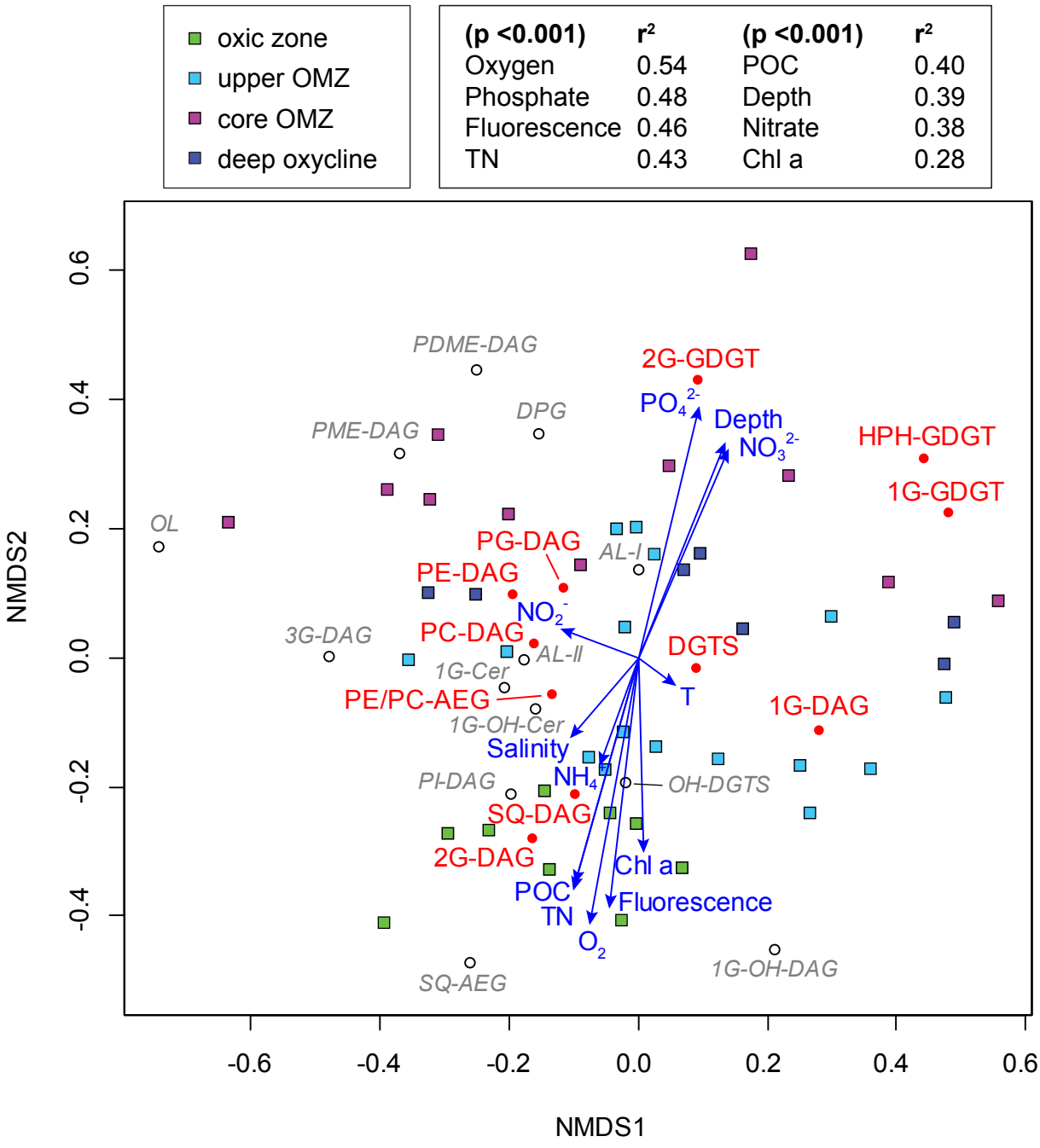

1G-DAG (CA)
2G-DAG (CA)
SQ-DAG (CA)
PC-DAG (CA)
PE-DAG (CA)
PG-DAG (CA)
DGTS (CA)
1G-DAG (DBE)
2G-DAG (DBE)
SQ-DAG (DBE)
PC-DAG (DBE)
PE-DAG (DBE)
PG-DAG (DBE)
DGTS (DBE)
Water column (m)
Upper OMZ
Core OMZ
Deep oxycline
Station 1
Station 2
Station 5
Station 8

fig06


oxic zone
upper OMZ
core OMZ
deep oxycline
(p <0.001) r²
Oxygen 0.54
Phosphate 0.48
Fluorescence 0.46
TN 0.43
(p <0.001) r²
POC 0.40
Depth 0.39
Nitrate 0.38
Chl a 0.28
PDME-DAG
DPG
PME-DAG
OL
3G-DAG
1G-Cer
1G-OH-Cer
PI-DAG
SQ-AEG
AL-I
AL-II
OH-DGTS
1G-OH-DAG
2G-GDGT
HPH-GDGT
1G-GDGT
PG-DAG
PE-DAG
PC-DAG
PE/PC-AEG
SQ-DAG
2G-DAG
1G-DAG
DGTS
T
Depth
Salinity
Chl a
POC
TN
Fluorescence
NMDS1
NMDS2