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Interactive comment on "Functional diversity of microbial communities in pristine aquifers inferred by PLFA – and sequencing – based approaches" *by* Valerie F. Schwab et al.

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We thank referee #1 for the constructive and helpful comments. We will carefully consider each of them in the revised manuscript (RM).

Answers general comments:

An important point brings by reviewer 1 and 2 is the separation between the glycolipids and the phospholipids. As mentioned by reviewer 1, an "incomplete separation can result to a significant proportion of glycolipids, betaine lipids and sulfoquinovosyldiacylglycerols (SQDGs) in the phospholipids fraction. Consequently, the PLFAs fractions might also contain fatty acids derived from glycolipids, betaine lipids, and to some ex-

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tent SQDGs, and thus might not only reflect the active biomass", i.e. fatty acids (FA) –derived from phospholipid head groups. As suggested by reviewer 2, we checked the efficiency of the separation by simply running a glycolipid (digalactosyl diglyceride) and a phospholipid (1,2-dinonadecanoyl-sn-glycero-3-phosphatidyl-choline) standard thought the SPE column using the written protocol. After hydrolyze and methylation of the FA, no phospholipid derived FA (C17:0) and glycolipids derived FA (C17:2) was detected in the glycolipids and phospholipids fractions, respectively. We additionally test the glycolipid and phospholipid fractions of the samples for the presence of Glycerol Dialkyl Glycerol Tetratether Lipids (DGDTs). Those lipids could only be detected in the phospholipid fractions. Therefore, it is likely that the phospholipid fractions also contain long chain FA (DGDTs $\geq$ 30 C) derived from glycolipids. However, since in this study we focused on short chain PLFAs ($\leq$ 20 C), we expect these compounds mainly derived from phospholipid head groups and thus represent active organisms. As mentioned by Heinzelmann et al. (2014) an incomplete separation is likely recurrent when using the commonly used PLFA extraction method. Such PLFA extraction/separation protocol has been used for most previous PLFA studies and the development of most PLFA biomarkers. Therefore, in order to be able to compare our study with the previous ones, the similar extraction/separation protocol has been preferred here. The suggestion from Heinzelmann et al. (2014) to study the FA from two fractions (FA eluting in the phospholipid fraction using the common PLFA separation and FA derived from the entire intact polar lipids eluted in a second fraction with MeOH) was not possible, since lipid concentration in such aquifer samples was very low.

However, as asked by referee # 1, this problem will be discussed in the RM. The reader will be informed of such a possible bias. The last part of the introduction will be changed into "Despite PLFAs are widely used in microbial biology, their potential to asses change in microbial structure still remain the topic of much research efforts. A definitive identification of the lipid sources remains often limited because many PLFAs that are commonly associated to a group or genus of bacteria may also be found, albeit in smaller amounts, in cell membranes of other organisms (Frostegård et al.,

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2011). Only a few PLFAs are highly specific, for example ladderanes are characteristic membrane constituents of anammox bacteria (Sinninghe Damsté et al., 2005; Sinninghe Damste et al., 2002) and have commonly been used to infer the presence of active anammox bacteria in diverse environments (Kuypers et al., 2003; Jaeschke et al., 2009). As these organisms are capable of anaerobically oxidizing ammonium with nitrite to molecular N2, they play an essential role in N removal from marine (Dalsgaard et al., 2003; Burgin and Hamilton, 2007) and lacustrine environments (Yoshinaga et al., 2011). Yet, their role in aquifer environments is only starting to be considered (Humbert et al., 2009). Another important limitation of PLFA-based studies resides in the fact that the proposed method to separate the glycolipids and phospholipids using a silicic acid column is incomplete and may result in significant proportion of glycolipids, betaine lipids and sulfoquinovosyldiacylglycerols (SQDGs) in the phospholipids fraction (Heinzelmann et al. 2014). Therefore, PLFAs fractions may also contain fatty acids derived from glycolipids, betaine lipids, and to some extent SQDGs, and thus might not only reflect the active biomass. In attempt to overcome these limitations, we combined a detailed multivariate statistical analysis of PLFAs with PLFA δ13C values, and DNA and RNA-based Illumina MiSeq amplicon sequencing of bacterial 16S rRNA genes in groundwaters with very different redox conditions and water chemistry (Kohlhepp et al., 2016). This approach allows parallel study of microbial community composition and specific substrate consumption by evidencing specific PLFAs that respond significantly to change of the groundwater chemistry. Microbial community structure and potential function assessed by PLFAs were confirmed by Illumina MiSeq amplicon sequencing targeting 16S rRNA genes and transcripts, providing a more detailed insight into bacterial community structure and taxonomic affiliation (Kozich et al., 2013). We showed that such PLFA-based study has particular relevance and importance when trying to understand how micro-organisms in groundwater interact with their environment. This study provides baselines for future studies investigating the impact of changes in surface conditions on microorganism in carbonate-rock aquifer ecosystems."

Additionally in the method section, we will add.

"To test the efficiency of the separation between the glycolipids and the phospholipids, the glycolipid standard (digalactosyl diglyceride; Sigma Aldrich) and the phospholipid standards (1,2-dinonadecanoyl-sn-glycero-3-phosphatidyl-choline) were run through the SPE column using the above protocol. The absence of phospholipid derived FA (C17:0) in the glycolipids fraction and glycolipids derived FA (C17:2) in the phospholipids fractions points to an efficient separation and thus a major origin of the studied FAME from phospholipid head groups"

Minor comments referee 1: Text related to PCA analyses will be clarified. L308- 318 "The PCA analyses using the physicochemical parameters of the groundwater separate the wells in three main groups (Figure 3) with 73.6% of the variability explained by the first three principal components (PC): PC1, 32.8%; PC2, 23.8% and PC3, 16.9%. The conductivity, redox potential and the concentrations of Ca2+, SO42-, St and O2 positively correlated (response > 0.5) with PC1 separating the oxic to sub-oxic wells H5.1, H4.1, H3.1 and H3.2 from the anoxic wells H4.2/3 and H5.2/3. The concentrations of NH4+, K+ and Mg2+ inversely correlated (response < 0.5) with PC1, separating wells H5.2/3 from the others. The Fet, TIC and HCO3- positively correlated along PC2 and mainly separated the anoxic wells between location H4 and H5. Groundwaters in location H5 have lower Fet, TIC and HCO3- concentrations but higher NH4+ and K+ concentrations, whereas higher Fet, TIC and HCO3- concentrations but lower NH4+ and K+ concentrations were measured in location H4." And L336-342 "A PCA analysis explained 56.5% of the PLFA variation with PC1 explaining 29.1%; PC2, 15.9% and PC3, 11.5% of overall variability (Figure 4). It separated the wells into the same groups evidenced by PCA analysis of the groundwater chemistry (Figure 3). The wells of the upper aquifer assemblage were separated along PC1; wells from sites H4 separated from those of the sites H5/H3. Along PC2, the wells were separated between the oxic (well H3.1, H4.1 and H5.1), sub-oxic (well H3.2) and anoxic groundwater (H4.2, H4.3, H5.2, H.5.3)."

Abstract will be shortened. L68: thank you for this. This sentence will be changed

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to “Phospholipid fatty acids (PLFAs) are important constituents of microbial cell membranes.” L74-90: This part will be deleted L116: The description of the sampling site will be included into the Material and Methods. L179: TLE will be changed to BDE

Figure 1: Make it more clear which of the wells are HTU and HTL. I found it a bit confusing. Also make it a bit more clear in the Figure that you didn’t sample from H1 and H2 (you never mentioned them in the text but in the figure they suddenly show up). Additionally, make it more clear which well is H3.1 or H3.2 etc.

Figure 1 will be modified to clarify the sampled wells, see attached figure. The following sentence will be added in the RM “Due to very low groundwater level, location H1 and H2 were not sampled.”

Technical comments L59: SO42- will be corrected L76: prefixes of fatty acids like iso (i) and anteiso (a or ai) will be in italic L143, L154-L155 L will be noted in the MS L170: Co2- will be corrected L171: We will add a space between the units m mol an and wrote L-1 L219: the – will be deleted L280: space after – L289: space before - L303: insert space before - L312: St will be corrected: t subscript L364-367: space after - L402: NH4+ will be corrected L403: sulphate will be corrected in the MS L413: A dot after the) will be added L417: sulphur will be corrected in the MS L421: the % after 1.1 will be removed; similar error will be corrected in the MS L439: Fet will be corrected: t: Subcript L507: The C in 10MeC12:0 will be removed and similar error will be corrected in the MS L508: Fe2+ will be corrected L566: The space before % will be remove in the all the MS L615-933: Reference list will be changed according to Journal style. doi will be added when possible. L632: space after the will be added L659-660: Author name will be corrected in the MS L665: The 2 will be noted in subscript L677 Names of microorganisms will be noted in italic L682: space missing before Nitrospira will be added L692-694: Authors initials will be the same L901: Capital D for Desulfovibrio L907: the name Sinninghe Damsté will be corrected Figure 2: HCO3- will be corrected Figure 3: Na will be corrected

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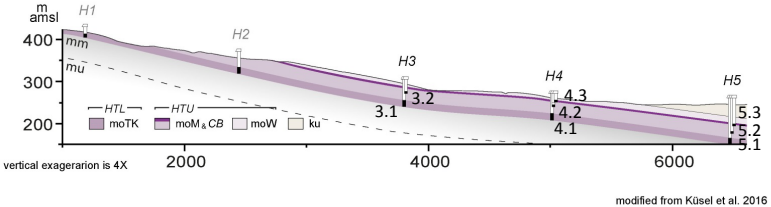

Figure. 1: Schematic representation of the geologic cross section of the Hainich monitoring well transect. The wells sampled for this study are numbered in black. The black colours in the wells indicate screen sections and accessed depths of the aquifer assemblages. Abbreviation; mu: Lower Muschelkalk; mm: Middle Muschelkalk; mo: Upper Muschelkalk; moTK: Trochitenkalk formation; moM: Meissner formation; CB: Cycloides-Bank; moW: Warburg formation; ku: Lower Keuper.

Fig. 1.

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