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## In-depth prospection of Avène Thermal Spring Water reveals an uncommon and stable microbial community

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## **ABSTRACT**

### **Background**

Avène Thermal Spring Water (TSW) exhibits therapeutic properties in the treatment of skin pathologies. Arising from a dolomitic aquifer system, its physico-chemical properties are well-established and its bacteriological quality regularly monitored. The microbiote of this aquifer has been characterized.

### **Objectives**

We aimed to describe the structure of the bacterial community inhabiting the deep aquifer and to examine its dynamics over time.

### **Methods**

The Avène TSW was collected at the catchment point and filtered through 0.1 µm pore size filters. The sampling was carried out every 3 months to generate a four-year time series. The DNA extracted from filters was analyzed using high-throughput 16S rRNA gene amplicon sequencing and the microorganisms and their contribution were characterized by the taxonomic assignment of sequence variants generated from each sample.

### **Results**

Bacteria were distributed into 39 phyla. Nitrospirae and Proteobacteria were the most prevalent, accounting for 38% and 23% of the total community on average, respectively. A stable pattern was observed throughout the study. A few bacterial species were always detected, forming a core community of likely chemolithoautotrophic organisms which might use energy sources and nutrients produced from water-bedrock interactions. Most of species were distantly related to organisms described to date.

### **Conclusions**

Avène TSW provided by the deep aquifer system harbours a unique microbial community, shaped by the physico-chemical characteristics of the deep environment. Its remarkable stability over time has revealed a high-level of confinement of the water resource.

## INTRODUCTION

Life on Earth depends on microorganisms. For a very long time, our understanding of the microbial world was related to diseases and the food applications. Probably because they are invisible, microbes were not seriously considered unless they affected human life. At the end of the 20th century, the emergence of molecular and genetic tools have revealed a new vision of the microbial world, revealing the ubiquity and cosmopolitan distribution of microorganisms in all ecosystems including the most extreme natural environments and in association to higher organisms including humans <sup>1,2</sup>. Their high concentrations and large diversity associated with broad functional capacities make them important players on our planet. They provide two third of the oxygen living organisms breath and they play an essential role in the recycling of all elements. Microorganisms also benefit human health and well-being by contributing to digestion, producing vitamin K, promoting development of the immune system, and detoxifying harmful chemicals <sup>3</sup>. As a very efficient chemical factory for the myriad of molecules they produce to survive or thrive, they are also a source of innovative bioactive molecules for industrial, biotechnological or therapeutic applications including the treatment of skin diseases <sup>4,5</sup>.

The deep continental groundwaters are highly oligotrophic environments (low organic carbon concentration) that limit microbial growth, but they remain interesting study sites to investigate the metabolic capabilities and diversity of the microbes that are able to subsist under these conditions. Little is known about the way they impact upon groundwater chemistry, and bioprospecting from this environment may also lead to the discovery of novel bioactive compounds as therapeutic agents <sup>6,7</sup>. Several studies provide evidence of a microbial active life in the deep continental groundwaters <sup>8,9</sup>. Microorganisms inhabiting these ecosystems are very different from those usually encountered in surface waters and their distribution is dependent on the geochemical and hydrological characteristics <sup>10,11</sup>. They are mostly characterized by small size and streamlined genomes <sup>12,13</sup>. Therefore, to survive in these extreme habitats they may have developed specific functions and adopted a chemolithotrophic lifestyle using inorganic resources to provide them with the energy and nutrients necessary for survival <sup>13</sup>. The microbial composition of groundwaters and their stability with time are good indicators of ecosystem confinement and of the good quality of resource management <sup>14</sup>. Some studies have shown a direct impact on groundwater microbial communities with the modification of discharge conditions, or long term diversity changes after drilling <sup>15,16</sup>. However, studies using next-generation sequencing tools for the long-term monitoring of microbial communities are scarce, and are required to understand the geobiological functional interactions that take place in these ecosystems.

Well-known for its benefits for patients suffering from skin diseases, through hydrotherapy treatments or dermocosmetic products, Avène TSW has received a lot of attention regarding its physico-chemical properties and biological safety over time <sup>6,7,17,18</sup>. High frequency and long-term quality controls have shown that the water arising from the deep aquifer has a constant temperature of 21°C, and a chemical composition controlled by the solubility of minerals (dolomite and quartz) which gives it a weak mineralization (conductivity 475  $\mu\text{S cm}^{-1}$ , pH 7.5), dominated by bicarbonate (271.5  $\text{mg.L}^{-1}$ ),  $\text{Ca}^{2+}$  (51.3  $\text{mg.L}^{-1}$ ) and  $\text{Mg}^{2+}$  (26.7  $\text{mg.L}^{-1}$ )

<sup>19,20</sup>. In addition to this traditional quality control, in-depth analysis of the groundwater microbial community has been carried out at the borehole for the past eighteen years using advanced gene sequencing - and more recently metagenomic approaches - aiming to understand the diversity of micro-organisms present, how bacterial populations metabolically interact and how they interact with their abiotic environment.

In the present study, we report an analysis of the microbial community of the Avène TSW at the catchment point using a molecular survey conducted every 3 months over a four-year period (from 2014 to 2018) via High-Throughput Sequencing (HTS) of 16S rRNA gene amplicons. Special focus has been given to the stable part of the community observed in the time series, as these persistent populations may be the key actors in the deep ecosystem functioning.

## **MATERIALS AND METHODS**

### *Study site and groundwater composition*

The thermal site of Avène-les-Bains is located in the north of the Hérault department in France at the eastern end of the "Montagne Noire" mountain. The TSW was collected at the Val d'Orb borehole which is a 180 m deep well that is screened between 160 and 170 m.

The residence time of this groundwater (time that takes rainfall to move through the system), has been estimated by tritium isotopic and atmospheric trace gases measurements to be higher than 200 years <sup>19</sup>. The TSW flows through Cambrian dolomites through a path which can reach 1000 meters deep.

### *Water sampling and processing*

A volume of 10 litres of Avène TSW was collected from the Val d'Orb well every 3 months from December 2014 until September 2018. Before sampling, the tap was chemically sterilized with a solution of isopropanol /alkylamine and the water allowed to flow for 1 min. All samples were collected in sterile bottles and immediately filtered through 0.1 µm pore size filters (Ultipor®, Pall Life Sciences). Filters were stored at -20°C until analysed.

### *DNA extraction, amplification and sequencing*

Bacterial composition was analysed using high-throughput 16S rRNA gene amplicon sequencing on the DNA extracted from filters. Total DNA was extracted using a NucleoSpin®Tissue, mini kit for DNA from cells and tissue (Macherey-Nagel, Germany) with minor modifications: at Step 1 of the manufacturer's protocol (6.2 "Support protocol for bacteria"), the filters were cut and introduced into sterile Eppendorf tubes, the lysis buffer was added and the tubes were heated at 94°C for 10 minutes. All other steps were performed according to the manufacturer's protocol.

DNA was amplified by MrDNA laboratories ([www.mrdnalab.com](http://www.mrdnalab.com), Shallowater, TX, USA) using custom double 7-mer barcodes attached to primers 341Fbar (CCTACGGGNGGCWGCAG) and 785Rbar (GACTACHVGGGTATCTAATCC) targeting the V3-V4 regions of the 16S Rrna gene <sup>21</sup>

and the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions : 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. The PCR products were pooled and purified using calibrated AMPure XP beads and the library was prepared using the TruSeq DNA Nano kit (Illumina). Sequencing was performed on a MiSeq sequencer (Illumina) following the manufacturer's guidelines. Three different runs were performed with samples 1) December 2014 to September 2016, 2) December 2016 to September 2017 and 3) December 2017 to September 2018.

#### *Sequence processing, taxonomy assignment.*

Sequences were essentially treated with a *qiime2* (version 2018.8) pipeline <sup>22</sup> based on amplicon sequence variants (ASVs) generated by DADA2 <sup>23</sup>, ASV tables were constructed for each individual run and combined in a final table. Phylogenetic affiliation of representative sequences was performed by the *qiime2* classifier using the SILVA SSU database (Version 132) as a reference <sup>24</sup>. The table was filtered to remove Chloroplasts, Mitochondria and Archaea sequences, and rarefied to 21430 sequences before estimating alpha diversity indices using *qiime2*.

#### *Phylogenetic analysis*

The partial 16S rRNA gene sequences were aligned using the BioEdit sequence alignment editor version 7.0.5.3 <sup>25</sup>. The evolutionary distances were computed using the Kimura 2-parameter method and a neighbor-joining phylogenetic tree was constructed using 398 positions using MEGA X <sup>26</sup>.

## **RESULTS**

### **Microbial diversity**

The microbial community of Avène TSW was investigated during a four-year sampling period at the catchment point to assess both its structure and temporal dynamics. The study of bacterial diversity was carried out from the analysis of amplicon sequence variants (ASV) <sup>27</sup> generated by next-generation sequencing targeting 16S ribosomal RNA genes, with rarefied data. For each sampling date, bacterial species richness was estimated by the ACE (Abundance-based Coverage Estimator), and the diversity by the Shannon index that weights each variant according to its frequency. A relatively constant richness within samples was observed (Table 1) with an average diversity of 613 species per datapoint. The Shannon index value varies from 6.44 to 7.69. These results suggest no major noticeable compositional shifts in the microbial community during the investigated period.

The clustering of sequencing reads from all samples generated a total of 2428 ASVs, and their taxonomic affiliation was defined by comparison to the reference database SILVA SSU 132. The results presented in Fig. 1 show that ASVs were assigned to 39 known phyla or candidate divisions. Nitrospirae and Proteobacteria were consistently the most prevalent

phyla and accounted on average for 38% and 23% of the total microbial community respectively. Elusimicrobia (8%), Bacteroidetes (5%), and Planctomycetes (5%) followed in phyla abundance ranking. All the 39 phyla were present at all sampling dates and with a relatively stable relative abundance, except for Firmicutes, Calditrachaeta, Lindowbacteria, and the candidate phylum FPB. The absence of the three latter phyla in some samples could be explained by their poor contribution to the bacterial community. As for the species richness estimation, the phylogenetic distribution of the bacterial community was also very stable, further supporting the temporal stability of the aquifer.

Surprisingly, a significant number of ASVs in the data set revealed low similarities when compared with sequences in available databases and could not be assigned to an existing class, order, family or genus of bacteria. As shown in Figure 2, an average 15% of the total community couldn't be assigned down to the genus level over the studied period. This result revealed the existence of a unique bacterial community in the aquifer water when compared to well described bacteria.

### **Identification of a core bacterial community**

When comparing all fifteen samples studied, 116 sequence variants were found in common among all samples, suggesting the existence of a core community in the aquifer. The proportional abundance of these persistent microorganisms and their taxonomic affiliation at the phylum level is shown in Fig. 3. Our results indicate that the core group represented a large part of the microbiota, accounting for 53% (December 2014 and November 2015) to 76% (June 2015) of all sequence variants. The phylogenetic distribution was very stable over time and was composed of 15 phyla. This core community was characterized by the predominance of microorganisms affiliated to the Nitrospirae phylum that accounted for up to 51% of all 16S rRNA gene reads in June 2015, followed by Proteobacteria (5% to 13%), Elusimicrobia (4% to 8%), and Bacteroidetes (3% to 7%). The variation of the contribution of this core community over time could mainly be explained by the variable contribution of the Nitrospirae group.

To assess the genetic diversity of organisms belonging to the dominant Nitrospirae phylum in the core community and their occurrence in other habitats, a phylogenetic analysis was carried out including the 16S rRNA gene sequences in this group and near reference strains or published sequences in the EBI sequence database (Fig. 4). The resulting phylogenetic tree showed that some of the 21 sequences were very similar and thus were probably members of the same bacterial species. When these sequences were clustered at a 98% cut-off, the taxonomic diversity could be reduced to 11 putative species and the Nitrospirae sequences identified in the core community (except for ASV 1510), were very distant from all officially described species in this phylum. The group formed by ASV 1139, ASV1339 and ASV1649 was by far the most dominant at every sampling date and represented on average 19.8% of the bacterial community living in the aquifer. This group was also closely related to the dominant members of a spring water microbial community of an alpine dolomitic-limestone karst aquifer with high water residence time<sup>28</sup>. ASV603 and the cluster composed of ASV288, ASV1021, ASV1095 and ASV2329 was next in the abundance ranking (4.2 and 3.5% respectively) and was also phylogenetically close to bacteria previously observed in

environments that are isolated from allochthonous organic inputs (groundwater in Icelandic lava fields, Genbank accession LR595597).

## DISCUSSION

The in-depth analysis of the bacterial community of Avène TSW using 16S rRNA genes analysis by HTS revealed a remarkable stability of the bacterial community at its catchment point throughout the four-year period investigated and corroborates previously unpublished results of monthly surveys of microbial diversity from 2001 to 2014 obtained using molecular fingerprinting and 454 pyrosequencing (data not shown). The absence of seasonal variability of this community likely reflects the isolation of the system that feeds the aquifer from organic matter or transient bacteria originating from rainfall runoff. The flow regime, favourable hydrogeological conditions (long water residence time, structure of the underground water system) combined with the stability of the biota - likely due to low metabolic activity of bacteria in karstic systems <sup>29</sup> - are possible explanations of the constancy of Avène TSW composition.

Maintenance of the bacterial community structure was driven by a core assemblage characterized by persistent bacterial species which could contribute up to 76% of the total microbiota where most were affiliated to the Nitrospirae phylum. Members of the Nitrospirae have been reported in various natural environments and have been shown to be particularly prevalent in karst systems <sup>28,30</sup>. At present, the few species that have been isolated and characterized in this phylum are chemolithoautotrophic and include ferrous iron oxidizers and nitrifiers. Among those, *Nitrospira inopinata* has developed outstanding metabolic capabilities allowing the complete oxidation of ammonia to nitrate <sup>31</sup>. Others are also capable of magnetotaxis by the intracellular biomineralization of magnetic crystals <sup>32</sup>, or are metabolically versatile in being able to subsist on limited energetic resources <sup>33</sup>. In our study, a member of Nitrospirae was overwhelmingly represented in the core community, contributing to about 20% of the overall community. This phylotype shared 99% sequence similarity to the 16S rRNA gene of a microorganism that dominated an alpine dolomitic-limestone karst aquifer spring of high average water residence time and relatively constant flow <sup>28</sup>. The prevalence of closely related phlotypes in distant areas that show similar hydrological patterns not only support a very good adaptation to the prevailing abiotic conditions, but also suggests an important contribution to the biogeochemical processes within the karstic environment.

In the last decade, several studies using molecular surveys have described the microbial diversity of karst systems <sup>14</sup>. Surprisingly, most of the phlotypes affiliated to Nitrospirae in the core community were not closely related (identity < 97 %) to 16S rRNA gene sequences reported in this and other studies and likely represent previously undescribed bacterial species. More broadly, a high proportion of ASVs in all the phyla found in the present study could not be taxonomically classified. Overall these observations support the existence of a unique bacterial community in Avène TSW driven by the biogeochemical specificities of this deep aquifer system. Successful cultivation or metagenomic sequencing of this largely unknown fraction of the microbiota could greatly improve our understanding of their functions and interactions among members of the microbiome in the Avène TSW reservoir.



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**TABLE**

**Table 1 Diversity and richness of the bacterial community in Avène TSW at different sampling dates.**

Estimations of diversity and richness were computed after normalization of the ASVs count table at a depth of 21430 ASVs per sample. ACE (Abundance-based Coverage Estimator).

		<b>No. of ASV</b>	<b>ACE</b>	<b>Shannon</b>
<b>2014</b>	<i>December</i>	1901	527	7.50
<b>2015</b>	<i>March</i>	1883	545	7.50
	<i>June</i>	1993	435	6.44
	<i>September</i>	1909	519	7.31
	<i>November</i>	1734	697	7.35
<b>2016</b>	<i>April</i>	1826	606	6.56
	<i>June</i>	1727	704	7.69
	<i>September</i>	1865	564	7.35
	<i>November</i>	1708	736	7.15
<b>2017</b>	<i>May</i>	1823	610	6.67
	<i>September</i>	1756	676	7.39
	<i>November</i>	1878	551	6.78
<b>2018</b>	<i>March</i>	1727	703	7.36
	<i>May</i>	1785	647	7.37
	<i>September</i>	1754	678	7.03
<b>Mean ± STD</b>		<b>1818 ± 85</b>	<b>613 ± 87</b>	<b>7.16 ± 0.38</b>

## LEGENDS

**Table 1** Diversity and richness of the bacterial community in Avène TSW at different sampling dates.

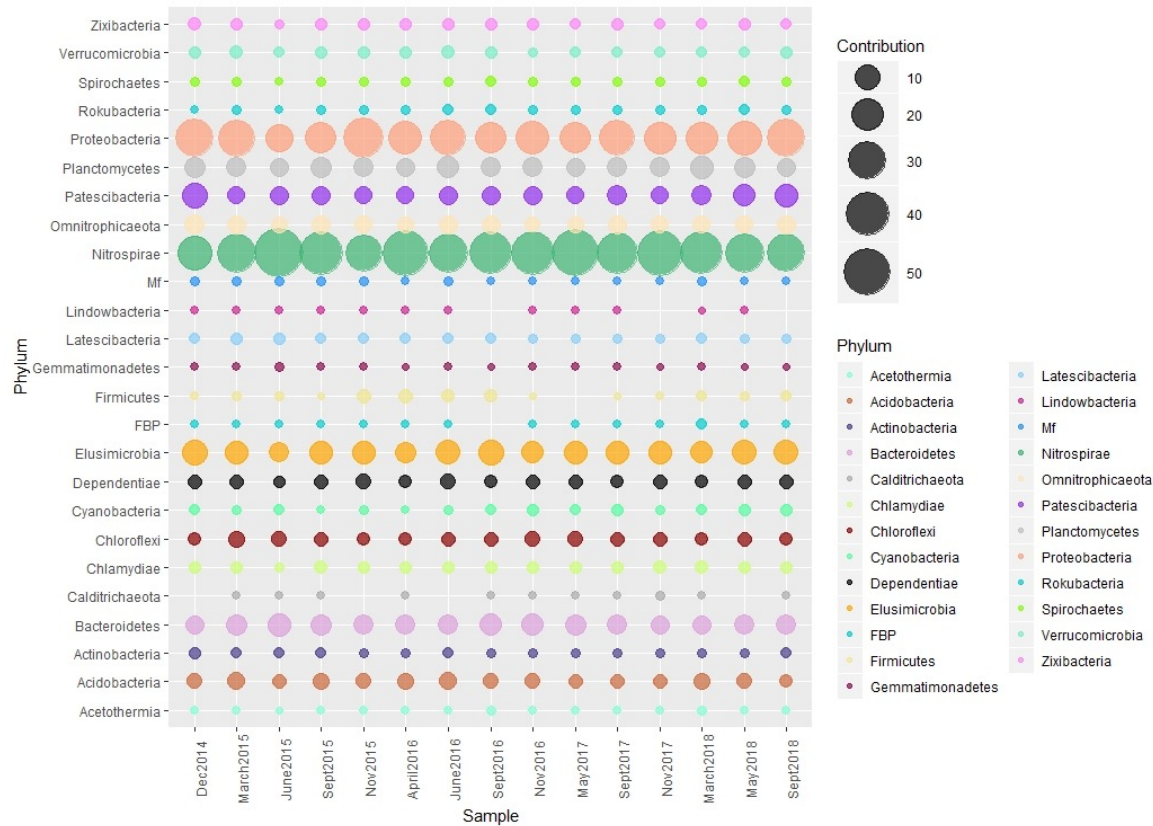
Estimations of diversity and richness were computed after normalization of the ASVs count table at a depth of 21430 ASVs per sample. ACE (Abundance-based Coverage Estimator).

**Figure 1** Taxonomic community composition analysed at different sampling dates by 16S rRNA gene amplicon sequencing. The contribution is given in % of the total community. (Mf: Cumulative contribution of minor phyla).

**Figure 2** Proportion of identified Amplicon Sequence Variants (ASVs) detected at each taxonomic level.

**Figure 3** Taxonomic composition and dynamics of the core community over four years of monitoring.

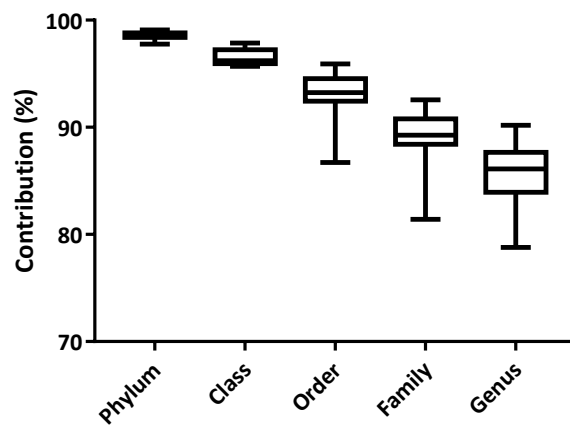
**Figure 4** Phylogenetic relationships between the Amplicon Sequence Variants (ASVs) belonging to the Nitrospirae phylum and the nearest sequences in *the EBI* database based on partial 16S rRNA gene sequence. Bubble plots illustrate the relative abundance (in %) over time from December 2014 to September 2018. Bootstrap values based on 1000 replicates are shown next to the branches.



**Figure 1**

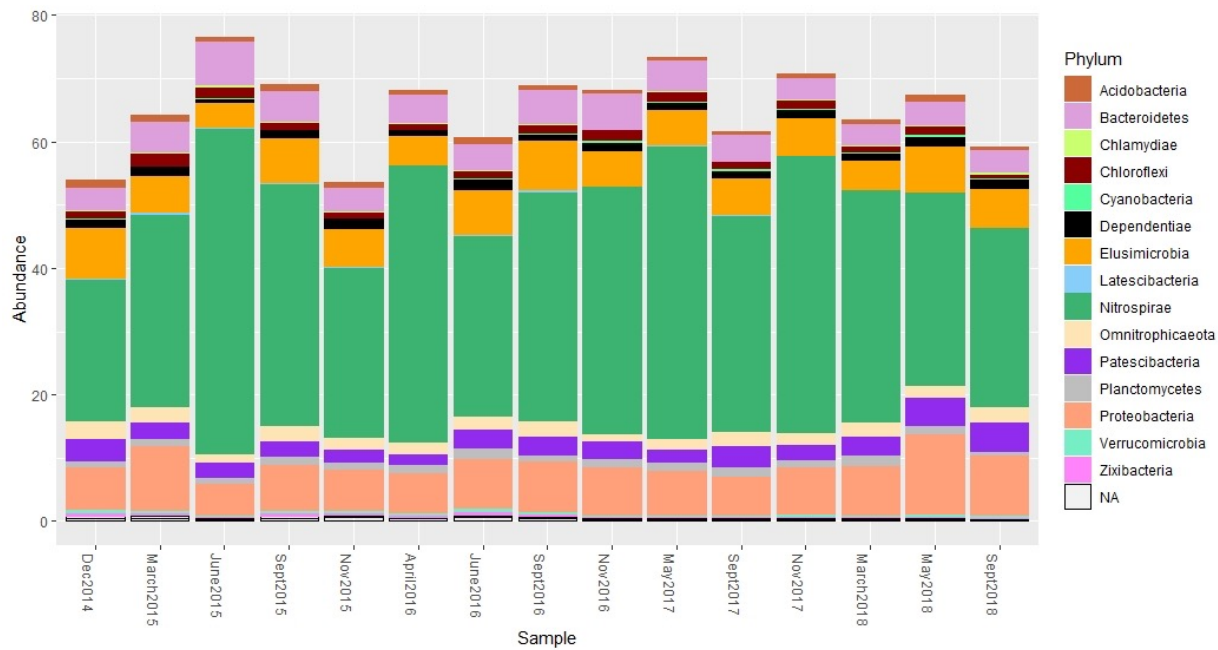
Taxonomic community composition analysed at different sampling dates by 16S rRNA gene amplicon sequencing. The contribution is given in % of the total community. (Mf: Cumulative contribution of minor phyla).

Proportion of ASVs assigned to a given taxonomic level



**Figure 2**

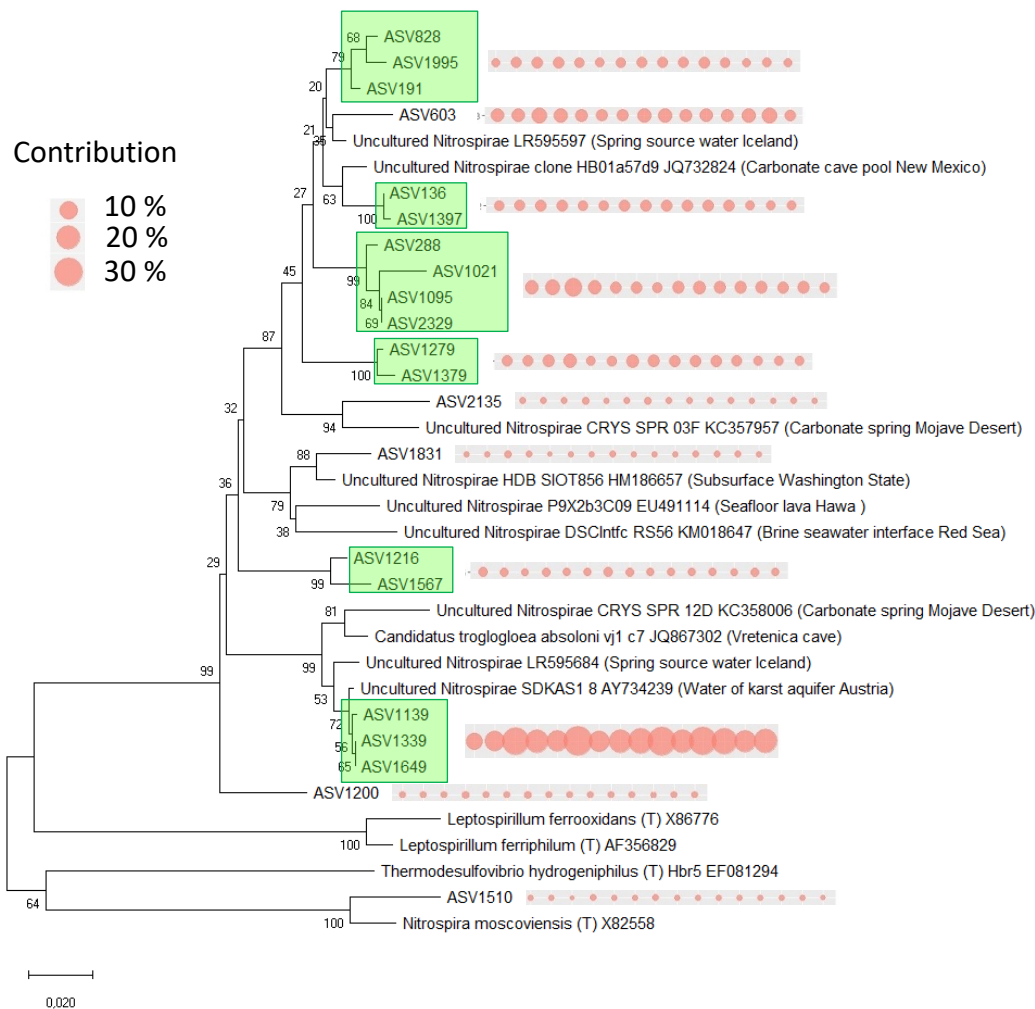
Contribution of Amplicon Sequence Variants (ASVs) assigned to a given taxonomic level.



**Figure 3**

Taxonomic composition and dynamics of the core community over four years of monitoring. NA, not assigned.





**Figure 4**

Phylogenetic relationships between the Amplicon Sequence Variants (ASVs) belonging to the Nitrospirae phylum and the nearest sequences in the EBI database based on the partial 16S rRNA gene sequences. Bubble plots illustrate the relative abundance (in %) over time from December 2014 to September 2018. Bootstrap values based on 1000 replicates are shown next to the branches.