Functional diversity of microbial communities in pristine aquifers inferred by PLFA- and sequencing -based approaches

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Abstract: Microorganisms in groundwater play an important role in aquifer biogeochemical cycles and water quality. However, the mechanisms linking the functional diversity of microbial populations and the groundwater physicochemistry are still not well understood due to the complexity of interactions between surface and subsurface. Within the framework of Hainich (north-western Thuringia, central Germany) Critical Zone Exploratory of the Collaborative Research Centre AquaDiva, we used phospholipid derived fatty acids (PLFAs) relative abundances to link specific biochemical markers within the microbial communities to the spatio-temporal changes of the groundwater physicochemistry. The functional diversities of the

microbial communities were mainly correlated with groundwater chemistry, including dissolved O_2, Fe_t and NH_4^+ concentrations. Abundances of PLFAs derived from eukaryotes and potential nitrite-oxidizing bacteria (11Me16:0 as biomarker for *Nitrospira moscoviensis*) were high at sites with elevated O_2 concentration where groundwater recharge supplies bioavailable substrates. In anoxic groundwaters more rich in Fe_t, PLFAs abundant in sulphate-reducing bacteria (SRB), iron-reducing bacteria and fungi increased with Fe_t and HCO_3^- concentrations suggesting the occurrence of active iron-reduction and the possible role of fungi in meditating iron solubilisation and transport in those aquifer domains. In NH_4^+ richer anoxic groundwaters, anammox bacteria and SRB- derived PLFAs increased with NH_4^+ concentration further evidencing the dependence of the anammox process on ammonium concentration and potential links between SRB and anammox bacteria. Additional support of the PLFA-based bacterial communities was found in DNA and RNA-based Illumina MiSeq amplicon sequencing of bacterial 16S rRNA genes, which showed high predominance of nitrite-oxidizing bacteria *Nitrospira* e.g. *Nitrospira moscoviensis* in oxic aquifer zones and of anammox bacteria in NH_4^+ richer anoxic groundwater. Higher relative abundances of sequence reads in the RNA-based data sets affiliated with iron-reducing bacteria in Fe_t richer groundwater supported the occurrence of active dissimilatory iron-reduction. The functional diversity of the microbial communities in the biogeochemically distinct groundwater assemblages can be largely attributed to the redox conditions linked to changes in bioavailable substrates and input of substrates with the seepage. Our results demonstrate the power of complementary information derived from PLFA-based and sequencing-based approaches.

1. Introduction

Continental and marine subsurface environments represent the largest habitat on Earth for microbial life and therefore are of primary importance for energy fluxes on a global scale (Edwards et al., 2012). In terrestrial ecosystems, complex interactions between the surface and subsurface compartments, including aquifers, such as groundwater recharge and rainfall event-driven flow, influence the availability of O_2, and the nature and abundance of bioavailable organic matter (OM; Benner et al., 1995; Kalbus et al., 2006). Young and shallow groundwaters tend to maintain the chemical characteristics of surface, i.e. higher O_2 levels and greater amounts of bioavailable substrates (e.g. labile OM) which support aerobic heterotrophic microbial activity (Landmeyer et al., 1996). In contrast, deep and especially fossil groundwaters tend to reflect the chemistry of the aquifer materials. They have typically lower concentrations of O_2 and bioavailable substrates which cause facultative anaerobes to switch to terminal electron acceptors with lower energy yield such as NO_3^-, MnO_2, FeOOH and SO_4^{2-} (Chapelle and Lovley, 1992). In pristine aquifers low amount of OM typically results in a higher amount of terminal electron acceptors than electron donors (Chapelle, 2001). As many chemolithoautotrophs can use a variety of compounds to meet their energy needs in the dark subsurface, increasing number of studies report the importance chemolithoautotrophy in groundwater (Stevens and McKinley, 1995; Emerson et al., 2015; Herrmann et al., 2015). However, it is still not well understood how the composition and function of microbial communities in groundwaters depend on hydrology, chemistry and the relationship to groundwater recharge dynamics.

There are a number of ways to assess the composition and function of microbial communities in groundwaters. Intact polar lipids, mainly phospholipids, are important constituents of bacterial and eukaryotic cell membranes. They consist of a polar head group linked to a glycerol backbone with two fatty acids esterified to it. Because various phospholipids derived fatty acids (PLFAs) are indicative of specific types or groups of bacteria in soil (e.g.,

Frostegård and Bååth, 1996; Frostegård et al., 2011) and aquifers (Green and Scow, 2000), PLFA-based studies are recognised as a valuable approach to infer the presence of specific microbial groups and to show trends in the spatial distribution of active microbial populations related to specific substrate utilization patterns in environments (e.g., Torsvik and Øvreås, 2002; Schneider et al., 2012). PLFAs that are commonly associated to a group or genus of bacteria are listed in Table 1. Additionally, the stable carbon isotope ratios ($\delta^{13}C$ values) of PLFAs reflect a combination of the source of microbial carbon and kinetic isotope fractionation effects associated with the carbon assimilation pathway (e.g., heterotrophy, autotrophy, methanotrophy; Teece et al., 1999; Zhang et al., 2003; Londry et al., 2004). Although a wide range of carbon isotope effects have been measured, in general autotrophs are expected to have PLFA $\delta^{13}C$ values more negative than heterotrophs (Blair et al., 1985; Teece et al., 1999; van der Meer et al., 2001; Zhang et al., 2003; Londry et al., 2004; Schouten et al., 2004). In particular, large isotope effects have been associated with anammox bacteria that have PLFA $\delta^{13}C$ values as much as 47‰ more negative than the dissolved inorganic carbon (DIC) source (Schouten et al., 2004).

Despite PLFAs being widely used in microbial ecology, their potential to assess changes in microbial communities still remains the topic of much research efforts. One of the major limitations of PLFA based studies is the definitive identification of the lipid sources, since many PLFAs commonly associated to a group or genus of bacteria (Table 1) may also be found, albeit in smaller amounts, in cell membranes of other organisms (Frostegård et al., 2011). A few PLFAs are highly specific, for example ladderanes are characteristic membrane constituents of anammox bacteria (Sinninghe Damsté et al., 2002; 2005) 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 N_2, 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 may reside in the PLFA extraction technique. Heinzelmann et al. (2014) showed that the proposed method to separate the glycolipids and phospholipids is incomplete and results in a significant proportion of glycolipids, betaine lipids and sulfoquinovosyldiacylglycerols (SQDGs) in the phospholipids fraction. Therefore, PLFA fractions might 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 some of these limitations, we combined a detailed multivariate statistical analysis of PLFAs with PLFA $\delta^{13}C$ values, and DNA and RNA-based Illumina MiSeq amplicon sequencing of bacterial 16S rRNA genes in groundwaters with 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 changes in 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.

2. Sampling and methods

2.1. Study site

The sampled groundwater wells are part of the monitoring well transect of the Hainich Critical Zone Exploratory (CZE: north-western Thuringia, central Germany) of the Collaborative Research Centre (CRC) AquaDiva. AquaDiva aims to determine how deep can signals of surface environmental conditions be traced into the Critical Zone (Küsel et al., 2016). The wells access two distinct aquifer assemblages in marine sediments of the Upper Muschelkalk (mo) lithostratigraphic subgroup (Germanic Triassic, Middle Triassic epoch) at different depths and locations (Figure 1). Wells in the hilltop recharge areas (H1, H2) were not sampled, due to very low groundwater levels or desaturation. Aquifers predominantly receive surface recharge in their outcrop areas at the eastern Hainich hillslope. The lower aquifer assemblage (subsequently referred to as HTL) represents one aquifer hosted in the Trochitenkalk formation (moTK), whereas the upper aquifer assemblage (referred to as HTU) comprises several aquifers and aquitards of the Meissner formation. The HTL, sampled at depths ranging from 41 to 88 m below the surface, is rich in O_2, whereas the upper aquifer found at depths from 12 to 50 m below surface, is anoxic to sub-oxic. Both aquifer assemblages are found in alternating sequences of limestones and marlstones that are partly karstified (Kohlhepp et al., 2016). More details on the CZE and well constructions can be found in Küsel et al. (2016) and Kohlhepp et al. (2016).

2.2. Groundwater sampling

Groundwater was sampled for chemical analyses and colloidal/particulate organic matter (POM) in June, September and December of 2014 (Table 2) during regular sampling campaigns within the coordinated joint monitoring program of the CRC. Groundwater samples were collected at locations H3, H4, and H5 (i.e. the lower topographic positions of the well transect,

Figure 1). Wells H3.2, H4.2, H4.3, H5.2 and H5.3 reach into the HTU, while wells H3.1, H4.1 and H5.1 access the HTL aquifer (Figure 1). The wells were originally drilled between 2009 and 2011, and were specifically designed for sampling groundwater (micro)-organisms and particles. Prior to sampling, stagnant water (at least three well volumes) was pumped out and discarded until the physicochemical parameters pH, dissolved O_2 concentration, redox potential and specific electrical conductivity remained constant. Subsequently, ~1000 L of groundwater were filtered on site using a submersible pump (Grundfos SQE 5-70, Grundfos, Denmark) connected to a stainless steel filter device (diameter 293mm, Millipore USA) equipped with a removable pre-combusted (5 h at 500°C) glass fiber filter (Sterlitech, USA) of fine porosity (0.3 µm) allowing a water flow of ca. 20 $Lmin^{-1}$. Filters with the collected particulates were carefully removed and immediately stored at -80°C until analysis. Groundwater extraction temperature, redox potential, specific electrical conductivity, pH and dissolved O_2 concentration were monitored continuously during pumping in a flow-through cell equipped with the probes TetraCon 925, FDO 925, Sentix 980, ORP 900 (WTW GmbH, Germany) and meter (Multi 3430 IDS, WTW GmbH, Germany).

During the sampling campaign of June 2014, groundwater was additionally sampled for nucleic acid extraction. The groundwater was transferred to sterile glass bottles and kept at 4 °C. Within a few hours after sampling, five to six litres of groundwater were filtered through 0.2 µm pore size polyethersulfone (PES) filters (Pall Corporation, USA), and 2 litres were filtered through 0.2 µm pore size polycarbonate filters (Nuclepore, Whatman, United Kingdom) for extraction of DNA and RNA, respectively. Filters were immediately transferred to dry ice and stored at -80 °C until nucleic acid extraction.

2.3. *Groundwater chemistry analyses*

Concentration of the major anions (SO_4^{2-}, Cl^-, NO_3^-, PO_4^{3-}, PES filter <0.45 µm) were determined according to DIN EN ISO 10304-1 (2009a) using an ion chromatograph (DX-120, DIONEX, USA; equipped with an IonPac AS11-HC column and an IonPac AG11-HC pre-column). The redox sensitive parameters (Fe^{2+}, NO_2^-, NH_4^+) were determined by colorimetry (DR 890, Hach Company, USA) according to manufacturer's protocol following APHA (1981) and Reardon et al. (1966). The concentration of DOC and DIC (filter <0.45 µm) were determined by high temperature catalytic oxidation (multi 18 N/C 2100S, Analytik Jena, Germany) according to DIN EN 1484 (1997). Total S (S_t), Mn (Mn_t) and iron (Fe_t) were analysed by ICP-OES (725 ES, Varian/Agilent, USA) according to DIN EN ISO 11885 (2009b). The acid and base neutralizing capacity (ANC, BNC) by acid/base endpoint-titration was determined according to DIN 38409-7 (2005). The approximated concentrations of HCO_3^- and CO_2^- were converted from $ANC_{4.3}$ and $BNC_{8.2}$ by simple replacement (cCO_2^- (mmol L^{-1}) = $BNC_{8.2}$(mmol L^{-1}); $cHCO_3^-$ (mmol L^{-1}) = $BNC_{4.3}$ (mmol L^{-1})), assuming that other buffering species than those are negligible, in the nearly pH-neutral waters (Wisotzky, 2011).

2.4. *PLFA extraction and pre-treatment*

To minimize external contamination, all material (including filters) and glass in contact with the samples during extraction and purification were baked at 500 °C for 5 h to remove organic contaminants. Only trace level of 16:0 fatty acid methyl ester (FAME) has been detected in blank extracts. PLFAs were extracted from filters using a method slightly modified from Bligh and Dyer (1959) and Seifert et al. (2013). The filters were cut into small pieces and extracted in a phase solution of chloroform-methanol (2:1; v/v) with 0.005 M phosphate buffer. The solution

was rotated and shaken for 4 h. Chloroform and water (1:1; v/v) were then added to the mixture. After shaking, the chloroform phase, containing the Bligh-Dyer-extract (BDE), was separated from the water-MeOH phase and concentrated by a rotary evaporator. The BDE was then partitioned into the conventionally defined neutral lipids (NL), glycolipid (GL) and phospholipid (PL) fractions by chromatography (SPE 6 mL column) on pre-activated silica gel (Merck silica mesh 230-400, 2 g pre-activated 1 h et 100 °C) using chloroform (12 mL), acetone (12 mL) and methanol (48 mL), respectively. The phospholipids were converted to FAMEs using mild-alkaline hydrolysis and methylation (White et al., 1979). The different fatty acids were then separated using NH_2 column (Chromabond 3mL, 500 mg) with 3 mL of hexane/DCM (3:1; v/v) for eluting the unsubstituted FAMEs; 3 mL of DCM/ ethylacetate (9:1; v/v) for the hydroxy FAME and 6 mL of 2% acetic acid in methanol for unsaponifiable lipids. To quantify the recovery the standard, 1,2-dinonadecanoyl-sn-glycero-3-phosphatidyl-choline (Avanti Polar Lipids, Inc. USA), was added on clean pre-combusted glass filters that were treated exactly as the samples following the above protocol. The formed C17:0 FAME was quantified to calculate a mean recovery of 82%. To test the efficiency of the separation between the glycolipids and the phospholipids, the glycolipid standard digalactosyl diglyceride (Sigma Aldrich) and the phospholipid standard 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 glycolipid fraction and glycolipid derived FA (C17:2) in the phospholipid fractions points to an efficient separation and thus a major origin of the studied FAME from phospholipid head groups.

2.5. Nucleic acid extraction, amplicon sequencing, and sequence analysis

DNA was extracted from the PES filters using the Power Soil DNA extraction kit (Mo Bio, CA, USA) following the manufacturer's instructions. RNA was extracted from polycarbonate filters using the Power Water RNA Isolation Kit (Mo Bio, CA, USA). Traces of co-extracted genomic DNA were removed using Turbo DNA free (Thermo Fisher Scientific, Germany), and reverse transcription to cDNA was performed using ArrayScript Reverse Transcriptase (Thermo Fisher Scientific) as described previously (Herrmann et al., 2012). DNA and cDNA obtained from the groundwater samples from PNK51 were shipped to LGC Genomic GmbH (Berlin, Germany) for Illumina MiSeq amplicon sequencing of the V3-V5 region of 16S rRNA genes and transcripts, using the primer combination Bakt_341F/Bakt_805R (Herlemann et al., 2011). Sequence analysis was performed using Mothur v. 1.36 (Schloss et al., 2009), following the MiSeq SOP (http://www.mothur.org/wiki/MiSeq_SOP; Kozich et al., 2013). Quality-trimmed sequence reads were aligned to the SILVA reference database (v 119; Quast et al., 2013). Potential chimeric sequences were detected and removed using the uchime algorithm implemented in Mothur. Taxonomic classification of sequence reads was based on the SILVA reference database (v 119). To facilitate comparisons across samples, sequence read numbers per sample were normalized to the smallest number of sequence reads obtained across all samples using the subsample command implemented in Mothur. Raw data from 16S rRNA amplicon Illumina sequencing were submitted to the European Nucleotide Archive database under the study accession number PRJEB14968 and sample accession numbers ERS1270616 to ERS1270631.

2.6. Gas chromatography and gas chromatography-mass spectrometry

Ten percent of the PLFA extracts were used for peak identification and relative quantification using a gas chromatograph (Trace 1310 GC) coupled to a triple quadrupole mass spectrometer (TSQ-8000; Thermo-Fisher, Bremen, Germany) at the Friedrich Schiller University Jena, Institute of Inorganic and Analytical Chemistry (Germany). The GC was equipped with a TG 5silms capillary column (60 m, 0.25 mm, 0.25-µm film thickness). Helium was used as carrier gas at a constant flow of 1.2 mL min^{-1}, and the GC oven was programmed to have an initial temperature of 70 °C (hold 2 min), a heating rate of 11 °C min^{-1}, and a final temperature of 320 °C, held for 21 min. The PTV injector was operated in splitless mode at an initial temperature of 70 °C. Upon injection, the injector was heated to 300 °C at a programmed rate of 720 °C min^{-1} and held at this temperature for 2.5 minutes. FAMEs were quantified relative to an internal standard nonadecanoic acid-methyl ester (19:0) added prior to GC analysis. FAMEs were identified based on the mass spectra and on retention time of standards. Standard nomenclature is used to describe PLFAs. The number before the colon refers to the total number of C atoms; the number(s) following the colon refers to the number of double bonds and their location (after the 'ω') in the fatty acid molecule. The prefixes "me," "cy," "*i*" and "*a*" refer to the methyl group, cyclopropane groups, and iso- and anteiso-branched fatty acids, respectively.

2.7. PLFA distribution and statistical analyses

The forty-seven PLFAs, expressed in %, were investigated in the different wells (Supplement Table S1). The sum of the PLFAs considered to be predominantly of bacterial origin (BactPLFA; *i*15:0, *a15*:0, 15:0, 16:1ω7, 16:0, cy17:0, 18:1ω7, 18:0 and cy19:0) was used as an index of the bacterial biomass (Bossio and Scow, 1998; Frostegård and Bååth, 1996). The fungal biomass

(FunPLFA) was estimated from the sum of the relative abundance of the 18:2ω6c (Bååth et al., 1995), 18:3ω6c (Hamman et al., 2007) and 18:1ω9c (Myers et al., 2001); these were all significantly correlated with each other. Gram-positive (G+) bacteria were represented by the sum of PLFAs: *i*12:0, *i*13:0, *a*15:0, *i*15:0 (Kaur et al., 2005). Gram-negative (G-) bacteria included 16:1ω7c, cy17:0, 18:1ω7c and cy19:0 (Kaur et al., 2005). The ratios of FunPLFA/BactPLFA and G^+/G- were calculated from the above PLFAs.

The PLFA data and twenty-nine environmental parameters were used for principal component analysis (PCA) and redundancy analyses (RDA) using CANOCO for Windows, version 5 (Microcomputer Power, Ithaca, New York, United States). Before regression, the data were centered and standardized. We used PCA to emphasise strong variations and similarities of the PLFA distributions between the wells and identify patterns in the dataset. RDA is used to determine PLFA variations and similarities (response variables) that can be significantly explained by different environmental parameters (explanatory variables). This technique helps to identify the environmental parameters that have the highest effects on the PLFA distribution, i.e. on the microbial communities in the different wells.

Additionally, we used variation partitioning analyses with conditional effects to determine the variations in PLFA composition between the different wells that can be explained significantly by the preselected environmental variables. To visualise the PLFAs acting significantly with the environmental variables (predictor), we used PLFA-environmental variables t-value biplots (Šmilauer and Lepš, 2014). These plots can be used to approximate the t-value of the regression between a particular PLFA and an environmental variable. The PLFAs are represented by arrows projecting from the origin. Those with a preference for higher values of the environmental variable are enclosed by a red (indicating positive relationship) circle. Inversely,

those with preference for low values of the corresponding environmental variable have their arrow-tips enclosed by a blue (indicating negative relationship) circle.

2.8. Compound-specific stable isotope carbon measurements

The carbon stable isotope composition of pre-purified PLFAs were determined using a GC-C-IRMS system (Deltaplus XL, Finnigan MAT, Bremen, Germany) at the Max-Planck-Institute (MPI) for Biogeochemistry, Jena. Analyses were performed using 50% of the total amount of PLFA extracts. The gas chromatograph (HP5890 GC, Agilent Technologies, Palo Alto USA) was equipped with a DB1-ms column (60 m, 0.25 mm ID, 0.52 um film thickness, Agilent). The injector at 280 °C was operated in splitless mode with a constant flow of 1 mL min^{-1}. The oven temperature was maintained for 1 min at 70 °C, heated with 5 °C min^{-1} to 300 °C and held for 15 min, then heated with 30 °C min^{-1} to 330 °C and hold 3 min. Isotope values, expressed in the delta notation (‰), were calculated with ISODAT version software relative to the reference CO_2. Offset correction factor was determined on a daily basis using a reference mixture of *n*-alkanes (*n*-C_{17} to *n*-C_{33}) of known isotopic composition. The carbon isotopic composition of the reference *n*-alkanes were determined off-line using a thermal conversion elemental analyser (TC/EA) (Thermo-Fisher, Bremen, Germany) interfaced to the DELTA V PLUS irMS system via a Conflo III combustion interface (Thermo-Fisher, Bremen, Germany; Werner and Brand, 2001). The contribution of the methyl carbon derived from the methanol after mild- alkaline hydrolysis and methylation of the PLFAs to the FAME was removed by isotopic mass balance, with $\delta^{13}C_{PLFA} = [(N_{PLFA} + 1) \times \delta^{13}C_{FAME} - \delta^{13}C_{MeOH}] / N_{PLFA}$ where N is the number of carbon atoms in the PLFA and $\delta^{13}C_{FAME}$ stands for the measured values of the methylated PLFAs (Kramer and Gleixner, 2006). The carbon isotope composition of MeOH used for derivatisation ($\delta^{13}C$ value = -31.13 ± 0.03‰) was determined off-line using a thermal conversion

293 elemental analyzer (TC/EA) (Thermo-Fisher, Bremen, Germany) interfaced to the DELTA V
294 PLUS irMS system via a Conflo III combustion interface (Thermo-Fisher, Bremen, Germany).

295 **3. Results**

296 *3.1. Groundwater physicochemistry*

297 The deeper aquifer assemblage, HTL (wells H3.1, H4.1 and H5.1), had higher mean
298 concentration of O_2 (3.7 ± 1.0 mgL^{-1}) than the shallow aquifer assemblage, HTU (wells H4.2,
299 H4.3, H5.2 and H5.3). Groundwater extracted from HTU wells were anoxic with O_2 < 0.02 mgL^{-1}
300 (Supplement Table S2 and Figure 2) except for well H3.2 that had mean O_2 = 2.4 ± 0.7 mgL^{-1}.
301 No significant differences in the content of dissolved organic carbon (DOC: mean = 2.3 ± 1.0
302 mgL^{-1}) were measured between the different aquifers. The HTL had higher mean concentration
303 of sulphate (183.5 ± 110.9 mgL^{-1}) than the anoxic HTU (76.4 ± 3.1 mgL^{-1}). The highest
304 concentrations of nitrate were measured in the well H3.2 (30.0 ± 3.3 mgL^{-1}) of the HTU. Higher
305 mean concentrations of total iron (Fe_t = 0.1 ± 0.08 mgL^{-1}), TIC (86.6 ± 7.0 mgL^{-1}) and HCO_3^-
306 (4.69 ± 0.07 mgL^{-1}), the latter measured as acid neutralizing capacity (Wisotzky, 2011), were
307 found in the anoxic groundwater of the wells H4.2 and H4.3 than of the wells H5.2 and H5.3 that
308 had mean Fe_t = 0.01 ± 0.00 mgL^{-1}, TIC = 75.6 ± 5.4 mgL^{-1} and HCO_3^- = 4.02 ± 0.2 mgL^{-1} (Figure
309 2). Inversely, mean concentrations of total sulphur (S_t = 26.1 ± 4.9 mgL^{-1}), sulphate (76.7 ± 14.8
310 mgL^{-1}) and ammonium (0.62 ± 0.16 mgL^{-1}) were higher in the anoxic groundwater of the wells
311 H5.2 and H5.3 than of the wells H4.2 and H4.3 that had mean S_t = 12.3 ± 0.5 mgL^{-1}, SO_4^{2-} = 37.6
312 ± 2.0 mgL^{-1} and NH_4^+ = 0.13 ± 0.06 mgL^{-1} (Figure 2 and Supplement Table S2).

313 The PCA analyses using the physicochemical parameters of the groundwater separate the
314 wells in three main groups (Figure 3) with 73.6% of the variability explained by the first three
315 principal components (PC): PC1, 32.8%; PC2, 23.8% and PC3, 16.9%. The conductivity, redox
316 potential and the concentrations of Ca^{2+}, SO_4^{2-}, S_t and O_2 positively correlated (response > 0.5)
317 with PC1 separating the oxic to sub-oxic wells H5.1, H4.1, H3.1 and H3.2 from the anoxic wells
318 H4.2/3 and H5.2/3. The concentrations of NH_4^+, K^+ and Mg^{2+} inversely correlated (response <
319 0.5) with PC1, separating wells H5.2/3 from the others. The Fe_t, TIC and HCO_3^- positively
320 correlated along PC2 and mainly separated the anoxic wells between location H4 and H5.
321 Groundwaters in location H5 have lower Fe_t, TIC and HCO_3^- concentrations but higher $NH_4^+$and
322 K^+ concentrations, whereas higher Fe_t, TIC and HCO_3^- concentrations but lower NH_4^+ and K^+
323 concentrations were measured at location H4.
324

325 *3.2. PLFA distribution and statistical analyses*

326 The 16:1ω7c (mean 22.2 ± 8.9%), 16:0 (mean 13.4 ± 2.3%) and 18:1ω7c (mean 5.2 ±
327 2.6%), common in most bacteria, were the most abundant PLFAs in both aquifer assemblages
328 (Supplement Table S1). The PLFAs 10Me16:0 (mean 7.8 ± 5.6%), 17:1ω6c (mean 1.2 ± 1.0%)
329 and i17:1 (mean 0.8 ± 0.7%) derived from Deltaproteobacteria mainly encompassing SRB, iron-
330 reducing or iron-oxidizing bacteria were dominant only in the anoxic groundwater, whereas the
331 11Me16:0 (mean 4.2 ± 4.7%) were found in high relative abundance in the oxic groundwaters.
332 The [3]- and [5]- ladderane PLFAs specific to anammox bacteria were found in the anoxic wells
333 H5.2 and H5.3 and the sub-oxic well H3.2 in a relative abundance of up to 5.0%. The highest
334 fungal biomass, based on the FunPLFA ratios (Table 3), was observed in the anoxic wells H4.2
335 and H4.3 (mean 19.0 ± 7.8), whereas the lowest in the anoxic wells H5.2 and H5.3 (mean 1.9 ±

2). The Gram negative (G-) bacteria were more abundant than Gram positive bacteria (G+) in both HTU and HTL (Table 3: mean G+/G- ratio = 0.4 ± 0.2). The highest values of the G+/G- ratios were in the anoxic wells H4.2 and H4.3 (mean 0.7 ± 0.1).

A PCA analysis explained 56.5% of the PLFA variation. PC1 and PC2 explaining respectively 29.1% and 15.9% of overall variability (Figure 4), separated the wells into the same three groups evidenced by the PCA analysis of the groundwater chemistry (Figure 3). The wells of the HTU assemblage were separated along PC1; wells from sites H4.2/H4.3 separated from those of the sites H5.2/H5.3. Along PC2, the wells were separated between the oxic (well H3.1, H4.1 and H5.1), sub-oxic (well H3.2) and anoxic groundwaters (H4.2, H4.3, H5.2, H.5.3). The RDA analyses showed that O_2, Fe_t and NH_4^+ concentrations or O_2, HCO_3^- and NH_4^+ concentrations explained the greatest proportion (39.9%) of the PLFA variability (Figure 5). Well grouping obtained using the RDA analysis was consistent with the results of the PCA. The first RDA axis (21.5%) separated the anoxic wells of the upper aquifer according to Fe_t or HCO_3^- (wells H4.2 and H4.3) and NH_4^+ (wells H5.2 and H5.3) concentration. The second RDA axis (14.6%) separated suboxic to oxic (mainly lower aquifer) from anoxic groundwater (upper aquifer assemblage). In the following discussion, the wells are separated according the PCA and RDA analyses into these three main groups.

To identify the individual effects of O_2, Fe_t and NH_4^+ on the explained PLFA variation, we used variation partitioning with conditional effects implemented in Canoco 5 (Heikkinen et al., 2004; Roth et al., 2015). Because these environmental variables were the most significant factors, their combined variation was set to explain 100% of total PLFA variation in each RDA plot. In our case, the following eight fractions explained the PLFA distribution by effect of O_2 alone; a = 19.7%, effect of NH_4^+ alone; b = 22.0%, effect of Fe_t alone; c = 13.4%, and by combined effects of O_2 and NH_4^+; d = 22.3%, by combined effects of Fe_t and NH_4^+; e = 29.2%,

and by combined effect of O_2 and Fe_t; f = 25.9%. The fraction g (-32.4%) explained the combined effect of the three environmental variables (Figure 6). The PLFA-environmental variable O_2 t-plot (Figure 6A) showed that the % relative abundance of Me15:0, 16:1ω11c, cy17:0, 11Me16:0, 18:1, 22:5 and 22:6 increased significantly with O_2 concentration and the % relative abundance of 10Me12:0, *i*13:0, *a*15:0, 17:1 and [5]-ladderane decreased with O_2 concentration. The PLFA-environmental variable Fe_t t-values biplot (Figure 6B) showed that 10Me12:0, 17:1, 18:1ω9c, 18:1ω7c and 12:0 % relative abundance increased with Fe_t concentration, whereas 10Me16:0, i17:1, [3]-ladderane and [5]-ladderane % relative abundance decreased. Inversely, the PLFA-environmental variable NH_4^+ t-values biplot (Figure 6C) showed that 10Me16:0, 17:1, [3]-ladderane and [5]-ladderane % relative abundance increased with NH_4^+ concentration, whereas 10Me12:0, 12:0, 18:1ω9c, 18:1ω7c and 17:1 % relative abundance decreased.

3.3. PLFA $\delta^{13}C$ values

The PLFA $\delta^{13}C$ values for individual compounds ranged from -26 to - 68.8‰ (Supplement Table S3 and Figure 7). The most negative mean $\delta^{13}C$ values were found in the anoxic groundwater from location H5.2 and H 5.3 (-48.0 ± 10.5‰ and -45.9 ± 11.7‰, respectively) and in the suboxic groundwater at the location H3.2 (-45.4 ± 9.0‰) and coincided with the presence of the [5]- and [3]-ladderane. In those wells, the *i*13:0 (-52.4 ± 2.0‰), *i*15:0 (-55.6 ± 2.0‰), 10Me16:0 (-56.1 ±2.1‰) and i17:1 (-44.3 ± 2.0‰) were slightly ^{13}C-enriched compared to both [5]- and [3]-ladderane (-65.6 ± 2.0‰). More positive mean PLFA $\delta^{13}C$ values were measured in the anoxic wells H4.2 and H4.3 (-36.8 ± 2.1‰) and in the oxic wells H5.1, H4.1 and H3.1 (-35.3 ± 1.1‰). In those wells, the $\delta^{13}C$ values of the i13:0, *i*15:0 and 10Me16:0 were in the same range as the other PLFAs (Figure 7). The most positive $\delta^{13}C$ values were measured for 16:1ω11c and

11Me16:0 in the oxic wells H5.1 and H4.1 (mean -28.2 ± 2.5‰) and for 18:1ω9c (mean -30.2 ± 2.3‰) in the anoxic wells H4.2 and H4.3.

3.4. Bacterial community composition based on 16S rRNA gene sequences

Based on Illumina sequencing of DNA-based 16S rRNA gene amplicons, bacterial communities were largely dominated by members of the phylum Nitrospirae and of Candidate Division OD1, followed by Delta- and Betaproteobacteria, Planctomycetes, Alpha- and Gammaproteobacteria (Figure 8A). Members of the Nitrospirae were especially abundant in the groundwater of the anoxic wells H5.2 and H5.3 as well as the oxic wells H4.1 and H5.1, while this phylum only contributed a minor fraction in the groundwater of the anoxic wells H4.2 and H4.3 and the oxic wells H3.1 and H3.2 (Figure 8A). In addition, we performed sequencing of 16S rRNA amplicons derived from the extracted RNA to get insight into which taxonomic groups harbor protein synthesis potential as proposed by Blazewicz et al. (2013). RNA-based community analysis targeting 16S rRNA sequences has traditionally been used as an approximation of the currently active fraction of the microbial community. However, this interpretation is critical since many cells may retain high ribosome contents even in a dormant state (Filion et al., 2009; Sukenik et al., 2012) and thus, rRNA content of cells does not necessarily indicate current metabolic activity, especially in low-nutrient environments such as groundwater (reviewed in Blazewicz et al., 2013). Here, we used this approach to investigate whether key microbial groups identified by PLFA-based analysis were supported to be metabolically active or have the potential to resume metabolic activities based on the detection of the corresponding 16S rRNA gene sequences on the RNA level. In general, members of the Candidate Division OD1 formed only a minor part of the community obtained by RNA-based amplicon sequencing while members of the phyla Nitrospirae, Planctomycetes, and Proteobacteria showed the largest relative

abundances (Figure 8B). Members of the phylum Nitrospirae were especially highly represented in the RNA-based analyses of wells H3.2, H4.1, and H5.2 and H5.3. Among the Proteobacteria, Deltaproteobacteria were more frequently represented in the RNA-based analysis of communities of wells H3.1, H3.2, H5.2, and H5.3 while Alphaproteobacteria showed a higher relative abundance in the groundwater of wells H4.2, H4.3 and H5.1 (Figure 8B).

Bacterial phyla and classes may harbor organisms with a high diversity of different metabolisms. Therefore, as some source specific PLFA displayed strong relationships with the environmental variables O_2, NH_4^+, and Fe_t, we specifically focused on groups potentially involved in iron oxdiation and reduction, sulphate reduction, anammox, and nitrite oxidation. Here, relative fractions of reads assigned to bacterial genera known to be involved in either of these processes were summed up to get an estimation of the potential for these processes within the microbial community with both DNA- and RNA-based analyses. On the level of DNA-based sequencing, bacteria involved in iron oxidation accounted for 0.25 to 6.2% of the sequence reads across sites (Figure 9A) while they accounted for 0.24 to 2.8% on the level of the RNA-based analyses with the highest relative fraction of bacteria potentially involved in iron oxidation at wells H5.1 and H5.3 (Figure 9B). Differences across sites and aquifers were more pronounced for bacteria involved in iron reduction, which were accounted for by 0.16 to 3.7% of the sequence reads on the DNA level but for 0.15 to 20.4% on the RNA level with the highest number of sequence reads affiliated with known iron reducers in the groundwater of well H4.3 (Figure 9B). Bacteria related to the genera *Acidiferrobacter*, *Gallionella*, and *Sideroxydans* were the most frequent genera among the known iron oxidizers while members of the genera *Albidiferax* and *Ferribacterium* dominated the iron reducing groups. Bacterial groups potentially involved in sulphur reduction included the genera *Desulfacinum*, *Desulfovibrio*, *Desulfosporosinus*, *Desulfatiferula* as the most frequent groups and accounted for 0.2 to 2.8% of the sequence reads

on the DNA level and 0.4 to 10.4% on the RNA level with the maximum in the anoxic well H4.2 (Figure 9). Anammox bacteria mostly represented by the Candidatus genera *Brocadia* and *Kuenenia* accounted for 0.6 to 3.0% of the sequence reads on the DNA level and for 1.1 to 16.8% on the RNA level with the highest fractions in the groundwater of the wells H3.1, H5.1, H5.2 and H5.3 (Figure 9). Finally, we observed large fractions of potential nitrite oxidizers mostly related to the genus *Nitrospira* with the vast majority of the *Nitrospira*-affiliated reads especially in the lower aquifer assemblage showing a high sequence similarity to the 16S rRNA gene sequence of *Nitrospira moscoviensis* (96 – 99%). Moreover, reads associated with the genus *Nitrospira* may also include potential comammox organisms (Pinto et al., 2016). Relative fractions of sequence reads affiliated with this genus on the DNA and RNA level were highest in the oxic groundwater as the well H4.1 and lowest in the anoxic groundwater of wells H4.2 and H5.2 (Figure 9). Since nitrifiers such as *Nitrospira* are known to retain a high ribosome content even if cells are not active (Morgenroth et al., 2000), these results do not necessarily indicate high nitrite oxidation activity at the time point of sampling but point to nitrite oxidizers forming a large fraction of the microbial community with protein synthesis potential.

4. Discussion

4.1. PLFAs distribution

The PCA of PLFAs indicated that the oxic/suboxic and anoxic groundwaters had distinct bacterial communities, with the anoxic groundwater additionally differentiated into two distinct bacterial communities (Figure 4). Of the environmental variables tested, the variation partitioning showed that NH_4^+, O_2 and Fe_t concentration explained 22.0, 19.7 and 13.4% of the PLFA variations, respectively (Figure 6), and differentiated those three bacterial communities. Variation

partitioning analyses revealed, along those environmental variables, clusters of covarying PLFAs that may originate from the same functional group of organisms or closely affiliated organisms that react similarly to certain environmental conditions. While the ladderanes are unequivocally attributed to anammox bacteria (Sinninghe Damsté et al., 2002; 2005), the other PLFAs are not exclusive to a phylogenetic or functional microbial group which complicates their use to understand the role of microbes in environments. The t-value biplots of variation partitioning analyses evidenced the PLFAs that significantly correlated with the environmental variables O_2 (Figure 6A) Fe_t (Figure 6B) and NH_4^+ (Figure 6C), and provided better insights into the functional diversity of active microorganisms in the subdivided groundwaters. Additional supports of the bacterial community structure, assessed by the PLFA patterns, were found in the 16S rRNA-based results. Although a large fraction of the microbial community remains poorly classified and thus precludes the knowledge of the metabolic capacities, high sequence similarity to genera known to be involved in iron oxidation or reduction, sulphate reduction, anammox and nitrite oxidation allowed an estimation of the fraction of the microbial population potentially involved in these processes. By combining the PLFA-based and sequencing-based approaches, we aimed, here, to compensate for biases introduced by PCR as well as for the limited phylogenetic resolution of PLFA-based analysis. This combined approach resulted in highly supported evidences of some key microbial players and associated biogeochemical processes in physicochemical distinct aquifer assemblages of the aquifer transect.

4.1.1. PLFA cluster in oxic to suboxic groundwater (wells 5.1, 4.1, 3.1 and (3.2))

A cluster of the covarying 20:4, 20:5, 22:5 and 22:6 PLFAs has to our knowledge heretofore never been observed in groundwater. Associations of those PLFAs have been commonly found in eukaryotes such as microalgae etc. (Volkman et al., 1989; Shinmen et al., 1989; Kennedy et al., 1993; Olsson, 1999; Qi et al., 2004) and protozoans (White, 1988). Protozoa act as detritivores

and are expected to be key predators in the microbial loop feeding on different subsets of the bacterial communities and other protozoa (Brad et al., 2008; Akob and Küsel, 2011). Consistently, sessile and free swimming suspension feeding flagellates, e.g., *Spumella* sp., mobile naked amoebae and ciliates could be detected in this aquifer with a cultivable protist abundance of up to 8.000 cells L^{-1} (Risse-Buhl et al., 2013). 18S rRNA gene sequences also revealed high relative fractions of *Spumella*-like Stramenopiles, and sequences affiliated with fungi and metazoan grazers. DNA based pyro-tag sequencing of fungal internal transcribed spacer (ITS) sequences revealed a fungi community structure dominated by Ascomycota and Basidiomycota (Nawaz et al., 2016) with the majority of the observed fungal groups being involved in ectomycorrhizal symbioses. In general, the abundance of micro-eukaryotes in pristine groundwater is estimated to be low, because they are limited in nutrients, space, and are unable to cope with oxygen limitations (Akob and Küsel, 2011). Consistently, they are commonly found in higher concentrations in OM-rich contaminated groundwaters (Ludvigsen et al., 1997). In pristine aquifers, the origin of those eukaryotic organisms is difficult to determine as they may be autochthonous, allochthonous or both. In the studied sites, the close relation of eukaryotic PLFA biomarkers with O_2 concentrations (Figure 6A) suggests their association with recharging groundwater within larger conduits prone to faster water flow. Freshly introduced surface OC and O_2 could fuel the heterotrophic bacterial growth in groundwater. This may subsequently stimulate protists that selectively graze on the prokaryotic biomass and result in the observed relationship between the eukaryotic PLFAs and the O_2 concentration. It is possible to speculate that some surface microorganisms would also survive the transport from surface to the aquifer (Dibbern et al., 2014), especially if the transport is fast. In this case, high cy17:0 to 16:1ω7c ratios (Table 3) may show physiological stress due to change of the environmental conditions within the gram negative communities (Balkwill et al., 1998).

The 16:1ω11c and particularly the 11Me16:0 are major components of *Nitrospira moscoviensis* (Lipski et al., 2001) cell membranes, an obligatory chemolithoautotrophic nitrite-oxidizing bacterium (NOB: Ehrich et al., 1995). In the oxic groundwater, the occurrence of 16S rRNA gene sequence reads closely related to *Nitrospira moscoviensis* (Herrmann et al., 2015) further supports the potential of 11Me16:0 as biomarker for *Nitrospira moscoviensis* and confirms previous assumptions about an important role of nitrite oxidizers within the autotrophic community of the lower aquifers (Herrmann et al., 2015). The correlation of 11Me16:0 and 16:1ω11c with O_2 (Figure 6A) indicated the occurrence of active nitrification in oxic zones of the aquifers in agreement with observation of experiments (Satoh et al., 2003). *Nitrospira* use the reverse tricarboxylic acid cycle as the pathway for CO_2 fixation (Lücker et al., 2010) which leads to small ^{13}C fractionation (2 - 6‰) between biomass and CO_2 (van der Meer et al., 1998). The ^{13}C-enrichment of 11Me16:0 and 16:1ω11c relative to the other PLFAs (up to 18‰ in well H4.1) supports thus major *Nitrospira* contribution to those PLFAs found in oxic groundwaters (Figure 7).

4.1.2. PLFA cluster in anoxic Fe_t richer groundwater (wells H4.2 and H4.3)

In groundwater the concentration of dissolved iron is often inversely related to oxygen as O_2 in water will chemically oxidize iron that will precipitate as insoluble iron-hydroxides at neutral pH. In the wells H4.2/4.3, the increase of the PLFAs 10Me12:0, 12:0, 17:1, 18:1ω7c and 18:1ω9c with concentrations of Fe_t, Fe_2^+ and HCO_3^- (Figure 5 and 6B) and the DNA- and RNA-based analyses (Figure 9) suggested degradation of OM by anaerobic iron-reducing bacteria (IRB). Because many IRB are highly versatile, i.e. they can use different metal substrates as electron acceptors coupled to the oxidation of the OM (Coleman et al., 1993; Lovley et al., 1993; Holmes et al., 2004), specific PLFAs linked to the reduction of iron in anoxic environments are poorly described. The two most studied genera of IRB are *Geobacter* and

524 *Shewanella* which contain most of those PLFAs (Coleman et al., 1993; Lovley et al., 1993;
525 Hedrick et al., 2009). However none of these PLFAs are specific to a certain genus or species.
526 The 17:1 is generally related to anaerobic SRB (Dowling et al., 1986) as *Desulfobulbus* (Parkes
527 and Calder, 1985; Macalady et al., 2000) but also occur in dissimilatory IRB as *Shewanella*
528 (Coleman et al., 1993). The ability of some sulphate reducers to reduce iron rather than sulphate
529 has long been recognized in groundwater (Coleman et al., 1993).

530 The 18:1ω9c is common and abundant in fungi (Frostegård and Bååth, 1996; Kaiser et al.,
531 2010), but may also occur in micro-algae (Arts et al., 2001) and gram-negative bacteria
532 (Kandeler, 2007). The 18:1ω9c, 18:2ω6,9 and 18:3ω6 are typically used as fungi biomarkers in
533 soil (Frostegård and Bååth, 1996; Ruzicka et al., 2000; Bååth and Anderson, 2003) and more
534 particularly for saprotrophs (Etingoff, 2014). The correlations between 18:1ω9c, 18:2ω6,9 and
535 18:3ω6 suggested a major fungal origin of those PLFAs in the studied groundwaters. In soil,
536 fungi are well known for their role in accelerating weathering and solubilisation of iron-
537 containing minerals by excreting organic acids including phenolic compounds, siderophores,
538 and protons (Arrieta and Grez, 1971; Landeweert et al., 2001). By forming dense hyphae
539 tunnelling in soils and shallow rocks, fungi mediate and facilitate iron transport in plants and
540 increase iron availability in the environment (van Schöll et al., 2008). Therefore, several studies
541 have linked enhanced rates of iron cycling to the presence of fungal biomass (Gadd, 2010).
542 Moreover, in a recent study, it is been shown that rhizoplanes are important root channels for
543 preferential vertical transport from soil to seepage area of soil colloids including microbes
544 (Dibbern et al., 2014). Limitation of ferric iron may restrain the growth and activity of IRB in
545 subsurface (O'Neil et al., 2008). In the groundwater of wells H4.2 and H4.3, the close relation of
546 18:1ω9c and 18:2ω6,9 with Fe_t concentration (Figure 6B) suggested that fungal biomass may,

by mediating and facilitating the transport of different types of organic/inorganic particles and colloids, play a key role in iron bioavailability and thus sustain IRB growth and activity.

4.1.3. PLFA cluster in anoxic NH_4^+ richer groundwater (wells H5.2 and H5.3 and (3.2))

To our knowledge, this is the first time phospholipid [3]-ladderane and [5]-ladderane, which attest the presence of viable or recently degraded anammox bacteria (Jaeschke et al., 2009), have been identified in groundwater. The occurrence of anammox bacteria in those groundwaters is consistent with the DNA- and RNA-based analyses (Figure 9) and coincided with higher concentrations of ammonium (Figure 2). The difference between DIC and ladderanes $\delta^{13}C$ values of 55‰ was within the range previously reported for anammox in Black Sea (Schouten et al., 2004), further suggesting that autotrophic carbon fixation pathways within the diverse group of anaerobic ammonium-oxidizing bacteria are similar. In the sub-oxic (well H3.2) and anoxic groundwaters (well H5.2 and H5.3), the increasing concentration of ladderane lipids derived from anammox bacteria with decreasing O_2 concentration (Figure 6A) agrees well with the reported high sensitivity of the anammox process to O_2 (Kalvelage et al., 2011). Denitrification and anammox are the dominant nitrogen loss pathways in aquatic ecosystems (Burgin and Hamilton, 2008; Koeve and Kähler, 2010). The occurrence of lipids derived from anammox bacteria in those groundwaters indicates that the anammox process may be critically important in the nitrogen loss from this part of the aquifer assemblage.

High amounts of 10Me16:0 are typically found in SRB (Dowling et al., 1986; Vainshtein et al., 1992; Kohring et al., 1994) but also occur in anammox bacteria (Sinninghe Damsté et al., 2002). Anammox bacteria strongly fractionate against ^{13}C, producing ladderane lipids which are ^{13}C-depleted by 47‰ compared to the inorganic carbon source (Schouten et al., 2004). Relative to ladderanes, SRB-derived lipids are expected to be ^{13}C-enriched as cultured SRB under heterotrophic and autotrophic growth fractionated against ^{13}C by up 27‰ (Londry et al., 2004).

Therefore, the ^{13}C-enrichment of 10Me16:0 (up to 19‰) relative to the ladderanes supported major SRB contribution to the 10Me16:0 found in these groundwaters. The i13:0, *i*15:0 and i17:1 are typically, as 10Me16:0, associated with SRB (Edlund et al., 1985; Kohring et al., 1994). In those groundwaters, similar $\delta^{13}C$ values, in the -44 to -56‰ range, also supported a common SRB origin of those PLFAs.

Variation partitioning analyses showed that the concentrations of [3]-ladderane, [5]-ladderane, 10Me16:0 and i17:1 correlated with NH_4^+ concentration (Figure 6C). Many studies in other aquatic environments showed that the relative importance of the anammox process is directly related to the availability of NH_4^+ (Dalsgaard and Thamdrup, 2002; Kuypers et al., 2003). Commonly, the breakdown of OM via ammonification or dissimilatory nitrate reduction to ammonia (DNRA) is presumed the major sources of NH_4^+ for anammox (Kartal et al., 2007). However, the recent discovery of comammox organisms capable of complete nitrification underlines the complexity of the nitrogen cycle and the variability of ammonium sources for anammox (van Kessel et al., 2015). The availability of OM is known as an additional important factor influencing the anammox process. Higher anammox activity has been observed in OM-poor environments and interpreted as a decrease in competition for NO_2^- by heterotrophic denitrifiers (Hu et al., 2011). Consistently, high anammox activity was observed in redox zones associated to sulphate reduction or sulphur oxidation (Mills et al., 2006; Canfield et al., 2010; Prokopenko et al., 2013; Wenk et al., 2013). In the groundwater of the wells H5.2 and H5.3, the occurrence of anammox bacteria and SRB supported thus low groundwater-surface interactions which likely threatened the availability of generically favourable electron acceptors and labile OM.

5. Conclusion

In this study, we used constrained ordination to show environmental variables that significantly correlated with PLFA relative abundances in groundwater of distinct carbonate-rock aquifer assemblages. This technique shows that the active subsurface microbial communities were mainly affected by variations in dissolved O_2, Fe_t and NH_4^+ concentrations. Variation partitioning identified PLFA-based microbial functional groups that were directly supported by results of DNA- and RNA-based amplicon sequencing targeting bacterial 16S rRNA genes. Higher O_2 concentration resulted in increased eukaryotic biomass and higher relative fractions of nitrite oxidizing bacteria (e.g. *Nitrospira moscoviensis*) but impeded anammox bacteria, sulphate-reducing bacteria and iron-reducing bacteria. In anoxic groundwater, concomitant increase of total iron (Fe_t), HCO_3^- and PLFAs abundant in gram-negative bacteria and fungi suggested the occurrence of active dissimilatory iron-reduction and a possible role of fungi in meditating iron solubilisation and transport in those aquifer assemblages. The relative abundance of PLFA derived from anammox bacteria correlated with NH_4^+ concentrations, showing the dependence of the anammox process on the availability of NH_4^+. Our study shows that different relationships among the microbial community structures, estimated based on both the PLFA patterns and 16S rRNA gene-targeted next generation sequencing, reflected changes in the physiological strategies of microorganisms related to a decrease in substrate bioavailability and redox potential of the groundwater.

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Table 1. Lipid markers proposed to represent a group or genus of microorganisms

Lipid marker	Interpretation	references
branched PLFAs	Gram-positive bacteria	Harwood and Russell (1984)
mono-saturated PLFAs	Gram-negative bacteria	Wilkinson (1988)
10Me18:0	Actinomycetes	Zogg et al. (1997); Zelles (1999)
18:1ω9c. 18:2ω6c, 18:3ω6c, 18:3ω3c	Fungi	Frostegard and Bååth (1996)
10Me16:0, cy18:0(ω7,8)	*Desulfobacter*	Dowling et al. (1986)
11Me16:0. 16:1ω11	*Nitrospira moscoviensis*	Lipski et al. (2001)
i17 :1ω7c, i15 :1ω7c, i19:1ω7c	*Desulfovibrio*	Edlung et al. (1985), Kohring et al. (1994)
17:1ω6, 15:1	*Desulfobulbus*	Parkes and Calder (1985), Macalady et al. (2000)
i17 :1ω5, 10Me18:1ω6, 11Me18:1ω6	*Thiobacillus*	Kerger et al. (1986, 1987)
20:2ω6, 20:3ω6, 20:4ω6, 22:5, 22:6	Fungi, *Protozoa,* Algea	Kennedy et al.(1993), Olsson (1999), White (1988); Volkman et al. (1989)
[3]-ladderane, [5]-ladderane	anammox	Sinninghe Damsté et al. (2002, 2005)

Table 2: Well depths, sampling dates and stratigraphic units of the studied monitoring wells.

well name	aquifer assemblage	well depth* (m)	Sampling dates	Stratigraphic unit	Notes
H3.1	HTL	42.7-46.7	June 14	moTK	well almost dry. Pumped only 100L
H3.2	HTU	15-22	June, September 14	moM	well dry in December 14
H4.1	HTL	44.5-47.5	June, September 14	moTK	well not accessible in December 14
H4.2	HTU	8.5-11.5	June, September 14	moM	well not accessible in December 14
H4.3	HTU	8.5-12.5	June, September 14	moM	well not accessible in December 14
H5.1	HTL	84-88	June, September, December 14	moTK	
H5.2	HTU	65-69	June, September, December 14	moM	
H5.3	HTU	47-50	June, September, December 14	moM	

*depth of well screen section below surface; HTL: Hainich transect lower aquifer assemblage; HTU: Hainich transect upper aquifer assemblage; moTK: Upper Muschelkalk, Trochitenkalk formation; moM: Upper Muschelkalk, Meissner formation

Table 3: FunPLFA/BactPLFA, G-/G+ and cy17:0/16:1ω7c ratios averaged in the upper aquifer (HTU) and lower aquifer (HTL) and in the anoxic groundwater at location H4 and H5.

	FunPLFA	std	G-	std	G+	std	FunPLFA /BactPLFA	std	G+/G-	std	cy17:0/16ω7c	std
HTL	7.8	3.3	27.2	7.1	8.9	2.6	0.2	0.1	0.4	0.2	0.2	0.1
HTU	8.2	9.2	29.4	8.0	11.4	4.4	0.2	0.2	0.4	0.2	0.0	0.0
H4.2/H4.3	19.0	7.8	25.5	4.5	16.3	1.5	0.5	0.2	0.7	0.1	0.0	0.0
H5.2/H5.3	1.9	2.0	34.0	8.4	9.5	3.2	0.1	0.1	0.3	0.1	0.0	0.0

Table 4: PLFA $\delta^{13}C$ values averaged for the sampled month in the different wells

PLFA	H3.2	std	H4.2	std	H4.3	std	H5.2	std	H5.3	std	H3.1	std	H4.1	std	H5.1	std
i 13:0	-39.2	3.5	-42.9		-37.7	0.8	-53.6	1.6	-51.2	10.3	-38.8		-40.7	1.8	-40.4	3.4
a 15:0	-40.3	0.7	-40.8		-35.9	0.3	-45.4	1.4	-43.0	2.8			-40.5	0.8	-37.1	1.5
i 15:0	-43.7	0.0	-47.4		-36.3	0.8	-55.0	3.3	-56.8	12.7			-46.0	0.8	-42.1	2.5
16:17ωc	-47.4	0.6	-37.9	1.6	-36.0	2.3	-44.9	2.3	-44.3	1.3	-42.1		-40.0	3.8	-40.2	2.1
16:1ω11c	-44.4	2.4	-40.5	1.8	-36.0	4.0	-36.0	1.7	-34.6	4.1	-39.4		-27.8	2.4	-28.1	0.7
C16:0	-45.2	3.0	-34.8	2.6	-34.4	1.2	-45.3	2.4	-42.9	3.4	-36.4		-34.3	1.0	-35.4	2.2
10Me16:0	-49.9	0.9			-42.7		-57.6	3.3	-54.6	7.4	-42.2				-40.0	2.2
11Me16:0	-30.5										-28.3		-26.5	0.6	-25.7	2.5
cy17:0											-33.2				-26.3	1.6
i17:1	-45.1						-42.6	2.0	-46.1							
18:1ω9c	-34.0	0.5	-30.2	0.1	-30.9	3.5	-36.7	1.8	-33.4	0.0	-32.1		-31.4	0.0	-31.0	1.4
18:1ω7c	-42.5	0.3	-32.5	0.3	-32.2	1.8	-39.6	2.3	-40.2	2.1	-32.4		-34.9	2.5	-34.6	1.2
18:1ω5c			-33.5		-30.0				-31.7		-35.1		-31.6			
18:0	-35.5	4.4	-42.1	1.0	-36.7	1.3	-36.1		-33.5		-32.9		-32.9		-31.5	1.7
cy19:0	-45.2															
20:4	-42.3												-33.1	1.0	-34.7	0.9
20:5	-41.0												-33.4	0.0		
[3]-lad	-62.9						-64.0	0.3	-63.7	0.8						
[5]-lad	-68.8						-67.0	0.9	-67.7	1.5						
mean	-44.6		-38.3	1.2	-35.4		-48.0		-46.0		-35.7		-34.9		-34.4	
std	9.4		5.4	1.0	3.5		10.5		11.4		4.5		5.5		5.5	

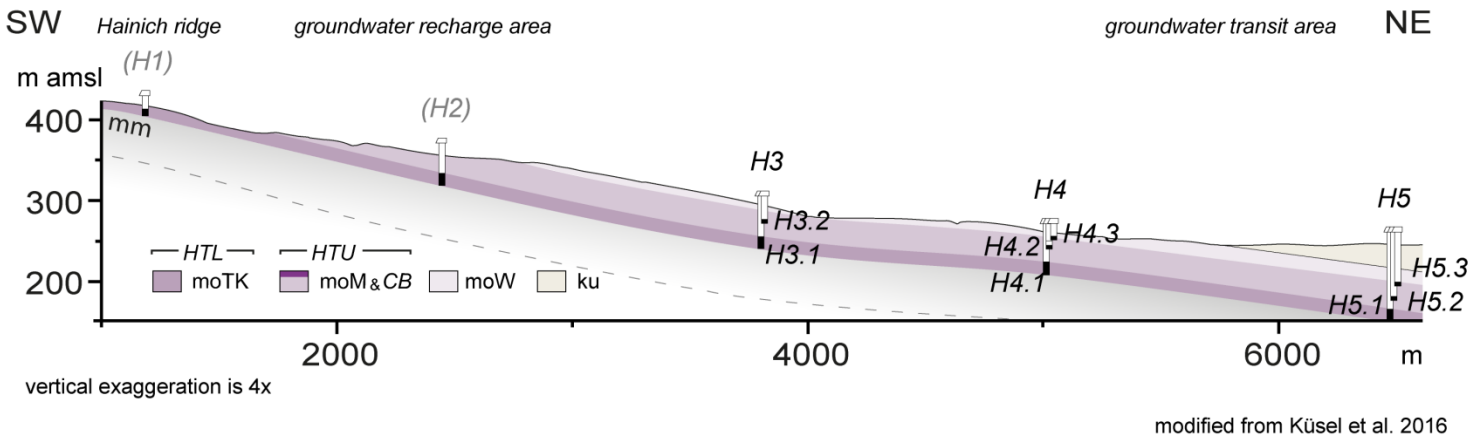
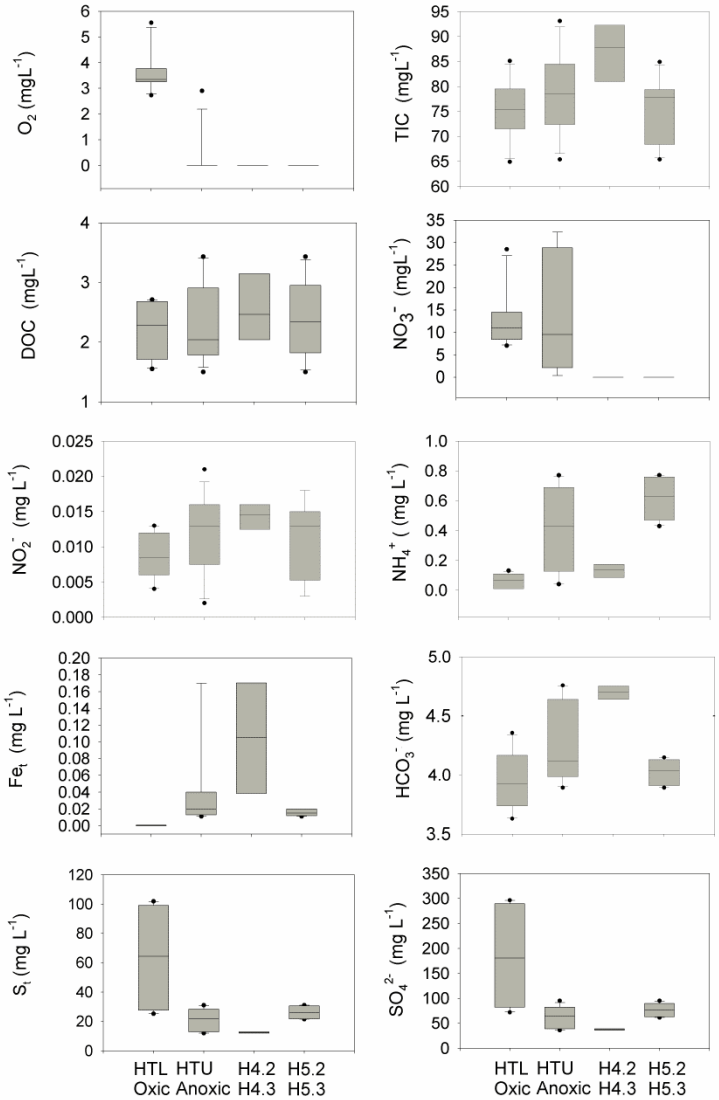


Figure. 1: Schematic geological cross section of the Hainich monitoring well transect (without karst features). The wells sampled for this study are numbered in black. The black colors in the wells indicate screen sections and accessed depths of the aquifer assemblages. Abbreviations: mm: Middle Muschelkalk; mo: Upper Muschelkalk; moTK: Trochitenkalk formation; moM & CB: Meissner formation with Cycloides-Bank; moW: Warburg formation; ku: Lower Keuper.



Schwab and al.,
FIG. 2

Figure 2: Variations of the chemical compositions of the groundwaters relevant for the discussion. HTL and HTU refer to the wells of the lower and upper aquifer assemblage, respectively.

Chemical compositions of the groundwater of the wells H4.2/4.3 and H5.2/5.3 of the HTU are given separately for comparison.

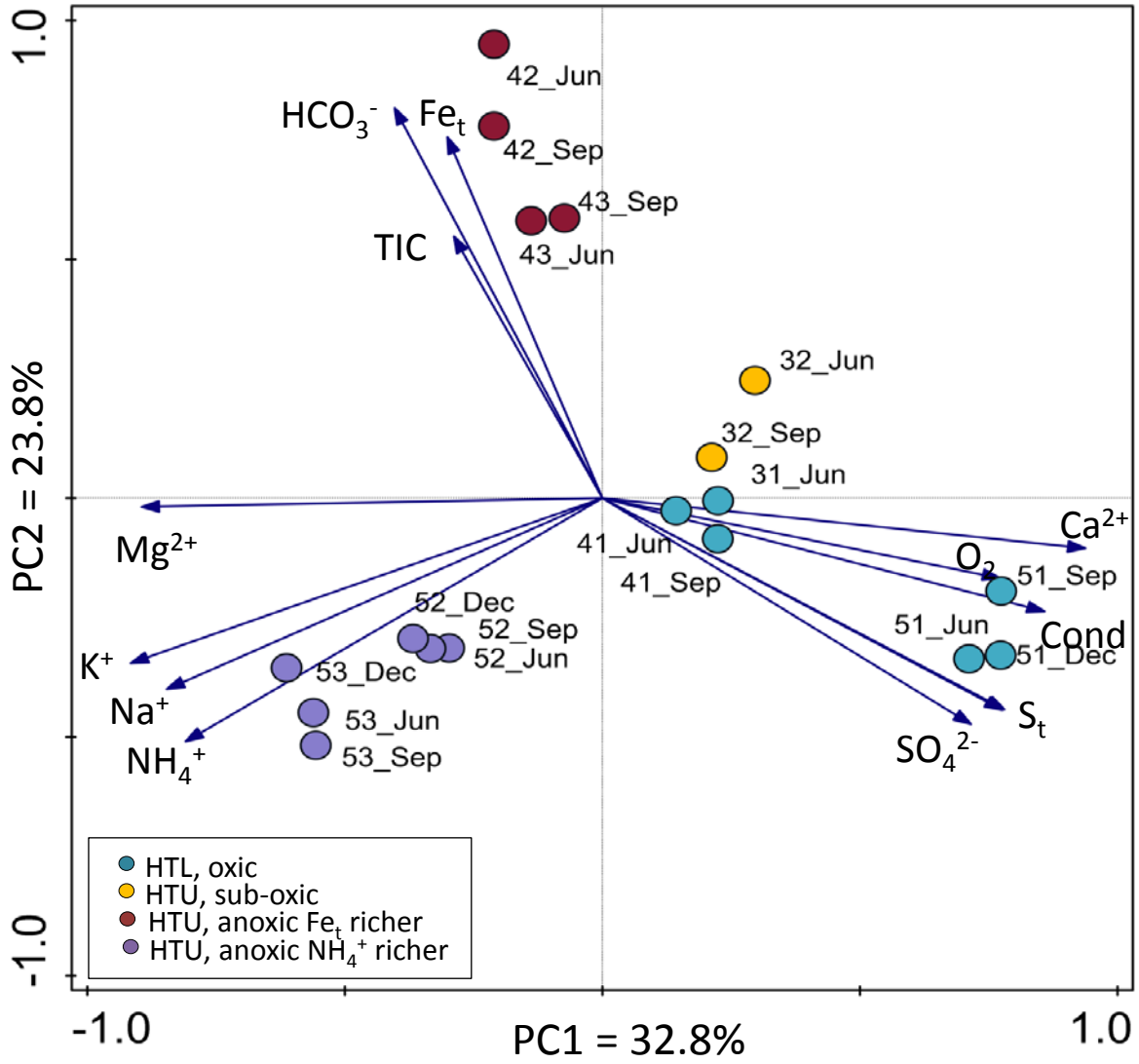


Figure 3: Principal component analysis (PCA) of the groundwater physicochemical compositions. Vectors indicate the steepest increase of the respective physicochemical parameter. The different wells are represented by dots with different colours: blue for oxic groundwater, yellow for sub-oxic groundwater, dark red and violet for anoxic groundwater richer in Fe_t and NH_4^+. Note the separation between the lower and upper aquifer (HTL and HTU, respectively) and the anoxic wells at location H4.2/4.3 and H5.2/5.3.

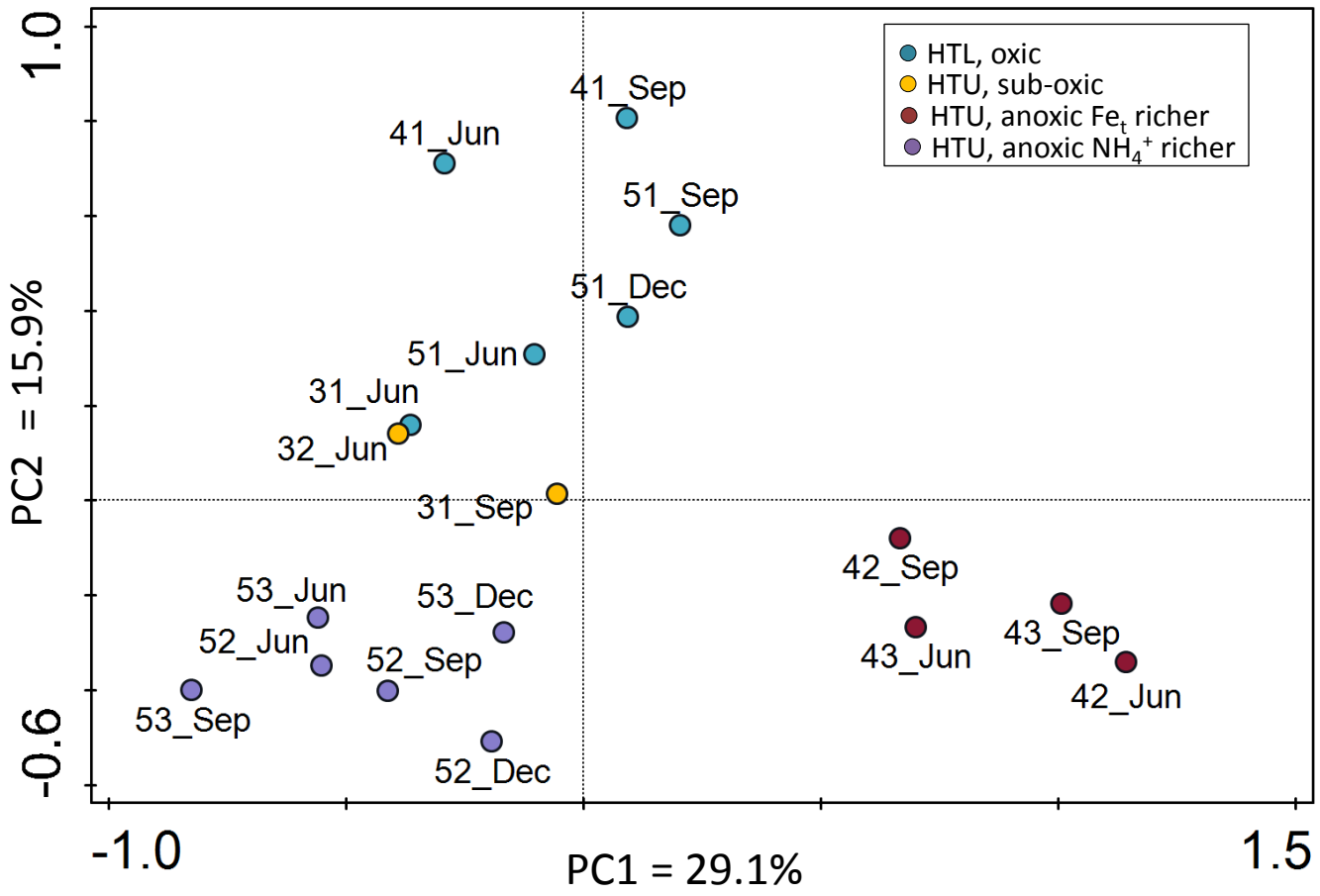


Figure 4: Principal component analysis (PCA) of PLFAs composition. The different wells are represented by dots with different colours: blue for oxic groundwater, yellow for sub-oxic/oxic groundwater, dark red and violet for anoxic groundwater richer in Fe_t and NH_4^+. Note the separation between the lower and upper aquifer (HTL and HTU, respectively) and the anoxic wells at location H4.2/4.3 and H5.2/5.3.

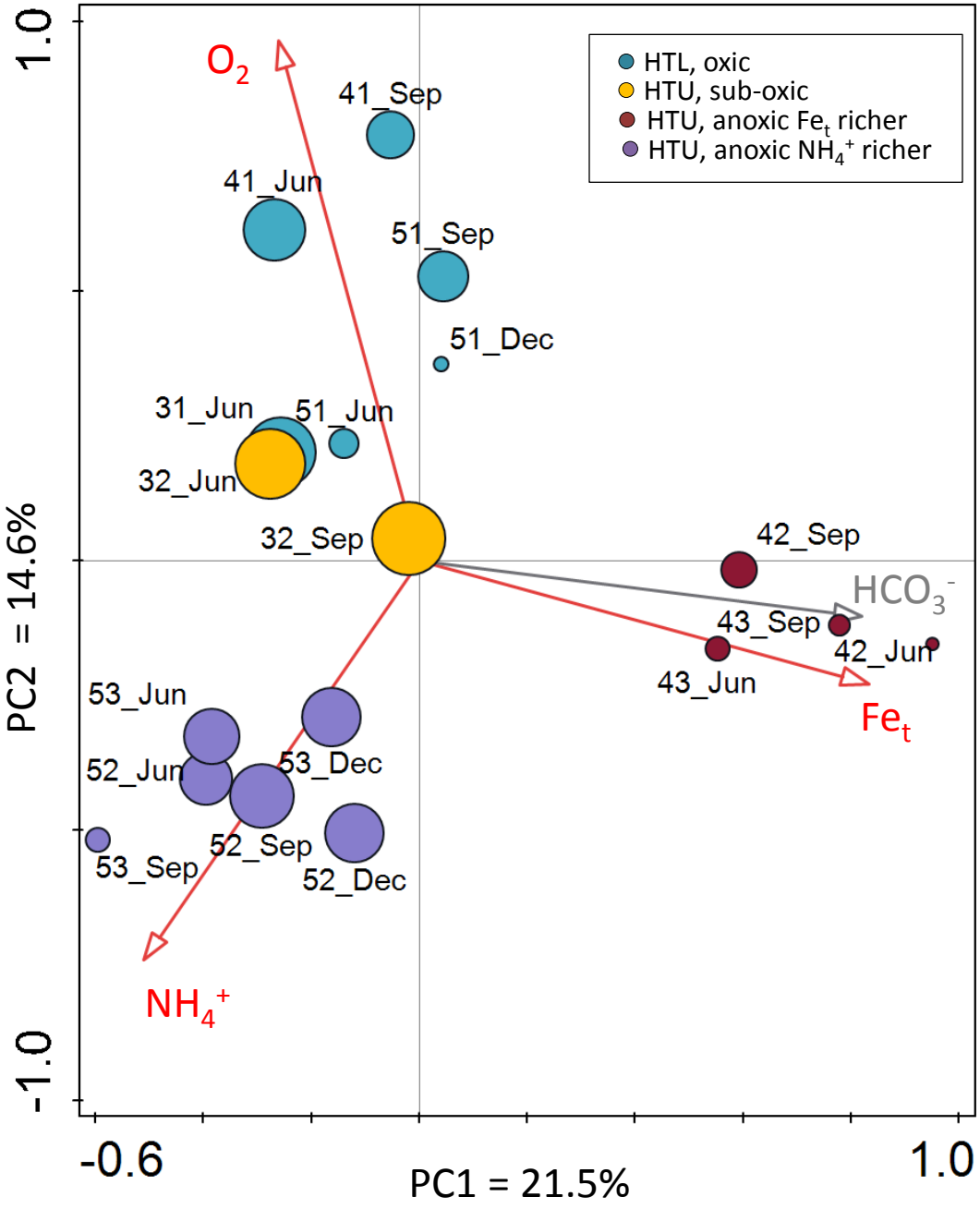
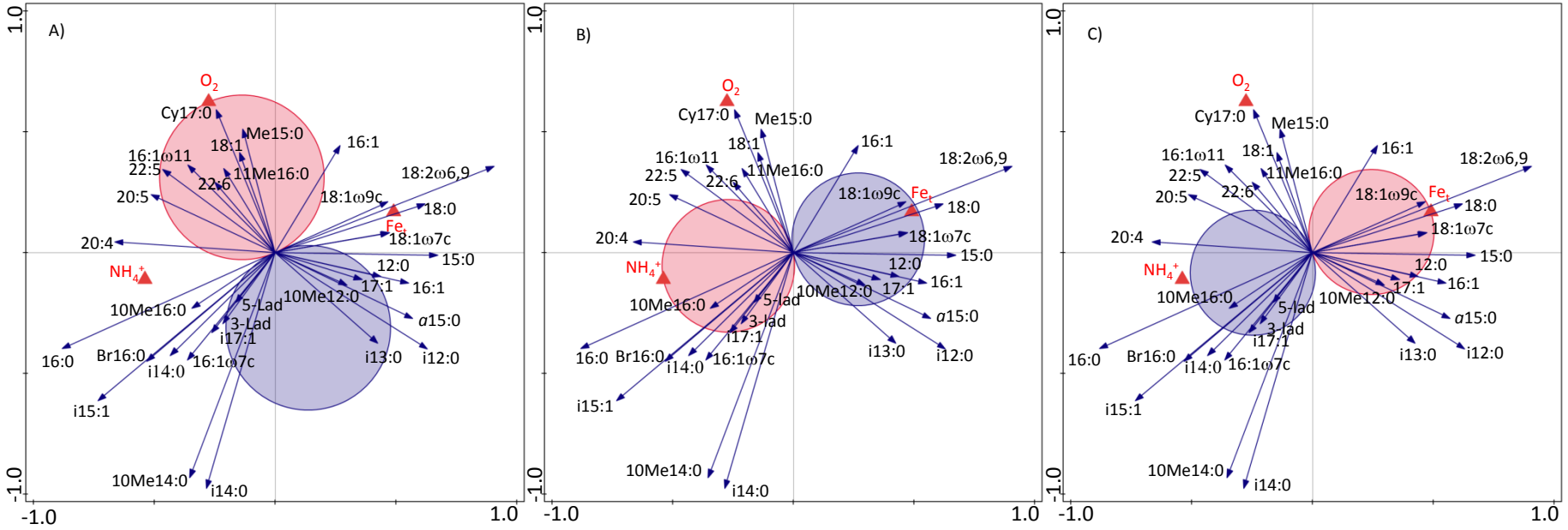


Figure 5: Redundancy analysis (RDA) of PLFAs, used as species, and the most significant environmental parameters O_2, NH_4^+ and Fe_t that explained 39.9% of the variability. The different wells are represented by dots with different colours: blue for oxic groundwater, yellow for sub-oxic groundwater, dark red and violet for anoxic groundwater richer in Fe_t and NH_4^+.



Significance test for variation partitioning

Tested Fraction	% of explained variation	F	P
a+b+c+d+e+f+g	100	3.1	0.002
a	19.7	2.2	0.008
b	22.0	2.1	0.034
c	13.4	1.9	0.016
d	22.3	3.1	0.001
e	29.2	3.6	0.004
f	25.9	3.5	0.002

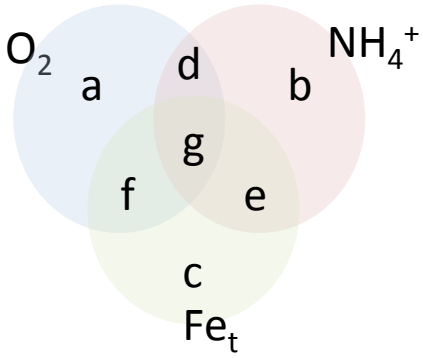


Figure 6: Variation partitioning t-value biplots showing the PLFAs significantly correlated with the environmental variables (A) O_2, (B) Fe_t and (C) NH_4^+. Results of the significance test of the variation partitioning are shown in the associated table. The PLFAs are represented by arrows projecting from the origin. Concentration changes, between sampling data, of a particular PLFA is significantly related to concentration changes of the environmental variables, when the arrow-tip of those PLFA is enclosed within circles. The arrow-tip is enclosed within the red circle for positive correlation and inversely within the blue circle for negative correlation.

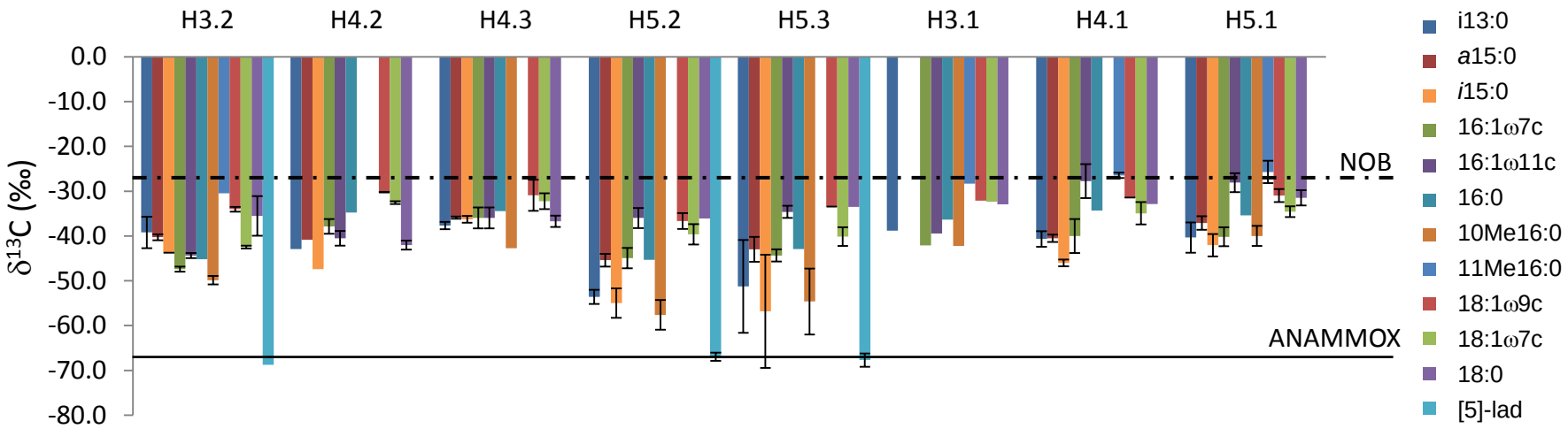


Figure 7: PLFA $\delta^{13}C$ values averaged in the different wells for June, September and December. The dotted and full lines represent the $\delta^{13}C$ values of 11Me16:0 and ladderanes associated with nitrite oxidizing bacteria (e.g. *Nitrospira moscoviensis*) and anammox bacteria, respectively.

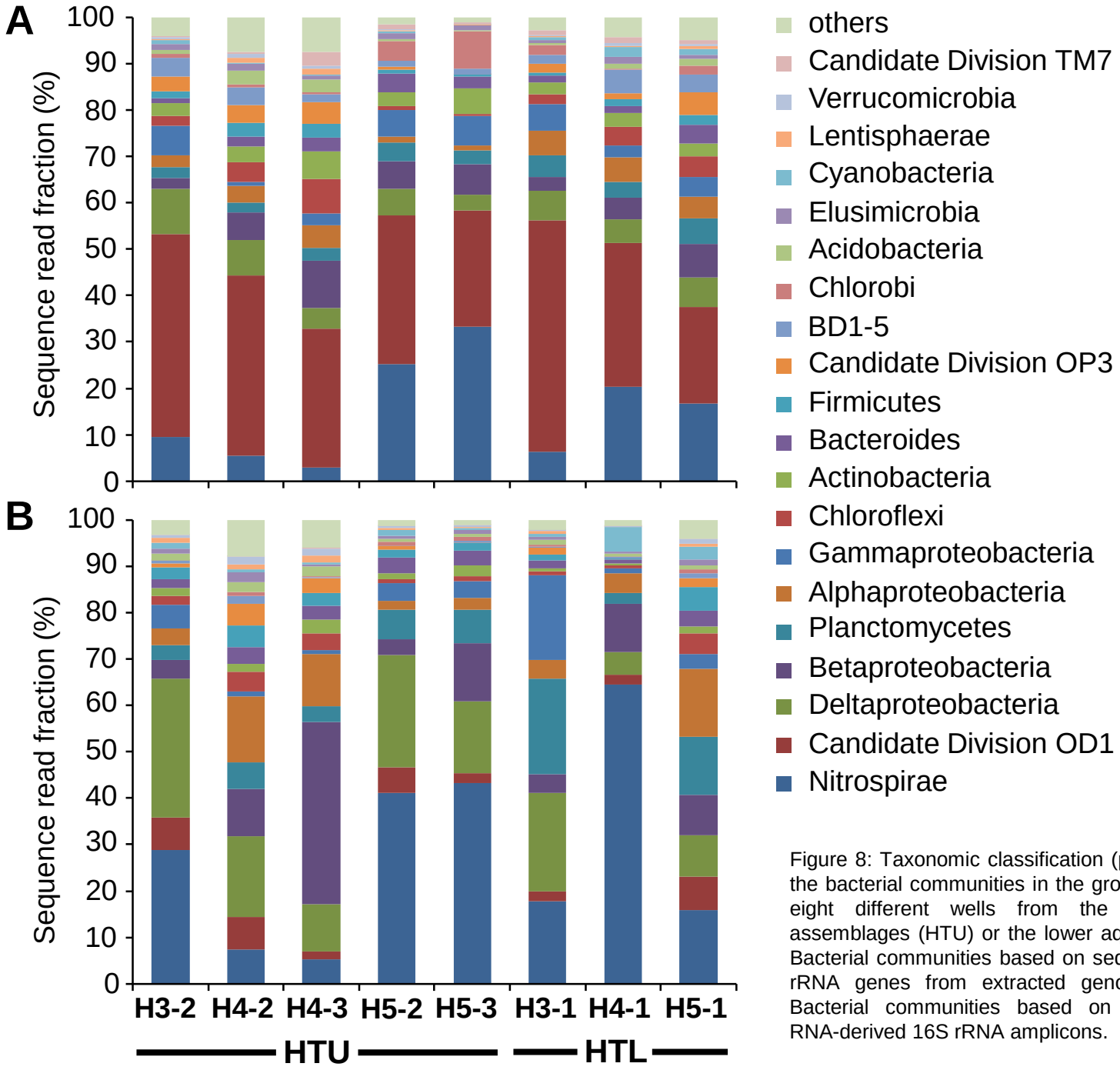


Figure 8: Taxonomic classification (phylum level) of the bacterial communities in the groundwater of the eight different wells from the upper aquifer assemblages (HTU) or the lower aquifer (HTL). (A) Bacterial communities based on sequencing of 16S rRNA genes from extracted genomic DNA. (B) Bacterial communities based on sequencing of RNA-derived 16S rRNA amplicons.

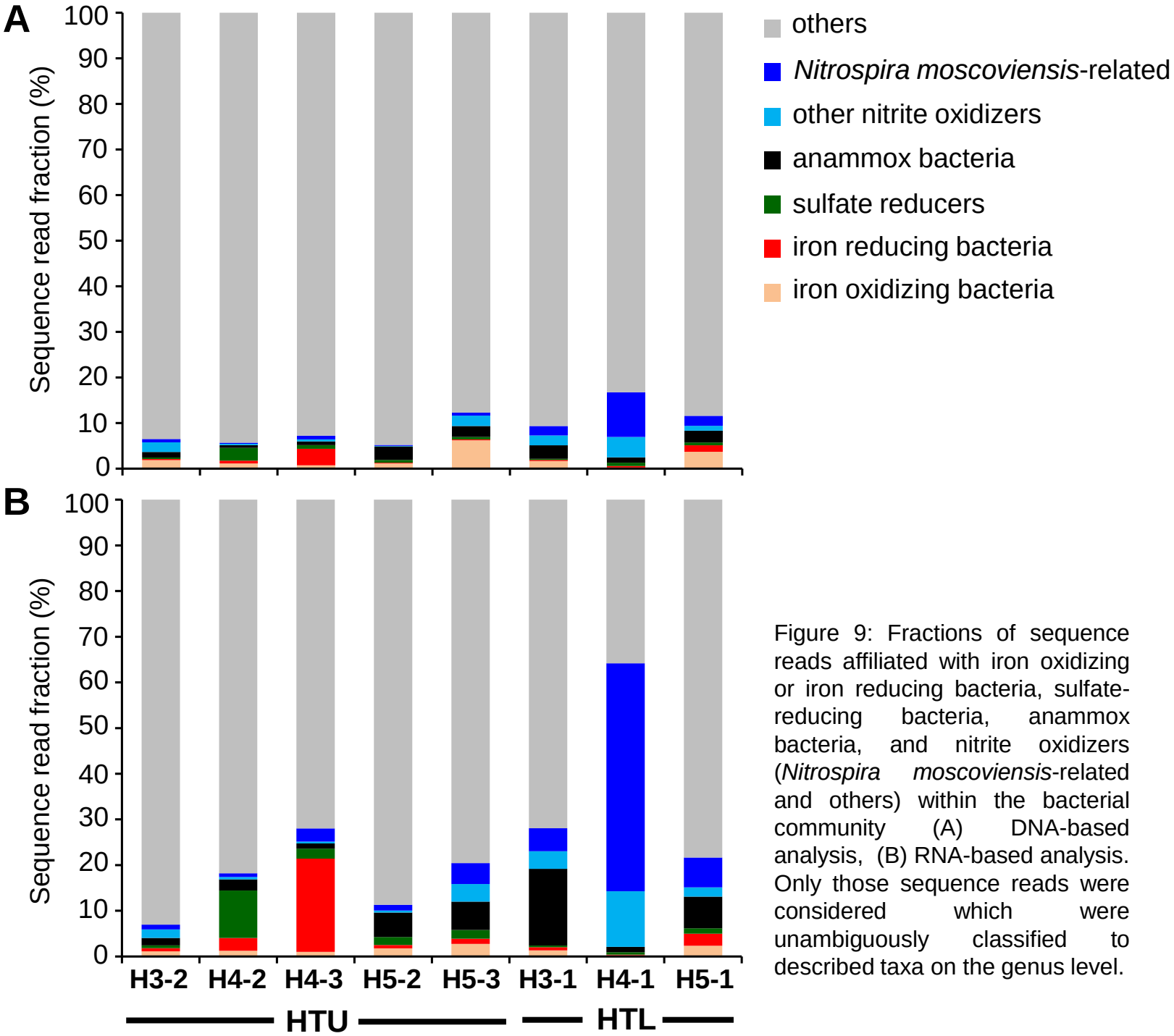


Figure 9: Fractions of sequence reads affiliated with iron oxidizing or iron reducing bacteria, sulfate-reducing bacteria, anammox bacteria, and nitrite oxidizers (*Nitrospira moscoviensis*-related and others) within the bacterial community (A) DNA-based analysis, (B) RNA-based analysis. Only those sequence reads were considered which were unambiguously classified to described taxa on the genus level.