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Effects of radioactivity and other variables on aquatic microorganisms: case study in springwaters of Auvergne

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Salomé Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e do Doutor David Biron, Investigador Sénior do Laboratoire Microorganismes: Génome et Environnement (LMGE) da Université de Clermont-Auvergne.

“Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.”

Louis Pasteur

o júri

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palavras-chave

fontes de água; radioactividade; variáveis ambientais; citometria de fluxo; PCA; vírus; procariotas

resumo

O presente trabalho propõe-se a mostrar as informações mais recentes a respeito dos microrganismos presentes em fontes minerais e termais, falando sobre a influência que diferentes variáveis ambientais, com foco na radioatividade, têm nas comunidades procariotas e virais. A dissertação consiste na Parte 1, que é uma revisão da literatura que aborda procariotas e vírus em fontes de água. A revisão aborda o impacto de variáveis como temperatura, pH e radioatividade em ambos os grupos, tanto no contexto de água doce em geral quanto especificamente nas fontes de água. Em seguida, na Parte 2, é discutido o que foi feito no trabalho prático, que consiste no tratamento de dados de citometria de fluxo de várias fontes de água na região de Auverge, França. O trabalho prático consistiu em um estágio Erasmus + de três meses na LMGE, Université Clermont-Auvergne, França. Uma análise dos parâmetros bióticos e abióticos foi então realizada em várias fontes de água na área vulcânica do Maciço Central, região de Auvergne. A análise estatística dos dados da citometria de fluxo foi realizada no Excel e XLSTAT e com o objetivo de analisar a interação entre as diversas variáveis nas fontes estudadas. Para isso, foi realizada uma PCA para todas as variáveis e todas as fontes, juntamente com um dendrograma para análise de similaridade / dissimilaridade entre as fontes. Os resultados da PCA permitiram fazer algumas observações: uma forte correlação positiva entre a concentração de procariotas e a radiação gama e condutividade, mas uma correlação negativa entre essas variáveis e a concentração de radon; a concentração do vírus teve uma correlação fraca com a concentração de procariotas e radiação gama, e uma correlação negativa com a atividade do radon. A correlação negativa entre radão e radiação gama, juntamente com a forte correlação entre radiação gama e procariotas, foram as observações que mais chamaram a atenção, e o entendimento atual das interações entre essas variáveis ainda é pobre. Não foi encontrada literatura que explicasse essas duas observações. Para a correlação negativa entre radão e radiação gama, sabe-se que a atividade do radon é caracterizada pela libertação de radiação alfa, que é libertada pelas filhas do radão, como o 214Pb. Portanto, pela minha hipótese, a quantidade de radiação gama seria diretamente correlacionada com a quantidade dessas filhas que se formam a partir do decaimento do radão. Os resultados do dendrograma permitiram observar três separações de fontes em termos de dissimilaridade. Havia uma fonte com maior dissimilaridade, Par, e dois outros grupos de fontes. O isolamento da fonte Par deve-se ao fato de ser por uma boa margem a fonte mais quente, com 78ºC. Outro grupo de fontes é separado principalmente pela ação da variável concentração de radon, enquanto o outro é separado pela ação de outras variáveis como radiação gama e condutividade. A pesquisa sobre a influência da radioatividade em microrganismos em água doce e nascente ainda é um assunto de interesse limitado, e há carência de pesquisa. Além disso, os resultados mencionados ainda são preliminares e limitados pela ação do COVID. Ainda assim, isto oferece um ponto de partida em termos de pesquisas futuras, nomeadamente sobre os impactos da radioatividade em diferentes grupos de microrganismos nas fontes de água. Futuros estudos são necessários e que incluam análise das comunidades procarióticas e virais (espécies e estirpes), o que permitirá obter uma perspectiva total da influência da radioatividade nestes microorganismos.

keywords

springwater; radioactivity; environmental variables; flux cytometry; PCA; viruses; prokaryotes

abstract

The present work proposes to show the most recent information regarding the microorganisms present in mineral and thermal springs, talking about the influence that different environmental variables, with a focus on radioactivity, have on the prokaryote and virus communities. The dissertation consists of Part 1, which is a literature review that addresses prokaryotes and viruses in water sources. The review addresses the impact of variables such as temperature, pH and radioactivity on both groups, both in the freshwater context in general and specifically in water sources. Then, in Part 2, what was done in the practical work is discussed, which consists of the treatment of flow cytometry data from various water sources in the Auverge region, France. The practical work consisted of a three-month Erasmus+ internship at the LMGE, Université Clermont-Auvergne, France. An analysis of biotic and abiotic parameters was then carried out in various water sources in the volcanic area of the Massif Central, Auvergne region. Statistical analysis of flow cytometry data was performed using Excel and XLSTAT and with the aim of analyzing the interaction between the various variables in the studied sources. For this, a PCA was performed for all variables and all sources, along with a dendrogram for analysis of similarity/dissimilarity between sources. The PCA results allowed us to make some observations: a strong positive correlation between the concentration of prokaryotes and gamma radiation and conductivity, but a negative correlation between these variables and the concentration of radon; virus concentration had a weak correlation with both prokaryote concentration and gamma radiation, and a negative correlation with radon activity. The negative correlation between radon and gamma radiation, together with the strong correlation between gamma radiation and prokaryotes, were the observations that drew the most attention and the current understanding of the interactions between these variables is still poor. No literature was found that explained these two observations. For the negative correlation between radon and gamma radiation, it is known that radon activity is characterized by the release of alpha radiation, while gamma radiation is released by radon daughters such as 214Pb. Therefore, in my hypothesis, the amount of gamma radiation would be directly correlated with the quantity of these daughters that form from radon decay. The results of the dendrogram allowed to observe three separations of sources in terms of dissimilarity. There was a font with greater dissimilarity, Par, and two other font groups. The isolation of the Par source is due to the fact that it is by a good margin the hottest source, with 78ºC. Another group of sources is separated mainly by the action of the radon concentration variable, while the other is separated by the action of other variables such as gamma radiation and conductivity. Research about the influence of radioactivity in microorganisms in freshwater and springwater is still a subject of limited interest, and research is lacking. Also, the results mentioned are still preliminary and limited by the action of COVID. Still, this offers a start in terms of future research, namely on the impacts of radioactivity on different groups of microorganisms in water sources. Still, this offers a starting point in terms of future research, namely on the impacts of radioactivity on different groups of microorganisms in water sources. Future studies are needed that include analysis of prokaryotic and viral communities (species and strains), which will allow to obtain a full perspective of the influence of radioactivity on these microorganisms.

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List of abbreviations

DNA - Deoxyribonucleic acid

RNA – Ribonucleic acid

DOM – Dissolved Organic Matter

POM – Particulate Organic Matter

QS – Quorum Sensing

E. coli – *Escherichia coli*

(CRISP)/CAS - Clustered regularly interspaced short palindromic repeats (CRISPR) and associated proteins (Cas)

nGy/h – nanoGray per hour

μGy/h – microGray per hour

kGy - kiloGray

^{222}Rn – Radon-222

^{226}Ra – Radium-226

^{238}U – Uranium-238

^{214}Pb – Lead-214

Bq – Becquerel

Bq/L – Becquerel per liter

Sv – Sievert

nSv/h – nanoSievert per hour

Cs – Cesium

Sr – Strontium

ROS – Reactive Oxygen Species

Mn – Manganesium

Fe – Iron

Mn/Fe – Ratio of Manganesium to iron

D10 – Decimal reduction dose

D. radiodurans – *Deinococcus radiodurans*

UV – Ultraviolet rays

UV_A - Ultraviolet A rays

O_2 - Oxygen

OH – Hydroxyl

CO_2 - Carbon dyoxide

Na^+ - Sodium

Ca^{2+} - Calcium

K^+ - Potassium

Mg^{2+} - Magnesium

SO_4^{2-} - Sulfate

CO_3^{2-} - Carbonate

HCO_3^- - Bicarbonate

H_2O_2 – Oxygen peroxide

$^1O2$ – Singlet Oxygen

FeSOD - Iron superoxide dismutase

MnSOD - Manganese superoxide dismutase

(HPI, HPII) - Hydroperoxidases

GR - Glutathione reductase

G-C – Guanine-Citosine

NGS – Next Generation Sequencing

UV_B – Ultraviolet B rays

Nm – Nanometer

DO – Dissolved Oxygen

AMD – Acid Mine Drainage

Cu – Cooper

H+ - Hydrogen ion

Zn - Zinc

Cr - Chromium

Cd - Cadmium

Al - Aluminum

As - Arsenic

Pb – Lead

As(III) – Arsenic (3+)

P – Phosphorus

OTU – Operational Taxonomic Unit

ssDNA – Single stranded deoxyribonucleic acid

mGy – miliGray

mGy/y – miliGray per year

FCM – Flow Cytometry

PCA – Principal Components Analysis

PC1/PC2 – Principal Component 1 / 2

µS/cm - microSiemens per centimeter

mg/L – Miligrams per liter

HCl - Hydrogen chloride

EDTA - Ethylenediamine tetraacetic acid

Nm - Nanometer

Part 1 – Influence of radioactivity and other parameters on microorganisms present in freshwater

Contextualization

The practical project in this dissertation analyzes different variables and their interactions in the springs of Massif Central, France. Biotic (prokaryotic and viral concentrations) and abiotic (e.g. temperature, pH, gamma radiation, Radon, oxygen level) variables were analysed and will be presented in Part 2 of the present thesis. In Part 2, springs will also be addressed. Part 1 is a literature review on some topics related to the practical work (Part 2) such as prokaryotic and viral ecology in springs. The influence that different abiotic and biotic variables have on prokaryotic, viral and diatom communities of water systems, particularly springs was also explored.

1. Introduction to prokaryotes and viruses in freshwater

1.1. Bacteria in freshwater

Bacteria in freshwater drive transformation and the cycling of most of the biological active elements, therefore being the principal degraders and mineralizers of organic compounds (e.g. nitrogen, carbon) to their inorganic constituents in aquatic systems (Newton et al., 2011). Many bacteria in freshwater systems have a cosmopolitan character (Humbert et al., 2009).

In freshwater ecosystems, the main bacterial phyla are Proteobacteria, Cyanobacteria, Bacteroidetes, Actinobacteria and Verrucomicrobia (Okazaki et al., 2017; Wu et al., 2019). Betaproteobacteria, which are gram-negative and aerobic or facultative bacteria, are a key group in freshwater bacterioplankton and have an important role in freshwaters (Wu et al., 2019). They can be abundant in lakes and wastewater environments (Wu et al., 2019). Actinobacteria have global distribution and can be the most abundant in some freshwater systems. Firmicutes have a small share in the prokaryotic community of some freshwater systems. Many of its species, particularly of the class Bacilli, have strong environmental adaptability and can produce resistant spores (Wu et al., 2019).

1.2. Archaea in freshwater

Archaea is one the three life domains and these microorganisms are distributed in diverse habitats, such as the water column and the sediment of both freshwater and marine waters (Hu et al., 2016). They are not restricted to extreme environments as originally thought (Hu et al., 2016). Archaea have an important role in the biogeochemical cycling (Wang et al., 2020), as they have evolved a variety of energy metabolisms using organic and/or inorganic electron donors and acceptors (Wang et al., 2020). Considering cell counting and molecular studies, Archaea represent more than 20% of all marine prokaryotes and they are also abundant in extreme environments (Wang et al., 2020).

Currently, there is limited information regarding freshwater archaeal communities, their composition and diversity compared to other aquatic and terrestrial systems (Hu et al., 2016; Wang et al., 2020). Studies have revealed high phylogenetic diversity of archaeal communities in planktonic freshwater biomes (Hu et al., 2016).

Wang et al. (2020) speculate that Archaea may depend on the interaction with other microorganisms (such as Bacteria and microalgae). The authors point out that even if these biotic interactions are not well studied, observations show that the occurrence and abundance of Archaea coincide with bacteria, diatoms and viruses.

1.3. Viruses in freshwater

Viruses are biological entities with DNA or RNA genome encapsulated by a protein coat named capsid (Weinbauer, 2004). Viruses are acellular and small infectious agents, also being the most abundant living entities on Earth (Zhang et al., 2018). They infect all types of living forms and are present in every ecosystem (Zhang et al., 2018).

Globally, the number of viral particles is estimated to be 1e31 and their average half-life is 48h. Most of them are thought to be phages, viruses that infect bacteria (Rohwer et al., 2009). Many viruses have been discovered in the last decades, both in freshwater and marine waters, including cyanophages, phycoviruses, archaea viruses, giant viruses and virophages (Zhang et al., 2018).

2. Effects of abiotic and biotic variables on prokaryotes and phages in freshwater systems

Freshwater microorganisms have global importance, but our current understanding of them is fragmented (Bock et al., 2020). In particular, we understand little about the factors that shape community patterns (Bock et al., 2020; Ruhl et al., 2018).

Yet, several studies demonstrate that prokaryotic and viral communities are controlled by a combination of geographic, biotic and abiotic variables (Bock et al., 2020; Ferrera et al., 2017, Moresco et al., 2016). Seasonal variations of biotic and abiotic conditions change bacterial abundance, activity and community composition (Santos et al., 2019). As for geographical factors, there is still little consistency among different studies as to what extent they influence bacterial communities (Bock et al., 2020). The consequences of these multiple stressors on microbial communities can vary because of functional redundancy and various resistance mechanisms, which vary across different taxonomic and functional groups (Sagova-Mareckova et al., 2021).

With Archaea, community composition in aquatic systems can also be associated with physicochemical variables and biotic variables (Hue et al., 2016). Laskar et al. (2017) note contrasting reports on the influence of environmental variables on archaeal communities compared to bacterial ones, while referring that there has been little effort on the research of the impact of environmental factors in the archaeal community. In the case of viral communities, abiotic and biotic factors can contribute to the loss of viral infectivity (Moresco et al., 2016).

Understanding the interaction of biological communities with environmental variation is one of the main goals of Ecology (Machado et al., 2020). Studying microbial community diversity and its changes is useful to evaluate the effects of human perturbation on the ecosystem (Cibic et al., 2019). Some abiotic variables and their influence in prokaryotes and viruses in freshwater were studied: gamma radiation and radon activity (the two main variables in study), pH, temperature, conductivity, oxygen availability; as well as the interaction of viral and prokaryotic communities (biotic variables). These variables were explored in a freshwater/springwater context (the springwater approach was necessary since the work in Part 2 took place in springwaters of Auvergne). Some complemental but also relevant variables, such as nutrient availability, metal contamination and light were also explored in this review.

2.1. Biotic factors

2.1.1. Interactions between viruses and prokaryotic host cells

2.1.1.1. What are viruses?

A virus is a small parasite that is dependent on others for its reproduction (Lodish et al., 2000). Once it infects a susceptible cell, the virus directs the cell machinery to produce more viruses. Their genetic material is either DNA or RNA, and double-stranded or single-stranded. The viral particle, the virion, consists of nucleic acid and an outer shell of protein (Lodish et al., 2000).

Viruses are relevant microbial predators, they influence global biogeochemical cycles, primary productivity and marine carbon cycle, control the structure and function of ecosystems and drive microbial evolution (Palermo et al., 2019; Rohwer et al., 2009). They reproduce by attaching themselves to a host cell and transferring their genetic material. This material is then used by the cellular machinery of the cell to produce more viruses (Rohwer et al., 2009). Viral predation, together with protist grazing, is important to control microbial communities (Rohwer et al., 2009). By focusing on the abundant members of the community and lysing them, they allow less competitive species to thrive (Palermo et al., 2019). Viruses act as key gene transfer agents, allowing hosts to adapt and microbial communities to change (Palermo et al., 2019).

Viruses are important in aquatic environments (Palermo et al., 2019) and most of our knowledge of viruses in aquatic environments comes from a marine context (Rohwer et al., 2009). They cause 20-40% of daily prokaryotic mortality (Kavagutti et al., 2019). In surface marine waters, they kill 10% of phytoplankton population and up to 50% of bacteria, and their impact increases with higher nutrient concentration (Palermo et al., 2019). Besides bacteria, viruses kill diatoms and dinoflagellates and release nutrients (Rohwer et al., 2009). Besides marine ecosystems, viruses are also common in harsh environments such as hot springs and hypersaline waters (Rohwer et al., 2009). Viruses are more active in eutrophic aquatic systems and are potentially capable of controlling host abundance, respiration and production (Palermo et al., 2019).

In freshwaters, viruses are the most abundant entities, with up to 10^6-10^8 viruses per milliliter (Kavagutti et al., 2019). They are incredibly diverse and they influence microbial community ecology by increasing the transfer of carbon and phosphorus to microorganisms, which in turn modulates microbial lineages and maintains the diversity of the community (Kavagutti et al., 2019). In freshwater eutrophic lakes, the abundance of phages varies widely and, in some environments, it is reportedly abundant. Freshwater viruses are still less studied compared to marine viruses and many fundamental aspects, such as distribution and patchiness are unknown (Palermo et al., 2019).

2.1.1.2. Antagonistic relationships between bacteria and viruses

Viruses are the major cause of mortality of prokaryotes, making them as important as grazing by protists (Jacquet et al., 2018; Vaqué et al., 2017). Microbial community composition can be altered by viruses, as it was found in marine cyanobacteria and in freshwater bacterial communities (Jacquet et al., 2018). Viral cell lysis is an important cause of prokaryote and phytoplankton mortality. Viral lysis destroys the cells and also converts them into Dissolved Organic Matter (DOM) and Particulate Organic Matter (POM) (Heinrichs et al., 2020; Zhao et al., 2019). These conversions limit the spread of nutrients to higher trophic levels, and the cellular debris is instead consumed by heterotrophic bacteria, stimulating bacterial growth (Jacquet et al., 2018). Hosts can use different strategies to defend against viruses. They can produce specific enzymes (e.g. endonuclease), change, reduce or eliminate cell surface receptors to block the entrance of viruses and they are able to reduce cell size, growth rate and abundance (Jacquet et al., 2018). Viruses will in response develop solutions to anti-viral systems (Jacquet et al., 2018).

Despite the documented forms of attack-defense of microorganisms and viruses, resistance to viral infection does not seem dominant in the environment (Jacquet et al., 2018). Hosts with faster growth rate and/or bigger cells will invest less in defense and will be more susceptible to infection and lysis (Jacquet et al., 2018). Cells can use Quorum Sensing (QS) signal compounds for the regulation of their population size, and viral lysis is one of the phenotypes that can be induced by QS (Liang and Radosevich, 2019; Liang et al., 2020). QS is proved to be involved in phage defence in *E. coli*, with these bacteria reducing the number of virus-receptors on cell surfaces as a response (Jacquet et al., 2018; Rémy et al., 2018). Virus-host interaction could then be regulated by QS, and QS signal compounds have been detected in water and waterborne cyanobacteria (Herrera et al., 2019; Jacquet et al., 2018). The entire QS network could regulate viral lysis and host resistance (Jacquet et al., 2018). Changes in environmental conditions can lead to growth of competitive populations, which could then be controlled by viral lysis (Jacquet et al., 2018).

Regarding bacterial defence mechanisms, there has been a greater interest on their study in recent years (Azam and Tanji, 2019). The discovery of the defence mechanism (CRISP)/CAS system has provided us with an efficient and precise genome editing tool for the engineering of a wide range of organisms (Azam and Tanji, 2019). Other phage defence mechanisms were also identified (Azam and Tanji, 2019).

2.1.1.3. Mutualistic, synergistic relationships between prokaryotes and viruses

Some other interesting findings were made about host-phage relationships. Viruses can stimulate primary production making phytoplankton population achieve higher growth. Viruses and grazers can compete for prey, as grazing stimulates viral infection of bacteria (Blanc-Mathieu et al., 2019; Jacquet et al., 2018). Also, experimental studies have demonstrated that by lysing algae-killing bacteria, bacteriophages will indirectly impact microalgae (Cai et al., 2011; Jacquet et al., 2018). According to a hypothesis formulated by Jacquet et al. (2018), primary production would decrease in aquatic ecosystems if viruses were not present. The presence of viruses and their viral lysis would promote nutrient cycling, production and diversity in these aquatic environments (Jacquet et al., 2018).

Viruses can mediate the structure of the host community, which could be due to lysis of some particular species. The release of cell debris by lysis could also change the composition of organic materials and nutrients, thus changing the microbial community further (Jacquet et al., 2018; Keshri et al., 2017). Bacterial fitness and physiological states are relevant to host-virus interaction (Fister et al., 2016; Thingstad and Våge, 2019).

Changes in the physiological status of bacteria can result in transcriptional responses that alter the cell wall, a receptor for phages. This can create difficulties for viruses, as they fail to bind to their host. Difficulties for the phages can happen also because of external factors (Fister et al., 2016). Environmental parameters such as temperature and salinity interfere in the virus–host interactions (Finke et al., 2017).

2.1.1.4. Outlook and future directions in the study of phage-bacteria interaction

The advent of novel technologies and experimental approaches, together with an increased interest in phages, has boosted knowledge in bacterial defence of parasites (Rostøl and Marraffini, 2019). How phages interfere with the life cycle of other phages is still not sufficiently understood (Rostøl and Marraffini, 2019). It is also necessary to better study defence mechanisms of bacteria and other prokaryotes against phages, to better understand prokaryotic evolution (Rostøl and Marraffini, 2019).Freshwater viruses are still understudied and untouched by advances in microbial culturing techniques and genomics (Kavagutti et al., 2019). Very few isolate phage genomes from freshwater habitats are available (Kavagutti et al., 2019).

Most studies of viruses have been made in marine ecosystems, however, there has also been an increase in the study of freshwater ecosystems (Jacquet et al., 2010). Research has also been conducted in groundwater, high salinity ponds, hyperthermal vents and acidic geothermal environments (Jacquet et al., 2010).

There are still critical gaps in our knowledge of molecular mechanisms of bacterial defences (Hampton et al., 2020).

2.2. Abiotic factors

2.2.1. Radiation and radionuclides

There are growing concerns with the threat of radioactivity to people and environment (Jeong and Choi, 2017). Nuclear accidents have leaked large amounts of radioactive materials to the environment (Jeong and Choi, 2017). Radiation level varies across the planet, existing in low levels in underground spaces (20nGy/h), in ambient levels (60-100 nGy/h) and the high levels present in nuclear disaster zones (up to 30 μGy/h in radon-rich groundwater) (Lampe et al., 2017).

Ionizing radiation is well known as a source of free radicals via radiolysis of water (Kottemann, 2005). These free radicals are responsible for 80% of DNA damage, with the remaining 20% being direct effect of c-photons (Kottemann, 2005).

2.2.1.1. Radionuclides

Radioactivity implies the spontaneous transformation or decay of an atomic nuclei, resulting in the emission of radiation. This can also be called radioactive decay of radionuclides (Luig et al., 2011). Radionuclides are unstable and emit high energy radiation (Campbell, 2009). As the nucleus relaxes into a more stable state, it emits energy in the form of ionizing radiation (Campbell, 2009). Their presence in the environment is mainly due to human activities such as mining of Uranium and nuclear emissions, and represent a danger for the environment and health, as they contaminate the soil and water (Burow et al., 2014; Lopez-Fernandez et al., 2020; Sivaperumal et al., 2020). Most radionuclides decay until becoming stable, and some decay in more than one step and are called series radionuclides and form a decay chain. The decay chain that begins with Uranium-238 culminates in Lead-206 after forming intermediates such as Thorium-230, Radium-226 and Radon-222 (EPA, 2019).

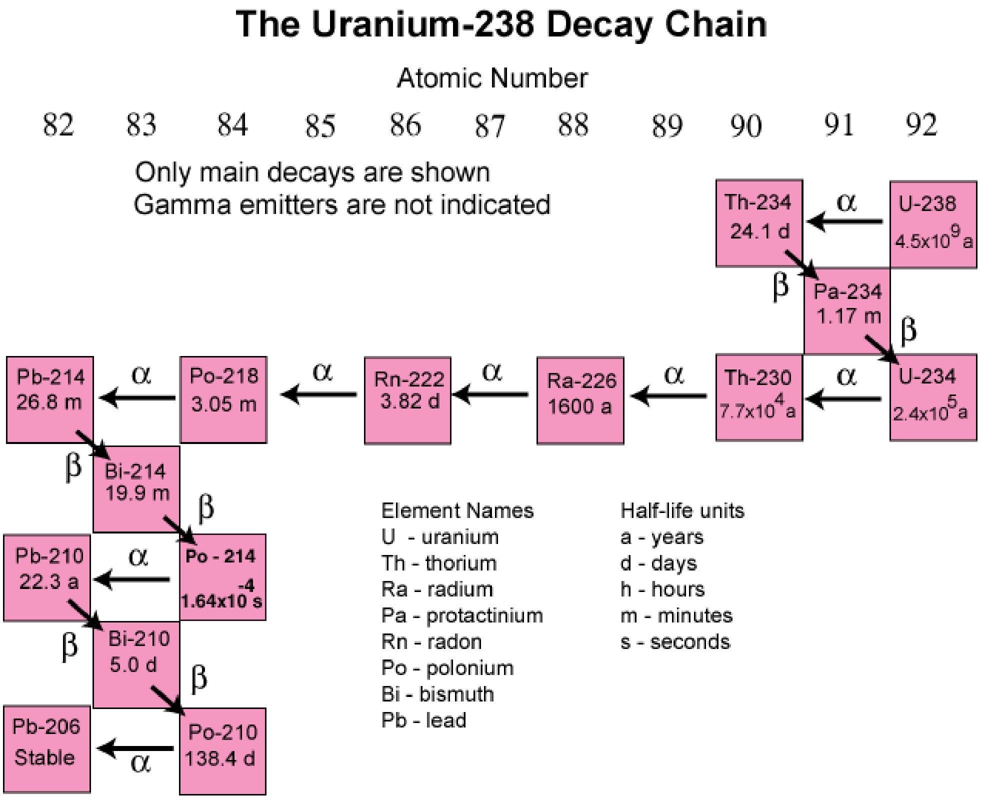

Figure 1 - Representation of the Uranium-238 decay chain, showing the important radionuclides in the decay series. Radon (Rn-222) comes after radium (Ra-226) and its decay mode is via alpha emission. Source: pubs.ugs.gov

For radionuclides there is the physical quantity ‘’activity’’, with the measure of the number of decays per unit time (Luig et al., 2011). This activity is the number of decays per unit of time (second) and its name is Becquerel (Bq) (Luig et al., 2011). Other measure, the absorbed dose, D, can be expressed by gray (Gy) (Luig et al., 2011). Another unit is the sievert, used for measuring ionizing radiation effective dose. One dose is generally defined as the amount of radiation roughly equivalent in biological effectiveness to one gray (1Gy=1Sv) (Britannica, 1998; United States Nuclear Regulatory Commission, 2020).

In freshwater bodies, natural radionuclides can be more variable if compared to marine ones, as the local geochemistry will have a heavy influence (Brown et al., 2004). Data on natural radionuclides in freshwater bodies is sparse in Europe and it is necessary to collect more data on the concentrations of radionuclides in European freshwater organisms in order to understand their exposure to radioactivity (Brown et al., 2004).

The most important radionuclides are Cesium (Cs) and Strontium (Sr) due to their half-life and transference through living beings in the food chain (Sivaperumal et al., 2020). Another particular example is radon (^{222}Rn) which is a colorless, water soluble and odorless noble gas that results from the decay of uranium (^{238}U), and which releases radioactive high-energetic α-particles (Anda et al., 2014; Nayak et al., 2019). As ^{222}Rn is not a γ-emitting nuclide, its activity is evaluated on the basis of the activity of its daughter ^{214}Pb, a γ-emitting radionuclide (Millan et al., 2019). Much of the work done with radon is the measure of radon concentration in groundwater, hot springs, soil, thermal plants and houses (Nayak et al., 2019). Not many attempts have been made regarding microbiological studies of radon-contaminated water (Nayak et al., 2019).

2.2.1.2. Effects of radioactivity on microorganisms

Natural radioactivity mainly comes from members of the ^{238}U decay chain, and if this radiation occurs in an oligotrophic environment it puts selection pressure on microorganisms, altering their diversity (Ding et al., 2019; Doyi et al., 2017). Radioactive substances have lethal implication, with gamma rays and other ionizing radiations inhibiting reproduction of microorganisms as they alter their DNA (Shukla et al., 2017). Different types of radiation have different efficiency in the cell. Gamma rays, for example, are sparsely ionizing radiation, and cause only a few dozen ionizations along their path through the cell. The resulting damage can therefore be repaired (Luig et al., 2011). Densely ionizing particles (e.g. alpha particles) cause thousands of ionizations as they pass through the cell. Damage to DNA is imperfectly repaired (Luig et al., 2011).

Ionizing radiation such as gamma rays indirectly damages the cellular integrity by producing reactive oxygen species (ROS) (e.g. hydroxyl radical) from water radiolysis, which cause cell death (Kish et al., 2009; Singh, 2018; Matusiak, 2016; Srinivasan et al., 2015). These ROS can cause DNA damage by direct ionization reactions that modify DNA bases (Kish et al., 2009; Kottemann et al., 2005; Matusiak, 2016; Park et al., 2018) and severely damage intracellular proteins (by oxidation) and lipids, which are controlled by the manganese ion complex (Kish et al., 2009; Srinivasan et al., 2015). Hydrogen peroxide and ozone are the most reactive and harmful ROS (Matusiak, 2016; Park et al., 2018).

Responses from aquatic microbial communities to ionizing radiation are quite complex (Fuma et al., 2010). In Fuma et al. (2010), some effects observed were not adverse but stimulative or lacked dose-dependency, having been labelled as indirect effects. The authors hypothesized that the indirect effects were caused by interspecies competition and prey-predator relationships between producers and consumers, but further clarification was considered necessary.

Microbiological environmental and laboratorial studies can have different responses to radionuclide contamination, as effects of radionuclides are also modulated by surrounding variables (Ding et al., 2019; Shuryak and Dadachova, 2016). Studies using combinations of multiple stresses are still lacking (Shuryak and Dadachova, 2016). Shuryak and Dadachova (2016) indicate that the effects of radioactive contamination in microorganisms might become more severe if other unfavourable environmental conditions are included. The study concludes that laboratorial studies are insufficient to determine microbial responses to radioactive contamination. Nazina et al. (2010) also list other variables that influence the effects of radioactive contamination, such as redox conditions, pH and the presence of complexing agents and microorganisms. Microorganisms, when interacting with radionuclides, affect their solubility by changing their oxidation state or forming reduced compounds (Nazina et al., 2010).

Overall, sites contaminated by radionuclides normally show less microbial concentration due to their toxicity (Ding et al., 2019). Yet, despite the toxicity of environments with radionuclides, many microorganisms manage to survive and thrive under these conditions (Ding et al., 2019). Microorganisms are able to adapt fast to radioactive contamination, as they have not only short generation times (Geras'kin, 2016) but also tolerance, abundance and high surface area to volume ratio (Ding et al., 2019). Microorganisms can interact with these radionuclides, adsorbing them through extracellular binding involving physical adsorption, ion exchange, complexation and precipitation (Sivaperumal et al., 2020). Both prokaryotes and eukaryotes play a role in the biogeochemical cycling of radionuclides (Ding et al., 2019). Radiation resistance can also come from DNA repair abilities and protection of proteins (Fuma et al., 2010). Prokaryotes with resistance to radiation are shown to have a high Mn/Fe ratio and are also able to scavenge ROS by intracellular Mn complexes (Fuma et al., 2010). Microorganisms can be radiotolerant and extremophilic and can withstand high doses of radiation in a vegetative state (Matusiak et al., 2016).

Fuma et al. (2012), indicate low risk of radioactive contamination to microbial communities, with chronic irradiation having smaller effects than acute irradiation. In Lampe et al. (2017), environmental radiation did not significantly contribute to the rate of beneficial mutation supply and was therefore not considered an abiotic stressor.

Other factors that can influence radiation effects include intensity and exposure time of radiation, the species or strain of the microorganism and its growth phase, metabolic state, and on the culture medium used (Matusiak, 2016). It is believed that gram-positive bacteria are less sensitive to radiation than gram-negative bacteria (Abo-State et al., 2014; Matusiak, 2016). Bacterial spores also show good resistance (Matusiak, 2016).

2.2.1.3. Examples of resistant microorganisms (prokaryotes) to radioactivity

Extremophile radiation-resistant microorganisms have been receiving attention as candidate organisms for bioremediation of these materials (Jeong and Choi, 2017) due to their survival mechanisms (Fuma et al., 2010). Most bacterial genera and species are considered radiosensitive, with only 0,1-0,5 kGy of radiation dose necessary for a 1log cycle reduction in survival (D10) (the radiation dose needed to inactivate 90% of bacterial load)(Singh, 2018). Only some bacteria exhibit radiation tolerance (Singh, 2018).

An example of a resistant microorganism is the bacteria *Deinococcus radiodurans*, well-known for its high resistance to various environmental stresses, including gamma radiation (Jeong and Choi, 2017). *D. radiodurans* gets its extreme resistance from a powerful DNA repair and an efficient antioxidation mechanism (Jeong and Choi, 2017). It can survive against acute exposure to more than 10-20 kGy (Jeong and Choi, 2017). *D. radiodurans* could potentially be used for bioremediation of radioactive heavy metals and radionuclides (Jeong and Choi, 2017). *Deinococcus radiodurans* and *Deinococcus geothermalis* are known to have higher level of manganese ions (Asker et al., 2011). *Deinococcus* has 62 of its 70 species with reported gamma radiation resistance (Park et al., 2018) and at least 4 species described as sensitive (Asker et al., 2011).

Another example is the bacterial genus *Shewanella* (Grouzdev et al., 2018). These bacteria have been found in radionuclide-contaminated environments, and they can potentially alter solubility of a broad range of radionuclides, including uranium (Grouzdev et al., 2018).

The genus *Hymenobacter*, from the family Cytophagaceae, has several species reported as resistant to gamma radiation, along with UV radiation (Srinivasan et al., 2015).

Halobacterium demonstrates extreme resistance to up to 20 days of ionizing radiation, together with desiccation and vacuum conditions. Its D10 is 5kGy (Moreira et al., 2010; Kottemann et al., 2005). Resistance to gamma radiation seems to be based on similar mechanisms as those used to withstand desiccation, based on observations of *D. radiodurans* (Kottemann et al., 2005).

Chroococcidopsis is an extremophilic cyanobacteria with wide distribution. It can be isolated from freshwaters, including hot springs. *Chroococcidopsis* shows good tolerance to drying and ionizing radiation (can resist up to 15 kGy) (Matusiak, 2016). Their mechanisms of radiation resistance have yet to be better understood (Matusiak, 2016). This cyanobacterial genus includes members with the ability to withstand X-rays up to 15 kGy, as well as to resist desiccation (Heidari et al., 2018).

The gram negative bacterium *Acinetobacter radioresistans* is reported to support up to 25 kGy of gamma radiation (Singh, 2018).

Several other prokaryotic groups have been reported as radiation resistant, such as the genera, *Kinoeococcus*, *Kocuria*, *Methylobacterium* and *Rubrobacter* from Bacteria, and the genera *Pyrococcus* and *Thermococcus* from Archaea (Egas et al., 2014; Park et al., 2018). Particular examples of species include *Acinetobacter radioresistens*, *Rubrobacter radiotolerans*, *Kineococcus radiotolerans*, *Ralstonia* sp. and *Burkholderia* sp. (living in biofilm communities from spent fuel pools) and the archaea *Thermococcus gammatolerans* (Egas et al., 2014; Matusiak, 2016).

Figure 2 - Coloured scanning electron micrograph (SEM) of four *Deinococcus radiodurans* bacteria forming a tetrad. Source: Science Photo Library

2.2.1.4. Effects of radioactivity on viruses

An inverse correlation between viral genome size and susceptibility to inactivation by gamma irradiation has been suggested although this correlation is not universal, as there are exceptions to the trend (Hume et al., 2016). The inactivation of viruses by gamma radiation can be direct or indirect. In the direct inactivation, there is likely radiolytic cleavage or crosslinking of genetic material. In the indirect inactivation, radicals like the OH are formed by radiolytic cleavage of water, together with ozone, formed by radiolytic cleavage of O_2 and which will act on the viral nucleic acids and proteins (Hume et al., 2016).

Jebri et al. (2013) noted a larger resistance to radiation on bacteriophages compared to bacteria. From these phages, naturally occurring somatic coliphages were more resistant than naturally occurring F-specific RNA phages. Different phages have different resistance to radiation (Sommer, 2001). The authors also reported that the nature of the matrix had much more influence on the effect of gamma radiation on bacteriophages compared to a bacterium such as *Escherichia coli*. Furthermore, the presence of organic matter, probably by scavenging of free radicals, greatly increased the resistance of bacteriophages to gamma radiation.

Sommer (2001) indicates that the characteristics of water had a significant impact on microorganism inactivation by radiation, and bacteria could not serve as reliable indicators for the prediction of bacteriophage resistance.

2.2.1.5. Research and limitations

Interest in the study of biodiversity and activity of microorganisms in environments contaminated with radionuclides has greatly increased in the last decades (Lopez-Fernandez et al., 2020). However, limitations still exist.

Most research on the effects of ionizing radiation has been done in a laboratorial context with single species, where biotic factors are excluded and abiotic factors are controlled (Geras'kin, 2016; Hevrøy et al., 2019). Most of these laboratorial studies have used acute exposures to gamma radiation to investigate biological endpoints up to the individual level, making them limited in ecological relevance (Hevrøy et al., 2019). These studies have less capacity to understand a real-world scenario of radioactive contamination, where organisms have different capacity to cope with radiation (Geras'kin, 2016). Furthermore, as most of this research is with single-species, it fails to evaluate community-level effects (Fuma et al., 2012). Another difficulty is that microorganisms, such as bacteria, are difficult to culture in laboratory media (e.g. less than 1% of environmental bacteria can be cultured on laboratory media) (Ishii et al., 2011). Culture-independent studies are a solution, as they can give new insight into the microbiology of radionuclides (Lopez-Fernandez et al., 2020).

As for field studies, these are sometimes confused by environmental variables other than the toxic agents in question (Fuma et al., 2012b). Additionally, it can be difficult to estimate the radiation effects at the ecosystem level since ecosystems have biological communities, physico-chemical factors and their interactions (Ishii et al., 2011). For example, an ecosystem contaminated with radiation can be contaminated with other toxic agents (Hevrøy et al., 2019). A better understanding of this ecological situation must require comparing the effects of radiation with the effects from other contaminants (Hevrøy et al., 2019).

Studies of radiation effect on microbial communities have concentrated on terrestrial ecosystems, with limited attention to aquatic communities (Fuma et al., 2010).

Regarding radioecological research on organisms in general, Beresford et al. (2019) comments on reported observation of effects at extremely low dose rates of radiation. These effects could also be contributed by many other factors, including: failure to account to relevant confounding factors; residual influence of historic acute/high exposure; whether the observed effects are the result of previous acute exposure to higher level of radiation, rather than the current low dose rate; and incorrect interpretation of statistical results, as correlation does not necessarily mean causation. Beresford et al. (2019) recommends a robust and open scientific research, open sharing of expertise and data sets and independent study replication, so as to address the disagreement between different radiation effects studies.

2.2.1.6. Bioremediation

The interaction between microbes and radionuclides can bring benefits such as bioremediation, as they control and detoxify radionuclides (Ding et al., 2019). Microbes can change the radionuclide species or alter the physiochemical nature of the environment, therefore impacting the toxicity of radionuclides (Ding et al., 2019). The stress from ionizing radiation leads to genetic mutations, and if bacteria are able to accept and undergo stable mutations, they will form recombinant mutants with self-sustaining capability (Ding et al., 2019). These mutants could be used for bioremediation (Ding et al., 2019).

The bioremediation of radionuclides in a polluted environment requires organisms capable of effective accumulation of radionuclides while resisting their toxicity and being radio-resistant (Heidari et al., 2017). One of the most radiation-resistant organisms discovered so far is the bacterium previously mentioned, *Deinococcus radiodurans*, which is Gamma and UV radiation-resistant (Tapias et al. 2009). Cyanobacteria are also considered potential biomaterial for the removal of radionuclides due to their high metal-complexation capability, rapid equilibrium and great availability in freshwater ecosystems (Heidari et al., 2018b).

2.2.2. Temperature

Warming should increase rates of respiration and decomposition, leading to a predominance of heterotrophic organisms compared to autotrophic ones (Machado et al., 2020). For their reduced size and short life cycle, prokaryotes become important models for understanding the consequences of global warming (Machado et al., 2020).

Temperature has a significant effect on taxa involved in primary productivity (Guo et al., 2020). High temperatures require microorganisms to adapt (Uribe-Lorío et al., 2019). Richness and diversity will normally decrease with increasing temperatures (Guo et al., 2020; Li et al., 2015; Uribe-Lorío et al., 2019). But this decrease happens in temperatures above 40°C to 45°C, probably due to the inactivation of many proteins (Uribe-Lorío et al., 2019) Below this point (45°C) diversity positively correlates with temperature (unimodal effect) (Uribe-Lorío et al-. 2019).

At temperatures above 75°C (e.g. thermal springs), bacterial phyla with thermophilic/hyperthermophilic characteristics are favored, such as *Aquifiae*, *Deinococcus-thermus*, *Thermosulfobacteria*, *Thermotogae* and also thermophilic members of Proteobacteria and Firmicutes. Archaeal phyla found include Crenarchaeota, Euryarchaeota and Thaumarchaeota. For temperatures below 75°C, Proteobacteria and photosynthetic bacteria such as cyanobacteria and Chloroflexi are expected to be the most common populations (Guo et al., 2020). Archaea were thought as the main microorganisms in environments with higher temperatures, but recent findings prove Bacteria as predominant in prokaryotic communities (Guo et al., 2020).

Temperature plays a critical role in the cyanobacterial community, as most cyanobacteria are positively correlated with temperature (Zhang et al., 2021). Increasing temperatures stimulate growth of most cyanobacteria, and their optimum growth temperature is above 25°C (Zhang et al., 2021). However, photosynthesis is limited to temperatures below 75°C, and the presence of Cyanobacteria and Chloroflexi decreases. Oxygenic photosynthesis is affected at such temperatures, and cyanobacteria richness and diversity consequently decrease (Guo et al., 2020).

For viruses in aquatic systems, the increase in temperature contributes to their faster inactivation, according to Pinon & Vialette (2018). Even so, bacteriophages have demonstrated the ability to form plaques at low temperature (4°C) and resistance to high temperatures (62°C-95°C) (Jurczak-Kurek et al., 2016).

The influence of temperature on phage infection is difficult to discern in oligotrophic waters with high temperatures and low host abundance (Ashy and Agustí, 2020). Ashy and Agustí (2020), suggested that higher temperatures could indirectly increase lysogeny while also suggesting further studies on the impact of other environmental variables on lysogeny. Other sources indicate a shift to a lytic cycle with higher temperatures, such as Finke et al. (2017), and Egilmez et al. (2018), which also indicates lower host abundance as a factor. Changes in temperature might also impact viral infectivity in an indirect way, with the changes in the host's metabolism become the actual direct factor (Demory et al., 2017).

2.2.3. pH

Freshwater bacterial community composition is especially driven by the pH of the environment (Bååth and Kritzberg, 2015; Sagova-Mareckova et al., 2021). It will often be the most important parameter compared to others such as temperature, organic matter and nutrient concentration in freshwater systems (Bååth and Kritzberg, 2015). pH is an environmental parameter that varies greatly in normal freshwater systems, and during these episodes of fast change, the microbial communities (e.g. bacteria) might not be optimally adapted (Bååth and Kritzberg, 2015).

Cyanobacteria are known to be sensitive to changes in pH since they have preference for either bicarbonate or CO_2 as a source of carbon (Uribe-Lorío et al., 2019). In the case of thermal springs, evidence shows a potential positive correlation between pH and species richness, which indicates an influence of this variable on microbial community (Uribe-Lorío et al., 2019). The effects of pH in thermal springs have been extensively studied, most of them acid (pH=2-4) and circum-neutral (pH=6-8), with a few of them being alkaline (pH>8). One of the conclusions drawn was that temperature would have a bigger influence on diversity of neutral springs, while pH has a bigger influence on acid springs (Uribe-Lorío et al., 2019). Bacteriophages exist in environments with various pH values and ionic strengths (Fister et al., 2016).

Acidophiles, being a part of extremophiles, are organisms with the capability of growing at a pH lower than 5-6, and they exist in all three Domains within the Tree of Life (Rampelotto, 2013). Acidophiles have been discovered in several extreme environments such as hot springs and active or abandoned mines. The acidophilic microbial communities are mostly composed of bacteria and archaea. Examples include *Thermoplasma* spp., *Leptospirillum* spp., *Acidithiobacillus* spp., *Sulfobacillus* spp., *Ferroplasma* spp., *Aciduliprofundum boonei*, and *Acidianus* spp. (Dhakar and Pandey, 2015). Acidophiles have many mechanisms for homeostasis to maintain their balance between internal and environmental pH (Dhakar and Pandey, 2015). Other examples of extremely acidophilic prokaryotes include Alpha-Proteobacteria, Beta-Proteobacteria and Gamma-Proteobacteria, together with many species from the class Acidithiobacillia, gram-negative phyla Nitrospirae, Aquificae and Verrucomicrobia, and gram-positive phyla Actinobacteria and Firmicutes (Quatrini and Johnson, 2018). In Archaea, acidophiles can be found in the phyla Euryarchaeota, which has specifically the *Picrophilus* spp., most acidophilic life-form discovered. Crenarchaeota and Eurkarya also have examples of these life-forms (Quatrini & Johnson, 2018).

2.2.4. Conductivity/Salinity

Salts dissolve in water and break into positively and negatively charged ions (CWT, 2004). Water conductivity means the ability of water to conduct an electric current, with ions being the conductors (Apera Instruments, 2018; CWT, 2004). The major positively charged ions are sodium (Na^+), calcium (Ca^{2+}), potassium (K^+) and magnesium (Mg^{2+}), and the major negatively charged ones include sulfate (SO_4^{2-}), carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) (Apera Instruments, 2018; CWT, 2004). Meanwhile, salinity is the measure of the amount of salts in the water, and as both factors are positively correlated with ion concentration, they become related (WT, 2004).

Salinity is a dominant environmental selective force for prokaryotic communities in aquatic systems, being possibly more important than temperature, pH and other physicochemical factors (Ruhl et al., 2018; Zhong et al., 2016). It was shown to be inversely related with diversity of all three domains of life, particularly to Bacteria (Ruhl et al., 2018).

However, microorganisms with more efficient osmotolerance strategies are less affected (e.g. Halobacteriaceae) (Ruhl et al., 2018).

Other environmental factors that can correlate with salinity and prokaryotic community composition are pH and oxygen availability (Zhong et al., 2016).

As for viruses, the increase in water salinity was shown to significantly increase viral inactivation (e.g. for influenza A H1N1) (Pinon and Vialette, 2018; Poulson et al., 2016). For Poulson et al. (2016), the increased osmotic pressure could disrupt the integrity of the viral membrane and/or cause premature inactivation.

2.2.5. Light

Sunlight is a well-known disinfectant and is relevant in many aquatic systems (Nelson et al., 2018). UV radiation in sunlight is a highly detrimental abiotic factor which impacts microorganisms at both community and cellular levels. It changes the microbial diversity and dynamics of community structure and causes damage to important biomolecules such as lipids, DNA and proteins (Pérez et al., 2017). Therefore, sunlight influences microbial ecology in aquatic environments, including human pathogens and indicator organisms in contaminated waters (Nelson et al., 2018).

Bacteria can be inactivated by sunlight (Nelson et al., 2018; Oppezzo, 2012; Reed, 2004). Light availability can directly affect heterotrophic activity of bacteria, as it photodegrades organic matter, and this matter is important to stimulate bacterial growth and decomposition activity (Pope et al., 2019).

Compared to viruses, bacteria are vastly more complex, with more potential targets of photochemical damage but also with an adaptive regulatory response (Nelson et al., 2018). It is widely accepted that UV_A radiation (400 – 315 nm) mediates its biological effects on bacteria mainly via reactive oxygen species (ROS), including the superoxide radical, hydroxyl radical, hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O2$) (Hoerter et al., 2005).

Bacteria can have many stress responses to help protect them against or repair damage (Pérez et al., 2017). Some enzymes have a key role in catalyzing reactions to avoid the accumulation of ROS. Examples are iron and manganese superoxide dismutase (FeSOD, MnSOD), hydroperoxidases (HPI, HPII) and glutathione reductase (GR) (Hoerter et al., 2005;

Rastogi et al., 2014). Bacteria inhabiting harsh environments (e.g. extremophiles) permanently exposed to damaging solar radiation, demonstrate different strategies to deal with UV stress. They have efficient DNA repair mechanisms and/or active defence against UV-induced oxidative stress, having a tightly regulated proteome (Pérez et al., 2017). Bacteria in freshwater can obtain UV stress resistance, as is shown by the Actinobacteria in Newton et al. (2011). In this case, Actinobacteria have pigmentation, high G-C content and spore formation could be the causes for this resistance. Another example is in *Synechococcus* and *Prochlorococcus*, two oxygenic phototrophs. Mella-Flores et al. (2013), showed results suggesting that *Synechococcus* has developed efficient ways to cope with light and UV stress, while *Prochlorococcus* cells seemingly survive stressful hours of the day using protection mechanisms and temporarily bringing down several key metabolic processes. These strategies can nevertheless be overwhelmed, which results in cell death (Nelson et al., 2018).

Stress response by bacteria is still quite difficult to characterize, and we cannot be sure of all the mechanisms that lead to inactivation. Many types of stress and damage can occur at a particular time, making it difficult to know which mechanisms lead to damage. Solar inactivation of microorganisms can vary with different environmental conditions, such as pH, oxygen level and salinity (Nelson et al., 2018). For some bacteria grown in laboratorial cultures, such as *E. coli*, faster growth rates led to an increased susceptibility to sunlight (Nelson et al., 2018).

Bacterial and viral assemblages are strongly influenced by sunlight in surface waters. Bacteria can be inhibited or stimulated by exposure to sunlight. Bacterial growth can be stimulated by sunlight, with the production of bioavailable organic matter (Nelson et al., 2018). This organic matter supports growth of some bacterial groups together with repair after sunlight exposure, therefore changing the bacterial community (Nelson et al., 2018). Analysing the influence of sunlight in laboratory can be challenging in the case of bacteria since the susceptibility of bacteria to this parameter is altered by prior growth conditions. For example, bacteria originating from wastewater were proved to be less susceptible to sunlight then lab-grown bacteria (Nelson et al., 2018). Advances in next-generation sequencing (NGS) technologies will help in the research of bacterial communities and in the further understanding of the role that sunlight has (Nelson et al., 2018).

Viruses, particularly phages, have been found in environments with high UV irradiation (Fister et al., 2016). The longest wavelengths are UV_A (315–400 nm) and UV_B (280–315 nm), found in normal sunlight. UV_A and UV_B light rays have limited germ-killing ability because viruses, together with bacteria, have had millions of years to adapt to them (Mackenzie, 2020). Still, sunlight controls bacteriophage populations in surface waters by destruction of viral particles or the reduction of viral infectivity. Light, therefore, has a negative effect on viral replication, as UV radiation is one of the main causes of viral decay, and decay rates for viruses of different microorganisms increase in proportion to irradiance (Finke et al., 2017). Rezaie et al. (2020), found that UV_A exhibits antiviral effects against positive sense, single-stranded RNA viruses including coxsackievirus group B and coronavirus-229E. The antiviral process influences bacterial community composition (Nelson et al., 2018).

2.2.6. Oxygen availability

Concentration of dissolved oxygen (DO) is a relevant factor in water and low DO levels can threaten resident macroorganisms, but some Bacteria and Archaea can remain active in these waters and perform important functions, such as mineralization of organic matter and other biogeochemical cycling (Spietz et al., 2015). Dissolved Oxygen is then a key parameter for change in bacterial communities in many marine and freshwater systems. Faull et al. (2020), for instance, demonstrated a strong influence of a vertical oxygen concentration gradient on prokaryotic assemblage composition. Low DO levels can increase richness of the bacterial community, as energy is transferred from macrofauna to microbes, as macrofauna is more oxygen-dependent. The subsequent increase in bioavailable energy allows the coexistence of multiple taxa, and therefore greater biodiversity (Faull et al., 2020; Spietz et al., 2015; Wu et al., 2019). Just like on many other variables, DO can exert action by co-variance with other variables (Spietz et al., 2015).

Studies on the influence of oxygen level on pathogen survival in groundwater are still limited. In the case of viruses, some studies showed stronger virus inactivation with stronger oxygen levels. One possible explanation was that the increased oxygen levels directly increase oxidation of the viral capsid (Gordon & Toze, 2003).

2.2.7. Metal contamination

Metals are known to decrease diversity and richness of prokaryotic assemblages, and some few taxa become dominant by their higher tolerance to such conditions or due to metabolization of the contaminants, and therefore manage to have a competitive advantage (Mohapatra et al., 2011; Tangherlini et al., 2020). However, this situation might not be a general rule, as there are studies that report no significant reaction to metals. The combined effects of multiple metals are also not entirely known (Mohapatra et al., 2011; Tangherlini et al., 2020). Chemical contaminants, such as metal ions, are essential at low concentrations for many life processes (e.g. enzyme production). However, increasing concentrations of metals will eventually make them toxic for many life forms (Jebelli et al., 2017).

Metal contamination may occur naturally (e.g. a geological context) or be caused by human activities (e.g. mining and industrial activities, wood processing, urbanization) (Jacquiod et al., 2018). How metals influence microbial response will depend on the external factors, such as site-specific physicochemical differences (e.g. pH and organic matter), microbial interactions and genetic modalities of metal resistance/tolerance (e.g. chromosomes, plasmids) (Jacquiod et al., 2018).

Prokaryotes haven’t been a priority in the ecological monitoring of the impact of heavy metals (Tangherlini et al., 2020). Nevertheless, there are many studies regarding metal contamination on microbial assemblages, especially in the marine context. Results show that high metal concentrations perturb bacterial metabolism and turnover, decrease the ability to decompose organic matter, respiration and activity of many enzymes. In the case of diversity, it changes in favor of the more tolerant taxa (Almeida et al., 2008; Tangherlini et al., 2020). Besides being affected by these contaminants, prokaryotes can also modify them by influencing speciation and mobility of metals and degrading organic contaminants (Tangherlini et al., 2020). An example of metal contamination is Acid Mine Drainage (AMD), combined with acidity/low pH. Viruses need metals for the functioning of their DNA and RNA replication enzymes. But metals can have antimicrobial and antiviral properties, such as copper (Cu). It was suggested that copper can inactivate viruses by damaging its DNA (Gledhill et al., 2012). Cu exposure has been deemed as a factor for the decline in virus particles, and it was suggested that its antiviral properties are due to damage of viral DNA and RNA (Gledhill et al., 2012).

Adaptation to conditions of limitation and stress of metals is complex (Chandrangsu et al., 2017). In the case of metal ion limitation, bacteria will respond by activating the expression of specific sets of genes in regulons. These genes are controlled by a regulatory transcription factor, a metalloregulatory protein, which senses metals and binds to them, undergoing an allosteric transition (Chandrangsu et al., 2017).

But even with the many responses, bacteria will still have their growth limited and can die from metal limitation and excess (Chandrangsu et al., 2017).

There is still lack of knowledge on the influence of metal in archaeal community (Guo et al., 2019).

2.2.7.1. The case of Acid Mine Drainage

Mineral activity can cause contamination, such as acid mine drainage (AMD) (Carlier et al., 2020). Contamination originating from mine sites is a major environmental problem with negative consequences for aquatic and terrestrial ecosystems (Aguinaga et al., 2018). This event is characterized by a strong acidic wastewater with high concentrations of metals, methaloids and sulfates that contaminate water systems and damage the health of aquatic species (Carlier et al., 2020). AMD will be caused mainly by oxidation of sulfide minerals, which results from exposure to oxygen, water and microorganisms. This event can have natural causes, but mining activities usually accelerate it by increasing exposure of the minerals (Carlier et al., 2020). Acidic drainage starts when sulfides get exposed to O_2, creating soluble sulphates and H+. Minerals with high concentrations of ferrous iron oxidize when they contact with water, creating ferric ions and H_2 as a result. These ions will cause a decrease in the pH of waters. Metal ions will also have abnormal concentrations in these contaminated waters, such as Zn, Cr, Cd, Cu, Mn, Al, As and Pb (Almeida et al., 2008).

Metal hydroxides and other metal compounds are soluble at low pH but precipitate at higher pH in streams. The acidic waters increase in pH when in contact to stream water through buffering or dilution, which leads to precipitation and deposition of metal hydroxides until the water reaches a neutral pH. Such a deposition of metal hydroxides can cause more serious effects on biomass of streams compared to concentrations of dissolved metals in water (Bermanec et al., 2018). Prokaryotes can be found in acidic waters containing heavy metals (Almeida et al., 2008). Microbial community structure has been studied in some categories of AMD, such as iron, sulfide, copper, lead, zinc and coal mine waste (Almeida et al., 2008).

Diversity of Archaea and Bacteria was expected to be extremely low in AMDs, but some culture methods and culture-independent methods have demonstrated a great diversity (Almeida et al., 2008). Some phyla such as Proteobacteria, Acidobacteria, Actinobacteria are among the most abundant in the sediments of water bodies affected by AMD (Carlier et al., 2020). Other phyla such as Bacteroidetes, Euryarchaeota (archaea) and Nitrospirae can also be found (Carlier et al., 2020). At the species level, bacteria found in AMDs include chemolithotrophs such as *Acidithiobacillus ferrooxidans*, *Acidithiobacillus caldus* and *Leptospirillum ferrooxidans*. In the case of Archaea, Sulfolobus, Acidianus, Metallosphaera, Sulfurisphaera and Ferroplasma have been reported in these environments (Almeida et al., 2008). It is important to note that microbial activity can itself originate AMDs.

2.2.7.2. Examples of metals and their effects

2.2.7.2.1. Arsenic and Zinc

One of the most abundant metals/metalloids is the metalloid Arsenic, occurring primarily in forms of arsenite and inorganic oxyanions arsenate (Jebelli et al., 2017). Arsenic levels in water systems can increase either due to natural contamination by the parent rock or due to anthropogenic contamination (e.g. acid mine drainage) (Halter et al., 2011). Normal levels in natural waters are usually less than 10 micrograms per liter but can get as high as 5 000 micrograms per liter in some places (Halter et al., 2011). Since As has high toxicity, its chemical behavior in natural systems and AMD scenarios has been widely studied (Casiot et al., 2003). Even if As is toxic to the majority of organisms, some prokaryotes adapted to this metal by gaining mechanisms that oxidize or reduce it (Jebelli et al., 2017). In environments with arsenic, microorganisms influence the biochemical cycle of arsenic by converting it to other forms with different toxicity, using reduction, oxidation (e.g. As(III) oxidation) and methylation mechanisms (Jebelli et al., 2017). Some bacteria have proved to catalyze As oxidation and can be found in acidic hot springs and mining effluents (Casiot et al., 2003). Bacteria isolated from As-rich environments include *Achromobacter*, *Pseudomonas*, *Alcaligenes* and *Thiobacillus* (Jebelli et al., 2017).

Among heavy metals, zinc (Zn) can be included as an example; since it is the 24rd most abundant element on earth and it is an essential trace element for all living beings, including, for example, heterotrophic bacteria (Bong et al., 2010; Guarino et al., 2020). Zinc ions are essential for organisms, as they maintain proper function of many proteins (Almeida et al., 2008). At higher concentrations, this metal becomes toxic for most organisms, as excess load of Zn shows toxicity and inhibition to microbial processes (Almeida et al., 2008; Bong et al., 2010). Acidophilus microorganisms demonstrate high tolerance for these conditions (Almeida et al., 2008).

2.2.8. Nutrient availability

As prokaryotes drive transformation and cycling of most biologically active elements they regenerate and mobilize nutrients in freshwater food webs (Wu et al., 2019). Heterotrophic bacteria in aquatic systems alter the mineralization of nutrients and organic carbon, increasing the ecosystems' productivity (Philipps et al., 2017). The increase of nutrient availability is one of the most pervasive human impacts on aquatic systems and is mainly caused by the use of fertilizers (Biasi et al., 2017). An intensification of agriculture to feed a growing human population will lead to an increase in nutrient availability in ecosystems (Biasi et al., 2017).

Microbial activity can be affected, as more nutrients coming from human activities result in a higher concentration of inorganic nutrients in water (Biasi et al., 2017). Nutrient enrichment in freshwater systems, for instance, stimulates microbial metabolism, growth and reproduction (Biasi et al., 2017). Cell size and morphology will be strongly affected by nutrient limitation and the ratios between carbon and other components (Philipps et al., 2017). How bacteria deal with nutrients will depend on other factors, such as temperature. Higher temperatures lead to less growth efficiency and higher nutrient regeneration efficiency in bacteria. A change in temperature alters the concentration of some nutrients such as phosphorus (P) and changes the ratios of important nutrients (Philipps et al., 2017).

Anthropogenic eutrophication in natural freshwaters is a process that is increasing and leads to considerable changes in microbial community composition (Kiersztyn et al., 2019). Many studies have been carried out on the influence of the trophic state in the diversity of bacteria in lakes (Kiersztyn et al., 2019). Bacterial abundance, biomass and metabolic rate are reported to increase with the deterioration of freshwater trophic status, caused by eutrophic and highly polluted environments (Santos et al., 2019). Bacterial OTU (Operational Taxonomic Units) richness also increases with eutrophication. Planktonic cyanobacteria, for example, increase with eutrophication, and their higher abundance influences taxonomic composition of bacteria (Sagova-Mareckova et al., 2021).

Regarding community composition of bacteria in freshwater systems with differing nutrient loads, *Flavobacterium* can be found in high abundance in eutrophic and hypertrophic urban rivers (Wu et al., 2019). Cyanobacteria could potentially dominate in shallow, non-stratified lakes with high nutrient load combined with high temperature (Wu et al., 2019).

Hershey et al. (2018) observed that under low nutrient conditions, microbial diversity was higher. Bacterial community was more diverse despite the lower concentration of organic carbon. A potential explanation to this high species richness under a starved environment was the environmental heterogeneity, mixing and predation that prevent the formation of true competitive exclusion. This in turn allows the existence of high species richness even in low nutrient conditions.

3. Study and biomonitoring of prokaryotic and viral communities in freshwater

Freshwater systems have high species diversity and higher functional diversity than other ecosystems (Sagova-Mareckova et al., 2021). In these systems, aquatic microorganisms with high metabolic and growth rates are strongly influenced by local and global pressures (Sagova-Mareckova et al., 2021).

Natural and anthropogenic activities threaten freshwater quality, so regulatory networks were enforced worldwide for the protection of these waters (Liao et al., 2018). In water for human consumption, for example, bacteriological communities are monitored for water quality assessment (Liao et al., 2018). Some bacteria such as enterobacteria and faecal bacteria are used as indicators for protective standards. They must exist in concentrations below a reference concentration (Liao et al., 2018).

As bacteria are relevant for nutrient, energy and carbon cycling in aquatic systems, they could be used to develop bioassessment tools based on their community composition, bacterial-mediated processes and population dynamics (Garrido et al., 2014). Together with this, information on bacterial responses to external disturbance could be used to determine effects of pollution, as quantification of microbiological changes are shown to be more sensitive than the physicochemical characterization of water (Sharuddin et al., 2018). However, studies are still lacking (Garrido et al., 2014).

The use of prokaryotes also comes with limitations. Only a small fraction of the prokaryotes (both Bacteria and Archaea) can grow on agar plates, increasing the difficulty to study prokaryotic diversity, and consequently, the study of viral diversity (Wang et al., 2020; Weinbauer, 2004). The advent of culture independent techniques helped mitigate this problem (Weinbauer, 2004). The approach of culture-independent methods for the study of prokaryotic diversity has advantages, as they avoid cultivation bias and produces a detailed insight (Wu et al., 2019).

Archaea are still underrepresented in molecular surveys and undersampled, since they represent a lower fraction of the microbial community (Juottonen et al., 2020).

One of the limitations in using viruses is that they don´t share universally conserved genes that can be targeted to survey their communities, in contrast to prokaryotes and eukaryotes (Palermo et al., 2019). Yet, due to their evolutionary history, viruses and hosts can share the same genes, and viral homologues in host genomes must be specifically annotated as such (Palermo et al., 2019).

In marine systems, viral sequence databases and knowledge of marine virus ecology have greatly expanded, but viral ecology in freshwaters remains untapped (Palermo et al., 2019).

Still, existent research on freshwater viruses shows distinct viral communities from the marine ones (Palermo et al., 2019).

To study viruses, a tool such as shotgun metagenomic sequencing could help advance viral ecology (Palermo et al., 2019) and metagenomics has been used in the study of freshwater viral communities (Palermo et al., 2019; Wu et al., 2019).

Part 2 – Influence of radioactivity and other parameters on microorganisms present in springwater: case study in Auvergne, France

4. Introduction

4.1. Springs

A spring is characterized as a groundwater-dependent ecosystem, strictly delimited and defined, with global distribution. Springs are considered as an interface between groundwater and surface freshwater habitats (Cantonati et al., 2006; Manenti and Pezolli, 2019; Paffett et al., 2018). Until recently, springs weren´t properly classified (Springer and Stevens, 2008). Springer & Stevens (2008) created an index of 12 spheres of discharge, differentiated by their hydrogeology of occurrence and their microhabitats. It became a way of comparing these spheres of discharge and, according to Springs Stewardship Institute, by having a common lexicon, hydrogeologists can offer a more consistent guidance for springs. The 12 types of springs, according to this index, are limnocrenic and rheocrenic (as shown in Fig. 1), helocrenic, hypocrenic, cave, hillslope, mound form, gushet, geyser, fountain, exposure and hanging garden. The springs are described relative to their hydrogeology of occurrence, and the microhabitats and ecosystems they support. According to Stevens et al. (2020) springs have great ecological relevance in most of the landscapes. They help control critical flow, water quality and provide ecosystem goods and services for Man.

With all the importance they have for biodiversity and water quality, springs are still less studied than other water systems (Cantonati et al., 2012; Mogna et al., 2015; Paffett et al., 2018; Teittinen and Soininen, 2015). The lower level of study is partially explained by the under recognized influence of spring types, and by the individuality of each spring in terms of ecosystem characteristics, assemblage structure and response to anthropogenic influence (Paffett et al., 2018; Stevens et al., 2019). Additionally, protective legislation does not cover enough of these ecosystems, which leads to their destruction (Cantonati et al., 2012).

Figure 3 - Examples of limnocrene springs (a,b) and rheocrene springs (c,d). Source: Fattorini *et al*., (2016).

4.2. Use of prokaryotes and phages for water quality assessment in springwater

Different microorganisms are used in groundwater and springwater environments as indicators of their water quality, such as prokaryotes (e.g. *Escherichia coli* and *Enterococci)* and viruses (Méndez et al., 2004; Wen et al., 2020). Estimates point to 40% of Earth's prokaryotic biomass being in aquifer systems (Smith et al., 2016).

Each spring has unique prokaryotic and viral communities, and one reason for that could be differing nutrient inputs, related to distinct land use around the springs (Malki et al., 2020). Prokaryotes are important to mediate biogeochemical cycling, as they remove organic carbon, nitrates and micropollutants, therefore helping to maintain the water quality in aquifers (Malki et al., 2020). Bacteria in mineral waters are important for ecosystem functioning, as they produce and transform organic matter and participate in geochemical cycles of elements (Lebedeva et al., 2020). Knowledge about prokaryotic communities in these ecosystems is still limited (Malki et al., 2020), yet it is known that the prevalence of *E. coli* strains is increasing worldwide (Allocati et al., 2013). For example, microbial investigation in Vietnam springs revealed high concentrations of the bacterium *E. coli*, with microbial groundwater quality being deemed as unsafe for drinking (Nguyet et al., 2016). The rise of multidrug-resistant strains of *E. coli* also occurs in Europe (Allocati et al., 2013).

Viruses are gradually being more considered as members of aquatic ecosystems, but just like prokaryotes, studies are still scarce in groundwater and springwater ecosystems (Malki et al., 2020). Nevertheless, more studies are taking place with viruses in these environments (Parikka et al., 2016). Results show that viral particle abundance and virus-prokaryote ratio are low, which could be explained by the oligotrophic nature of groundwater (Parikka et al., 2016). Exploring the viral community composition of groundwater and springs, showed some few ssDNA viruses as the most abundant viruses, according to Malki et al. (2020). A previous study, by Smith et al. (2013), also showed increased levels of ssDNA viruses in aquifer systems.

In thermal springs, viral abundance is lower per host cell in relation to prokaryotic abundance and compared to temperate, mesothermal springs. Higher temperatures can increase instability of virions and lower their numbers (Parikka et al., 2016). In extreme thermal environments, viruses control prokaryotic communities by mediation of genetic transmission and predation (Sharma et al., 2018).

A relevant portion of the viruses studied belong to the order Caudovirales (Sharma et al., 2018). Hot spring virus communities include not only viruses of thermophilic bacteria (thermophages) but also archaeal viruses and probably eukaryotic viruses (e.g. algal viruses). Diversity of thermophages in hot springs is still, nevertheless under-sampled (Zablocki et al., 2018). Viruses and bacteria can end up in groundwater from faecal contaminants. The sources of this contamination can be failed septic systems, leaking sewer lines and land discharge (Lucena et al., 2005). Water supplies to households, agriculture and industry use an important fraction of the groundwater. Even if this groundwater might be more protected from contamination compared to surface water, contaminants can still reach it. When faecal contamination reaches groundwater, faecal microorganisms can start to thrive and thus create disease outbreaks in populations (Lucena et al., 2005). Possible indicators for faecal contamination include the use of one bacterium (e.g. *E. coli*) or dual use of a bacteria and a virus (bacteriophages) or one virus (e.g. coliphages) (Lucena et al., 2005).

4.3. Microbiology in radioactive springs

While worldwide radiation levels normally range from 10-200 nGy/h, several places in the world show abnormal high levels of natural radiation (Heidari et al., 2018). Examples include Ramsar, in Iran with 260 mGy of annual average radiation dose (which converts to approximately $3*10^4$ nGy/h) and measured specific activity of radon ranging from 0.9 Bq/L to 160.7 Bq/L (Asgarani et al., 2012; Bavarnegin et al., 2012; Heidari et al., 2018); Guarapari (dose rates range from 18 – $3.75*10^4$ nGy/h) (Vasconcelos et al., 2013) and Minas Gerais, (e.g. one of the springs, Venâncio, reaches 7.4 mGy/y, which converts to 830 nGy/h, and 222Rn activity concentration surpasses the recommended value of 100 Bq/L in two springs named Villela and Dona Beja) in Brazil (Bonotto, 2014; Negrão and Oliveira, 2011).

There are some examples of studies with microorganisms in radioactive springs. These include Ramsar, in Iran, which contains many hot springs with high amounts of radium (^{226}Ra), and many extremophile microorganisms with specific characteristics inhabit this place (Heidari et al., 2018). In such springs, *Graesiella emersonii* exhibited high capacity for the removal of ^{238}U and ^{226}Ra (Heidari et al., 2017). Cyanobacteria, coccus and *Bacillus* bacteria in the Misasa Hot Springs in Japan show the capacity to adsorb ^{226}Ra in their capsular surfaces (Tazaki, 2009). This results in capsules and slime layers of extracellular polymers around the cells protecting not only against radionuclides but also against metals (Tazaki., 2009). The capability of bacteria to immobilize heavy metals could be used to offset the impact of radionuclides (Tazaki, 2009). Řezanka et al. (2019) described their work with the bacterial genus *Kocuria*, collected from radioactive springs of Jáchymov in northwestern Bohemia (Czech Republic), which are characterized by the production of gas radon. Makk et al. (2019), isolated from a radioactive thermal spring a gram-negative, aerobic and coccus-shaped strain of *Deinococcus fonticola* after exposure to 5 kGy of gamma radiation. The isolate showed good resistance to gamma radiation (D10 < 8kGy). Wang et al. (2017) isolated the strain TS3-9 (*Geobacillus*) from a hot spring with 55Bq/L of radon radiation. Its cells were gram-negative, sporulating rods and possessing particular characteristics of radiation resistance. The strain was compared to *D. radiodurans* and also demonstrated good resistance, growing weakly under radiation doses as high as 14 kGy. Another particular example of radon-containing spring is Paralana hot spring, which supports a flourishing microbial community (Anitori et al., 2002). The spring also contains measurable levels of gamma radiation (Anitori et al., 2002). Anitori et al. (2002) discovered in such spring a halophile, *Halobacterium salinarum*, resistant to ionizing radiation and also made the first report on Archaea in a radon-containing hot spring. While searching for literature, I could not find papers regarding studies of prokaryotic and viral communities in Auvergne springs.

4.4. Flow cytometry

Flow cytometry (FCM) works by collecting information on total cell concentration and nucleic acid content of bacterial populations (Santos et al., 2019). Flow cytometers use lasers to produce scattered and fluorescent light signals that are read by detectors such as photodiodes or photomultiplier tubes (see Figure 4). The signals are then converted to electronic signals that are analyzed by a computer and written to a standardized format data file (McKinnon, 2019). Various fluorescent reagents are used in flow cytometry, and examples are DNA binding dyes, viability dyes, ion indicator dyes, fluorescently conjugated antibodies and fluorescent expression proteins (McKinnon, 2019).

With this technique, cell populations can be analyzed and/or purified based on their fluorescent or light scattering characteristics (McKinnon, 2019). By discriminating microorganisms such as bacteria, based on their high or low nucleic acid content, FCM creates a bacterial community fingerprint (Santos et al., 2019). These fingerprints give information on the characteristics of bacterial community and its changes (Santos et al., 2019). The high throughput permits to decrease analysis time and increase accuracy of the measurement, helping flow cytometry be a method of choice for bacterial enumeration (Buysschaert et al., 2017). With the improved resolution of the instrumentation, the technique became popular in the last decades for microbiology (Buysschaert et al., 2017).

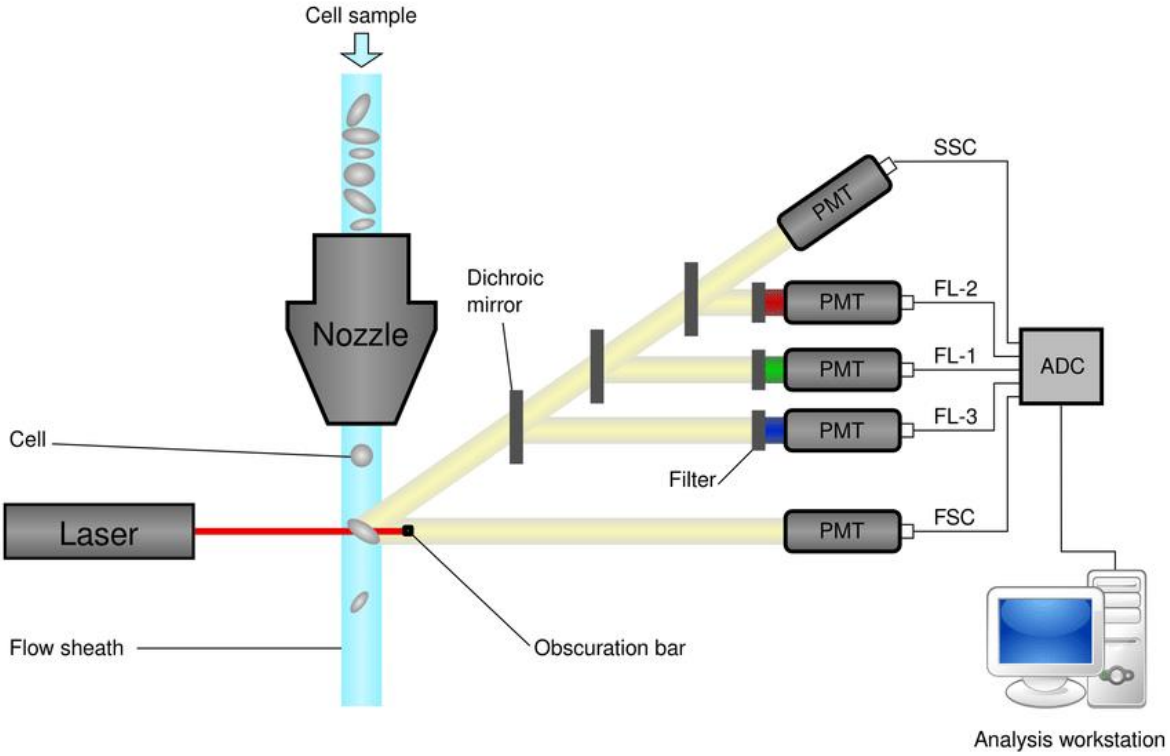

Figure 4 - Schematic diagram of a flow cytometer, consisting of the fluid sheath, laser, optics, photomultiplier tubes (PMTs), analogue-to-digital converter (ADC), and analysis workstation. Source: O'Neill et al. (2013)

FCM has potential use for microbial water quality monitoring, detection of changes in microbial communities and viral enumeration (Santos et al., 2019; Safford and Bischel, 2018). This tool could be applied for automated, cultivation-independent diagnosis (Santos et al., 2019). FCM based analysis can be highly repeatable and allows discrimination between water samples from different places (Santos et al., 2019). Bacteria with differing quantities of nucleic acid content could be quantified and discriminated from each other with this technique (Santos et al., 2019). Applications include the investigation of nucleic acid, cytokines, hormones and protein contents (Adan et al., 2017). This methodology was validated and used for reporting bacterial and viral counts in reservoir systems (Pradeep et al., 2019).

4.5. Objectives

In the present study, fifteen springs were analyzed in Auvergne, a region rich in springs with natural radioactivity. The analysis included the study of its physicochemical (abiotic) parameters, with a focus on the parameter of natural radioactivity (radon activity and gamma radiation). The other parameters included temperature, pH, oxygen level (concentration and percentage), conductivity and the concentration of one of the minerals, carbonate. Together with these parameters, the concentrations of prokaryotes and of viruses (biotic parameters) were included in order to observe how these two groups are influenced by radioactivity and by the other parameters. The study was done with the help of flow cytometry. Due to the pandemic COVID-19, the data presented are preliminary.

5. Materials and methods

5.1. Study sites

Many mineral springs emerged in Massif Central due to its volcanic history. Internal activity and carbon dioxide emissions led the groundwater to rise to the surface via the faults in the rocks. During the flow through the faults, the water is enriched with chemical elements (Millan et al., 2019). More than 600 mineral springs exist in Auvergne region of France, and 16 of them are saline and host flora and fauna normally encountered in marine systems (Richin et al., 2012). The springs are mainly helocrenic and rheocrenic. The present study took place in the springs present in Table 1. Samples were collected from these springs from 16 to 30 of October, 2019 and analyzed by flow cytometry. In Figure 1, the location for all the springs can be seen in the map.

Table 1 - Location, altitude and features of the springs

Name	Location	Altitude (m)	Features	Source (*)
Par	Chaudes-Aigues	763.69	Highest temperatures of all springs in Europe (around 82°C)	(Lopes, 2020)
Font Salée	Apchon	851.16	High mineralization and conductivity	(Millan et al., 2019)
Croizat	Mont-Dore	932.63	Strongly mineralized, hot spring (40°C)	(Rebours, 2017c)
Combris	Ambert	642.61	Ferruginous deposit Releases gas bubbles	(Rebours, 2017a)
Mariol	Mariol	324.05	High amount of iron Abundant flow rate	(Rebours, 2018)
La Montagne1	Chateldon	364.83	Most radioactive spring in France ; high amounts of radon	(Rebours, 2017b) (Millan et al., 2019)
Bard 1/2	Boude	496.70 (1) 499.03 (2)	High concentration of ions, iron and carbon dioxide	(Rebours, 2016) (Poustache, 2020)
Dourioux	Dourioux	711.47	Spring managed by people	Direct source
Les Estreys	Polignac	680.58	High radioactivity High mineralization and carbonic acid	(Down, 2018) (Auvergne Vacances)

Chemin	Ternant les eaux	634.49	Highly gaseous water	(Marie, 2015)
Salut	Ternant les eaux	646.62	Highly gaseous water	(Marie, 2015)
Felix	Mont-Dore	918.32	Close to Croizat, but with less temperature (30°C) and mineralization	(Rebours, 2017d)
Trois Sauts	Leyvaux	736.17	Saline spring Contains halophilic flora	(Dupuy and Chouzet, 2017)
Salins	Drugeac	584	Cold and gaseous spring	(BRGM, 1975)

(*) - Note : Direct source - information directly provided by the advisors

Figure 5 - Location of the springs studied in Auvergne region, France.

Figure 6 - Pictures from some of the studied springs. a) Par; b) Dourioux; c) La Montagne 1; d) Les trois sauts; e) Mariol; f) Salins; g) Les Estreys (measure of conductivity).

Figure 7 - Pictures taken from the studied springs (2). a) Graviers (not included in the results); b) Font salée; c) Daguilon (not included in the results); d) Croizat; e) Combris; f) Chemin

Figure 8 - Pictures taken from the studied springs: a) and b) - measure of radon level in Salut spring); c) - Ours spring; d) - measure of gamma radiation in Bard 1 spring

5.2. Flow cytometry

For the determination of bacterial and viral abundances, samples (1 mL each) were fixed with paraformaldehyde (0.5 % final concentration) and maintained in the dark at 4 °C during 30 min. Following this process, the samples were then passed through FACS Calibur flow cytometer. The equipment used an air-cooled laser of 15 mW at 488 nm with the standard filter set-up. The extracted samples were diluted in 0.02 µm filtered TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8), and stained with SYBR Green I (10,000-fold dilution of commercial stock, Molecular Probes, Oregon, USA). Before the counting, validity check and standardization were carried out, as dilution of water samples with TE buffer depends on the *in situ* bacterial and viral abundances.

5.3. Statistical analysis

Flow cytometry data were analyzed and used to understand and interpret the interaction between multiple environmental and biotic variables (temperature, pH, oxygen concentration and percentage, radon activity, gamma radiation activity). Initially, flow cytometry data were standardized using XLSTAT on Excel. The standardized values of the different variables were used on a Principal Components Analysis. The clustering analysis was also made using XLSTAT to understand how the studied springs related to each other.

6. Results

Due to the unavailability of data on the variable “Radon”, four sampling sites were suppressed. Preliminary results are made available as a variables table, with the values for each variable, the PCA analysis of those values and a dendrogram. The results of the physico-chemical and biotic variables for all springs, together with the variables of prokaryotic and viral concentrations, are presented in Table 2. Temperature in the spring waters ranged from a minimum of 9.9°C in Font Salée to a maximum of 78.1°C in Par spring. Most springs had temperatures between 10-18°C. “Par” spring is an outlier compared to the other springs, reaching a much higher temperature. Felix spring, with 27.8°C, and Croizat spring, with 36.4°C were also noticeably higher in temperature. Conductivity values ranged from a minimum of 255 µS/cm in Dourioux to a maximum of 11 830 µS/cm in Croizat. pH values varied slightly among the springs, ranging from a minimum of 6.3 in Combris spring and a maximum of 7.36 in Par spring. Most pH values varied between 6.3 and 6.9 and Par spring had a higher pH value. For oxygen content, Bard 2 spring had the lowest concentration and percentage (0.32mg/L and 3.1%) while Combris spring showed the highest concentration (2.8 mg/L and 26.5%) and Par spring the highest percentage (52.5%). Oxygen concentrations were low overall. Carbonate concentration varied widely between springs, with minimum in Dourioux (85 mg/L) and maximum in Bard 2 (3 013 mg/L). As for gamma radiation (nSv/h) and radon activity (Bq/L), those two variables have shown wide variations. Gamma radiation (1m) reached a minimum of 90 nSv/h in Salins spring and a maximum of 600 nSv/h in Les Estreys. For gamma radiation (ground) the values went from a minimum of 120 nSv/h in Bard 2 and a maximum of 870 nSv/h in La Montagne 2. Radon activity had its minimum level in Par spring (2.1 Bq/L) and maximum level in La Montagne (3 452.5 Bq/L). Seven of the fifteen springs analyzed had radon activity values surpassing 100 Bq/L, which is the World Health Organization's maximum limit (Bonotto and Padron-Armada, 2008).

From visual inspection of the PCA biplot (Fig. 9) we can observe that:

- The springs of Felix, Croizat, Chemin, Salut, Les Trois Sauts and Bard 1-2 can be expected to have higher values of prokaryotic concentration, conductivity, carbonate concentration and gamma radiation activity relative to other springs. The springs of Par, Salins and Combris are expected to have higher values of oxygen, temperature and pH relative to other springs. The springs of Mariol, Les Estreys, Dourioux, La Montagne 1 and Font Salée are expected to have higher levels of radon activity relative to other springs;

Table 2 - Values for all the biotic and abiotic variables of the sampling sites

Sites	Viruses/mL	Prok/mL	Temperature (ºC)	Conductivity (µS/cm)	pH	Oxygen (mg/L)	Oxygen (%)	Carbonate (mg/L)	Gamma (nSv/h) (at 1m)	Gamma (nSv/h) (ground)	Radon (Bq/l)
Font salée	177761	1233	9.9	1139	6.53	0.52	4.6	640	150	210	39.5
Salins	191568	2712	13.9	3090	6.83	2.05	20.1	1948	90	150	11.9
Par	578156	5917	78.1	1314	7.36	2.59	52.5	586	180	240	2.1
Montagne 1	53994	6903	12.5	693	6.54	0.65	6.8	340	360	870	3452.5
Mariol	60651	1233	17.4	811	6.44	0.41	4.3	442	270	210	138.8
Dourioux	42160	23915	10.7	255	6.73	1.12	10.1	85	330	480	3110
Bard 1	168393	12574	15.1	6560	6.83	0.35	3.5	2955	120	180	5.8
Bard 2	294379	37229	14.7	6380	6.81	0.32	3.1	3013	150	120	10
Les trois sauts	885108	104290	13.1	3890	6.66	1.35	12.8	1940	180	270	321.6
Les Estreys	118343	3452	16.0	3480	6.38	1.62	16.4	1185	600	210	1272.6
Combris	27120	1233	12.1	1675	6.3	2.8	26.5	955	240	210	270.3
Source du chemin	284024	204142	14.2	4860	6.41	1.58	15.3	2515	240	420	121.6
Source du salut	111686	63363	12.1	5850	6.46	1.27	11.7	2740	180	210	5.6
Felix	278600	32298	27.8	6960	6.43	1.13	14,0	1450	180	210	2.5
Croizat	182692	47584	36.4	11830	6.49	1.05	14.2	1785	150	390	28.8

- As the cosines of angles between vectors assess the covariance between the variables they represent (Buttigieg and Ramette, 2014), we can observe that a single variable, radon activity, has an angle relative to other variables ranging from 90° to 180°, suggesting a neutral to moderately negative covariance with all those variables. A group of variables, which includes pH, temperature and oxygen level have angles between each other significantly less than 90º, suggesting a strong positive covariance between them. The same conclusion can be made for the group of variables of conductivity, gamma radiation, carbonate concentration and prokaryote concentration. At last, the variable of viral concentration has an angle relative to all the other variables minus radon activity closer to 90º, which suggests very little covariance.

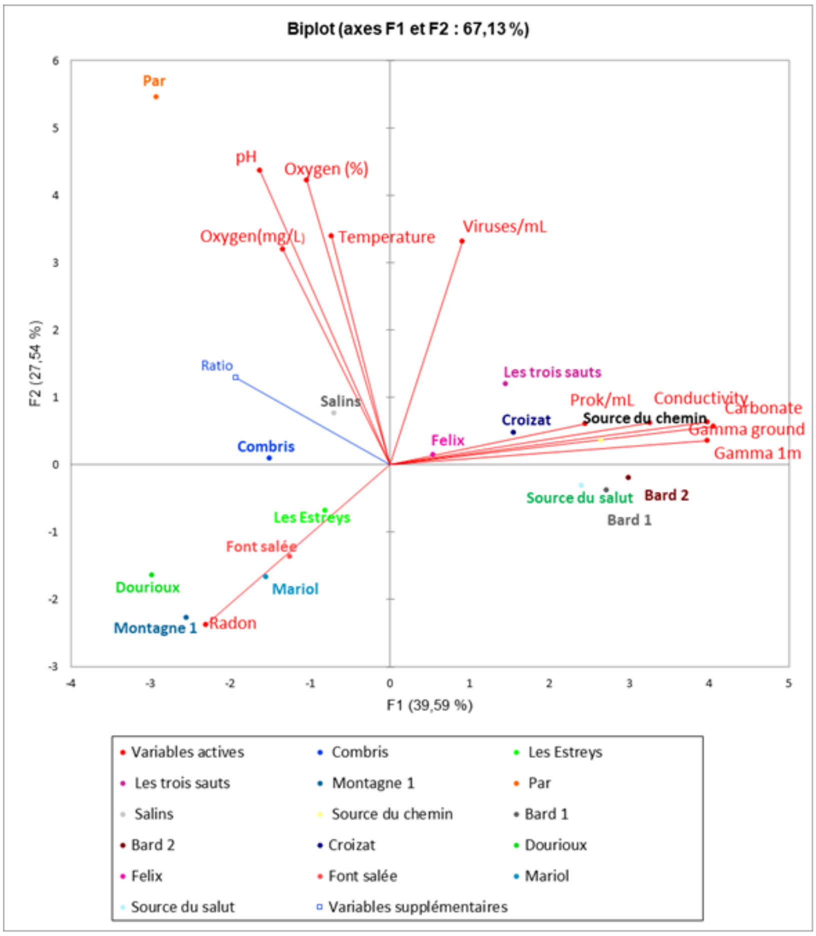

Figure 9 - PCA results (PC1 and PC2) of all variables and springs analysed

Table 3 - Principal components. Considering the information obtained, we can use the first four principal components

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Eigenvalue	4.355	3.030	1.108	0.970	0.561	0.554	0.259	0.143	0.020	0.001
Variability (%)	39.592	27.541	10.071	8.815	5.097	5.036	2.358	1.304	0.178	0.007
% cumulative	39.592	67.134	77.204	86.020	91.117	96.153	98.511	99.815	99.993	100.000

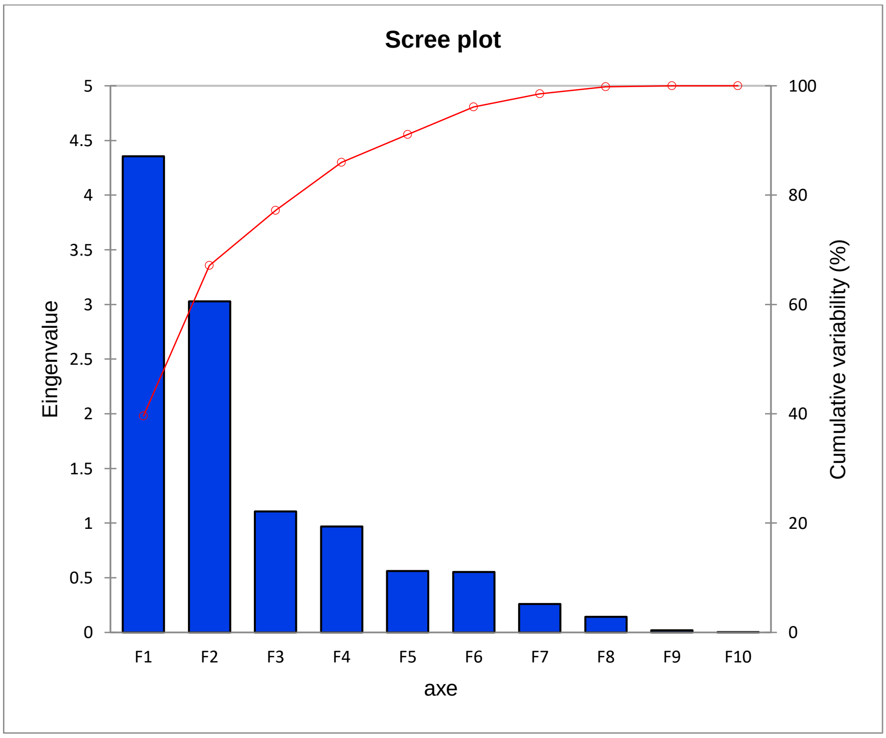

Figure 10 - Scree plot

Interpreting principal components is finding which variables are most strongly correlated with each component. The important correlations are considered those above 0.5. In Table 3, the highest correlations are in boldface (in red boldface, if the correlation is negative). The first PCA axis strongly correlates with the variables of carbonate concentration, gamma radiation, conductivity, prokaryotic concentration and radon activity. This suggests that these variables vary together in the springs and if one increases, the others also increase.

The opposite is for radon activity, with a negative value, suggesting that it decreases with the other variables increasing. For carbonate concentration and gamma radiation, the first component has stronger connection. As for the second component, F2, the correlation is strong with viral concentration, temperature, pH, oxygen (mg/L) and oxygen (%), implying that these five variables vary together in the springs.

For F3, the variables of prokaryotic concentration and oxygen (mg/L) vary positively and the variable pH varies negatively, implying that springs with higher prokaryotic concentration might have lower pH and higher oxygen concentration. The fourth principal component, F4, increases only with viral concentration.

Table 4 - Correlation between variables and Principal Components of the PCA

	F1	F2	F3	F4
Viruses/mL	0,215	**0,658**	-0,047	**0,583**
Prok/mL	**0,581**	0,121	**0,526**	0,497
Temperature	-0,248	**0,838**	-0,244	-0,130
Conductivity	**0,775**	0,123	-0,104	-0,325
pH	-0,174	**0,673**	**-0,581**	0,232
Oxygen(mg/L)	-0,321	**0,635**	**0,604**	-0,218
Oxygen (%)	-0,387	**0,867**	0,230	-0,150
Carbonate	**0,948**	0,127	-0,038	-0,059
Gamma 1m	**0,948**	0,071	-0,028	0,012
Gamma ground	**0,964**	0,114	-0,036	-0,041
Radon	**-0,548**	-0,472	-0,007	0,363

Observing the dendrogram (Figure 11), two groups of springs with important levels of dissimilarity standout (blue and green). “Par” spring is shown as an outlier, with strong dissimilarity from the other springs. The strongest levels of similarity are observed in the groups of Bard 1/Bard 2 and Montagne 1/Dourioux, closely followed by Felix/Croizat and Salins/Les Estreys.

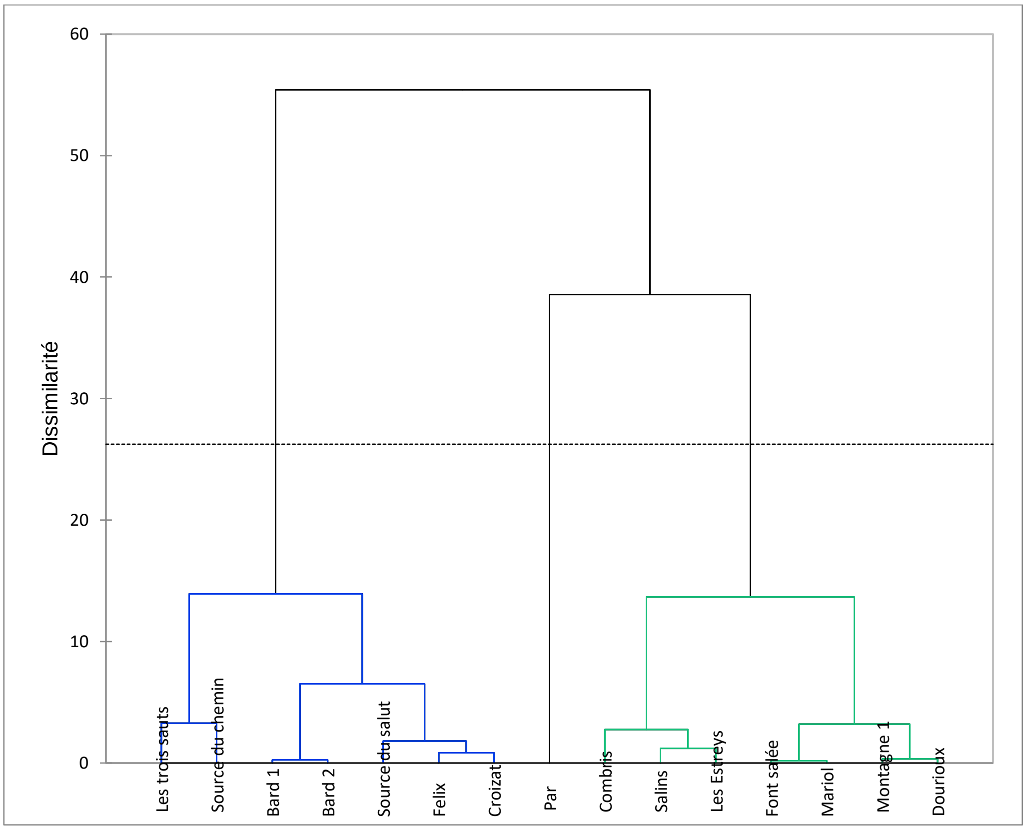

Figure 11 - Dendrogram for all the springs, showing the dissimilarity between them.

7. Discussion

7.1. Gamma radiation and prokaryotic concentration

There was a strong positive correlation between gamma radiation and prokaryotic concentration in water, with prokaryotic community structure yet to be confirmed. Ionizing radiation is proved to cause negative effects on organisms, but little is known about its ecological and evolutionary effects. Low radiation levels are believed to be relatively benign for organisms, and several microorganisms have developed a wide range of tolerance to ionizing radiation (Ruiz-Gonzalez et al., 2016). In this study, the levels of gamma radiation do not seem at least high enough to decrease prokaryotic concentration.

7.2. Radon activity interaction with other parameters

Some points regarding results with radon must be made. Girault et al. (2016) referred the variability of radon concentration in water. Radon is released in waters by natural processes such as decay of its parent radionuclide radium (^{226}Ra) and dissolution of the surrounding geological environment (Jobbagy et al., 2016). Radon concentration will be higher in areas of uranium- and radium-enriched acidic crystalline rocks, and near geologic fault zones (Akkaya et al., 2016).

Radon solubility in water is relatively low (0.01 mol/kg at 293 Kelvin) but its activity concentration in waters can be orders of magnitude higher than that of other natural radionuclides (Jobbagy et al., 2016). In surface waters, radon will have less chance to accumulate and more chance to escape (Jobbagy et al., 2016). However, in groundwater, the constant contact with geological formation of uranium and its existence in closed or nearly closed environment will make radon achieve higher activity concentration (Jobbagy et al., 2016). As radon has a short half-life of less than four days, decay correction must be considered (Jobbagy et al., 2016). In case of samples with low radon activity concentration, measurement should be made *in situ* or a short time after sampling, in the laboratory (Jobbagy et al., 2016). As radon is an inert, noble gas it can easily escape the measurement volume, turning the methods less robust (Talha et al., 2008; Jobbagy et al., 2016). It is important to consider the decrease in radon activity after water sampling and the need for correction factors in order to quote the results (Jalili-Majareshin et al., 2012; Millan et al., 2019).

No strong correlation was observed between radon and the other physicochemical variables, being instead neutral to moderately negative. Radon level varies with physical factors, such as temperature of the water (Akkaya et al., 2016) although no correlation can also be found between radon and physicochemical variables, as stated by Nagabhushana et al. (2020). In Radolic et al. (2005), no correlation of radon solubility was found with other parameters such as pH and conductivity; Ye et al. (2019) found a weak correlation of pH and radon solubility. Radon is an inert gas, so it’s not surprising that no relationship between its concentration and pH values were found (Qadir et al., 2020). In Schubert et al. (2012), a correlation of radon solubility with salinity was found in the waters with high levels of salinity (e.g. some groundwaters). Ye et al. (2019) found negative correlation of radon solubility with salinity. Salinity is regarded as one of the main influencing environmental parameters for prokaryotic community composition (Marine et al., 2013). Zhong et al. (2020) found correlation between oxidation of groundwater and enrichment with radium, which leads to enrichment in radon. Radon will transfer from water to air mainly through out-gassing, which increases in agitated or waters with higher temperature. This relationship can explain the negative correlation between Radon and temperature (Fouladi-Fard et al., 2020; Radolic et al., 2005; Roba et al., 2010; Ye et al., 2019). It is important to note that radioactivity in surface water is mainly due to radioactive components in the earth's crust (Fouladi-Fard et al., 2020). The abundance of parent radionuclides such as ^{238}U (uranium) and ^{226}Ra (radium) in soil and rocks will play a key role in radon concentration in groundwater (Fouladi-Fard et al., 2020; Zhong et al., 2020). Radioactivity of radionuclides in groundwater will strongly depend on the time periods of water-rock interaction (Zhong et al., 2020).

In the present study, radon activity concentration observed was high in many springs, particularly in La Montagne 1 with around 4 000 Bq/L of radon activity. Maximum contaminant level (MCL) for radon in water is around 11 Bq/L (proposed by US Environmental Protection Agency (USEPA)) (Rangaswamy et al., 2015; Rani and Dugall, 2013) or 100 Bq/L (WHO) (Ting, 2010). Seven of the fifteen springs had values of radon activity above or significantly above the maximum recommended by WHO (100Bq/L). Comparing with the literature example of Ramsar (Iran), with maximum radon activity level observed of 160.7 Bq/L, five springs exceeded or significantly exceeded this value. A similar comparison can also be made with the springs of Villela and Dona Beja (Minas Gerais, Brazil) which had values exceeding 100 Bq/L. Considering the values of gamma radiation in nSv/h (with equal conversion to nGy/h), none of the spring analyzed had values close to the ones mentioned for the literature springs. For example, the maximum observed value of gamma radiation was for La Montagne, 870 nSv/h was still much lower than those in Ramsar ($3*10^4$ nGy/h) and Guarapari ($3.75*10^4$ nGy/h), and equivalent to Venâncio spring in Minas Gerais (830 nGy/h). Places like thermal springs can be privileged for radon emanation (Belgacem et al., 2015). Auvergne region is famous for its mineral springs, and some of them, particularly La Montagne, are naturally radioactive (Millan et al., 2019).

7.3. Radon activity and viral concentration

Viral concentration had no strong positive correlation with the other variables. A moderately negative correlation was observed with radon activity concentration and with prokaryotic concentration a weak positive correlation was found. Viral lysis is deemed to control prokaryotic community diversity and not so much its abundance (Meunier and Jacquet, 2015). The role of environmental factors in controlling virus-prokaryote interactions is still not clearly understood (Marine et al., 2013).

7.4. Dissimilarity between springs

Analysing the dendrogram, “Par” spring is presented as an outlier to the two colored groups but with stronger similarity with the green group. The much higher temperature than all the other springs (Lopes, 2020) might explain such strong distinction. Felix and Croizat are located closely, because they show similar features such as temperature and mineralization (Rebours, 2017). Salut spring and the two Bard springs (which have very strong similarity as they are in the same location and have similar features) are present in the same cluster. The other cluster in the blue group contains Les Trois Sauts and Chemin. Font salée and Mariol show strong similarity, as is also shown on the PCA. The springs, La Montagne 1 and Dourioux, are another pair with very strong similarity (dissimilarity value very close to 0). Both have the highest values of radon activity, which might be the strongest influence for such similarity. La Montagne 1 and Dourioux are located in the same cluster as Font Salée and Mariol, meaning that these pairs have stronger similarity with each other compared to all the other springs. Just like in La Montagne 1 and Dourioux, Font Salée/Mariol are influenced by radon activity. Combris, Salins and Les Estreys form the other cluster with close similarity to each other. I could not find similar studies with these springs to compare the results of their dissimilarity.

8. Conclusion

Due to COVID-19, laboratorial work, which was initially planned for three months was reduced to less than a month. The objective of the practical part became solely the statistical analysis of data sent to me by LMGE, located in the campus of the University of Clermont-Auvergne. The theme also had to be changed, it started with diatoms and ended up using prokaryotes and viruses, since I could only have data of these two groups in the springs of Auvergne. COVID-19 limited the amount of work and data to use for Part 2. The results present here are preliminary and a complement to the main part of the thesis, the bibliographical review.

Nevertheless, the results show that there was some effect of radioactivity on prokaryotes and viruses inhabiting the springs as correlations were observed between the variables of radon activity, gamma radiation and the groups of prokaryotes and viruses (although those correlations were overall not very strong). The variable of radioactivity was of particular interest in this study, as its influence on microorganisms in water systems is not well known. Ecological studies on the influence of radioactivity in microorganisms are scarce, and even more so in aquatic environments, so that also makes the conclusions from these results not very clear.

After the bibliographical review and preliminary results concerning the effects of natural radioactivity in aquatic prokaryotic and virus communities, it is possible to identify further research ideas: information regarding other microorganisms such as diatoms, as Millan et al. (2019) worked with them in La Montagne and Mariol springs and showed that they are influenced by radioactivity, forming morphological deformities, therefore being potential organisms to use in future studies; analysis of the community structure of diatoms, prokaryotes and viruses in the springs. As community structure was shown to be changed by radioactivity in past studies, it is important to advance to the next stage by applying this variable in ecological studies. A clearer understanding of the effects on the microbes' genetic material by radioactivity and radiation is crucial, since we cannot yet comprehend well the mechanisms impacting microbes and how they can cope. Analysing genetic diversity in the communities could be done. Other potential studies with much higher doses of gamma radiation and radon activity would also be important so as to compare with the thesis's results.

We also need to know the impact of environmental variables and biotic interactions on different species of each group (e.g. interaction of specific species with radiation). Considering other physicochemical variables (e.g. other minerals, metals and radionuclide activities) and an additional number of springs would also be good and enrich the study.

Finally, future research has the potential address these limitations, as the interest towards natural radioactivity in water is increasing (Alharbia and El-Taher, 2015). Living organisms have evolved to manage the stress induced by many variables such as ionizing radiation, but the evolutionary effects of chronic exposure to natural radioactivity are not fully understood (Millan et al., 2019). The lack of studies in this area is clearly visible so more studies are necessary.

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Figure 1 from Part 1 - Fattorini, S., Borges, P. A. V., Fiasca, B., & Galassi, D. M. P. (2016). Examples of limnocrene (a, b) and rheocrene (c, d) springs [Photograph]. In *Trapped in the web of water: Groundwater-fed springs are island-like ecosystems for the meiofauna* (p. 6).