Bacterial diversity in the sulfur and iron springs at Ponts-de-Martel, Neuchâtel

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BACTERIAL DIVERSITY IN THE SULFUR AND IRON SPRINGS AT PONTS-DE-MARTEL, NEUCHÂTEL

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Summary

Two interesting sites that serve as an example of the effects of microbial activity on a landscape can be found near the village of Les Ponts-de-Martel, Neuchâtel. The emergence of anaerobic water enriched in either hydrogen sulfide or ferrous iron offers unique conditions for the development of microorganisms that gain their energy for life by metabolizing these chemically reduced inorganic compounds. Two entirely different microbial ecosystems develop in these chemically different, yet spatially close springs, which are fuelled by the activity of these microorganisms. These ecosystems have now been studied by the genomic sequencing of bacterial molecular markers, and the diversity of the bacterial communities in both springs is described herein. For the sulfur spring the most abundant identified bacterial groups corresponded to photo- and chemotrophic sulfur-oxidizing bacteria, as well as sulfate-reducing bacteria. In the iron spring a few genera known to be involved in iron oxidation and iron reduction could be identified. However, the relationship between the diversity of the bacteria and their potential role in the iron cycle was not as clear as it was for the bacterial community observed in the sulfur spring. Overall, the results presented here shed light on the microbial processes occurring in these remarkable microbial ecosystems and might prompt further interest from the general population and for future generations of scientists to study in detail the relationship between microbial diversity and iron and sulfur cycling.

INTRODUCTION

Microorganisms play a key role in the global cycling of most elements on our planet. Two very important elements are sulfur and iron. While the former is an essential macronutrient for life, the latter is usually considered to be a micronutrient. However, the low bioavailability of iron makes it a common limiting factor for the development of life. Besides their nutritional role, sulfur and iron can be used in what is called *dissimilatory* metabolism. In this case, these elements intervene in the production of energy by the cell, but are not incorporated into the cellular biomass.

Chemically, sulfur can be found in nature in a variety of oxidation-reduction states that range from sulfides (-II) and organic reduced sulfur (-I) to sulfate (+VI). Most of the time, elemental sulfur (0) is an intermediate in the

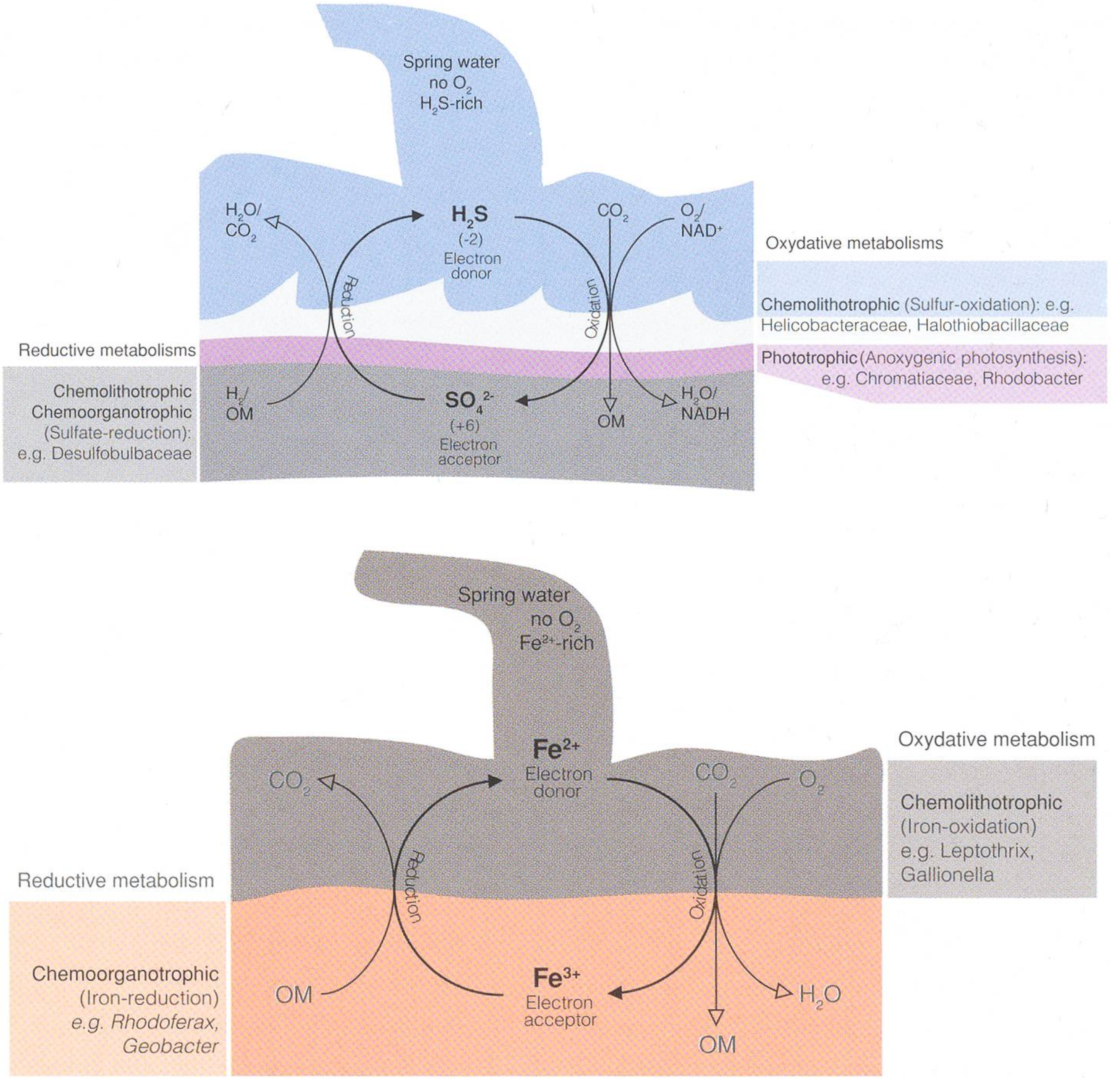

Figure 1 : Schematic representation of the microbial metabolisms involved in the cycling of the sulfur (A) and iron (B) potentially occurring in the sulfur and iron springs at Ponts-de-Martel, Neuchâtel. Specific key microbial groups at the different stages are indicated in the figure.

oxidation-reduction of sulfur (Zopfi *et al.*, 2004). The oxidation-reduction cycle of iron is much simpler, as this element is only found in two forms: ferrous (+II) or ferric (+III) iron. Chemical or biological agents contribute to the transformation of sulfur or iron from one state to another. A simplified depiction of the sulfur and iron cycles is shown in Figure 1. It can be observed that two other major elements play a key role in the fate of sulfur and iron: carbon and oxygen. Normally, under aerobic conditions, the reduced forms of either sulfur or iron are rapidly oxidized by a purely chemical process, and therefore are rarely found in sufficient concentrations to sustain the development of microorganisms able to utilize these elements as an energy source. Specific geochemical settings, however, allow the establishment of environments enriched in these elements. Two of these systems are found in surprisingly close proximity to each other in the ancient fen area near the village of Les Ponts-de-Martel, Neuchâtel. The first spring is characterized by the release of water with a strong smell of sulfide, while the second one releases water rich in dissolved iron, and visible rusty deposits can be observed in the outlet. The understanding of the origin of one of these springs, the sulfur spring, was the starting point for a theoretical manuscript published in 1992 by Michel Aragno (Aragno, 1992). Michel Aragno presented a series of processes that combine the activity of microorganisms and the geology of the site to explain the elevated concentrations of hydrogen sulfide emerging at the site. Although the origin of the iron-rich spring was not discussed in that manuscript, one can imagine that equivalent processes dealing with iron metabolism are responsible for the existence of the iron spring at Les Ponts-de-Martel.

The Laboratory of Microbiology at the University of Neuchâtel has been using these sites as an open-air laboratory to illustrate to geomicrobiology students the processes that have shaped our planet, and that interconnect biology and geology. As part of this work, we have measured proxies to microbial activity *in situ*, and more recently we completed a comprehensive analysis of the diversity of the bacterial communities in the water and sediments at both sites. The results of these analyses, as well as a discussion of their meaning, are presented in this contribution, which sheds additional light on the microbial processes occurring in these microbial ecosystems. We hope that this contribution might prompt further interest from the local population to visit these two prime microbial ecosystems.

MATERIALS AND METHODS

Description of the site

The sulfur and iron mineral springs are located in the Vallée des Ponts et de la Sagne, Neuchâtel (46°59.264'N, 06°44.243'E) at an altitude of 988 m above sea level. At the sulfur spring a characteristic smell and grey deposits can be observed. Below the water surface a pink mat can be found which covers another black one. The iron spring runs into a small concrete-based basin where thick rusty slimy deposits cover the walls, and no smell or purple bacterial mat can be observed. Although these reddish-brownish deposits prevail, additional greenish mats can also be found. In addition to this, foamy raft-like structures with an orange-brownish color float on the surface of the water.

Physicochemical measurements made in situ

The temperature, pH, conductivity and oxygen level of the mineral water were measured on site using a HACH HQ40d portable multimeter (HACH, Loveland CO, USA). Hydrogen sulfide, total iron and ferrous iron were measured using a DR890 HACH colorimeter (HACH, USA) and the corresponding commercial measuring kits.

Microsensor analysis

Samples of the biofilms found at the source of the water spring were taken from the sulfur and iron springs and brought back to the laboratory. The biofilms were placed in a container with tap water and air was bubbled into the water to homogenize the oxygen concentrations in the water-mat interface. Measurements were taken every 500 µm starting from the underlying water, down to the water-mat interface and into the mat itself. Microsensor probes for oxygen and hydrogen sulfide were purchased from Unisense (Unisense, Denmark). The profiles were recorded with a picoammeter PA2000 and analyzed using SensorTrace Basic 2.0 software (Unisense, Denmark).

Samples for molecular studies

Samples of water, biofilm and microbial mats were collected in 1 L sterile bottles, then stored at -20°C for molecular methods.

DNA extraction, sequencing and analysis

The soil, sediment and biofilm samples were subjected to an indirect DNA extraction, as previously described (Wunderlin *et al.*, 2013). Water from the samples was also filtered through 0.22 µm membranes to collect the biomass. The DNA was extracted using a FastDNA Spin Kit for Soil (MP Biomedicals, California), and using a modified protocol in order to ensure that the DNA was not only extracted from vegetative cells but also from spores and other cells that were difficult to lyse (Wunderlin *et al.*, 2013). The DNA concentration was measured with a Qubit Fluorometer using a dsDNA BR Assay Kit (Invitrogen, California).

In order to study the diversity of the bacterial communities 454 amplicon sequencing of the 16S rRNA gene was performed using the services of Eurofins MWG Operon (Germany). Fragments of approximately 500 bp were retrieved using the Eub8f (5'-AGAGTTTGATCCTGGCTCAG-3') and Eub519r (5' GTATTACCGCGGC-TGCTGG-3') primers, as previously described (Li *et al.*, 2009). The raw sequence data were analyzed with QIIME (Caporaso *et al.*, 2010), using the pipeline for *de novo* OTU picking and diversity analyses from 454 data, as suggested in the QIIME tutorials. The amplicon sequencing resulted in 8050 and 9397 sequence reads after quality filtering for the sulfur and iron spring samples, respectively. The sequences were de-noised with the split_library.py function implemented in QIIME, and checked for chimera using USEARCH version 6.1 with the reference database used by in the 1.8.0 version of QIIME. To the rest of the trimmed and processed sequences, an alignment was performed through the RDP website (https://rdp.cme.msu.edu/tutorials/aligner/RDPtutorial_ALIGNER.html) using Infernal Aligner (Nawrocki & Eddy, 2007). OTUs were identified using a threshold of 97% sequence similarity with USEARCH version 6.1. Alpha diversity within the samples was calculated in rarefied subsets of sequences in order to have equal sequence coverage, as suggested by the QIIME tutorial for 454-sequencing analysis. The parameters retained for the analysis were the Richness, Shannon and Simpson diversity indices, and the percentage of the ratio OTUs/chao1 (coverage) (Ray *et al.*, 2012)2012.

RESULTS AND DISCUSSION

The sulfur spring (NeSul)

The sulfur spring in Les Ponts-de-Martel is an example of a *sulfuretum* (Camacho, 2009), a unique type of environment usually associated with a source of sulfide-rich water (Figure 2). This water is initially anoxic and contains dissolved sulfide, but it oxygenates rapidly once it comes into contact with the atmosphere at the release point. However, the slow flow of the water allows the establishment of an oxygen gradient towards the bottom of the stream. Under these conditions three elements co-exist: light, oxygen and sulfide. Hence, both the chemical and biological oxidation of sulfide can occur, associated with either photosynthesis or chemosynthesis (Camacho, 2009). Visual signs of the suitable environmental conditions for sulfur bacteria were easily recognized at the Les Ponts-de-Martel site. First, the characteristic smell of sulfide indicated the presence of this compound. Second, white patches of elemental sulfur resulting from the chemical or biological oxidation of sulfide with atmospheric oxygen covered certain areas of the stream and the surrounding rocks (Figure 2A). Likewise, the formation of colored microbial mats was an indication of photosynthetic sulfur oxidation (Figure 2A). The microscopic observation of these different elements clearly indicated the presence of both phototrophic (Figure 2B-C) and chemotrophic colorless (Figure 2D-E) sulfur-oxidizing bacteria. Both groups are recognizable by the deposition of elemental sulfur granules, but in

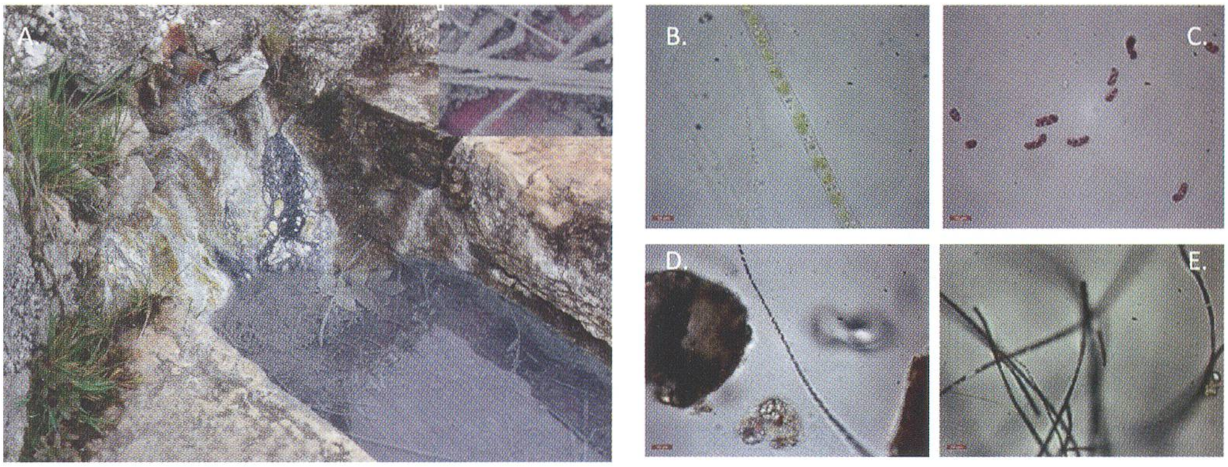

Figure 2: Macro- and microphotographic images of the sulfur spring at Ponts-de-Martel. A. The source and its surroundings with a close-up image of the purple mat at the bottom of the source (right insert). B-E. Microphotographs of phototrophic (B-C) and colorless (D-E) sulfur-oxidizing bacteria. In both cases granules of elemental sulfur are clearly distinguishable inside the cells. Scale bar: 10µm. The images were taken during the field excursion and laboratory work with the Biogeosciences students in May 2016.

the case of the phototrophic groups, they are distinguishable by the presence of pigments.

Since a wide variety of microorganisms are able to oxidize, reduce or disproportionate sulfur species, the microbial community structure of sulfur-rich habitats is clearly influenced by the environmental conditions at the site (Elshahed *et al.*, 2003). In the case of Les Ponts-de-Martel, the sulfur spring is characterized by a pH of about 7.83 and a temperature of 15 °C at the time of sampling, which in this case corresponded to the source of the spring in late April (Table 1). The measured concentration of hydrogen sulfide directly at the outlet of the source was high (266 mg/L, equivalent to 7.8 mM), and maintained low conditions of dissolved oxygen and iron (Table 1). From the source to the stream there was a gradient of increasing temperature and oxygen concentration (Table 2), thus creating the conditions for the development of specific microbial communities. However, the concentrations of dissolved hydrogen sulfide were not measured in the field.

A change in the concentrations of hydrogen sulfide and oxygen was monitored for one of the microbial mats developing at the spring by using a microsensor to obtain a high-resolution vertical profile (Figure 3). This analysis showed how hydrogen sulfide concentrations were high at the bottom of the mat (sulfidogenic conditions), but in the region where oxygen became available, the sulfide was rapidly oxidized and disappeared from the solution. Although at the site the underlying water is also rich in hydrogen sulfide, which is not the case for the mat analyzed under laboratory conditions, the observed results exemplified the hydrogen oxidation process that occurs on different scales and in different places at the site.

After sequencing the bacterial communities from the sulfur spring, which included a pooled sample of water and different mats, a total of 7954 high-quality sequences were retained for analysis (an average length of 579.57 bp). The sequences were grouped into 514 operational taxonomic units (OTUs). From these, 270 OTUs corresponded to a single sequence (singletons) and were removed from the analysis. From the remaining 244 OTUs the majority corresponded to OTUs represented by a very small number of sequences (Figure 4).

Table 1 : Site description and parameters measured *in situ*

Sample	**NeSul**	**NeFer**
Site	Ponts-de-Martel (NE)	Ponts-de-Martel (NE)
Description	Sulfur mineral spring in the Ponts-de-Martel valley, Neuchatel, Switzerland	Iron mineral spring in the Ponts-de-Martel valley, Neuchatel, Switzerland
GPS	47N, 6.73E	47N, 6.73E
Temperature	15°C	15°C
pH	7.83	6.94
Conductivity (µs/cm)	700	679
Dissolved O2 (mg/l)	2.28	1.96
O2 (µmol/l)	44	N.D.
H2S (mg/l)	266	N.D.
Fe+2 (mg/l)	0.04	2.03
Total Fe (mg/l)	0.04	2.62

Table 2 : Measurements of pH, O_2 concentration, conductivity and temperature at a distance gradient from the sulfur spring to the stream

Distance from the spring	**pH**	**O_2 (mg/L)**	**Conductivity (µS/sec)**	**Temperature (°C)**
0.5 cm	7.83	1.53	656	12.4
30 cm	7.75	1.46	645	12.5
30 cm, deeper	7.09	0.15	680	12.5
100 cm	7.76	0.98	680	13.6
100 cm, deeper	7.7	0.44	680	13.6
120 cm	7.68	0.58	680	14.2
250 cm	6.9	0.13	692	16.5
Inside the black reed mat	7.25	0.21	712	19.2
Stream entrance	8	8.8	543	20.3

Only 15 OTUs were represented by more than 76 sequences, thus corresponding to more than 1% of the community (a relative abundance of individual OTUs). The total cumulative frequency of these OTUs represented 67% of the total number of sequences obtained. These OTUs could be assigned to different taxonomic levels, and their relative abundance is shown in Figure 4B.

The most abundant group was identified as belonging to the Helicobacteraceae family, which is known to include genera involved in sulfur cycling such as *Sulfurimonas* and *Sulforovum* (JORGENSEN *et al.*, 2012). For some of the culturable representatives of these genera, a facultative mode of energy metabolism (either reducing or oxidizing sulfur compounds) has been shown (YAMAMOTO *et*

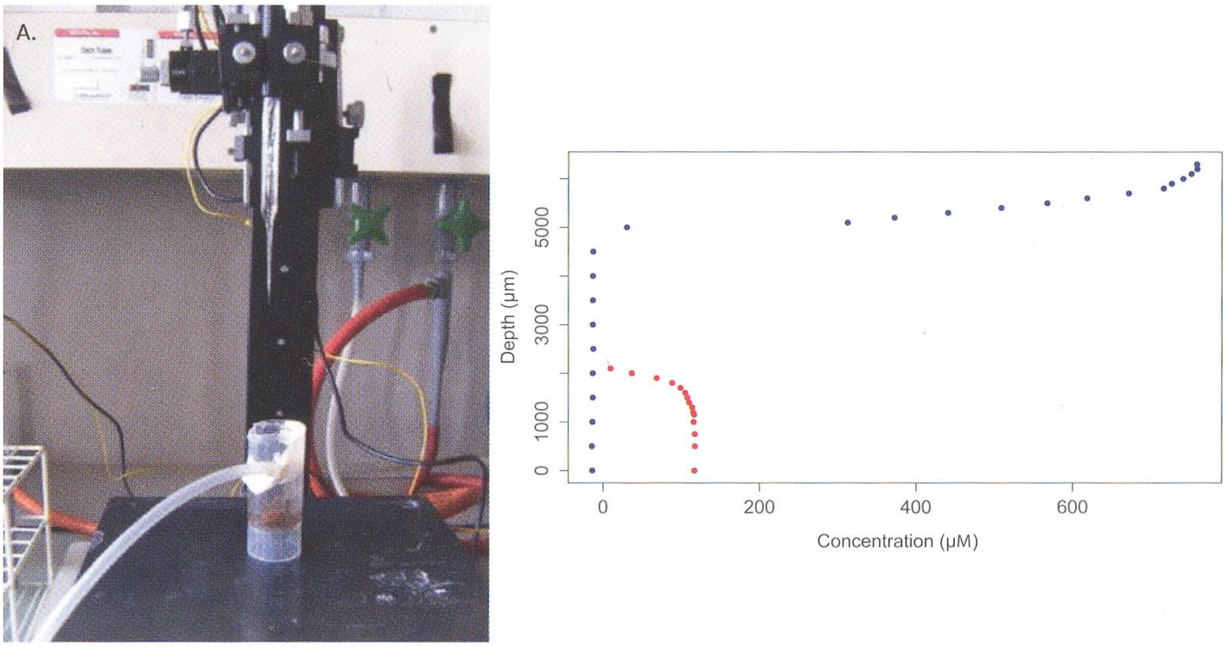

Figure 3: Measurements of the concentrations of hydrogen sulfide and oxygen made at a microscale for one of the microbial mats forming in the sulfur spring at Ponts-de-Martel. A. Images of the experimental set-up and of the microelectrode device. B. Gradients of the hydrogen sulfide (in red) and oxygen (in blue) measured in the mat. Image A was reproduced with the permission of the authors (L. Sauvain and F. Schindelholz).

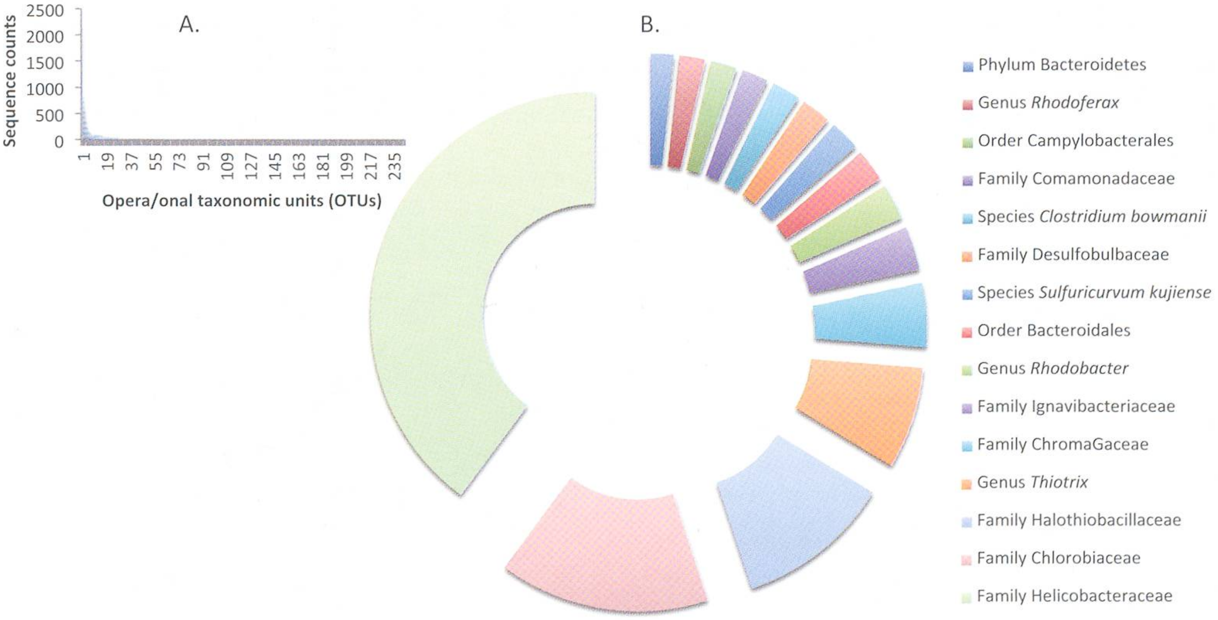

Figure 4: Bacterial community composition in the sulfur spring at Ponts-de-Martel analyzed using high-throughput amplicon sequencing of the 16S rRNA gene. A. The distribution of the sequence counts for the different taxonomic units (OTUs) obtained from the sample. B. Schematic representation of the relative abundance of the 15 most abundant OTUs (over 1% relative abundance).

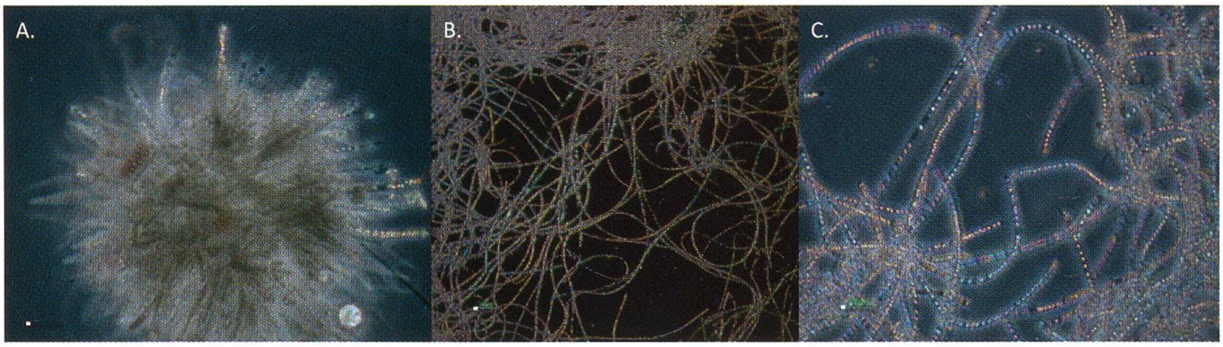

Figure 5: Images illustrating the accumulation of sulfur granules in the sulfur-oxidizing genus *Thiothrix*. A. Aspect of bundles of filaments associated with organic matter. B and C. Close-up images of filaments showing the accumulation of sulfur granules. Images courtesy of Jakob Zopfi.

al., 2010). One of the few groups that could be identified at a species level corresponded to *Sulfuricurvum kujiense*, which is a type species of the *Sulfuricurvum* genus (also belonging to the Helicobacteraceae family); it is a genus of interest because of its capability to utilize various reduced sulfur compounds such as sulfide, elemental sulfur or thiosulfate as energy sources, as previously observed in crude oil and oil sands (HAN *et al.*, 2012). The second most abundant group was identified as Chlorobiaceae only at a family level. This family includes numerous anoxygenic phototrophic green sulfur bacteria (CAMACHO, 2009). Sequences affiliated to Ignavibacteriaceae were also identified. The cultured representatives of this family are colorless and unable to grow phototrophically (INO *et al.*, 2010). The third most abundant OTUs were classified as belonging to the Halothiobacillaceae family, which is so far represented by halophilic obligate aerobic sulfur-oxidizing bacteria, some of which have been isolated from lead-contaminated sites (SHI *et al.*, 2011). One of the groups that could be classified at a genus level corresponded to *Thiothrix*, which are known gliding filamentous colorless sulfur-oxidizing bacteria (CAMACHO, 2009), which corresponded to some of the morphotypes observed microscopically at the site (Figure 2D-E), and which are characterized by their intracellular deposits of elemental sulfur (Figure 5). A second group of anoxygenic phototrophic sulfur oxidizers was also identified as Chromatiaceae at the family level. This family represents one of the purple sulfur bacteria (PSB) families belonging to the gamma-proteobacteria (CAMACHO, 2009). Along with the PSB, other phototrophic sulfur-oxidizing bacteria were represented by sequences identified as belonging to the *Rhodobacter* genus (FRIEDRICH *et al.*, 2001). In addition to the observed diversity of sulfur-oxidizing bacteria, sulfate-reducing bacteria were represented by sequences classified as Desulfobulbaceae, which contains sulfate reducers found in diverse environments (ASAMI *et al.*, 2005, GREEN-SAXENA *et al.*, 2012).

The iron spring (NeFer)

The release of anoxic reduced water rich in dissolved ferrous iron into an oxygenated environment leads to the spontaneous reaction of iron with dissolved oxygen at circumneutral pH, and to the rapid, predominantly abiotic precipitation of ferric hydroxides (KONHAUSER, 1998). However, bacteria can also exploit the oxidation of ferrous iron for growth under specific environmental conditions. For example, the capability of bacteria to oxidize iron at low pH (pH 1.5 to 3.5) is a well-characterized microbial metabolism occurring in acid mine drainages. At circumneutral pH, microaerophilic conditions are required

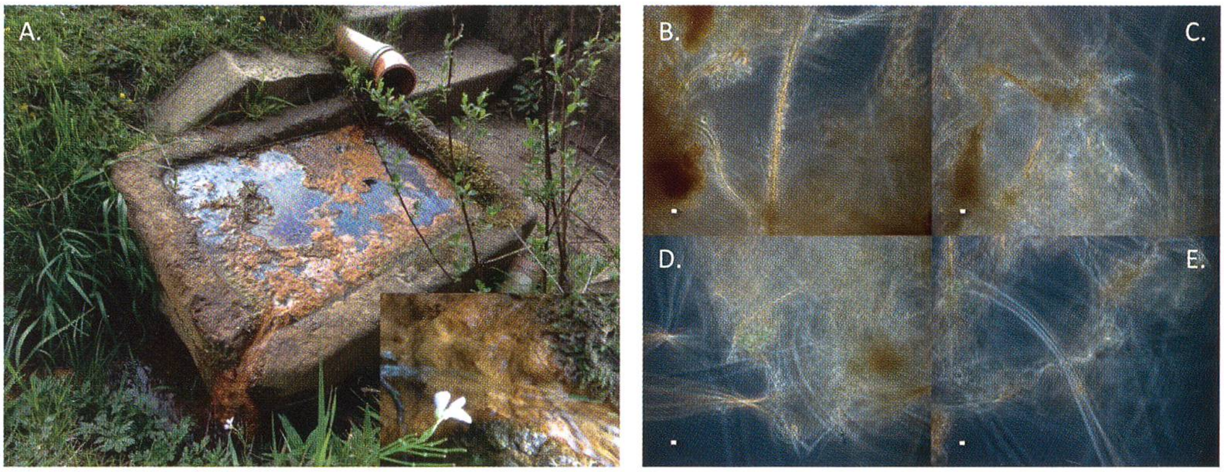

Figure 6: Macro- and microphotographic images of the iron spring at Ponts-de-Martel. A. The source and its surroundings, with a close-up image of the iron precipitates at the exit of the source (right insert). B-E. Microphotographs of the different morphologies of the iron hydroxides precipitating in the mat. Scale bar: 5µm. The images were taken during the field excursion and laboratory work with the Biogeosciences students in May 2011.

to favor biological iron reduction over purely abiotic oxidation (Konhauser, 1998). This situation (neutral pH and low oxygen availability) can be found in the iron spring located in close proximity to the sulfur spring in Ponts-de-Martel (Figure 6A; Table 1). Using a microsensor, a high-resolution vertical oxygen profile was established for one of the microbial mats collected from the site. The oxygen profile showed the establishment of microaerophilic conditions in the upper 4.5 mm of the mat (Figure 7). These microaerophilic conditions are the sort of environmental setting that favors biological iron oxidation at neutral pH.

Ferric iron issuing from the iron oxidation process (chemical or biological) is highly insoluble at neutral pH, and precipitates in the form of iron oxides. Bacteria can act as a passive charged surface that could serve as a nucleation center for the precipitation of these oxides. There are several examples of this type of iron-depositing bacteria in aquatic systems that can bind and precipitate ferric iron as encrusted sheaths, some of which were observed when analyzing the samples from the iron spring (Figure 6B-E).

The analysis of the microbial communities from the water and microbial mats in the iron spring was based on a total of 9320 high-quality sequences (an average length of 590.82 bp), which were grouped into 884 OTUs. As in the case of the sulfur spring, a majority (517 OTUs) corresponded to single sequences (singletons) and were removed from the analysis. From the remaining 367 OTUs the majority corresponded to OTUs represented by a small number of sequences (Figure 8). Only 20 OTUs were represented by more than 97 sequences, thus corresponding to more than 1% of the community (a relative abundance of individual OTUs). The total cumulative frequency of these OTUs represented 56% of the total number of the obtained sequences. These OTUs could be identified at different taxonomic levels, and their relative abundance is shown in Figure 8B.

On one hand, for many of the OTUs identified in the iron spring, a direct relationship with iron metabolism is difficult to be established

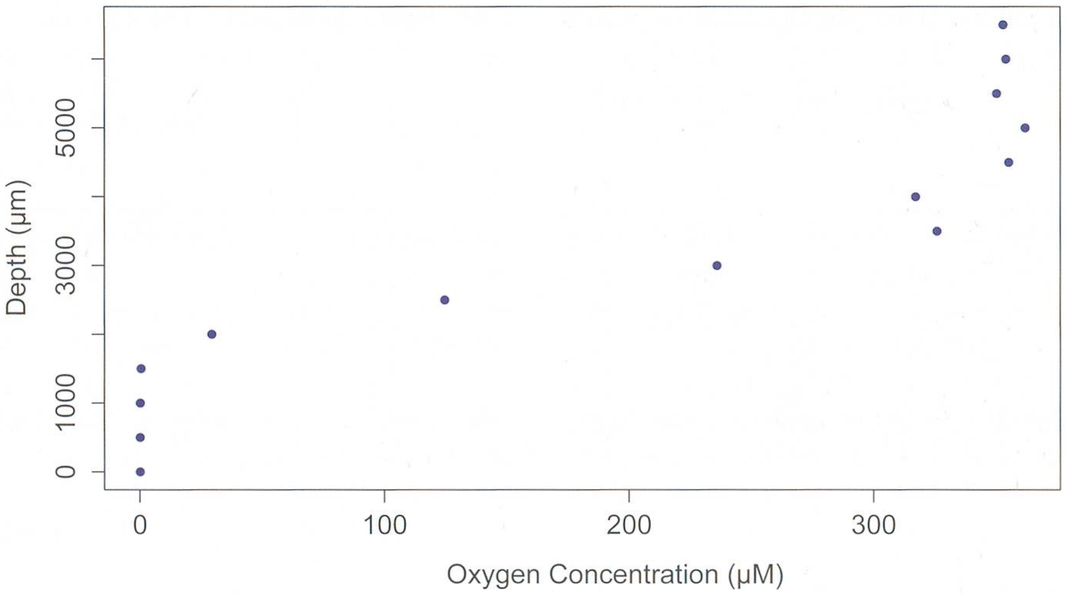

Figure 7: Measurements of the oxygen concentrations taken at a microscale for one of the microbial mats forming in the iron spring at Ponts-de-Martel.

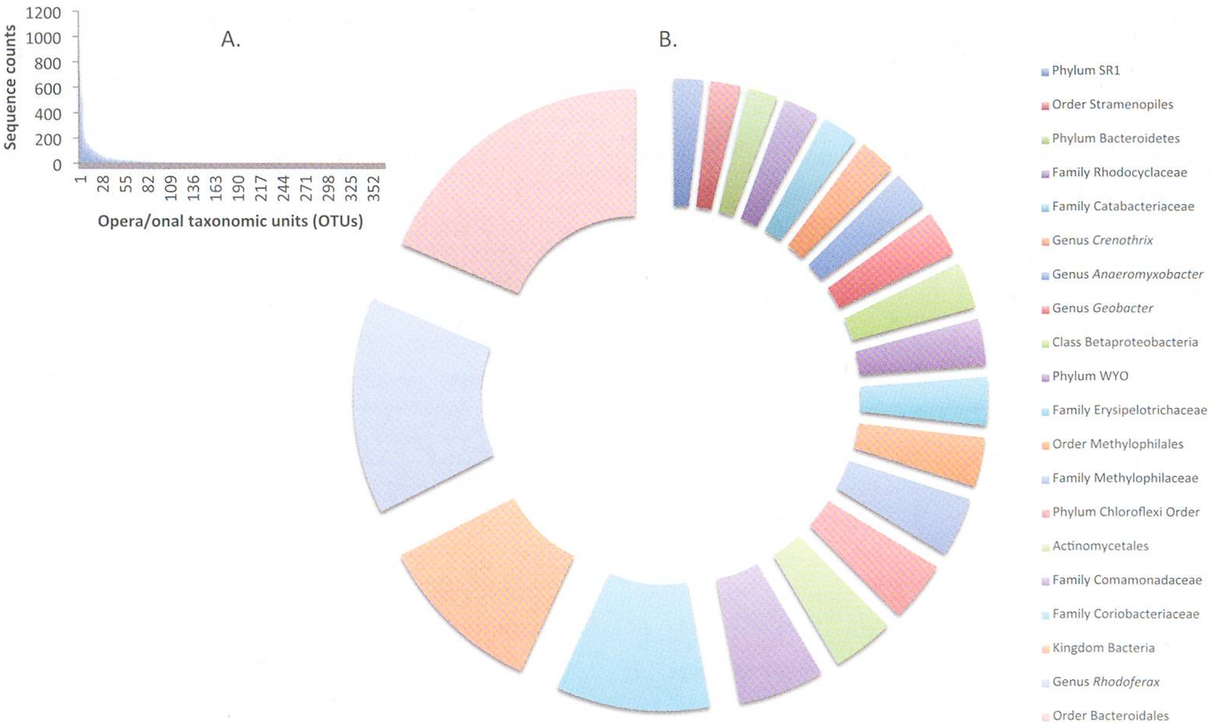

Figure 8: Bacterial community composition in the iron spring at Ponts-de-Martel analyzed using high-throughput amplicon sequencing of the 16S rRNA gene. A. The distribution of the sequence counts for the different taxonomic units (OTUs) obtained from the sample. B. Schematic representation of the relative abundance of the 20 most abundant OTUs (over 1% relative abundance).

because of the poor phylogenetic resolution obtained (e.g. OTUs identified to Kingdom, Phylum or even Order level). On the other hand, several well-known genera of iron-reducing bacteria such as the *Rhodoferax* (Risso *et al.*, 2009)2009, *Geobacter* (Childers *et al.*, 2002) and *Anaeromyxobacter* genera (He & Sanford, 2003) were identified. Other groups, such as the OTUs related to the Methylophilaceae family were previously observed in iron-rich waters (Kato *et al.*, 2013). Surprisingly, the most abundant group, which was identified at the order level of Bacteriodales, was recently reported in relation to the nitrogen cycle in a sulfur-rich system (Llorens-Mares *et al.*, 2015), but it was not reported for its activity in association with the cycling of iron. Despite the fact that organisms with the typical morphology of iron-oxidizing bacteria from the *Galionella* and *Leptothrix* genera have been observed in the iron source, sequences clearly affiliated to these two genera have not been obtained. Finally, one of the groups identified at the genus level was *Crenothrix*, which, although initially believed to be iron oxidizers (Konhauser, 1998), is currently known to be able to oxidize methane (Emerson *et al.*, 2010). This might indicate the presence of methane as an additional source of energy in the water, but this was not measured directly in this study.

Overall conclusion

It has been more than 20 years since Michel Aragno presented a theoretical framework for the formation of the sulfur springs in Les Ponts-de-Martel. During this entire period, these ecosystems have provided an ideal open-air laboratory where the role of microorganisms in the cycling of two very important elements for life, sulfur and iron, can be studied. The data presented here, which combine macro- and microscopic observations, *in situ* measurements and molecular analyses, offer a more complete view of the potential processes that explain the functioning of these ecosystems, and set the basis for more advanced studies focusing, for example, on the enrichment of specific metabolisms/microorganisms, or a demonstration of a coupling between the functional activity and the phylogenetic diversity of the bacteria found at each of the sites.

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