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IMPACT OF RADIOACTIVITY ON MICROORGANISMS LIVING IN MINERAL SPRINGS

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Although many studies have explored the responses of living organisms or ecosystems exposed to radioelements from human activities, very few studies have focused on the characterization of microbial biodiversity and the adaptations developed in environments characterized by natural, chronic and old radioactivity. Radioactive mineral springs are insulated ecosystems that have barely evolved in the last centuries. They therefore offer exceptional windows on the history of life on earth. Within the framework of the TIRAMISU project (biodiversiTy In RAdioactive MIneral Springs of the aUvergne region), biologists, ecologists, geologists, physicists, radiochemists and sociologists explore if and how natural ionizing radiation can be an "abiotic driver" impacting the diversity and structuring of microbial communities in food webs exposed to this radiation for long periods.

A INTRODUCTION

Where did life appear on Earth? Did it come from space, did it appear in an hydrothermal submarine spring deep down a primitive ocean or a geyser near its shores or *some warm simple pond* as suggested by Darwin in his letter to his friend J.D. Hooker in 1871? Since Stanley Miller and Alexander Ivanovich Oparin original works in the 50's, these fundamental questions are at the center of a multidisciplinary quest where (astro)biologists, (bio)chemists, geologists and (astro)physicists look everywhere on earth, in the solar system and beyond for traces of life and prebiotic processes. And life precursors have been found where they were not expected: analyses of the meteorites and comets composition has revealed the presence of prebiotic compounds on these celestial bodies that already populated the primitive solar system before earth formation. In parallel, the oldest fossils discovered on earth show that life began on earth more than 3.5 Ga ago [1-2]. Some of the earliest life on Earth flourished in terrestrial hot springs [3]. As a consequence, scenarii have emerged for hot spring origin versus the alternative oceanic hydrothermal vent scenario for the evolution of complex life on Earth [4].

A key player in these scenarii is natural radioactivity, present on Earth since its origin. A growing pattern of evidence suggests that current levels of natural radioactivity may affect mutational input and thereby the genetic constitution and composition of natural populations of animals and plants [5]. It also plays an important role in the evolution of terrestrial microbial

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biodiversity. At the bottom of mines or beneath the ocean floor, drillings reveal the presence of vast communities of microorganisms in the subsurface of our planet where the decay of radionuclides leads to hydrogen (H_2) and oxidant production via radiolysis of water [6]. This radiolysis could yield enough energy to fuel a large portion of this deep subsurface biome [7]. Moreover, experiments conducted in underground laboratories show that microbial life suffers when it is deprived of background levels of radiation [8]. In fact, terrestrial life began in a radiation field that is more intense than today: the radiation level today from natural β- and γ-emitters is about ten-fold lower than that during the Precambrian era [9].

Moving groundwater is able to mobilize, transport and accumulate matter, amongst others also radioactive elements [10]. Radioactivity is due to the presence of ^{226}Ra, ^{222}Rn, ^{228}Ra, and ^{220}Rn, and other daughter products of ^{238}U and ^{232}Th. Many springs form chemically precipitated mineral deposits at the Earth's surface. These deposits consist predominantly of calcite or aragonite in a rock form called travertine. The main sources of radioactivity of the spring sites are Ra and Rn because they are highly mobile in both acid and alkaline aqueous media and move upward into the hydrothermal cell and to the surface [11]. Deposits from the CO2-rich mineral waters that precipitate around the springs, such as iron hydroxides or calcareous travertines, are also rich of ^{238}U and ^{232}Th daughter products [21]. Mineral springs are therefore observatory sites for studying the influence of chronic radioactivity on the microbial ecosystems that have lived there for millions of years.

The Auvergne area in the French Massif Central, is known for its thermal spas for centuries including very radioactive mineral springs [12]. Within the framework of the French Long-Term Social and Ecological Research network, ZATU (Zone-Atelier Territoire Uranifère) is an observatory focused on life under natural radioactivity [13]. The TIRAMISU project (biodiversiTy In RAdioactive MIneral Springs of the aUvergne region) gathers biologists, ecologists, geologists, physicists, radiochemists and sociologists around the following goals:

- Identify and characterize naturally radioactive springs, from chemical, geological and physical properties to the analysis of the microbial communities in terms of diversity and structure
- Study the interactions between microorganisms and radioelements and develop models integrating biological, chemical and physical data for the different compartments (water, rocks and sediments)
- Integrate these observations into the historical and sociological contexts of the spring exploitation as thermal spas.

These goals are pursued in strong collaboration with local authorities to provide guidance on the possible risks related to the different usages of the spring water either for drinking, bathing or spreading on body parts. In the next section, we will give an overview of the properties of the springs studied and the methods used to assess the impact of radioactivity on their biodiversity. We will then introduce the first results and observations and describe briefly the new avenues under investigation.

B MATERIAL AND METHODS

Mineral springs are insulated ecosystems that have barely evolved in the last centuries. They therefore offer exceptional windows on the history of life on earth. To understand the interaction of radionuclides with microorganisms in these peculiar ecosystems, we need to characterize, analyze and compare the springs from many perspectives. Indeed, radioactivity is only one out of many abiotic factors that impact the assemblage of microorganisms and potentially induce stress on them. To disentangle the role of these abiotic factors, the TIRAMISU collaboration has identified 27 springs along a three-order of magnitude gradient of naturally enhanced radioactivity in the Auvergne region (figure 1) [15]. A multidisciplinary characterization of the water and sediment samples but also of the deposits from the CO2-rich mineral waters has been conducted to explore the interactions between microorganisms and radioelements.

Hydrogeological characterization

Mineral springs are surface manifestations of hydrothermal conduit systems which extend downward many kilometers to hot source rocks. Conduits are kept open by fluid pressure exerted by carbon dioxide-charged waters rising to the surface propelled by heat and gas (CO_2 and steam) pressure.

Application of classical and isotopic hydrochemistry both on water and gases on thermo-mineral water allows (1) to characterize its physico-chemical composition in its emergence, (2) to specify its origin and the conditions for its recharge, (3) to assess the interactions undergone by water during its underground

circuit (gas, rocks), and (4) to estimate potential mixing with surface water likely to alter its original properties.



Figure 1: geographical location of the 27 springs studied by TIRAMISU multidisciplinary teams [15].

Physico-chemical characterization

All the selected springs are accessible for sampling. Water samples are collected regularly to analyse water physical and chemical properties. Dissolved oxygen (%), conductivity (μS.cm^{-1}) and water temperature (°C) were measured *in situ* using an Ysi ProODO oxygen probe and a multi-parameter WTW probe FC 340i. The carbonate concentration (HCO_3^-) was also measured using a Hach carbonate kit. Additionally, during each sampling, a water sample was collected for further laboratory chemical analysis using a high-pressure Ion Chromatography technic. These samples were filtered using Whatmann GF/C filters. For cation and anion analysis, a Thermo Scientific Dionex ICS1100 and a Thermo Scientific Dionex Aquion system were respectively used to measure concentrations (mg.l^{-1}) in lithium (Li^+), sodium (Na^+), ammonium (NH_4^+), potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), fluorine (F^-), chloride (Cl^-), nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}) and sulphate (SO_4^{2-}) . Figure 2 displays the Piper diagram drawn from data collected on a subset of the springs the variety of water chemical properties that can be observed.

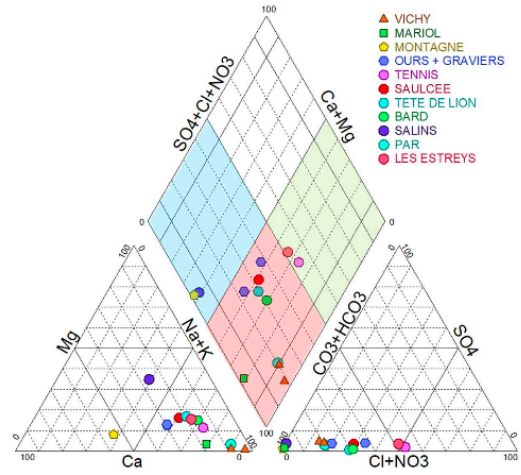


Figure 2: Piper diagram illustrating the variety of chemical properties observed in the spring waters.

Radiological characterization

Water samples have been collected from 27 mineral springs in Auvergne, Massif Central, France (Figure 1). Marinelli beakers of one-liter volume capacity were used to collect water from the springs. The beakers were sealed right after the sampling in order to avoid any Radon leakage. The radionuclide content as well as measurements for ^{223}Rn activity in the water were conducted within a few hours after the sampling using γ-spectroscopy. A High-Purity Germanium (HPGe) well-type detector of 35% relative efficiency coupled with the Genie2000™ gamma analysis software was used for the analysis at LPC (Clermont Ferrand, France). For the determination of the ^{222}Rn activity, the 352 keV γ-ray line of ^{214}Pb ($t_{1/2}$ = 27.06 min) was considered under the assumption of secular equilibrium.

Sediment samples were also collected at the different sites. The humid samples were sieved to remove parts greater than 2mm, then dried at 105°C for at least 24 hours and finally stored in sealed plastic tubes. The measurements for the determination of ^{226}Ra activity were conducted at least four weeks after the sealing, allowing for the secular equilibrium between ^{222}Rn and ^{226}Ra to be reached. These γ- spectroscopic analyses were conducted at Subatech (Nantes, France) using a coaxial HPGe detector of 45% relative efficiency coupled with the LabSOCS™ calibration software. The same non-destructive techniques were used to analyse the radionuclide content of core samples extracted from spring travertines.

In addition, the (bio)available share of radioelements/metals is evaluated by combining laboratory approaches, by selective extraction, and field approaches *via* the use of DGT (Diffusive Gradient in Thin film) sensors. Geochemical modeling approaches will accompany this work to predict, on the basis of current knowledge, the chemical forms of the metals/radioelements present and thus contribute to explaining the experimental observations.

All these data are then used as an input to the simulations used to assess the dose received by the microorganisms inhabiting the springs and build a biophysical model integrating biological, chemical and physical data.

Biological characterization

For each source, the genetic structure of microbial, prokaryotic (bacteria, archaea) and phototrophic eukaryotes (diatoms) an phages communities was characterized either in terms of abundance or richness and structure. The analysis of prokaryotic and eukaryotic microbial communities of radioactive springs was carried out over two distinct periods, spring and autumn.

A strain library of bacteria adapted to various levels of radiation was developed, in order to study their physiological and metabolic characteristics further. One of the specific goals of these microbial cultures was to isolate from these naturally radioactive springs potentially uranium-resistant strains and to study their interaction with uranium.

Bacteria and benthic diatom communities were analyzed using metabarcoding. A 312 pb region of the chloroplastic gene *rbc*L (coding for the large subunit of Ribulose Bisphosphate Carboxylase) was used as a barcode for targeting diatoms and a 470 pb region of the rDNA 16S gene for bacteria. In addition, raw materials were prepared for light microscopy (LM) observations, morphometric measurements, morphological analyses and evaluation of the relative abundance of diatom species. Diatom species identification was based on different taxonomical works [14].

For enumeration of phages and prokaryotes, water samples from mineral springs were fixed with paraformaldehyde at 0.5% final concentration and transported to the laboratory on ice and in the dark for immediate analysis using a FACS Aria Fusion SORP cytometer (BD Sciences, San Jose, CA).

Sociohistorical context

Diachronic analysis of the travertine cores is impossible without taking properly into account the work carried out on the spring sites for their exploitation. Most of the mineral springs in Auvergne have been or are still used by the local residents for their own water consumption. Many have also been exploited for industrial purposes during the XIXth and XXth centuries while thermalism was flourishing in the French Massif Central.
The study of public and private archives helps reconstructing the social history of springs and allows the precise identification of key dates linked to the operation of bottling plants, the identification of changes in the use and representations of springs, as well as qualification and regulation methods.

A sociological approach of collecting qualitative data from interviews gives access to the spring current uses and representations, their social and heritage visibility but also provides a better understanding of the uses of the water today, allowing to establish social vulnerability profiles.

Data analysis

Multivariate analyses of the data collected by the different experts allow to identify the key abiotic factors impacting the present biodiversity of bacteria, diatoms, phages and viruses within the springs.

The consideration and integration of all these data are essential to discriminate the effects of physico-chemical, radiochemical and radiological parameters on the microbial communities present in spring water, sediment and biofilm.

In addition, a multidisciplinary analysis of the core drilled in CO2-rich spring deposits, enlightened by sociohistorical data, gives access to a diachronic analysis of the biodiversity.

C RESULTS

Overall, 58% of the 27 springs sampled since 2019 have ^{222}Rn activity below 100 Bq/l while 11% are characterized by ^{222}Rn activity above 1000 Bq/l. According to the analyses, ^{210}Pb is present in the sediments in 80% of the mineral springs with a mean specific activity value of 1.4 Bq/g. ^{238}U and ^{226}Ra are present in the sediments of all the mineral springs with specific activities ranging from 7.6 10^{3} to 0.5 Bq/g, and from 0.028 to 52 Bq/g respectively [15].

Microdosimetric simulations have been conducted using the GATE Open Source Monte Carlo platform [16]. The results have shown among others that highest contribution to the dose received by the microorganisms is due to the alpha particles present in ^{238}U decay series. From this study, it was also evaluated that the most important contributor to the dose received by benthic diatoms living on the rocks and spring floor is the ^{226}Ra which is present in the sediments of the mineral spring ecosystem. Dissolved ^{222}Rn becomes the primary source of irradiation only in the springs where its activity reaches 4000 Bq/l and where the dose rate to benthic diatoms exceeds the 10 μGy/h dose rate threshold recommended for the protection of the environment [19].
Further evaluation of internal radiation exposure has highlighted the necessity of dedicated studies to assess the capacity of the radionuclides to penetrate the silicate frustule of the diatoms.

Despite their radioactivity, mineral springs are habitats where diatoms thrive. Some of the springs are natural, while others are man-made. Significant differences have been observed between diatom species richness of these two types of springs. The natural springs tend to present higher richness than man-made ones [17]. On the hand, multivariate analyses also show that radioactivity does not influence the species richness of diatom communities. Geographical location is the primary factor for explaining diatom species distribution [14]. Some springs host specific biocenoses (species adapted to the constraints imposed by these ecosystems). New taxa have been recently discovered in Massif Central springs ([18] and references therein).
The first observation of the stress induced by natural radioactivity to some diatom species came from the most radioactive spring in Auvergne [12]. As shown on figure 3, up to 30% of deformed valves were observed in the most radioactive spring (^{222}Rn activity in water ranging from 1500 to 4000 Bq/l) compared to less radioactive springs, here Mariol spring with ^{222}Rn activity of 130 Bq/l.

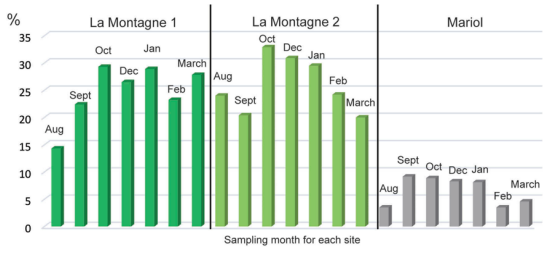


Figure 3: deformation rate of diatom valves from 2 mineral springs (Montagne and Mariol) between August 2018 and March 2019 (from [12]). Samples were collected in 2 locations at Montagne spring (La Montagne 1 and La Montagne 2)

Further studies have confirmed the correlation between the radon content of the water and the rate of deformation of specific diatom species, in particular, the *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot and *Crenotia angustior* (Grunow) Wojtal species that are abundant at Montagne spring. Some *Planothidium frequentissimum* strains could be isolated and cultivated in the perspective of laboratory experiments.
Analysis of the diatom assemblages in the Montagne sediment cores have confirmed that valve deformation was present already 70 years ago. Further analyses are under way to confront the biological, lithostratigraphic and radiological analyses of the cores.

The structure of the bacterial community differs between the studied springs. The difference in uranium concentration in these springs, with the lead, manganese and thorium concentrations, could explain the distribution of bacterial communities. In a subset of 6 springs, a total of 300 different bacterial strains were isolated. Some of them are resistant to these particular conditions and seem to interact with uranium (by biosorption mechanism). These strains can therefore modify the speciation of uranium in the environment, making it less mobile and therefore less toxic.

D CONCLUSION AND PERSPECTIVES

Started in 2019, the Tiramisu collaboration has selected 27 Auvergne mineral springs for a multidisciplinary study of the long term impact of natural radioactivity on microorganisms. The springs are characterized by ^{222}Rn activity in water ranging from 1.3 to 4000 Bq/l and ^{226}Ra activity in sediments up to 50 Bq/g.

Beyond the first results presented in this paper, the collaboration is currently pursuing the analysis of the abundant data collected for the last 3 years and exploring the correlations between the diversity and structure of microbial communities and the chemical, geological and physical properties of the springs.

In parallel to synchronic comparative analyses of the springs, diachronic studies are also underway to study how the microbial diversity and structure have evolved in relation to the spring industrial exploitation.

The extreme complexity of these unique environments also calls for *in vitro* complementary experiments. Culture of diatom strains isolated from the springs

allows further exploration of their response to acute irradiation using α and X-ray beam facilities, in an effort to build a full understanding of radiation impacts developed from acute exposure in the laboratory and chronic exposure in the field [20].

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