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 UCLouvain



Ph.D. thesis in Bioscience engineering

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Microbial dynamics in the aquatic environments of a nuclear reactor

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Valérie

Abstract

Nuclear reactors contain various watery environments, such as spent nuclear fuel pools for the intermediate storage of spent nuclear fuel underwater, the primary and secondary cooling circuits for the cooling of nuclear fuel in the reactor vessel and different ultrapure water tanks for the replenishing of evaporated water. These water systems are maintained at high purity levels through constant filtration and deionization in purification circuits, resulting in ultrapure waters with low conductivities and nutrient levels, occasionally exposed to high levels of radiation. Despite the extremely challenging conditions in these waters, microorganisms such as bacteria, fungi and microalgae have been previously detected. While most of the previous studies were performed on spent nuclear fuel pools, the aim of this work was to investigate the bacterial communities in different watery environments of the BR2 nuclear research reactor at SCK CEN in Mol, Belgium, with a particular focus on an open basin surrounding the reactor vessel.

In a first study, we investigated the viable microbial population in a range of interlinked watery environments using a cultivation-based approach. This yielded an extensive strain collection of 33 distinct bacterial species, which is the largest catalogue of isolates described so far in a single study. Furthermore, we attempted to characterize the radiation susceptibility of some of the isolated strains, which resulted in the identification of *Sphingomonas melonis* as the most radio-resistant species, as it survived an acute irradiation dose of 2.1 kGy.

As the BR2 reactor runs in successive cycles of operation and shutdown, this generates highly dynamic conditions in the basin surrounding the reactor core, with periodically shifting physico-chemical parameters such as temperature, radiation and flow rate. In the second part of our work, we characterized the long-term microbial community dynamics in this basin through 16S rRNA amplicon sequencing. Two sampling campaigns spanning several months were performed, which resulted in the characterization of a diverse bacterial population displaying clear shifts in community profiles: cycles were mostly dominated by an unclassified *Gammaproteobacterium* and *Pelomonas*, whereas *Methylobacterium* prevailed during shutdowns.

Finally, in order to dig deeper into the taxonomic and functional characteristics of the microbial community in the basin and characterize its dynamics more in depth, we adopted a shotgun metagenomics sequencing approach. To this aim, we designed a specialized filtration system in order to be able to collect a sufficient amount of cell material. With regard to the functional characterization of the community, several pathways believed to play a role in cell function recovery after irradiation were more highly represented during shutdowns. Furthermore, we managed to almost entirely reconstruct two MAGs from the metagenome, corresponding to *Bradyrhizobium* sp. BTAi1 and *Methylobacterium* sp. UNC378MF. These strains harbored significant adaptations in their genome allowing them to cope with the extremely challenging conditions prevailing in the basin.

In conclusion, we managed to uncover a large microbial diversity in the various watery environments of the BR2, which were shown to be mostly dominated by bacteria. Members of the community were believed to harbor significant evolutionary adaptations allowing them to survive in these extremely challenging environments.

Included manuscripts

Published research papers:

- Petit, P. C. M., Pible, O., Van Eesbeeck, V., Alban, C., Steinmetz, G., Mysara, M., Monsieurs, P., Armengaud, J. and Rivasseau, C. (2020). Direct Meta-Analyses Reveal Unexpected Microbial Life in the Highly Radioactive Water of an Operating Nuclear Reactor Core. *Microorganisms*, 8.

To be submitted research papers:

- Van Eesbeeck, V., Props, R., Mysara, M., Petit, P. C. M., Rivasseau, C., Armengaud, J., Monsieurs, P., Mahillon, J. and Leys, N. (2021). Cyclical patterns affect microbial dynamics in the water basin of a nuclear research reactor.
- Van Eesbeeck, V., Mysara, M., Goussarov, G., Monsieurs, P., Mahillon, J. and Leys, N. (2021). Microbial dynamics in the water basin surrounding a nuclear reactor in operation: a metagenomic approach.

List of abbreviations

AA	Aluminum Alloy
ALARA	As Low As Reasonably Achievable
ANI	Average Nucleotide Identity
ASV	Amplicon sequence Variants
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
Bq	Becquerel
BR2	Belgian Reactor 2
<i>ca.</i>	<i>circa</i>
CAHB	Culturable Aerobic Heterotrophic Bacteria
CEA	Atomic Energy Commission
CFU	Colony-Forming Unit
CMF	Core Mock-up Facility
D₁₀	Decimal reduction dose
DAPI	4',6-diamidino-2-phenylindole
DGGE	Denaturing Gradient Gel Electrophoresis
DOC	Dissolved Organic Carbon
DSB	Double-Strand Break
dsDNA	double-stranded DNA
DW	Demineralized Water
e.g.	example given
ECCS	Emergency Core Cooling System
eDNA	Extracellular DNA
ENA	European Nucleotide Archive
EPS	Extracellular Polymeric Substance
ESDSA	Extended Synthesis Dependent Strand Annealing
FBTR	Fast Breeder Test Reactor
FDA	Food and Drug Administration
FGMSP	First Generation Magnox Storage Pond
FTC	Fuel Transfer Channel
FT-IR	Fourier Transform Infrared Spectroscopy
GFP	Green Fluorescent Protein
GIF	Gamma Irradiation Facility

Gy	Gray
HEU	Highly Enriched Uranium
HR	Homologous Recombination
<i>i.e.</i>	<i>id est</i>
IAEA	International Atomic Energy Agency
ILL	Institut Laue Langevin
INTEC	Idaho Nuclear Technology Center
IOB	Iron-Oxidizing Bacteria
IRB	Iron Reducing Bacteria
ITS	Internal Transcribed Spacer
kb	kilobase
K_m	Michaelis-Menten constant
KO	Kegg Orthology
LB	Luria-Bertani medium
LET	Linear Energy Transfer
LMW	Low Molecular Weight
LPS	Lipopolysaccharide
MAG	Metagenome-Assembled Genome
MAPS	Madras Atomic Power Station
MIC	Microbioally Influenced Corrosion
MM	Minimal Medium
MS	Mass Spectrometry
MTR	Materials Testing Reactor
MW	MegaWatt, Molecular Weight
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
NHEJ	Non-Homologous End Joining
NMDS	Non-metric Multidimensional Scaling
NTD	Neutron Transmutation Doping
OM	Oligotrophic Medium
OTU	Operational Taxonomic Unit
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
PGC	Photosynthetic Gene Cluster
PI	Propidium Iodide
PVC	Polyvinyl Chloride

R2A	Reasoner's 2 Agar
RITA	Gamma Irradiation Facility
RO	Reverse Osmosis
ROS	Reactive Oxygen Species
rRNA	ribosomal RNA
SCK CEN	Belgian Nuclear Research Center
SEM	Scanning Electron Microscopy
SMRT	Single Molecule Real Time
SNFP	Spent Nuclear Fuel Pool
SRA	Sequence Read Archive
SRB	Sulfate-Reducing Bacteria
SRS	Savannah River Site
SS	Stainless Steel
SSA	Single-Strand Annealing
SSB	Single-Strand Break
ssDNA	single-stranded DNA
Sv	Sievert
TCA	Tricarboxylic Acid
TEM	Transmission Electron Microscopy
TFHX	Thermo-Fluid Heat Exchanger
TIC	Total Inorganic Carbon
TOC	Total Organic Carbon
TSA	Tryptic Soy Agar
UK	United Kingdom
USA	United States of America
UV	Ultra-Violet
VBNC	Viable But Non-Culturable
WNA	World Nuclear Association

Preamble

This Ph.D. thesis was funded by the Belgian Nuclear Research Centre (SCK CEN) Academy in Mol, Belgium. It was conducted in collaboration with the Laboratory of Food and Environmental Microbiology (MIAE) within the Earth and Life Institute (ELI) at the Université Catholique de Louvain (UCLouvain) in Belgium and the Commissariat à l’Energie Atomique (CEA) in France. The majority of the research was performed at SCK CEN.

The general aim of this work was to characterize the microbial diversity in the various watery environments of the BR2 nuclear research reactor located at SCK CEN in Mol, Belgium, with a particular focus on the basin surrounding the reactor core. Chapter I gives a general context on nuclear research reactors and the microbial communities inhabiting their various watery environments. Techniques typically used to analyze microbes in these settings are also reviewed, as well as microbial radiation resistance mechanisms. The objectives of this thesis are presented in Chapter II.

Chapter III comprises the results obtained during this thesis and is divided into three sections. Section 1 covers the isolation and characterization of bacteria from various watery environments of the BR2. Section 2 focuses on the effect of cyclical patterns in physico-chemical characteristics on the microbial dynamics in the basin water of the BR2. Finally, section 3 consists of the study of the microbial dynamics in the basin water of the BR2 through shotgun metagenomics. Chapter IV comprises a general discussion integrating and interpreting all the results obtained throughout this thesis. Final conclusions and perspectives for future research are given in Chapter V.

This thesis contains three manuscripts: one published in collaboration with CEA and two others ready to be submitted for publication. A list of other scientific communications (posters and oral presentations at conferences) is presented in Appendix B.

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Chapter I – General Introduction

Chapter I – General Introduction

1. Nuclear research reactors

Nuclear research reactors are used for research and training, various materials testing and the production of radioisotopes for medical and industrial purposes. They are generally not used for power generation, and are basically neutron factories, producing stable neutron beams for various purposes. They are relatively smaller than power reactors, whose primary function is the production of electricity. Their design is simpler and they operate at lower temperatures. Although they need far less nuclear fuel, they typically require Uranium-235 (^{235}U) enriched up to 20%, known as high-assay low-enriched uranium (HALEU) (WNA 2021). Just as in power reactors, the core needs to be cooled and a moderator is used to slow down the neutrons and to enhance the nuclear fission process. Most of them also use a reflector to reduce neutron loss from the core. Almost all of the world's nuclear research reactors operate with thermal (slow) neutrons. There are a total of about 220 nuclear research reactors in operation worldwide located in 53 different countries (Table 1). Three of them are located in Belgium: BR1, BR2 and VENUS.

Table 1. Operational research reactors (Adapted from WNA 2021).

Country	Operational research reactors	Country	Operational research reactors
Russia	52	Kazakhstan	4
USA	50	Belarus	3
China	16	Belgium	3
Argentina	5	Czech Republic	3
Canada	5	France	3
Germany	5	Indonesia	3
India	5	Japan	3
Italy	5	Ukraine	3
Brazil	4	Others	44
Iran	4	Total	220

Chapter I – General Introduction

1.1 Types of research reactors

Unlike power reactors, where 80% are built as either one of two similar types, research reactors have a much broader variety of designs and operating modes, which may be steady or pulsed (on-off).

One common design is the pool-type reactor, consisting of a core with a cluster of fuel elements submerged in a large pool of water. The core also contains control rods and empty channels for the testing of experimental materials. Each fuel element comprises several aluminum-clad fuel plates or rods, in a vertical box or bundle. Water is used both as a moderator and a coolant, and graphite or beryllium are typically used as the reflector material, although other options are also possible. The pool wall contains apertures to allow the neutron beams to access the core. Tank-type reactors (e.g. the BR2 reactor in Belgium) have a similar design to pool-type reactors, but cooling occurs in a more active manner by pumping the coolant directly through the tank at high flow rates (WNA 2021).

Another type is the TRIGA reactor (Training, Research, Isotopes, General Atomics), where the core comprises 60-100 cylinder-shaped fuel rods with aluminum or steel cladding containing self-moderating uranium zirconium hydride fuel with an enrichment level under 20%. The core is immersed in a pool of water and generally graphite is used as the reflector. These types of reactors can be spiked to very high power levels (up to 22,000 MW) for short periods of time (less than a second).

Other types use heavy water or graphite as a moderator. Others still are fast reactors, which do not require any moderator and use a mixture of uranium and plutonium as fuel. Lastly, homogenous-type reactors consist of a core containing a uranium salt solution enclosed in a large tank. These types generally have a very low power output and are not widely used anymore.

1.2 Uses of research reactors

One potential use of nuclear research reactors is medical radioisotopes production (MRP) through neutron activation, which is the only common way to render stable materials radioactive. Specific elements are bombarded with neutrons in order to add those neutrons to the target nucleus.

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One example is the production of yttrium-90 (^{90}Y) used to treat liver cancer through neutron bombardment of yttrium-89 (^{89}Y) (SCK CEN 2021). Neutron activation can also result in nuclear fission, which is the mechanism by which molybdenum-99 (^{99}Mo) and its decay product technetium-99m ($^{99\text{m}}\text{Tc}$), the most widely used radioisotope in nuclear medicine for a variety of applications, are produced. Uranium-235 (^{235}U) targets are irradiated with neutrons and ^{99}Mo (comprising approximately 6% of the fission products) is subsequently separated from the other fission products in a hot cell. The ^{99}Mo isotopes are supplied to hospitals in the form of lead containers enclosing glass tubes containing ^{99}Mo , which has a half-life of 66 h and progressively decays to $^{99\text{m}}\text{Tc}$ with a half-life of only 6h. When it is required, the $^{99\text{m}}\text{Tc}$ isotope is then chemically attached to a specific protein and the radiopharmaceutical is then used for administration to the patient.

Another use of research reactors is Neutron Transmutation Doping (NTD), which changes the properties of Silicon (Si), making it highly conductive to electricity. Doped Si is produced by irradiating large Si crystals inside a reactor reflector vessel. Neutron irradiation transforms one Si atom in every 10^9 into a P atom. The resulting irradiated silicon is cut into chips and subsequently used for a wide variety of applications in the electronics industry.

Some research reactors can also be used to test the properties of various materials, also known as Materials Testing Reactors (MTRs). In these types of reactors, materials such as steels and different alloys are subjected to intense neutron irradiation to study their changing characteristics (e.g. brittleness).

1.3 Water bodies in nuclear research reactors

1.3.1 Role of water

The majority of research reactors use water as a cooling fluid, moderator and biological shield. As a coolant, water removes the heat produced by the fission reaction occurring in the core and transports it to a heat exchanger system. As a moderator, it slows down the high energy neutrons produced during the fission process, making them energetically adequate for new fission reactions in order to sustain the reaction chain. As a biological shield, water greatly absorbs the emitted radiation in order to produce a safe environment for the reactor operators (IAEA 2011).

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1.3.2 Water purification

Maintaining the purity of the water is of crucial importance, as residual impurities might become activated by the emitted radiation. In addition, low water quality could increase the risk of corrosion of the fuel cladding, structural materials and measuring instruments in the reactor system, as well as the visibility needed for specific operations. In order to maintain the necessary level of purity in the water, it needs to be circulated through a purification system. The purpose of the purification system is the removal of particulate matter (including microbial contamination) as well as dissolved chemical (ionic) species. To this end, these water purification systems contain a filtration as well as a deionization component, usually built in on-line.

The primary purpose of filtration systems is to remove insoluble contaminants by physical adsorption or entrapment. Examples include i) sand filters (made of silica or anthracite) designed to remove particulates by physical entrapment, ii) mechanical filters consisting of metal foams, iii) activated charcoal which binds impurities through adsorption and iv) ultraviolet (UV) filters consisting of parallel flow-through columns containing UV lamps to eliminate microbes. Since these filters can hold lots of impurities removed from the water, they can potentially saturate. They need to be regularly regenerated through cleaning or replaced if a significant pressure drop is observed. In case of potential radioactive particulate entrapment in the filters or ion exchange resins, these systems also require provisions for radiation detection and/or shielding to ensure safe maintenance and replacement.

Deionization is used to remove residual ionic species and occurs through ion exchange resins, which consist of small, insoluble beads of polymeric material with functional group ions covalently attached to the polymeric structure of the resin. The functional groups are readily exchanged with the dissolved ions to be removed from the water. A typical co-polymer matrix is a polystyrene polymer combined with a divinyl benzene polymer to provide structural stability. Ion exchange resins are typically used to remove dissolved ionic impurities from the water in the primary circuit and spent fuel basins. They are generally mixed bed, forming a combination of cationic and anionic resins. They need to be regenerated by adding acidic and alkaline solutions (for cationic and anionic resins, respectively). Both pH and conductivity are based on ion concentrations, and therefore display a correspondence (IAEA 2011).

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They need to be monitored at all times in order to continually ensure ultrapure water quality. A low conductivity indicates a low potential for corrosion. Additionally, chemicals can be added as corrosion inhibitors. Each water system in a research reactor has different water quality requirements and therefore displays different physico-chemical properties.

1.3.3 Primary cooling system

The primary cooling system consists of the reactor tank or pool, depending on the type, the decay tank, circulation pumps, heat exchangers and circulation piping (Fig. 1). When the reactor is operational, all the components of this system are continuously exposed to water, which makes them potentially vulnerable to degradation processes, such as corrosion. Next to this, the primary cooling system is exposed to the highest levels of radiation out of all the reactor water systems, as it is in direct contact with the fuel elements in the core. Consequently, any remaining impurities might become activated and result in additional radiation. Maintaining ultrapure water quality in this system is therefore of crucial importance, as it has the most stringent requirements, out of all the water systems in the reactor. Conductivity should be maintained below $1.0\ \mu\text{S}/\text{cm}$ and pH between 5 and 6.5, whereas the temperature is typically below $100\ ^\circ\text{C}$ (IAEA 2011).

Despite the continuous water purification, some radioactive species are commonly found in the primary cooling circuit water. Significant water activation products include ^3H , ^{13}N , ^{16}N , ^{18}F , ^{24}Na and ^{38}Cl . On the other hand, if the integrity of the fuel elements is affected by corrosion, this might promote the release of fissile material and fission products directly into the water. Since this strongly depends on pH and temperature, these two parameters should also be well monitored and controlled. It is also recommended to check for radioisotopes in the water that might originate from activated corrosion products such as ^{54}Fe or from leaking fuel such as ^{137}Cs through gamma scans.

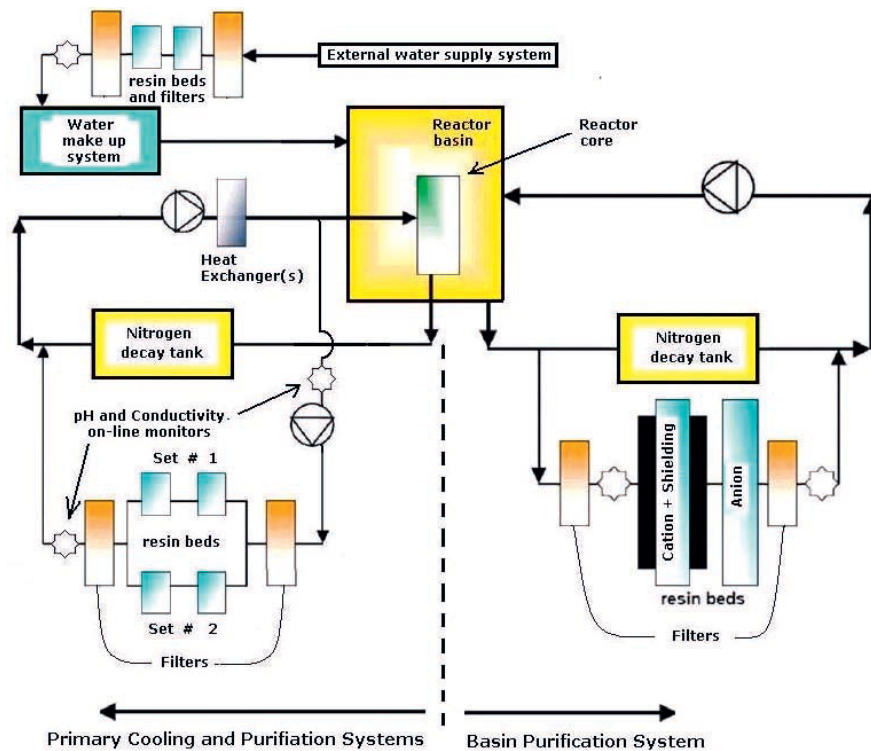


Figure 1. Schematic representation of a water purification system (filter-deionizer system) for high power research reactors. An external water supply (municipal water, well water or river water) is first filtered and deionized before being used. Two separate purification systems are established for the reactor core and basin (Adapted from IAEA 2011).

1.3.4 External tanks and reservoirs

Some external tanks are required in a research reactor, for example to replenish the primary cooling circuit with so-called make-up water in order to compensate for losses due, for instance, to evaporation. Since this water is directly incorporated in the primary coolant, it needs to comply with all the requirements associated with this specific type of water for quality and maintenance procedures (IAEA 2011).

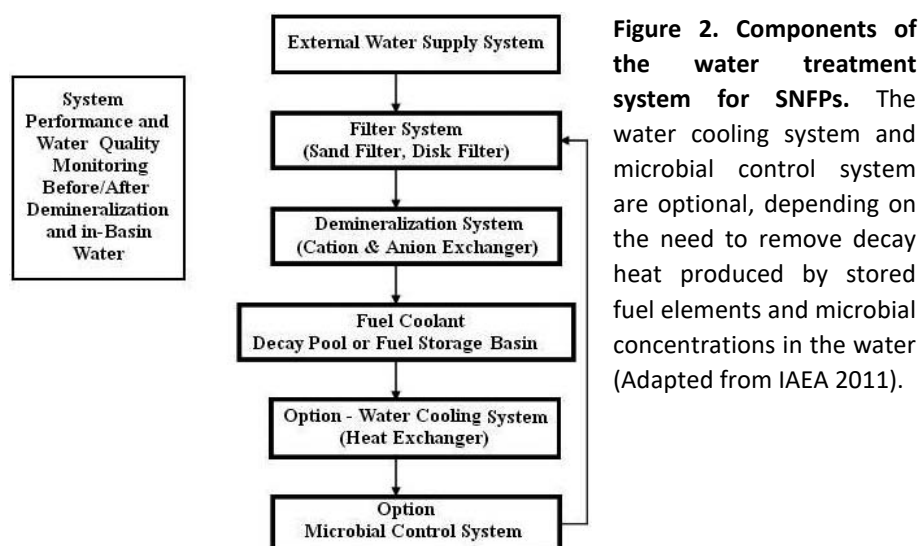
The Emergency Core Cooling System (ECCS) is designed to provide cooling and shielding to the reactor core in case of an emergency deficit of primary coolant. The principal aim of this system is to provide a sufficient volume of water to the reactor in the shortest possible amount of time, with the quality of the water being of lesser concern. However, low quality water might result in undesirable corrosion damage which might impede normal operation.

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For this reason, it is still recommended to maintain the water in ECCS tanks at a high quality. These waters generally require less maintenance, as they remain mostly stagnant and are only in contact with the reservoir lining material. It is recommended to keep these tanks closed, as ambient dust, loose particles, microbes and insects might otherwise pollute the water. Conductivity in these systems should be maintained below $3.0\ \mu\text{S}/\text{cm}$ and pH between 4.5 and 7.

1.3.5 Spent Nuclear Fuel Pools (SNFPs)

After being used in the reactor core, fuel immediately requires cooling. In order to remove heat and provide shielding from radiation, it is typically stored in Spent Nuclear Fuel Pools (SNFPs) prior to being transported to its ultimate disposal location. Depending on the reactor design, the SNFP may be part of the reactor pool or not. They may be either indoor or outdoor, depending on the activity level of the fuel (highly active fuel needs to be maintained indoor). It is important to maintain water purity in these pools as well, as some ionic species, undissolved particles and microorganisms can accelerate the corrosion rate of the fuel (IAEA 2011). This can potentially cause the water to become turbid and increase the concentration of radioisotopes (fission products). A purification system with filters and mixed bed ion exchange resins is therefore necessary in order to maintain the water quality within specified limits (Fig. 2). SNFPs may be indoor or outdoor, which affects their level of contamination with debris from inorganic or organic materials as well as microorganisms.



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Conductivity should be maintained below 10 $\mu\text{S}/\text{cm}$, pH between 4.5 and 7 and temperature below 45 °C. Gamma scans should also be performed to check for the presence of radioisotopes originating from failed fuel (^{137}Cs). Turbidity should be maintained to a minimum in order to ensure enough visual clarity for the reactor operators so that they can safely perform maintenance operations. Additionally, corrosion monitoring can be performed through the placement and periodic withdrawal of corrosion coupons from the SNFP. Those can be evaluated for certain characteristics in order to gauge the extent of potential corrosion. The results can be used to adjust the water quality monitoring. The life span of these pools is approximately 20 to 40 years (Karley *et al.* 2018).

1.3.6 Secondary cooling system

The secondary cooling system of a research reactor consists of a heat exchanger, piping, pumps and a cooling tower (Fig. 3). Heat from the primary circuit is transferred to the secondary circuit through the heat exchanger. The coolant used in this system is again typically water, whose quality needs to be monitored in order to prevent the fouling of metallic surfaces, corrosion of metallic components and accumulation of microbial matter. Both deposits and microbiological growth can reduce heat transfer efficiency and corrosion can lead to leakages and/or affect the integrity of structural materials typically made from carbon steel (IAEA 2011).

In an open cooling system, the same water is reused continually to ensure an adequate cooling process. Heat is dissipated through cooling towers, spray ponds or evaporative condensers. The main advantage of this system is that the water can be chemically treated to protect its components. The main disadvantage is the increase in concentration of chemicals and contaminants in the water due to evaporation losses, which results in the need for constant refilling. The most common method for dissipating heat in open circulating cooling systems are cooling towers. These are designed to provide maximal contact between water and air. Heat removal occurs primarily by evaporation of the cooling water. Some heat loss also occurs through direct cooling of the water by air, but to a minor extent.

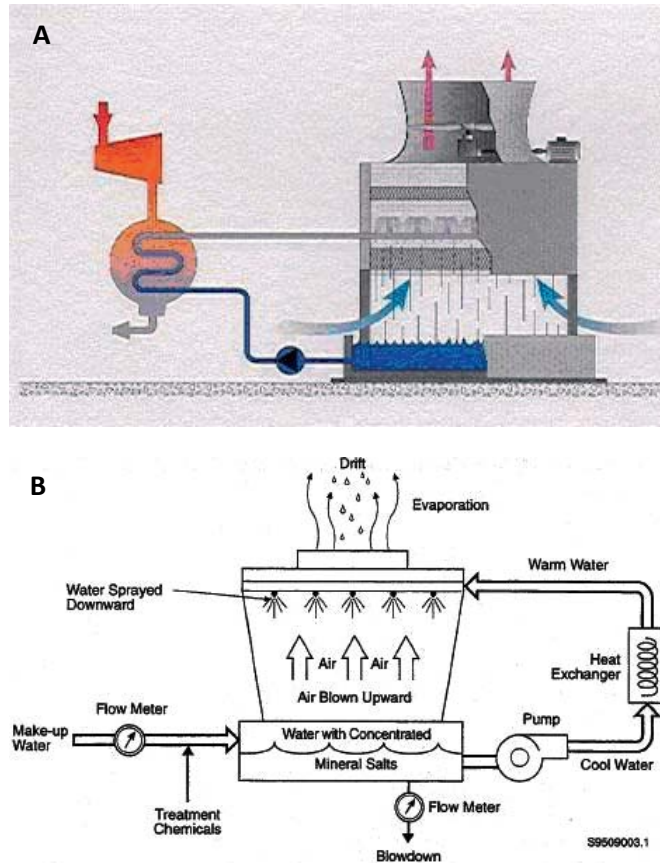


Figure 3. Secondary cooling circuit components. A. General overview of a typical secondary cooling circuit composed of a heat exchanger, piping and a cooling tower. B. Detailed representation of a cooling tower. Water loss occurs mainly through evaporation (Adapted from IAEA 2011).

Secondary cooling systems can use different water sources as their main water supply, such as municipal water, ground water, water from a reservoir, lake or river in the vicinity of the reactor. Water quality requirements are similar to those used in any other industry where heat is removed through cooling towers: pH should be maintained between 6 and 8 and conductivity below 2000 $\mu\text{S}/\text{cm}$. Microbial concentrations should also be controlled in order to avoid biofouling. Online monitoring of the secondary water radioactivity is also recommended to check for potential contamination of the secondary with the primary water.

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1.4 The Belgian Reactor 2 (BR2) at SCK CEN in Mol, Belgium

The BR2 reactor located at the Belgian Nuclear Research institute (SCK CEN) in Mol, Belgium, is a tank-type research reactor with a thermal power between 60 and 125 MW that first became operational in January 1963 (SCK CEN 2021). The reactor has been successfully refurbished four times over its lifetime, where the last refurbishment took place in 2015 – 2016, with an official restart in July 2016. It uses highly enriched uranium (HEU) as fuel, with a ^{235}U enrichment level of 74%. Fuel elements consist of a uranium-aluminum alloy bent into cylindrical shapes. The beryllium matrix in the reactor core is used as a neutron reflector and moderator. Water serves as the principal moderator and coolant. The core of the BR2 consists of the beryllium matrix, forming a vertical channel network of 200 mm diameter, surrounded by other smaller channels in a hyperboloid arrangement (Fig. 4). This design allows for an extremely high level of fission density, while also providing easy access to the top and bottom covers, thereby facilitating the insertion and withdrawal of irradiation devices. The reactor is capable of producing neutron fluxes (both thermal and fast) a hundred times higher than those produced in commercial reactors (up to 10^{15} n/cm².s).

The BR2 is currently the largest Materials Testing Reactor (MTR) in Europe. It is used for various research applications aimed at assessing and demonstrating the safety of nuclear cores or at developing new nuclear fuels. In addition, it produces essential radioisotopes used in nuclear medicine for diagnostics, cancer treatment and palliative care. Some produced radioisotopes include $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ used in oncology and cardiology, ^{131}I for the treatment of thyroid cancer, ^{177}Lu for the irradiation of solid tumors, ^{86}Re for the alleviation of metastatic bone pain, among others. The BR2 is also used for the production of neutron doped silicon for the semiconductor industry in the Silicon Doping by Neutron Irradiation Experiment facility (SIDONIE), designed to rotate and expose the silicon to the neutron beam evenly and continuously. Another facility (POSEIDON) was constructed in 2008 to increase the reactor's NTD silicon capability.

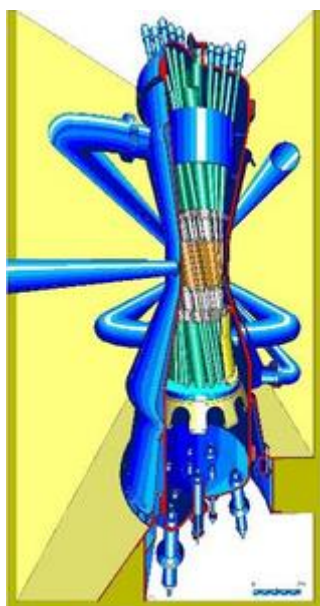


Figure 4. Core vessel of the BR2 reactor. The central vertical channel is surrounded by multiple smaller channels in which irradiation devices can be placed. The orange part in the middle is the reactor core, encased in a beryllium matrix. The horizontal conduit leads the neutron beams toward the core (Adapted from SCK CEN 2021).

Its primary cooling system consists of a closed loop around the core vessel through which water is pumped at a high flow rate ($7000 \text{ m}^3/\text{h}$) and pressure (12 bar) during operation. To account for evaporation, the primary cooling circuit is continuously replenished with demineralized water from an external tank located outside the reactor building. The core vessel of the BR2 sits in a large, open water basin, with a water level seven meters above the upper lid in order to shield technicians from radiation during reactor operation. The basin is in turn cooled by the secondary cooling system, which consists of heat exchangers, underground pipes, pumps and cooling towers. Spent fuel is stored intermediately in a SNFP connected to the basin via a transfer channel. When the reactor is not operating, the upper water layer of the basin is transferred to an external storage tank to allow technicians to access the lower areas for instrumentation and maintenance purposes. These different water systems are purified through purification circuits consisting of particulate filters and UV filters as well as mixed bed (cation and anion) ion exchangers. Most of the radioactivity in the primary water originates from the ^{24}Na radionuclide, with a half-life of approximately 15h. Other common radionuclides include ^{56}Mn , ^{188}Re , ^{41}Ar and ^{135}Xe . The main radionuclides found in the basin water are ^{24}Na , ^{51}Cr , ^{124}Sb , ^{186}Re and ^{60}Co , with ^{24}Na also being the primary contributor to the overall activity. Finally, the most dominant radionuclides detected in the SNFP include ^{60}Co , ^{137}Cs and ^{124}Sb .

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2. Microorganisms in nuclear reactor and other ultrapure waters

2.1 Microbes in Radioactive nuclear waters

Nuclear reactors contain different watery environments, some of which are exposed to varying doses of radiation, such as the very dynamic primary cooling system and the basin surrounding the reactor core, as well as the more static storage waters of SNFPs. These environments display different physico-chemical characteristics other than those from non-radioactive environments, and therefore exert different selection pressures on the resident microbial communities. Most of the studies analyzing microbes in nuclear water bodies focus on static SNFPs. As far as we know, only a single study has investigated the microorganisms in the dynamic cooling water systems of nuclear reactors, such as the pool directly surrounding the reactor core (Petit *et al.* 2020).

Most of the studied SNFPs displayed similar values for pH (between 5 and 7), temperature (between 18 °C and 37 °C) and conductivity (approximately 1 $\mu\text{S}/\text{cm}$). There were a few exceptions though, such as the interim wet storage system in Sweden (CLAB), which displayed a conductivity of $91.4 \pm 0.6 \mu\text{S}/\text{cm}$ (Masurat *et al.* 2005) and an SNFP in Brazil with a slightly more acidic pH, between 4 and 4.7 (Silva *et al.* 2018). The Sellafield site in the UK also contains various spent fuel storage facilities, such as an outdoor pond that showed seasonal temperature variations ranging from 7°C to 23°C, a pH reaching up to 8 and a conductivity averaging $3.9 \pm 0.6 \mu\text{S}/\text{cm}$ (McGraw *et al.* 2018). The First Generation Magnox Storage Pond (FGMSP) is another specialized outdoor SNFP in Sellafield where the pH is constantly maintained at 11.4 ± 0.1 by purging with demineralized water dosed with NaOH in order to minimize the fuel corrosion rate (Foster, Boothman, *et al.* 2020). Moreover, the temperature showed seasonal variations between 8°C and 19°C. Yet another high pH SNFP at Sellafield is located indoor, and the pH and temperature are maintained at 11.6 and 15°C, respectively (Ruiz-Lopez *et al.* 2020).

The reactor pool water surrounding the core of the Osiris reactor in France displayed a conductivity of 0.5 $\mu\text{S}/\text{cm}$ and the water temperature in the core unit reached up to 47 °C during reactor operation (Petit *et al.* 2020).

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2.1.1 Isolates obtained via cultivation-based approaches

Until recently, microbial communities in nuclear water environments such as SNFPs were mainly studied through cultivation-based approaches, for the isolation and characterization of individual microbes. These are effective to confirm viability and characterize specific biochemical properties of individual species, but do not cover most of the biodiversity inherent to these environments.

Despite the harsh conditions inherent to SNFPs, established by constant filtering and deionization as well as the residual radiation emanating from the fuel rods stored underwater combined with the presence of radionuclides, microorganisms have still been detected in these environments (Wolfram & Dirk 1997; Sarro *et al.* 2003; Chicote *et al.* 2004; Sarro *et al.* 2005; Chicote *et al.* 2005; Karley *et al.* 2018). Most of the identified microorganisms belonged to the bacterial kingdom, but some eukaryotic organisms such as fungi from the *Aspergillus* and *Ustilago* genera (Chicote *et al.* 2004; Silva *et al.* 2018) and micro-algae from the *Coccomyxa* and *Haematococcus* genera (Rivasseau *et al.* 2010; McGraw *et al.* 2018) have also been detected. Knowledge on archaea and viruses, including bacteriophages, is currently lacking.

The green micro-algae isolated from a spent nuclear fuel storage pool of the Institute Laue Langevin (ILL) research reactor in France was shown to withstand extremely high doses of ionizing radiation, up to 20 kGy, making it equivalent to the extremely radiation-resistant bacterial species *Deinococcus radiodurans* (Rivasseau *et al.* 2013). In addition, it was also shown to be capable of accumulating various radionuclides such as ^{238}U , ^{137}Cs , $^{110\text{m}}\text{Ag}$, ^{60}Co , ^{54}Mn , ^{65}Zn and ^{14}C . It proved to be as effective in the purification process of nuclear effluents as the conventionally used ion exchangers, and its capacity for bio-decontamination on a larger, real-life scale was also demonstrated (Rivasseau *et al.* 2010, 2013). The micro-alga was identified as *Coccomyxa actinabiotis*, an autotrophic freshwater microorganism from the Trebouxiophyceae class and Chlorophyta phylum (Fig. 5). Its main pigments are chlorophyll a and b, lutein, violaxanthin and β -carotene (Rivasseau *et al.* 2016).



Figure 5. A Scanning Electron Microscopy (SEM) image of a population of *C. actinabiotis*. Cells were grown in diluted BBM nutritive medium. Scale bar: 2 μm (Adapted from Rivasseau *et al.* 2016).

Bacteria were isolated from different SNFPs in planktonic form directly from the water by plating them on specific media and subsequently re-streaking them multiple times in order to obtain pure cultures (Bruhn *et al.* 1999; Galès *et al.* 2004; Chicote *et al.* 2005; Giacobone *et al.* 2011; Tisakova *et al.* 2013; Dekker *et al.* 2014; Pipiska *et al.* 2018; Karley *et al.* 2018, 2019). These bacteria included species from various genera such as *Bacillus*, *Burkholderia*, *Chryseobacterium*, *Comamonas*, *Corynebacterium*, *Curvibacter*, *Gluconobacter*, *Kocuria*, *Micrococcus*, *Ochrobactrum*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Serratia*, *Staphylococcus*, *Tardiphaga* and *Yersinia*, as well as several sulfate-reducing bacteria (SRB) (Table 2). One research group managed to isolate 21 different bacterial species from various SNFPs associated with the Cofrentes Nuclear Power Plant (NPP) in Spain belonging to several taxonomic groups (α -, β - and γ -Proteobacteria, Actinomycetales, *Flavobacterium*, and the *Bacillus/Staphylococcus* group) (Chicote *et al.* 2005).

The isolation of these bacterial strains allows for the characterization of various relevant biochemical properties, such as the bioaccumulation and/or resistance to radionuclides. Some studies have indeed identified bacterial species capable of accumulating ^{137}Cs , ^{60}Co and/or ^{54}Mn under laboratory conditions, either through biosorption on the surface or through active uptake in the cytoplasm (Tisakova *et al.* 2013; Pipiska *et al.* 2018). It was shown that living cells displayed a higher efficiency at removing radionuclides than dead biomass. Other bacterial strains closely related to the *Curvibacter*, *Serratia*, *Tardiphaga* and *Yersinia* genera were also shown to be highly resistant to $^{134}\text{Cs}^+$, $^{135}\text{Cs}^+$, $^{137}\text{Cs}^+$ and $^{60}\text{Co}^{2+}$, up to fourfold more than *Cupriavidus metallidurans* CH34 (Dekker *et al.* 2014). This could be of particular interest for their potential bioremediation capabilities of effluents contaminated with radionuclides.

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In addition, six morphologically different bacteria were isolated from a SNFP at the Atomic Energy Research Institute in Budapest, Hungary and were shown to absorb Cd, Co, Sr and Cr cations on their cell surface. They also proved to be slightly radioresistant, with D_{10} values ranging from 0.74 to 2.17 kGy (Diosi *et al.* 2003).

Other studies focused specifically on the radiation sensitivity of various isolated bacterial species, by exposing them to various doses of radiation (Bruhn *et al.* 1999; Karley *et al.* 2018; Foster, Muhamadali, *et al.* 2020). Some strains were shown to display a significant radio-tolerance (D_{10} values up to 2 kGy) when exposed to a variety of radiation doses ranging from 0.1 to 10 kGy using ^{60}Co (Karley *et al.* 2018). Another study investigated the radiation tolerance of *Pseudanabaena catenata*, a close relative of the major photosynthetic organism identified during a bloom event in a high pH SNFP at Sellafield, UK (Foster, Muhamadali, *et al.* 2020). It was subjected to X-ray irradiation for five consecutive days for a total dose of 95 Gy, thereby mimicking the conditions in the SNFP. This did not significantly affect its growth, as shown by turbidity measurements and cell counts. Metabolic variations during the post-irradiation recovery period were monitored through Fourier transform infrared spectroscopy (FT-IR), which revealed increased polysaccharide and decreased amide spectral intensities.

Another property that was tested in isolated strains was the capacity to grow autotrophically on H_2 (Galès *et al.* 2004). It is indeed known that H_2 can accumulate in SNFPs due to water radiolysis caused by the radiation continually emanating from the spent nuclear fuel rods stored underwater. One isolated *Ralstonia* and one *Burkholderia* strain were shown to efficiently grow in the presence of H_2 , O_2 and CO_2 , using oxygen as an electron acceptor and CO_2 as a carbon source.

It is also of interest to investigate the capacity of isolated bacteria to form biofilms on SNFP wall lining material, as biofilms are often observed on the walls and other metal surfaces. Some species isolated from an SNFP in India from the *Bacillus*, *Staphylococcus* and *Chryseobacterium* genera were capable of forming biofilms on stainless steel (SS-304L) as well as on glass under oligotrophic conditions and chronic radioactivity. The biofilms were also shown to be capable of cobalt and nickel uptake (Karley *et al.* 2018, 2019). Furthermore, *Bacillus cereus* RE 10 was found to be the predominant microorganism isolated from an SNFP in Argentina, among a total of 18 different bacterial species (Giacobone *et al.* 2011).

Aluminum coupons were exposed for 20 days to *Bacillus* cultures in highly diluted medium in order to simulate the environmental conditions. The coupons displayed biofilm formation as well as corrosion deposits, pitting and intermetallic inclusions (Fig. 6).

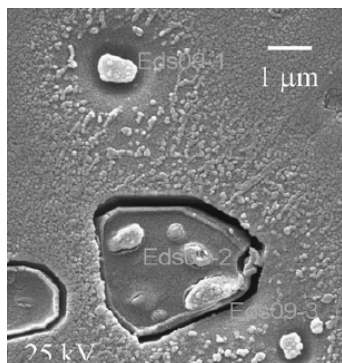


Figure 6. SEM-EDX image showing Al-Fe-Si and Al-Ti-Si inclusions on aluminum alloy after a 7-day exposure to *B. cereus* RE 10. Corrosion is visible around the intermetallic inclusions (Adapted from Giacobone *et al.* 2011).

In order to assess if microorganisms forming biofilms on various metal surfaces relevant to SNFP environments were able to survive radiation levels prevailing in these pools, this capacity was investigated on irradiated spent fuel cladding material within a hot cell environment (Bruhn *et al.* 2009). Twenty-two bacterial species were selected for this purpose, among which several isolated from SNFPs at the Idaho Nuclear Technology Center (INTEC), USA. They were introduced to test vessels containing irradiated cladding sections, which were then surrounded by radioactive source material. The total dose rate exceeded 2 Gy/h, with some bacteria receiving doses up to 5 kGy. Despite these conditions, biofilms were still observed.

Biofilms have also been isolated directly from the water and displayed a significant biodiversity, with bacteria belonging to a variety of genera (Table 2) (Sarro *et al.* 2003; Chicote *et al.* 2004; Sarro *et al.* 2005; Masurat *et al.* 2005; Sarro *et al.* 2007; Rivasseau *et al.* 2016). Microorganisms in extreme environments indeed tend to form biofilms as a survival strategy against unfavorable conditions (Costerton *et al.* 1995). These are composed of microorganisms attached to stable surfaces encased in a matrix of extracellular polymeric substance (EPS) containing polysaccharides, proteins and eDNA (extracellular DNA). In a mature biofilm, the EPS accounts for 50-90% of the total organic carbon in the structure (Christensen 1989). Biofilms are not homogeneously structured, contain pores, and may also include heavy metals and inorganic particles, as well as cellular components. Microorganisms in their planktonic form exhibit different physiological properties than those same organisms attached to a surface in biofilm form.

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The physico-chemical properties in the biofilm itself can radically differ from those in the free medium with regard to pH, oxygen concentration and the presence of organic and inorganic substances, creating microhabitats that can be exploited by a variety of different microorganisms.

Biofilms can also accelerate the rate of processes involved in biocorrosion, otherwise known as Microbiologically Influenced Corrosion (MIC) (Coetser & Cloete 2005). Corrosion processes such as MIC, environmentally assisted cracking, hydrogen-related mechanisms as well as galvanic and weld effects, may reduce the lifetime of nuclear fuel containers (King 2009). MIC is observed as corrosion underneath a biofilm, which is attached on the metal surface and could produce additional oxidants (Little & Wagner 1996). H⁺ ions produced by acid-producing bacteria could also enhance the corrosion process if it is not neutralized by a pH-buffering agent. Sulfide produced by Sulfate-Reducing Bacteria (SRB) could also promote the absorption of hydrogen on steel surfaces and thereby induce hydrogen-related degradation mechanisms.

Corrosion due to biofilms has been previously observed on aluminum alloys used as cladding for fuel rods coupled to stainless steel alloys commonly used in nuclear research reactors (Zhang *et al.* 1999). Pitting corrosion was observed under high as well as low bacterial densities, with pit depth being twice as high under high bacterial density. Another case of MIC was reported on copper cast iron canisters used as a model for spent nuclear fuel storage in a deep geological repository (KBS-3) in Sweden (Smart *et al.* 2014). Five miniaturized canisters were stored at a depth of 450 m at the Äspö Hard Rock Laboratory in Sweden and monitored for several parameters such as general corrosion, redox potential, pH, water chemistry, microbial numbers, diversity and activity. Five years of exposure led to rapid iron corrosion due to the extensive production of sulfide by SRB, as well as the formation of iron sulfide deposits on the exposed surfaces. Furthermore, the risk of MIC was also evaluated on aluminum alloy AA 6061, which is commonly used as a cladding material in nuclear fuel elements used in nuclear research reactors, and on pure aluminum (Al 99.99%) (Giacobone *et al.* 2011).

In order to investigate the potential risk of MIC on stainless steel and titanium surfaces, commonly used as an SNFP lining material, in storage racks and in other facilities, some studies analyzed the biofilm formation on metal coupons and balls submerged in SNFPs.

This was also done at the Cofrentes NPP in Spain for various periods of time (Sarro *et al.* 2003; Sarro *et al.* 2005; Sarro *et al.* 2007). The presence of biofilms on these coupons was investigated through epifluorescence and scanning electron microscopy, which revealed extensive colonization of the metal surfaces, despite the extremely oligotrophic conditions prevailing in these pools combined with the ambient radiation (Fig. 7). Different bacteria were isolated from these biofilms and subsequently identified through 16S rRNA Sanger sequencing. In one study, as much as 57 different bacterial species were identified, which belonged to the α -, β -, γ -Proteobacteria, Bacilli, Firmicutes and Actinobacteria phylogenetic groups (Sarro *et al.* 2005). The biofilms were also shown to accumulate radionuclides such as ^{60}Co , ^{65}Zn , ^{54}Mn , ^{58}Co and ^{95}Zr , which could be of potential use in bioremediation applications.

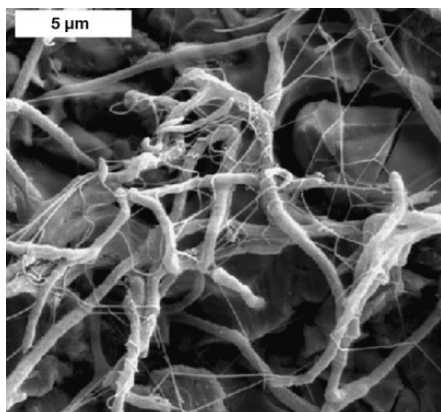


Figure 7. SEM image of biofilm formation on a stainless steel coupon immersed in the SNFP for 134 days. The biofilm is dominated by filamentous microbes (Adapted from Sarro *et al.* 2003).

Several microbial groups relevant to the development of biocorrosion such as SRB and acid-producing bacteria were detected in an SNFP at the Savannah River Site (SRS), USA. In order to investigate the biofilm forming capability of the microorganisms dwelling in this pool, metal coupons made out of stainless steel and aluminum alloys were used (Santo Domingo *et al.* 1998). Scanning electron microscopy and X-ray spectra analysis revealed that the coupons were fully covered by biofilms within 12 months, and some deterioration of the metal surface was also made evident.

Another study investigating the effect of biofilms on metal coupons found evidence of actual corrosion, with the formation of biocorrosion-induced pits of 100 nm depth detected on stainless steel coupons through atomic force microscopy (Diosi *et al.* 2003).

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2.1.2 Microbial profiles obtained by sequencing approaches

It is known that only a small percentage of environmental microorganisms can be cultivated under laboratory conditions (Whitman *et al.* 1998). Since most studies investigating the microbial communities in SNFPs focused on a cultivation-based approach, an important part of the diversity in these environments has not been studied. Indeed, it is known that perhaps only 0.1 to 10% of the microorganisms dwelling in water are culturable (Stokell & Steck 2012). Therefore, the use of sequencing techniques such as 16S rRNA amplicon sequencing, metaproteomics and metagenomics, have been recently implemented, which allowed to further study the taxonomic and functional characteristics of the entire microbial community.

In one SNFP located at the Savannah River Site (SRS), USA, a detailed characterization of the microbial community using 16S rRNA amplicon sequencing (454 Pyrosequencing) approach was performed following the proliferation of a white flocculent (Fig. 8) (Bagwell *et al.* 2018). A rich bacterial diversity associated with these precipitants was uncovered, with an estimated 4,000 OTUs/amplicon library, mainly belonging to the *Burkholderiaceae*, *Nitrospiraceae*, *Hyphomicrobiaceae* and *Comamonadaceae* bacterial families.

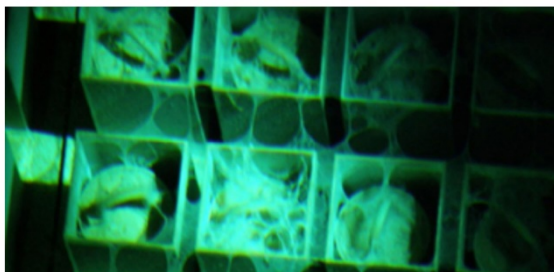


Figure 8. Underwater view of spent nuclear fuel canisters in a storage rack. Flocculent precipitates can be observed on top of and in between the storage racks (Adapted from Bagwell *et al.* 2018).

A small number of studies also used both 16S and 18S rRNA amplicon sequencing for the characterization of the microbial communities dwelling in several SNFPs located at Sellafield, UK. In one study, an outdoor SNFP that is subjected to seasonal algal blooms, causing plant downtime, was investigated. Sequencing of 18S rRNA amplicons revealed the dominant presence of a close relative of the alga *Haematococcus pluvialis*, whereas 16S rRNA amplicon sequencing uncovered a wide variety of freshwater bacteria, such as *Proteobacteria* and *Cyanobacteria* (McGraw *et al.* 2018).

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Additionally, FT-IR spectroscopy indicated that an environmentally relevant dose of ionizing radiation (80 Gy) spread over multiple days did not have a significant effect on the metabolic profile of *H. pluvialis*. Irradiated cultures did produce a significant amount of astaxanthin pigment, which protects the cells from radiation damage.

A similar study focused on the microbial characterization of a high pH (11.4) SNFP, the First Generation Magnox Storage Pond (FGMSP), which also undergoes blooms that can significantly reduce the visibility in the water (Fig. 9). The data obtained from 16S rRNA amplicon sequencing suggested that a single cyanobacterial genus was predominant during the bloom events, namely *Pseudanabaena* (Foster *et al.* 2020). An adjacent SNFP that is allowed to periodically overflow into the main pond displayed a different community profile. Blooms could be controlled by re-establishing a purging regime at a higher rate, which was performed by dosing the pools with NaOH to maintain the high pH.

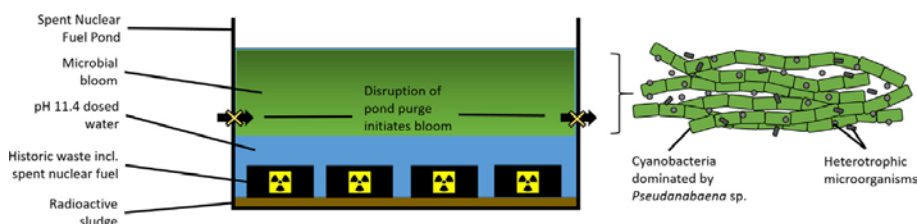


Figure 9. Microbial bloom events at the FGMSP storage pond at Sellafield, UK. The pond is dosed with NaOH to maintain a pH of 11.4. The main genus prevailing during the bloom events was *Pseudanabaena* sp. (Adapted from Foster *et al.* 2020).

A third study conducted at a high pH indoor SNFP at Sellafield (INP) found that the pond was dominated by prokaryotes, as only 16S rRNA gene fragments were successfully amplified for sequencing (Illumina MiSeq). Sequencing revealed that 91% of the OTUs identified in the water samples belonged to the phylum of the *Proteobacteria*, mainly from the α and β subgroups (Ruiz-Lopez *et al.* 2020). The most abundant genus overall was *Hydrogenophaga*, which suggests the utilization of hydrogen as an energy source. As indicated earlier, this is most likely linked with the phenomenon of water radiolysis due to the radiation emanating from the stored fuel. Culture-dependent techniques were also attempted, but yielded only relatively minor results, emphasizing the importance of DNA-based approaches for the assessment of microbial communities in nuclear facilities.

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A single study focused on the characterization of the microbiome in an SNFP and the Fuel Transfer Channel (FTC) of a nuclear power plant in Brazil, using a shotgun metagenomics sequencing approach. Biofilms were collected from the walls of these facilities, which revealed the uncommon presence of fungi (both Basidiomycota and Ascomycota) as the main organisms dwelling in these environments (Silva *et al.* 2018). Both facilities displayed low microbial diversities, with Ustilaginomycetes (Basidiomycota) being the main contributors to the communities. Nevertheless, *Proteobacteria*, *Actinobacteria* and *Firmicutes* were detected in small proportions.

Finally, the microbiome of a water pool directly cooling the nuclear reactor core at the Osiris reactor in France was investigated via 16S rRNA gene amplicon sequencing (Petit *et al.* 2020). This was the first study of its kind, as access restrictions and other constraints usually prevent the investigation of these types of environments. Sequencing of the 16S rRNA amplicons was combined with proteotyping to uncover the bacterial diversity in this extremely harsh environment that combines high levels of radioactivity and radionuclides with low nutrient concentrations. Twenty-five genera were identified, with the prevailing ones during reactor operation being *Variovorax* and *Sphingomonas*. These were replaced by *Methylobacterium*, *Asanoa* and *Streptomyces* species during the shutdown. *Variovorax* might also use hydrogen produced by water radiolysis as an energy source.

Table 2. Microorganisms identified in radioactive water environments in nuclear facilities.

Site	Microorganism	Sample origin Identification method	Reference
SNFP at Savannah River Site (SRS), USA	Sulfate-reducing bacteria (SRB) and acid-producing bacteria	Water samples and biofilms on metal coupons Cell counts, SEM of metal coupons	Santo Domingo <i>et al.</i> 1998
Two SNFPs at the Idaho Nuclear Technology Center (INTEC), USA	<i>Comamonas</i> , <i>Corynebacterium</i> , <i>Gluconobacter</i> , <i>Micrococcus</i> , <i>Pseudomonas</i> , <i>Rhodococcus</i> , two SRB strains	Water samples Cultivation, 16S rRNA Sanger sequencing	Bruhn <i>et al.</i> 1999
SNFP at the Atomic Energy Research Institute,	Six morphologically different bacteria, three aerobic and three anaerobic	Water samples No identification	Diosi <i>et al.</i> 2003

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Budapest, Hungary			
SNFPs at the Cofrentes Nuclear Power Plant in Valencia, Spain	<i>Bacillus</i> , <i>Brachybacterium</i> , <i>Bradyrhizobium</i> , <i>Burkholderia</i> , <i>Cellulomonas</i> , <i>Mesorhizobium</i> , <i>Methylobacterium</i> , <i>Microbacterium</i> , <i>Mycobacterium</i> , <i>Nocardia</i> , <i>Pseudomonas</i> , <i>Ralstonia</i> , <i>Staphylococcus</i> , <i>Strenotrophomonas</i> , <i>Variovorax</i> , <i>Xylophilus</i>	Biofilms on metal coupons and balls Cultivation, 16S rRNA Sanger sequencing	Sarró <i>et al.</i> 2003, 2005, 2007
SNFP at the Cofrentes Nuclear Power Plant in Valencia, Spain	<i>Aspergillus</i> (fungus), <i>Bacillus</i> , <i>Gordonia</i> , <i>Nocardia</i> , <i>Ralstonia</i> , <i>Staphylococcus</i>	Biofilms attached to the SNFP wall Cultivation, 16S rRNA Sanger sequencing	Chicote <i>et al.</i> 2004
SNFP in France	<i>Acinetobacter</i> , <i>Bacillus</i> , <i>Burkholderia</i> , <i>Delftia</i> , <i>Micrococcus</i> , <i>Pseudomonas</i> , <i>Ralstonia</i> , <i>Staphylococcus</i>	Water samples Cultivation, 16S rRNA Sanger sequencing	Galès <i>et al.</i> 2004
Two SNFPs at the Cofrentes Nuclear Power Plant in Valencia, Spain	<i>Afipia</i> , <i>Bradyrhizobium</i> , <i>Burkholderia</i> , <i>Chryseobacterium</i> , <i>Methylobacterium</i> , <i>Microbacterium</i> , <i>Nocardia</i> , <i>Pseudomonas</i> , <i>Ralstonia</i> , <i>Rhizobium</i> , <i>Sphingomonas</i> , <i>Staphylococcus</i> , <i>Strenotrophomonas</i> , <i>Streptococcus</i>	Water samples Cultivation, 16S rRNA Sanger sequencing	Chicote <i>et al.</i> 2005
SNFP (CLAB) in Sweden	<i>Meiothermus</i>	Biofilms in SNFP circulation systems Cultivation,	Masurat <i>et al.</i> 2005

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		16S rRNA Sanger sequencing	
Two SNFPs at the Idaho Nuclear Technology Center (INTEC), USA	<i>Aureobacterium, Micrococcus, Pseudomonas, Pseudonocardia, Rhizobium, Rhodococcus, Sphingomonas, Taxeobacter,</i>	Water samples Cultivation, 16S rRNA Sanger sequencing	Bruhn <i>et al.</i> 2009
SNFP in Argentina	<i>Acinetobacter, Aminobacter, Arthrobacter, Bacillus, Bosea, Leifsonia, Mesorhizobium, Micrococcus, Pseudomonas, Rhizobium, Rhodococcus, Sinorhizobium</i>	Water samples Cultivation, 16S rRNA Sanger sequencing	Giacobone <i>et al.</i> 2011
SNFP (JAVYS Inc.) in Slovak Republic	<i>Kocuria, Micrococcus, Ochrobactrum, Pseudomonas</i>	Water samples Cultivation, 16S rRNA Sanger sequencing	Tisakova <i>et al.</i> 2013
Outdoor SNFP at Sellafield, UK	<i>Curvibacter, Serratia, Tardiphaga, Yersinia</i>	Water samples Cultivation, 16S rRNA Sanger sequencing	Dekker <i>et al.</i> 2014
SNFP at the Institut Laue Langevin (ILL) research reactor in France	<i>Coccomyxa actinabiotis</i>	Biofilm on a spotlight SEM, Cultivation, 18S rRNA Sanger sequencing	Rivasseau <i>et al.</i> 2016
SNFP at Madras Atomic Power Station (MAPS) in India	Six aerobic bacterial isolates, three Gram-negative, three Gram-positive	Water samples Gram staining and fluorescence microscopy	Karley <i>et al.</i> 2018
SNFP (JAVYS Inc.) in Slovak Republic	<i>Kocuria palustris, Micrococcus luteus</i>	Water samples Cultivation, 16S rRNA Sanger sequencing	Pipiska <i>et al.</i> 2018

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Outdoor SNFP at Sellafield, UK	<i>Haematococcus pluvialis</i> (dominant green alga), <i>Flectobacillus</i> , <i>Flavobacterium</i> , <i>Chitinophaga</i> , <i>Sediminibacterium</i> , <i>Limnothrix</i> , <i>Phormidium</i> , <i>Polynucleobacter</i> , <i>Bosea</i> , <i>Herbaspirillum</i> , <i>Sphingomonas</i> , <i>Methylotheria</i> , <i>Chlorella</i> , <i>Micratinium</i> , <i>Actinastrium</i> , <i>Penicillium</i> ,...	Water samples 16S rRNA Sanger sequencing, 16S and 18S rRNA amplicon sequencing	McGraw <i>et al.</i> 2018
SNFP at Savannah River Site (SRS), USA	<i>Bradyrhizobium</i> , <i>Burkholderia</i> , <i>Curvibacter</i> , <i>Gemmata</i> , <i>Hyphomicrobium</i> , <i>Ideonella</i> , <i>Meiothermus</i> , <i>Methylobacterium</i> , <i>Mycobacterium</i> , <i>Nitrospira</i> , <i>Pedomicrobium</i> , <i>Pelomonas</i> , <i>Sideroxydans</i>	Water samples 16S rRNA amplicon sequencing	Bagwell <i>et al.</i> 2018
SNFP at the Angra 1 Nuclear Power Plant in Brazil	<i>Ustilago</i> (dominant fungus) <i>Acidovorax</i> , <i>Chelativorans</i> , <i>Cryptococcus</i> , <i>Erythrobacter</i> , <i>Gibberella</i> , <i>Malassezia</i> , <i>Mesorhizobium</i> , <i>Neurospora</i> , <i>Novosphingobium</i> , <i>Oceanicaulis</i> , <i>Pseudomonas</i> , <i>Sphingobium</i> , <i>Sphingomonas</i> , <i>Sphingopyxis</i> , <i>Schizosaccharomyces</i> , <i>Tilletia</i>	Biofilms on the SNFP walls Shotgun metagenomics sequencing	Silva <i>et al.</i> 2018
SNFP at Madras	<i>Bacillus</i> , <i>Chryseobacterium</i> ,	Water samples	Karley <i>et al.</i> 2019

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Atomic Power Station (MAPS) in India	<i>Staphylococcus</i>	Cultivation, 16S rRNA Sanger sequencing	
High-pH SNFP (FGMSP) and auxiliary pond at Sellafield, UK	<i>Pseudanabaena</i> (dominant cyanobacterium), <i>Mongoliitalea</i> , <i>Rhodobacter</i> , <i>Hydrogenophaga</i> , <i>Porphyrobacter</i> , <i>Roseococcus</i> ,...	Water samples 16S rRNA amplicon sequencing	Foster <i>et al.</i> 2020
High-pH indoor SNFP and purge water feeding tank (FT) at Sellafield, UK	<i>Hydrogenophaga</i> (dominant), <i>Curvibacter</i> , <i>Flavobacterium</i> , <i>Methylobacter</i> , <i>Methylophilus</i> , <i>Mongoliitalea</i> , <i>Novosphingobium</i> , <i>Polaromonas</i> , <i>Roseococcus</i> , <i>Sediminibacterium</i> , <i>Silanimonas</i> , <i>Sphingomonas</i> , <i>Unidibacterium</i> ,...	Water samples 16S rRNA amplicon sequencing	Ruiz-Lopez <i>et al.</i> 2020
Cooling pool of the Osiris nuclear reactor at CEA Saclay in France	<i>Asanoa</i> , <i>Methylobacterium</i> , <i>Pelomonas</i> , <i>Sphingomonas</i> , <i>Streptomyces</i> , <i>Variovorax</i> ,...	Water samples 16S rRNA amplicon sequencing, proteotyping	Petit <i>et al.</i> 2020

2.2 Microorganisms in non-radioactive nuclear waters

Next to the previously discussed watery environments in nuclear reactors exposed to varying doses of ionizing radiation, other environments exist in these facilities that are not subjected to radiation, as they never come into close contact with nuclear fuel. These include water bodies such as the secondary cooling circuit and the various purification circuits, reservoirs containing make-up water used to refill other systems and pools. The absence of radiation in these environments represents an important condition, as this removes a crucial environmental stressor which can heavily impact the microbial communities dwelling in these systems.

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Nevertheless, the water is still very oligotrophic, as it needs to comply with the strict standards imposed on these nuclear environments. These waters are therefore maintained at relatively high purity levels and low conductivities through constant filtering and deionization via ion exchangers.

A number of studies investigated the microbial communities in different cooling and purification systems within nuclear facilities displaying varying physicochemical properties (Rao *et al.* 2000, 2009; Balamurugan *et al.* 2011; Kéki *et al.* 2013; Props *et al.* 2016, 2019). Conductivities ranged between < 1 and 472 $\mu\text{S}/\text{cm}$, pH between 4.5 and 8.45 and temperatures between 15 and 50°C. Microorganisms were identified using both culture-dependent and culture-independent sequencing techniques (16S rRNA amplicon and shotgun metagenomics sequencing). Some studies focused on the phenomenon of microbiologically influenced corrosion (MIC) sporadically occurring in these systems (Rao *et al.* 2000, 2009; Balamurugan *et al.* 2011). Microbes can indeed accumulate in the pipes and on the surfaces of various circuits and reservoirs through biofilm formation.

Bacteria isolated from the cooling water system and thermo-fluid heat exchanger (TFHX) of the Fast Breeder Test Reactor (FBTR) located at Kalpakkam in India included various culturable aerobic heterotrophic bacteria (CAHB), iron-oxidizing bacteria (IOB) such as *Leptothrix*, iron reducing bacteria (IRB) such as the exopolymer producing *Pseudomonas aeruginosa* and several sulfate-reducing bacteria (SRB) such as *Desulfovibrio*, *Desulfomicrobium* and *Desulfococcus* (Table 3). These bacteria were identified in planktonic form as well as in biofilms formed on carbon steel coupons exposed online to the water in these systems. Scanning electron microscopy (SEM) revealed the presence of filamentous iron bacteria as well as evidence of corrosion in the form of pitting. A particular emphasis was put on SRB, as those have an important role in the development of MIC (Balamurugan *et al.* 2011). Bacteria identified in the TFHX were found to be thermo-tolerant, able to survive and grow under temperatures ranging between 45 and 50°C (Rao *et al.* 2009).

Bacteria inhabiting ultrapure water systems in nuclear facilities are very difficult to cultivate under laboratory conditions, as commonly used media are typically too selective. Therefore, one research group developed a range of customized media attempting to reproduce the oligotrophic conditions of the water purification system of a nuclear power plant in Hungary (Kéki *et al.* 2013).

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A total of 122 bacterial strains were isolated from the specialized media, out of which 27 were selected for 16S rRNA Sanger sequencing. The type of medium strongly affected the composition of the culturable bacteria, with a larger proportion of α -Proteobacteria such as *Ancylobacter*, *Mesorhizobium* and *Methylobacterium* and Actinobacteria such as *Leifsonia*, *Microbacterium* and *Mycobacterium* showing up on these customized media than on traditional media. Moreover, two novel bacteria taxa were also identified.

In another study, the bacterial community inhabiting the secondary cooling water system of the BR2 reactor in Belgium was investigated through 16S rRNA amplicon sequencing combined with flow cytometry, as the latter can provide faster assessments of microbial diversity dynamics using phenotypic fingerprints, as well as an accurate assessment of the concentration of bacterial cells present in the water (Fig. 10). It was demonstrated that a limited number of phenotypic features such as morphology and nucleic acid content can already provide sufficient information for the assessment of microbial dynamics at the species level (Props *et al.* 2016).

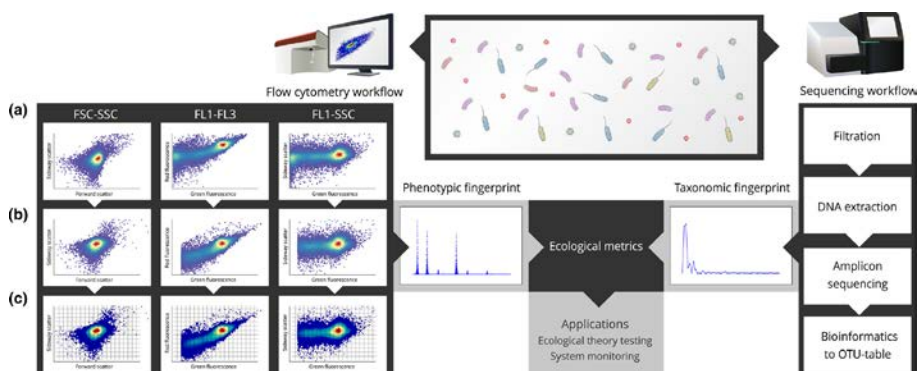


Figure 10. Overview of the microbial community analysis approach combining flow cytometry (left) and 16S rRNA amplicon sequencing (right). Phenotypic and taxonomic fingerprints are combined to generate relevant ecological metrics such as alpha diversity (Adapted from Props *et al.* 2016).

A follow-up study identified a novel *Ramlibacter* species as the dominant taxon (proposed name *R. aquaticus*), via shotgun metagenomics sequencing (Props *et al.* 2019). Genes with a role in phosphorus and carbon scavenging pathways were positively selected, and the accessory genome displayed a significant enrichment in environmental sensing pathways such as chemotaxis and motility, suggesting environmental adaptations through gene expansion. Cultivation of *R. aquaticus* revealed optimal growth at low nutrient concentrations and significant cell size plasticity.

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Finally, the water purification system of a nuclear power plant in Hungary was investigated in order to find adequate chemical treatment to remove microbial contaminants (Kéki *et al.* 2019). The mixed-bed ion-exchange resin containing unit proved to be the most contaminated site (Fig. 11), and was treated using Kathon WT in order to restore the water production capacity.

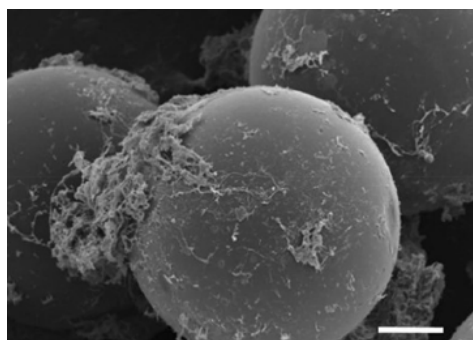


Figure 11. SEM image of mixed-bed ion exchange resin beads covered with biofilm. Scale bar = 100 μm (Adapted from Kéki *et al.* 2019).

Table 3. Microorganisms identified in various non-radioactive water environments in nuclear facilities.

Site	Microorganism	Sample origin Identification method	Reference
Cooling circuit of the Fast Breeder Test Reactor (FBTR) in India	<i>Leptothrix</i> , <i>Pseudomonas</i> , <i>Desulfovibrio</i> , Iron oxidizing bacteria (IOB) Sulfate-reducing bacteria (SRB) Culturable aerobic heterotrophic bacteria (CAHB)	Water samples and biofilms on carbon steel coupons SEM, biochemical and morphological characteristics	Rao <i>et al.</i> 2000
Thermo-Fluid Heat Exchanger (TFHX) and cooling water system of the Fast Breeder Test Reactor (FBTR) in India	<i>Pseudomonas</i> , <i>Desulfovibrio</i> , Iron reducing bacteria (IRB), IOB, SRB, Heterotrophic bacteria	Water samples and biofilms on carbon steel coupons SEM, biochemical and morphological characteristics	Rao <i>et al.</i> 2009
Cooling water system of the Fast Breeder Test Reactor (FBTR) in India	IRB, IOB, SRB: <i>Desulfotomaculum</i> , <i>Desulfonema</i> , <i>Desulfosarcina</i> , <i>Desulfococcus</i> ,	Water samples and biofilms on carbon steel coupons Biochemical and morphological	Balamurugan <i>et al.</i> 2011

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	<i>Desulfovibrio</i> , <i>Desulfomicrobium</i>	characteristics, SRB-specific PCR	
Water purification system of a nuclear power plant in Hungary	<i>Ancylobacter</i> , <i>Bosea</i> , <i>Burkholderia</i> , <i>Kaistia</i> , <i>Labrys</i> , <i>Leifsonia</i> , <i>Mesorhizobium</i> , <i>Methylobacterium</i> , <i>Microbacterium</i> , <i>Mycobacterium</i> , <i>Paenibacillus</i> , <i>Pelomonas</i> , <i>Rhizobium</i> , <i>Sphingomonas</i>	Water samples Cultivation, 16S rRNA Sanger sequencing	Kéki <i>et al.</i> 2013
Cooling water system of the Belgian Reactor 2 (BR2) nuclear research reactor in Belgium	Acidobacteria subdivision 3, Chitinophagaceae, Comamonadaceae, Sphingomonadaceae	Water samples 16S rRNA amplicon sequencing combined with flow cytometry	Props <i>et al.</i> 2016
Cooling water system of the Belgian Reactor 2 (BR2) nuclear research reactor in Belgium	<i>Ramlibacter</i> (dominant), Two <i>Bacteroides</i> species	Water samples Cultivation, Shotgun metagenomics sequencing	Props <i>et al.</i> 2019
Water purification system of a nuclear power plant in Hungary	<i>Ancylobacter</i> , <i>Bacillus</i> , <i>Bradyrhizobium</i> , <i>Brevibacterium</i> , <i>Burkholderia</i> , <i>Labrys</i> , <i>Leifsonia</i> , <i>Mesorhizobium</i> , <i>Methylobacterium</i> , <i>Microbacterium</i> , <i>Micrococcus</i> , <i>Mycobacterium</i> , <i>Paenibacillus</i> , <i>Pelomonas</i> , <i>Porphyrobacter</i> , <i>Ralstonia</i> , <i>Rhizobium</i> , <i>Rhodococcus</i> , <i>Sphingomonas</i>	Water samples and biofilms on ion exchange resins Cultivation, 16S rRNA Sanger sequencing	Kéki <i>et al.</i> 2019

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2.3 Microbes in non-nuclear ultrapure water systems

Ultrapure waters are utilized in a variety of different industries, such as the semi-conductor and pharmaceutical industry, as well as in hospitals. These waters may be prepared through multiple methods, such as distillation, ion exchange and reverse osmosis (RO). RO membranes provide a significant surface area for microorganisms to attach themselves onto, which can lead to biofouling (Flemming *et al.* 1996; Roth *et al.* 1998; Ridgway *et al.* 1999). Allowed contaminant levels for purified water used in the semiconductor and pharmaceutical industry are shown in Table 4 (SEMI International Standard 2013; USP 2014).

Table 4. Ultrapure water requirements for the semiconductor and pharmaceutical industry (Adapted from Mittelman & Jones 2018).

Analyte	Max. value (semiconductor industry)	Max. value (pharmaceutical Industry)
Conductivity ($\mu\text{S}/\text{cm}$)	0.05	1.3
TOC ($\mu\text{g}/\text{L}$)	2	500
Bacteria (aerobic heterotrophs), CFU/100 mL	0.1 (<1/L)	10
Endotoxin (EU/mL)	No specification	0.25

Diverse bacterial populations are observed in potable waters and other similar water systems, treated or untreated. Next to bacteria, some fungi have also been detected in potable waters (Arvanitidou *et al.* 1999; Cabral & Pinto 2002; Al-Gabr *et al.* 2014), which generally display higher levels of bio-available organic carbon (> 1 mg/L). Microorganisms observed in various purified water systems associated with the International Space Station (ISS) were similar to the ones detected in pharmaceutical and other industrial waters (Novikova *et al.* 2006).

The majority of the bacteria isolated from various ultrapure water systems are Gram-negative, aerobic heterotrophs, as is the case in most natural aquatic ecosystems (Mittelman & Jones 2018). The cell envelope of Gram-negative bacteria contains two phospholipid membranes. Contrary to the cytoplasmic membrane, the external membrane contains lipopolysaccharides (LPS) as outside layers.

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This may offer individual cells additional protection from the extremely hypotonic character inherent to ultrapure water systems. As these bacteria are mainly heterotrophic, they require reduced organic compounds (e.g. sugars, organic acids) as their energy source. These elements represent the main limiting growth factors in these nutrient-poor environments. Indeed, planktonic bacterial densities and concentrations of bio-available organic carbon generally display a positive correlation in these waters.

Different phylogenetic groups were identified from purified waters used in a variety of industries and even space-related environments, such as the Gram-negative *Afipia*, *Bradyrhizobium*, *Burkholderia*, *Chelatococcus*, *Cupriavidus*, *Mesorhizobium* and *Ralstonia*, and the Gram-positive *Brevibacillus*, *Microbacterium*, *Mycobacterium* and *Staphylococcus*, (Bohus *et al.* 2010, 2011; Mijndendonckx *et al.* 2013; Minogue *et al.* 2013).

2.3.1 Semiconductor industry

Ultrapure waters used in the semiconductor industry often contain extremely low concentrations of organic and inorganic solutes, under the detection limit of currently used measuring instruments (Table 4). Still, some oligotrophic bacteria were successfully isolated from these extreme environments and were shown to be able to survive on a much larger diversity of organic substrates than other bacteria originating from the same system (Kim *et al.* 1997). A single study investigated the bacterial populations in six different ultrapure water systems from different geographic locations, out of which three were associated with the semiconductor industry, and found a common core consisting of different genera such as *Ralstonia*, *Bradyrhizobium*, *Pseudomonas* and *Stenotrophomonas* (Kulakov *et al.* 2002). Other genera such as *Flavobacterium*, *Mycobacterium*, *Burkholderia*, *Bacillus* and *Sphingomonas* were also detected.

2.3.2 Pharmaceutical industry

Maintaining high water purity is of crucial importance in the pharmaceutical industry, as bacterial contamination and endotoxins can lead to non-sterile products and potential health hazards. The majority of FDA product recalls are associated with specific bacterial groups, such as *Burkholderia cepacia*, *Pseudomonas* spp. or *Ralstonia pickettii*, all commonly isolated from pharmaceutical-grade ultrapure water systems, such as water for injection (Jimenez 2007). The most frequently detected bacterium associated with these recalls was *B. cepacia*, a common isolate in these waters, together with some *Methylobacterium* species (Kressel & Kidd 2001; Kawai *et al.* 2004).

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The majority of the bacteria identified in pharmaceutical waters are found as biofilms attached to various surfaces. Desorption of the bacterial aggregates gives rise to the majority of planktonic bacteria inhabiting these systems (Mittelman 1998).

Ultrapure waters for the pharmaceutical industry are often produced by microporous membrane filtration, with pore sizes ranging from 0.1 to 0.45 μm . However, bacteria can often drastically reduce their cell volume under oligotrophic conditions and develop into so-called “ultramicrobacteria” (Mittelman & Jones 2018). As such, some bacteria have been described as being capable of passing through 0.45, 0.2 and even 0.1 μm pore-sized filter membranes (Tabor *et al.* 1981; Howard & Duberstein 1986; Christian & Meltzer 1986). However, this issue is still somewhat controversial.

2.3.3 Hospital and healthcare environments

Ultrapure waters are used in a variety of different applications in hospital and healthcare settings, such as water used for dialysis and in research laboratories (Mittelman 1991). Some bacteria were isolated from dialysis water, such as *R. pickettii* and *R. insidiosa*, which were able to utilize trace concentrations of Polyvinyl Chloride (PVC) compounds such as phthalates as carbon and energy sources (Dombrowsky *et al.* 2013). Other bacterial strains isolated from these waters include *P. aeruginosa*, *B. cepacia*, *Acinetobacter* spp. and *Mycobacterium* spp. (Gomila *et al.* 2005, 2006), which can also be found in potable waters (Brown-Elliott *et al.* 2011). These waterborne, Gram-negative bacteria can produce endotoxins, which can potentially cause pyrogenic responses in patients receiving dialysis treatments (Mangram *et al.* 1998; Roth & Jarvis 2000). The *Methylobacterium* genus has in turn been associated with nosocomial infections in healthcare environments as well as in hospital water (Gomila *et al.* 2006; Kovaleva *et al.* 2014). Microorganisms in these environments often form biofilms, which provide a protective barrier against microbial control agents, but can also produce bacterial endotoxins (Cappelli *et al.* 2003; Smeets *et al.* 2003).

2.4 Survival mechanisms of microbes in ultrapure waters

The mechanisms whereby microorganisms manage to survive in man-made ultrapure water systems are the same as the ones utilized in natural oligotrophic environments.

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These are defined by containing very low concentrations of dissolved organic carbon (DOC), with levels below 0.5 mg/L (Amy & Morita 1983; Amy *et al.* 1983; Cavicchioli *et al.* 2003), or 0.1 to 50 nM of low molecular weight (LMW) organic molecules such as amino acids and sugars (Fuhrman 1987; Williams 2000). This DOC is often only partly bioavailable (Barber 1968). Oligotrophic environments are prevalent everywhere on earth, be they freshwater, marine, or terrestrial, with up to one third of all the oceans being considered oligotrophic (Cavicchioli *et al.* 2003). With regard to the entire biosphere consisting of all different ecosystems, half of it is classified as oligotrophic (Morita 1997). Nevertheless, microbial concentrations and taxonomic diversities in oligotrophic aquatic environments can be high, with densities ranging up to 10^5 to 10^6 CFU/ml (Cavicchioli *et al.* 2003; Hobbie & Hobbie 2013) and relatively low growth rates of approximately 0.01 generation per day (i.e. generation time, *g*, of *ca.* 100 days) (Crump *et al.* 2013).

In order to mediate the nutritional constraints prevailing in oligotrophic environments, microorganisms have developed certain starvation-survival strategies, such as nutritional versatility, changes to cell morphology, the adoption of the viable but non-culturable state (VBNC), the formation of biofilms, as well as spores and cysts. These adaptations can be triggered by environmental stress factors including low organic carbon concentrations, high or low temperatures, extreme salinity, low nutrient availability and variable light conditions (Mittelman & Jones 2018). These strategies are implemented by microorganisms in order to efficiently reduce respiration rates as well as cell densities. For pathogenic microorganisms, this still enables individual cells to remain pathogenic. This is of crucial importance for ultrapure waters used in the pharmaceutical industry, for example.

Some bacteria unable to form spores have been observed to maintain a VBNC state for over seven years in oligotrophic marine environments (Lleò *et al.* 2005). Other spore-forming bacteria were successfully isolated from dried milk over 90 years old (Ronimus *et al.* 2006) and demonstrated to be viable, while others still were shown to be capable of surviving in space for more than six years (Horneck *et al.* 1994). Some spores have even been recovered and revived from amber aged between 25 and 40 million years (Cano & Borucki 1995), although these data should be subjected to extreme caution.

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2.4.1 Nutritional versatility

The ability to utilize multiple sources of energy and carbon, known as mixotrophy, is a widespread mechanism used by microorganisms to cope with the nutritional restraints in oligotrophic environments, as it provides them with greater nutritional flexibility. Mixotrophic organisms can adopt a variety of combinations of heterotrophy, (litho)autotrophy, phagotrophy and/or phototrophy, in order to provide for their required energy. Oligotrophic environments mainly favor mixotrophic microorganisms capable of nutritional versatility (Mittelman & Jones 2018). Mixotrophy confers a significant evolutionary advantage under oligotrophic conditions when low levels of DOC are available, but might conversely become a disadvantage in eutrophic environments when DOC levels are more abundant, as maintaining multiple trophic systems within one organism represents a metabolic cost. The golden brown alga *Ochromonas* is one example of a mixotrophic organism capable of utilizing a variety of carbon sources such as anthropogenic organic carbons as well as phagocytizing other microorganisms and performing photosynthesis to a limited extent (Aaronson 1973). Different *Euglena* species are also able to obtain their energy through phototrophy as well as heterotrophy (Hines *et al.* 1997). Other examples include sulfur bacteria from the *Thiobacillus* genus, which are capable of both autotrophic assimilation of CO₂ as well as heterotrophic assimilation of DOC. In addition, some marine bacteria can use light-driven proton pumps able to produce ATP, known as proteorhodopsins, embedded in their membranes. Bacteria with this particular capability were shown to survive for longer periods of time in oligotrophic marine environments (Gómez-Consarnau *et al.* 2010). In addition, microorganisms with high specific substrate affinities (low Michaelis-Menten constant, K_m) enabling them to rapidly absorb nutrients at (very) low concentrations have a competitive advantage over other microbes with lower substrate affinities under oligotrophic conditions (Hirsch *et al.* 1978; Button 1991).

2.4.2 Viable but Non-Culturable state (VBNC)

Microbial cells that are subjected to oligotrophic conditions are able to drastically alter their cellular metabolic rates, thereby entering the VBNC state. This “dormancy” state, characterized by the maintenance of cell viability but simultaneous loss of bacterial culturability, was described for the first time by Xu *et al.* in 1982. The VBNC state was subsequently defined as “a state in which viable bacteria do not divide sufficiently on non-selective media to identify visible growth” (McDougald *et al.* 2006).

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Microorganisms in the VBNC state display a low respiration rate as well as low energy requirements and slower growth rates. Microbes that adopted these mechanisms are better adapted to the low nutrient availability in oligotrophic environments than their faster growing counterparts (Moyer & Morita 1989).

The VBNC state is typically attained through three different stages, and the duration of each stage varies according to the species (Moyer & Morita 1989). First comes a transitional stage in which the concentrations of proteins, DNA and RNA rapidly decrease within the cell (Amy *et al.* 1983, 1993). Secondly, a reduction in microbial density follows of up to two-three log (Moyer & Morita 1989). The third stage represents the stabilization stage, with microbial concentrations becoming relatively constant. Since the ability to adapt to starvation conditions requires a lower respiration rate, microorganisms under oligotrophic conditions undergo a metabolism shift where most of the energy is redirected towards the essential biological functions instead of biosynthesis and reproduction-related processes (Hartke *et al.* 1998; Hobbie & Hobbie 2013). Another survival strategy adopted by some microorganisms is the temporary increase of budding and cell division processes just before entering the VBNC state, which leads to a higher number of smaller microbial cells. This increases the chances that a single cell will survive the harsh surrounding conditions (Álvarez *et al.* 2008). Bacteria in ultrapure water systems can be present in the VBNC state, but they are not performing active biosynthesis and cell proliferation. Therefore, the number of viable or potentially viable bacterial cells in oligotrophic environments is often significantly underestimated (McNamara *et al.* 2003).

2.4.3 Changes to cell morphology

Gram-negative bacteria commonly rely on cellular morphological changes as a starvation-survival mechanism. Typically, when triggered by environmental stressors, cells become more coccoid, cell length decreases and flagella may or may not develop for increased mobility. In addition, microorganisms may transform into so-called ultra-microcells (Tabor *et al.* 1981; Hartke *et al.* 1998). Some bacterial species belonging to the *Vibrio* genus have been demonstrated to decrease in volume down to $0.03 \mu\text{m}^3$ (Hartke *et al.* 1998). This strategy is used to optimize the ratio between surface area and volume in order to allow cellular transport processes to occur more efficiently. In addition, smaller cells are less subjected to predation.

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In oligotrophic freshwater and marine environments, microorganisms are prevalent mostly as these ultra-microcells (Moyer & Morita 1989). In addition, microbial cells have the potential to become more hydrophobic and adhesive by secreting extracellular polymeric substances that increase the rate of cellular aggregation (Declerck 2010). This would provide additional protection against predation as well as other environmental stressors and ensure that LMW organic molecules originating from neighboring cells can be adequately recycled (Álvarez *et al.* 2008).

2.4.4 Biofilm formation

As previously stated, microorganisms in ultrapure waters tend to form biofilms by adhering to various surfaces. The process of attachment to surfaces is mediated and stabilized by EPS (Fig. 12). At the molecular level, this occurs through a combination of electrostatic and hydrophobic interactions, which depend on a range of physico-chemical properties such as pH, temperature, ligand density or ionic strength. (Donlan 2002).

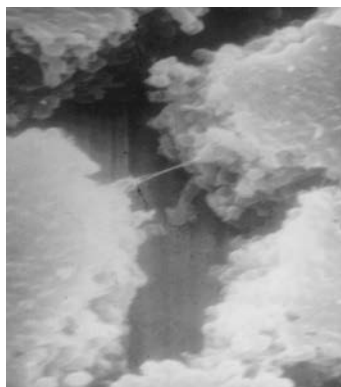


Figure 12. Biofilm formation on a stainless steel coupon submerged in purified water used in a pharmaceutical water system. Bar = 5 μm (Adapted from Mittelman & Jones 2018).

Surface characteristics are important for biofilm formation during the initial stages of cell attachment (Marshall *et al.* 1971; Dahlback *et al.* 1981). However, after this first adhesion event, these properties may not be of great importance anymore, as successively attaching bacteria may not come into contact with the underlying surface. Biofilms are found in various types of industrial water systems, where they can potentially cause health-related and economic problems (Kulakov *et al.* 2002). In ultra-pure pharmaceutical water systems, bacteria and their associated endotoxins represent the greatest threat to product quality.

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3. Analysis of microorganisms in nuclear facilities

As mentioned earlier, microorganisms inhabiting the waters of nuclear facilities have been studied through a variety of methods, both culture-dependent and culture-independent. Before the widespread use of next-generation sequencing techniques, studies relied mostly on isolation via cultivation and subsequent biochemical characterization and partial gene Sanger sequencing of microbes on various types of media. Gram staining was performed to determine bacterial cell wall properties and further identification was attained through the assessment of various biochemical properties such as the ability to metabolize different substrates, the utilization of oxygen, enzyme production or motility. Individual cell morphology could be observed through microscopy. With the broader accessibility of next-generation sequencing techniques such as whole population 16S and 18S rRNA amplicon sequencing and shotgun metagenomics, it became possible to study the dynamics of entire microbial communities on a larger scale.

3.1 Cultivation

The ultrapure waters typically used in nuclear facilities are considered to be extreme environments, and bacteria in these environments have adopted various strategies, described in the previous sections, in order to survive these oligotrophic conditions. Many of them are able to use dead cell components as a sole carbon and energy source (Poindexter 1981). Bacteria originating from ultrapure water environments remain difficult to culture under laboratory conditions, as they often reside in the VBNC state (Phung *et al.* 2004). The main reason why these bacteria are not easily cultivated is the difficulty to reproduce a natural environment combining all the conditions necessary for growth, where different species most likely display strong metabolic cooperations (Stewart 2012). This renders the obtaining of pure cultures rather difficult.

The most appropriate medium to detect bacteria in oligotrophic waters is commonly accepted to be R2A (Reasoner & Geldreich 1985). This medium is also recommended by the American Society for Testing and Materials (ASTM) as a means to test the quality of ultrapure water (ASTM 2000).

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Other commonly used media are Nutrient Broth agar medium (NB), undiluted or diluted 100-fold (Kim *et al.* 1997; Kim & Kye 2000), oligotrophic medium (OM) (McAlister *et al.* 2002), Tryptic Soy Agar (TSA) and M-27 medium (Stevenson *et al.* 2004). Other media such as (diluted) Luria-Bertani (LB) and Minimal Medium (MM) are also used, although they are not as efficient for the detection of oligotrophic bacteria.

In one particular study, five new media were developed to reveal the bacterial diversity in the water purification system of a power plant in Hungary which had been previously investigated using traditional media. These newly developed media attempted to reproduce the nutrient-deficient conditions as best as possible, resulting in highly oligotrophic compositions (Kéki *et al.* 2013). Instead of deionized water typically used in laboratories, the media were supplemented with refined saltless water from the water tank implemented in the water purification system as well as bacterial “extract” from bacteria previously isolated from the same system (Bohus *et al.* 2007, 2008). A much larger diversity of bacteria could be isolated on these media than on the previously used ones. Moreover, two novel bacterial strains were also detected.

3.2 Microscopy

3.2.1 Light and (epi)fluorescence microscopy

Light and epifluorescence microscopy are typically used for absolute cell enumerations in watery environments and for studying individual cell morphologies (Fliermans & Schmidt 1975). Light microscopy employs visible light in the 400-700 nm range to observe samples, which limits the obtainable resolution to this specific spectrum. Various dyes can be used to differentially color the cell nucleoid and cytoplasm.

Fluorescence microscopy on the other hand uses light with a much higher intensity and is used to visualize cells that have been tagged with a fluorescent dye, also known as fluorophore. These dyes are excited by the light emitted by the microscope, which causes them to become fluorescent and emit light with a lower energy and of longer wavelength. Fluorescence microscopy therefore rests on the detection of reflected rather than transmitted light. Most fluorescence microscopes used in microbiology are epifluorescence microscopes, where both the excitation light and emitted fluorescence pass through the same objective.

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A commonly used fluorophore is green fluorescent protein (GFP), which becomes excited by blue light and in turn emits green light with a longer wavelength. Filters are used to separate the fluorescent light from the surrounding light. Fluorescence microscopy can be used to visualize cell nucleoids stained with 4',6-diamidino-2-phenylindole (DAPI), which stains the DNA and confers it a blue color when observed under a fluorescent microscope. Its excitation wavelength lies in the violet region of the spectrum, i.e. at 405 nm. It is commonly used to stain fixed cells. Stained nucleoids can consecutively be enumerated after the imaging procedure using specialized image processing software. In addition, fluorescence microscopy can be employed to investigate the viability of bacterial cells in various samples. Other fluorescent dyes such as propidium iodide (PI) and SYTO9 can be used to this purpose. SYTO9 permeates the cell membrane and is therefore able to color all the cells present in the sample, whereas propidium iodide is membrane-impermeable and can consequently only stain dead cells whose membrane permeability has become compromised. Using this technique, living cells can easily be differentiated from dead cells under the microscope (Fig. 13).

Light and epifluorescence microscopy have both been used in a variety of studies investigating the microbial populations of watery environments in nuclear facilities, mainly for cell enumeration, the observation of biofilm formation on metal coupons and the studying of cell viability (Santo Domingo *et al.* 1998; Zhang *et al.* 1999; Kulakov *et al.* 2002; Sarró *et al.* 2003; Chicote *et al.* 2005; Masurat *et al.* 2005; Rivasseau *et al.* 2016; Karley *et al.* 2017; McGraw *et al.* 2018; Kéki *et al.* 2019; Foster *et al.* 2020).

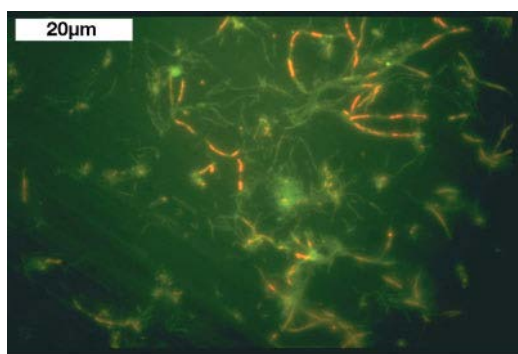


Figure 13. Epifluorescence microscopy image of biofilm formation on a stainless steel coupon submerged in an SNFP for 134 days. Live bacterial cells display a green stain, whereas dead ones are stained red (Adapted from Sarró *et al.* 2003).

3.2.2 Transmission and Scanning Electron Microscopy (TEM and SEM)

Transmission Electron Microscopy (TEM) is a microscopy technique using a beam of electrons which is transmitted through a sample to form an image of the internal structures of the microorganisms under study. Two-dimensional images are formed by the electrons interacting with the sample as the beam passes through, after which it is magnified and focused by a system of lenses onto an imaging device such as a fluorescent screen or a sensor. Samples are typically whole specimens or ultrathin sections less than 100 nm thick, which allow the transmission of at least 50% of the electron beam using energies in the range of 60-350 keV (Bancroft & Stevens 1996). The resolution obtained through TEM is typically much higher than the ones obtained through light and fluorescence microscopy, with a maximum of 1-2 nm for most biological material, but is limited by the thickness of the section, the nature of the specimen and its preparation techniques (Bergmans *et al.* 2005). Samples are first fixed and dehydrated, after which they are typically embedded in an epoxy resin and stained to improve contrast before sectioning using an ultramicrotome. The high resolution allows the instrument to capture images of microbial structures in much finer detail, which is of importance when observing individual species of interest.

TEM was used in two studies investigating the cellular structure of two different microbial species isolated from SNFPs in Sweden and France, respectively (Masurat *et al.* 2005; Rivasseau *et al.* 2016). TEM revealed the presence of sheath-like filaments containing rod-shaped cells in biofilms dominated by *Meiothermus* sp. isolated from the side drain of the CLAB SNFP in Sweden. This microscopy technique was also used to investigate the cellular structure of the extremely radiation-resistant green microalga *Coccymyxa actinabiotis* isolated by Rivasseau *et al.* (Fig. 14).

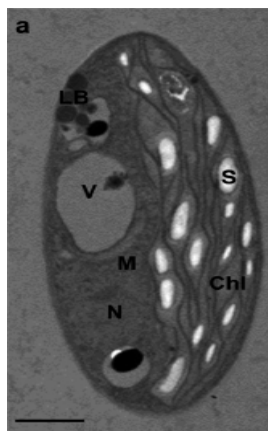


Figure 14. TEM image of *C. actinabiotis*. Chl = chloroplast, LB = lipid bodies, M = mitochondria, N = nucleus, S = starch granules, V = vacuoles (Adapted from Rivasseau *et al.* 2016).

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Scanning Electron Microscopy (SEM) on the other hand is typically used to study cell surfaces in high resolution. With this technique, an image is constructed by scanning across the sample surface with a focused electron beam. The electrons interact with the atoms on the sample surface and emit specific signals containing information about the sample topography and composition, which are in turn detected by the instrument. Secondary electrons emitted by excited atoms are detected through a secondary electron detector. Therefore, the entire instrument as well as the sample chamber need to operate under high vacuum conditions in order to prevent gas scattering of the primary beam or the emitted electrons. This implies that samples to be studied may not contain any volatile substances, as this might disrupt the formed image (Bergmans *et al.* 2005). Therefore, they need to be dehydrated or frozen before microscopic observation. In addition, the samples must be coated with a thin layer of electrically conductive material such as gold in order to prevent the build-up of surface charges generated by the electron beam. Some SEMs can achieve resolutions of down to 1 nm.

Various studies have employed SEM to study individual cell morphology from specific species isolated from nuclear facilities such as *Ramlibacter* sp. isolated from the secondary cooling water of the BR2 reactor in Belgium (Props *et al.* 2019) as well as complex interwoven networks of microbes in the form of biofilms on different metal surfaces. The latter was performed through the investigation of metal coupons submerged in a range of watery environments found in nuclear facilities, such as SNFPs and water purification systems in order to test, for example, the potential for MIC (Santo Domingo *et al.* 1998; Zhang *et al.* 1999; Rao *et al.* 2000; Sarró *et al.* 2003, 2007; Giacobone *et al.* 2011)

3.3 16S rRNA gene amplification

The 16S rRNA gene coding for the small subunit ribosomal RNA is commonly used to discriminate bacterial species from each other (Fox *et al.* 1992). Since this gene is involved in the translation of genes into proteins within individual cells, it is essential to cellular function among all prokaryotes and is therefore very well conserved over all bacteria. It displays evolutionarily conserved regions as well as nine hypervariable regions preceded by highly conserved elements, with the level of conservation depending on the taxonomic family (Mysara *et al.* 2017). Up to 67% of the bases internally paired, forming a compact structure (Ludwig *et al.* 2001) (Fig. 15).

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The structure of this gene is highly conserved among all bacteria, although its organization is slightly different for archaea, which still possess nine hypervariable regions that can be used for identification. The commonly accepted threshold nowadays for determining if different bacteria belong to the same species is 98.65% similarity between the full 16S rRNA gene sequences (Yarza *et al.* 2014). The thresholds for discriminating between different genera and families are 94.5% and 86.5%, respectively. Furthermore, the high level of conservation within the 16S rRNA gene allows for the development of universal primers targeting specific regions of the gene for a wide range of bacterial species.

Targeting this gene involves a polymerase chain reaction (PCR) step, allowing the amplification of a specific region, depending on the used oligonucleotide primers, which are typically around 20 bp in length. The selected primers attach to the targeted regions of the DNA, which are consecutively synthesized over multiple amplification cycles using a DNA polymerase enzyme. A commonly used primer pair is 27F-1492R (5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-TACGGYTACCTTGTTACGACTT-3'). This targets a fragment of nearly 1500 bp and covers all nine hypervariable regions contained in the gene (Frank *et al.* 2008). The resulting fragments can then be used for further downstream analyses such as Sanger sequencing or Denaturing Gradient Gel Electrophoresis (DGGE).

3.3.1 Sanger sequencing

16S rRNA gene fragments amplified through PCR can be sequenced to determine the identity of the bacterial species under study. A widespread method used for this purpose is the so-called Sanger sequencing developed by Sanger *et al.* in 1977. The method is based on the selective incorporation of chain-terminators (dideoxynucleotides) by a DNA polymerase in the DNA during the replication process. This technique rapidly became the gold standard for sequencing until the mid-2000s, when high-throughput sequencing became more readily accessible. It is still widely used to this day, as it has the advantage of producing relatively long read lengths ranging from approximately 450 to 900 bp. For the identification of bacterial species, the merged forward and reverse reads are mapped to a public database such as the National Center for Biotechnology Information (NCBI) containing a wide range of bacterial 16S rRNA gene sequences using the Basic Local Alignment Search Tool (BLAST).

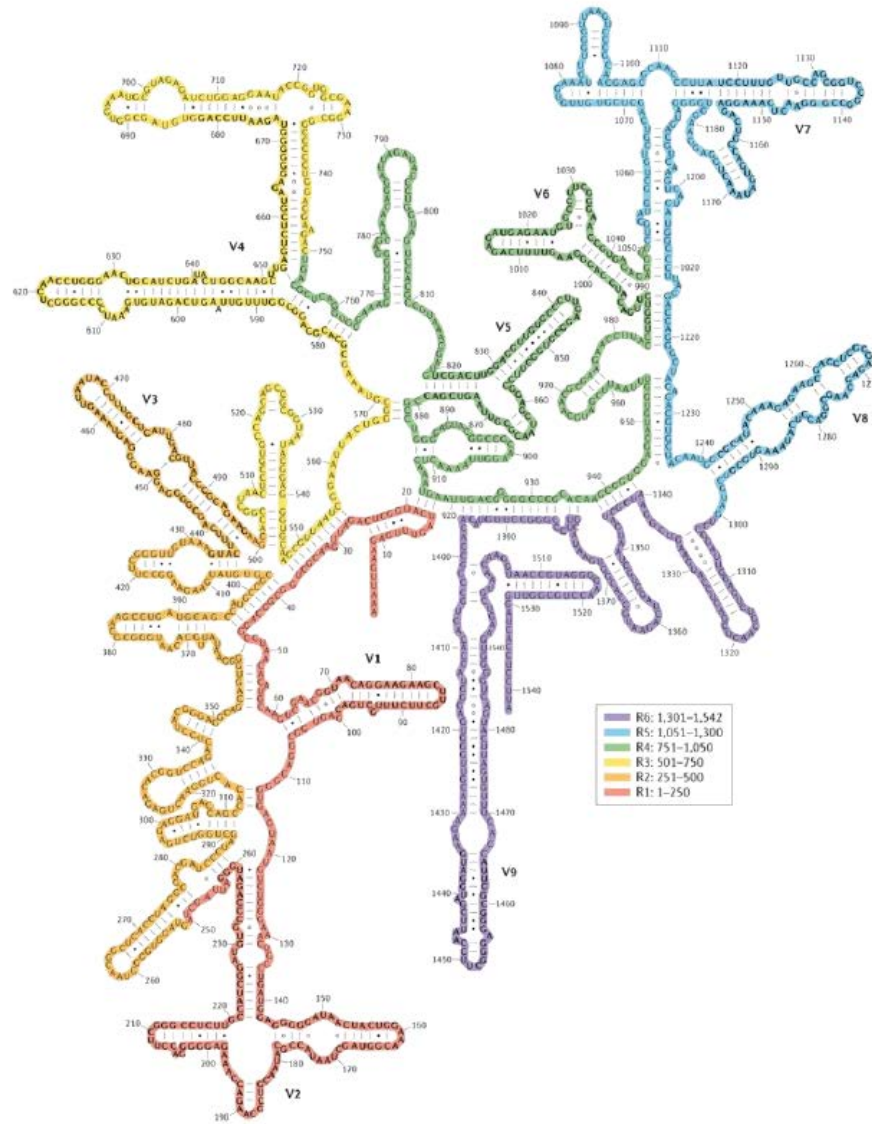


Figure 15. 16S rRNA gene structure. A schematic view of the secondary structure of the 16S rRNA gene for *Escherichia coli* is presented. A total of approximately 50 helices are visible. The nine hypervariable regions are designated as V1-V9 (Adapted from Yarza *et al.* 2014).

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3.3.2 Denaturing Gradient Gel Electrophoresis (DGGE)

Before the advent of next-generation sequencing techniques, DGGE could be used as a tool to assess global microbial diversity in various environments. It rests on the principle of adding DNA (e.g. extracted from a microbial community) to an electrophoresis gel containing a denaturing agent, which induces DNA melting at various stages. This allows for the determination of differences in DNA sequences of the same length. DNA extracted from microbial communities can be amplified through PCR by using universal primers targeting specific regions of the 16S rRNA gene for prokaryotes and the 18S rRNA gene for eukaryotes, resulting in a mixture of various amplicons. These mixed fragments of the same length are subsequently separated from each other on a gel by differences in GC content and distribution in their DNA sequences. The patterns observed on the gel can then be used to visualize microbial diversity in the environment under study and provide an estimate of the richness and evenness metrics of the community (Muyzer *et al.* 1993).

A couple of studies investigating the microbial communities in the waters of nuclear facilities have used DGGE as a means to evaluate microbial diversity in these environments (Balamurugan *et al.* 2011; Kéki *et al.* 2019). It was used as a molecular fingerprinting tool and formed a base for the calculation of Shannon diversity. In addition, it was used as a pre-screening method to select specific samples and microorganisms for sequencing based on their specific migration patterns on the gel (Sarró *et al.* 2003; Chicote *et al.* 2004, 2005).

3.4 Next-generation sequencing and –omics techniques

Recently, high-throughput sequencing methods, also called second- and third-generation or next-generation sequencing, have been utilized to investigate the microbial communities in various watery environments of nuclear facilities. The main advantage of these techniques is their ability to cover the entirety of the microbial diversity, which could not be studied through cultivation-dependent techniques alone. These approaches enable the generation of more sequencing data at a lower cost than the previously discussed techniques. They include approaches such as 16S and 18S rRNA amplicon sequencing as well as shotgun metagenomics. In addition, metaproteomics (see section 3.4.6 below) can be used to validate taxonomic and functional predictions obtained through sequencing data.

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3.4.1 16S and 18S rRNA amplicon sequencing

High-throughput sequencing of 16S and 18S rRNA amplicons is used as a method for the identification of prokaryotes and eukaryotes, respectively. For fungi, a commonly used marker is the Internal Transcribed Spacer (ITS) in the 18S rRNA gene (Kittelman *et al.* 2013). The method relies on the selective binding of previously selected universal primers to the highly conserved regions of the gene and the subsequent sequencing of the resulting amplicons obtained through PCR. These amplicons contain hypervariable regions that are taxon-specific and can therefore be used as a means to discriminate between different microbial genera (D'Amore *et al.* 2016). Different primer pairs result in the generation of amplicons of varying lengths whereby different hypervariable regions are amplified that are suitable to distinct sequencing platforms, such as Roche (454 Pyrosequencing), Ion Torrent (PGM), Illumina (MiSeq) (second-generation sequencing), PacBio (SMRT) and NanoPore (MinION) (third-generation sequencing) (Slatko *et al.* 2018). A commonly targeted fragment is the V3-V4 region, as this section offers a high discriminative power between different bacterial genera.

3.4.2 Sequencing platforms

In Roche 454 pyrosequencing, DNA fragments of approximately 400 to 700 bp are linked to adapters and amplified by PCR in a bead emulsion reaction. The DNA sequences of the adapters bind to the complementary sequences on the beads, ideally yielding one DNA fragment per bead. Hereafter comes a DNA synthesis step followed by detection of the synthesis reactions through measuring the release of pyrophosphate using a light-generating reaction. The intensity of the light reaction provides information on the number of homopolymers in the sequence, although this becomes challenging with larger numbers of the same nucleotide. This method is no longer in use, as the platform was discontinued in 2013 (Slatko *et al.* 2018).

The Ion Torrent PGM technology rests on the principle of nucleotides incorporated in a growing DNA chain releasing hydrogen ions, which changes the pH of the solution in which the synthesis reaction takes place. This pH change is then recorded as a voltage change by an ion sensor on a semiconductor chip (Rothberg *et al.* 2011). When the same nucleotide is incorporated multiple times, the voltage changes accordingly, although larger homopolymer strings present a higher difficulty to discern. DNA fragments of 200 to 1,500 bp are ligated to adapters, which bind to complementary DNA fragments attached to individual beads and are subsequently amplified on the bead through emulsion PCR.

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The sequencing reactions occur in millions of wells on the semiconductor chip that each contain one single bead. The direct recording of voltage is a major advantage of this technique, which greatly enhances the speed of the process.

The most commonly used platform by far for amplicon sequencing nowadays is Illumina MiSeq. This is in part due to the fact that it generates longer read lengths than other platforms such as HiSeq and NovaSeq, which are more useful for applications such as shotgun metagenomics or (meta)transcriptomics. In this technology, DNA fragments of about 500 bp long with ligated adapters on each end are amplified repeatedly on a glass slide containing oligonucleotides complementary to the ligated adapters through a process known as “bridge amplification”. After multiple amplification rounds, clonal clusters are created on the solid support, consisting of approximately 1000 copies of each synthesized fragment. During DNA synthesis, fluorescently labeled nucleotides acting as synthesis terminators are incorporated in the DNA chain and subsequently detected by direct imaging. After detection, the sequences are unblocked to allow for the next round of synthesis. The use of direct imaging greatly increases the detection speed, making this a highly competitive method. A large amount of data is typically generated using this technique, with sequencing errors generally amounting to 0.1% of the sequenced bases (Santos *et al.* 2020).

Third-generation sequencing methods typically generate longer reads than second-generation techniques. One example is the PacBio sequencing, also known as Single Molecule Real Time (SMRT) sequencing, able to generate fragments up to 30 to 50 kb or longer. This technology relies on the use of an engineered DNA polymerase bound to the bottom of a well in a SMRT flow cell. Nucleotides linked to fluorophores are incorporated into a growing DNA chain by the DNA polymerase, which generates light that can be detected in real time on a millisecond time scale each time a correct nucleotide is added. After nucleotide incorporation, the fluorophores are released and can no longer be detected. This leaves space for the next nucleotide to be added. This reaction occurs in parallel in millions of wells on a single chip within the SMRT cell. The DNA template is converted to a circular double-stranded molecule containing specific adapters that are complementary to the primers used to initiate the DNA synthesis process. This allows for the repeated reading of single large templates multiple times by passing through the circular molecule in each well until the polymerase stops.

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A consensus sequence can then be constructed that corrects for the inherent high rate in sequencing errors, which are stochastic rather than systematic in nature. This results in high accuracy consensus reads. Another advantage of the PacBio method is the ability to detect base modifications (such as methylation), as modified bases alter the rate of nucleotide incorporation measured in real-time. This is a useful feature for epigenetic studies (Slatko *et al.* 2018).

Finally, the NanoPore MinION system developed for DNA sequencing relies on the use of biological nanopores in the form of transmembrane proteins embedded in a lipid membrane or on metal substrates containing nanometer sized pores that allow the passage of long DNA and RNA molecules (Cherf *et al.* 2012; Liu *et al.* 2016). The technique involves the binding of long, double-stranded DNA molecules to a specific DNA polymerase such as the phi29 polymerase, which then attaches itself to an encountered nanopore and subsequently allows one DNA strand to pass through it. The translocation rate is controlled by the synthesis speed of the DNA polymerase. Nucleotides passing through a pore disrupt the current that was previously applied to it, thereby giving off an electronic signal characteristic for each nucleotide. Signal detection occurs in real time and read lengths of 10 kb are now considered a realistic output. When the DNA template has been removed from the nanopore, it becomes available again for a new DNA molecule. The MinION device used for this type of sequencing has the benefit of being small, allowing for many applications such as the sequencing of environmental and metagenomic samples in the field. The error rate is still relatively high, but this can be alleviated by creating consensus sequences from a large number of sequenced molecules. As with the PacBio technique, the nanopore technology can also be used to detect base modifications, allowing for the studying of epigenetics. As the latter two discussed sequencing techniques allow for the generation of longer reads in general, they are better adapted for metagenomics studies, although they can be used for amplicon sequencing as well in order to sequence the entire 16S or 18S rRNA gene. Figure 16 gives an overview of the differences between first-, second-, and third-generation sequencing.

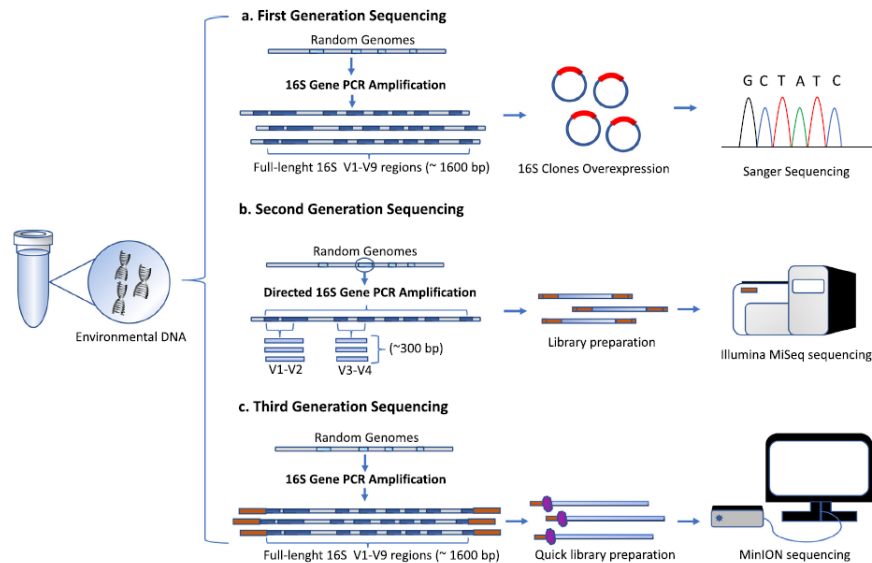


Figure 16. Comparison of different sequencing strategies for first-, second- and third-generation sequencing technologies. A. Sanger sequencing. B. Illumina sequencing. C. Nanopore sequencing (Adapted from Santos *et al.* 2020).

3.4.3 Sequencing data analysis

For 16S and 18S rRNA amplicon sequencing, the sequencing data generated by the described platforms must be processed through specific bioinformatics pipelines for the clustering of sequences into operational taxonomic units (OTUs). This OTU clustering is based on preemptively defined thresholds of sequence similarity, typically 97%, which reflect the boundaries of distinct phylogenetic groups, assuming a shared biological origin (Fricker *et al.* 2019). Clustering can be performed based on bacterial reference genomes (phylotyping) or *de novo* in order to identify previously undefined species (Rideout *et al.* 2014). With regard to fungi, the analysis of ITS amplicons follows the same pattern as for 16S and 18S rRNA amplicon analysis, with the difference that different fungal species display varying amplicon lengths and sequence similarities, which sensibly complicates sequence clustering and taxonomic classification (Halwachs *et al.* 2017). Therefore, an effort is being made to gather all the known ITS sequences in a consolidated repository known as the UNITE database (Nilsson *et al.* 2019). For bacteria and eukaryotes, the SILVA database is well established and contains the known sequences for the small and large subunit rRNA genes.

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Amplicon sequencing data are typically analyzed through open-source software packages such as MOTHUR (Schloss *et al.* 2009), QIIME (Caporaso *et al.* 2010), Phyloseq (McMurdie & Holmes 2013), and OCToPUS (Mysara *et al.* 2017). These packages typically include demultiplexing and quality control steps before OTU clustering (Fig. 17). QIIME2 has implemented specific denoising methods such as Deblur or Dada2 in order to discriminate between biological and technical sequence variations originating from the sequencing platforms (Callahan *et al.* 2016; Amir *et al.* 2017). These tools are used to account for sequencing errors and can achieve high resolutions for individual amplicon sequences up to a single nucleotide in order to assign taxonomic classifications up to the species or even strain level. Based on this, biological amplicon sequence variants (ASVs) are identified, which result in improved sensitivity and specificity. These avoid overinflating microbial datasets by misidentifying distinct OTUs originating from wrongly clustered sequences. Clustered OTUs are then compared to specific databases such as Greengenes (McDonald *et al.* 2012), SILVA (Quast *et al.* 2013), or RDP (Cole *et al.* 2014) for taxonomic classification. In a final step, various diversity metrics can be computed.

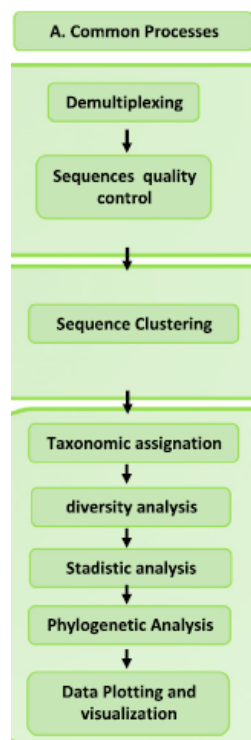


Figure 17. Typical bioinformatics workflow for the analysis of 16S rRNA amplicon sequencing data (Adapted from Santos *et al.* 2020).

3.4.4 Amplicon sequencing in nuclear reactor environments

So far, only a small number of recent studies have used high-throughput amplicon sequencing to study the microbial communities in nuclear reactor waters. In 2018, McGraw *et al.* used 16S and 18S rRNA amplicon sequencing to study bacterial and eukaryotic diversity in an outdoor SNFP in Sellafield undergoing seasonal algal blooms. With 18S rRNA amplicon sequencing they detected an algal species closely related to *Haematococcus pluvialis* as the dominant organism. 16S rRNA gene profiling yielded a broad diversity of Proteobacteria and Cyanobacteria. Another study utilizing 454 pyrosequencing uncovered a bacterial diversity of species mainly belonging to the Burkholderiaceae, Nitrospiraceae, Hyphomicrobiaceae and Comamonadaceae families in a SNFP in the US (Bagwell *et al.* 2018). Two other studies investigating different SNFPs in Sellafield employed 16S and 18S rRNA amplicon sequencing using the Illumina MiSeq technology to reveal that these ponds were mostly dominated by bacteria, with the most prevalent genera being *Pseudanabaena* and *Hydrogenophaga*, respectively (Foster *et al.* 2020; Ruiz-Lopez *et al.* 2020). Finally, 16S rRNA amplicon sequencing using the Illumina MiSeq platform was employed to study the bacterial community in a cooling pool surrounding an operating nuclear reactor in France (Petit *et al.* 2020). During operation, *Variovorax* was discovered to be the dominant genus, which was overtaken by *Methylobacterium* during shutdown.

3.4.5 Shotgun metagenomics sequencing

Instead of solely targeting the 16S or 18S rRNA genes, the whole-genome shotgun metagenomics approach consists in fragmenting and sequencing the entire genome from environmental samples in order to study the taxonomic and functional profiles of the entire microbial community. As such, all the different phylogenetic groups of microorganisms can be studied indiscriminately, including bacteria, archaea, eukaryotes (algae, fungi and others), viruses and phages. Shotgun reads can also originate from plasmids, chloroplasts and mitochondrial DNA. Contrary to 16S rRNA amplicon sequencing, this approach does not involve any PCR amplification. In order to attain a sufficient sequencing depth to reveal the entire microbial diversity, including rare species, this method therefore requires a much larger amount of sequencing data than the traditional 16S rRNA amplicon sequencing approach, with some studies reaching up to several Tb (Fricker *et al.* 2019).

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The bioinformatics analysis of shotgun sequencing data requires different quality control steps such as read trimming and filtering, which can be performed via tools such as KneadData (available at <http://huttenhower.sph.harvard.edu/kneaddata>). Reads are then assembled *de novo* or by comparing them to a reference database using a mapping-based approach (Nayfach & Pollard 2016). *De novo* assembly is typically used for the identification and functional characterization of previously unidentified microbial species (Brown *et al.* 2013). However, due to the significant sequencing depth required for this process, only the genomes of highly abundant species can typically be entirely reconstructed. For the taxonomic profiling of entire microbial communities, rare species included, a sequence mapping approach using specific marker genes can be adopted using bioinformatics tools such as MetaPhlAn2 (Truong *et al.* 2015).

Currently, only a single study exists that deployed a shotgun metagenomics approach to study the microbial community in a SNFP and fuel transfer channel (FTC) of a nuclear power plant in Brazil (Silva *et al.* 2018). Samples originated from biofilms collected on the walls of these facilities were subjected to massively parallel sequencing in order to obtain taxonomic and functional profiles of the entire microbial communities. DNA was sequenced using the Ion Torrent PGM platform and sequencing data were analyzed via the MG-RAST server. Fungi were detected in high abundances, as well as various bacterial taxa mainly belonging to the Proteobacteria, Actinobacteria and Firmicutes groups. Functional profiles were also determined, which revealed a high proportion of protein-coding sequences associated with respiration and protein metabolism.

3.4.6 Metaproteomics

Metaproteomics involves the extraction and subsequent analysis of all proteins contained in environmental samples. This technique can be used to detect the functional characteristics of microbial communities dwelling in various ecosystems, as well as for the identification of its individual members. After protein extraction, peptides are typically generated through proteolysis by a trypsin enzyme. The resulting peptides are then separated through reverse phase chromatography and subsequently processed by tandem mass spectrometry (MS/MS). Thanks to recent advancements in bioinformatics analysis techniques and mass spectrometry resolution, it is currently possible to link individual peptides with the microorganisms from which they originated (Armengaud & Pible 2015).

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MS/MS spectra generated by the mass spectrometer containing detailed amino acid sequences are typically compared to a public database containing known microbial protein sequences, allowing for accurate identification of the microorganisms under study. This can be achieved through specific bioinformatics software packages such as MASCOT, which compares the experimentally obtained MS/MS spectra with theoretically generated ones originating from the peptides obtained from the proteins in the database (Perkins *et al.* 1999; Bouyssié *et al.* 2007; Lacerda *et al.* 2008).

The metaproteomics approach can be complementary to amplicon sequencing methodologies, as the latter are known to introduce significant biases in the data through DNA extraction, PCR and sequencing errors, as well as the variable number of 16S rRNA gene copies present in a single genome, which depends on the species (Tourova 2003). These combined factors can sensibly affect the proportion of 16S rRNA genes and therefore OTUs in the final analyzed data (Brooks *et al.* 2015). However, bias can also be introduced in the metaproteomics approach through protein extraction.

One study has implemented a metaproteomics approach in combination with 16S rRNA amplicon sequencing in order to identify microbial taxa inhabiting the cooling pool surrounding the Osiris nuclear reactor in France (Petit *et al.* 2020). Tandem mass spectrometry data were used for the taxonomic identification of individual species through a method known as proteotyping, which can also be used to estimate the absolute proportion of each taxon in terms of biomass with relatively good confidence (Kleiner *et al.* 2017; Grenga *et al.* 2019). Both approaches are therefore complementary, as they provided distinct information on the microbial community.

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4. Radiation resistance mechanisms

4.1 Ionizing radiation

Radioactive atoms, also known as radionuclides, spontaneously decay, thereby emitting excess energy in the form of ionizing radiation. Each radionuclide emits a specific type of radiation at a certain energy and with a distinct half-life, which can be used for their identification. The half-life of a specific radionuclide consists of the time necessary for the radioactivity to decrease by 50% of its initial value, which can range from milliseconds to millions of years. Next to radionuclides, other sources of ionizing radiation include X-ray and cosmic radiation as well as nuclear fission reactions.

Ionizing radiation is a type of energy released by atoms that can be either electromagnetic (UV light, gamma and X-rays) or particulate (energetic electrons, protons, neutrons, α and β particles) in nature (Hall & Giaccia 2011). It has sufficient energy to cause the ionization of atoms and molecules by releasing energetic electrons from their atomic shell structure. Particles such as α and β particles cause ionization through collisions, as they cannot travel far in space and deposit their energy directly upon entering matter along their travelled tracks (Cox & Battista 2005). Gamma radiation on the other hand consists of photons that cause atoms to ionize through energy absorption or scattering. It can penetrate much deeper into cells or tissue since it does not interact with matter as intensely as α and β particles. Gamma radiation can deposit its energy through three different types of interaction (Desouky *et al.* 2015). Firstly through the so-called Compton scattering, where the photon energy is only partly absorbed by an encountered electron and a new photon is subsequently scattered with lower energy (increased wavelength) in a different direction. Secondly through the photoelectric interaction, where all the energy of a photon is transferred to an electron, mostly in the outer shell, which results in its ejection from the atom. Thirdly, photons can interact directly with the nucleus and generate a pair of particles through the conversion of energy into matter, resulting in the production of an electron as well as a positively charged positron. This phenomenon can only occur with high-energy photons (> 1.02 MeV) (Hall & Giaccia 2011).

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Ionizing radiation can be classified as either low linear energy transfer (LET), which is referred to as sparsely ionizing, or high LET (densely ionizing). Gamma radiation is low LET radiation, with a broad energy distribution in cells, whereas charged particles are high LET radiation and travel in straight tracks (Fig. 18).

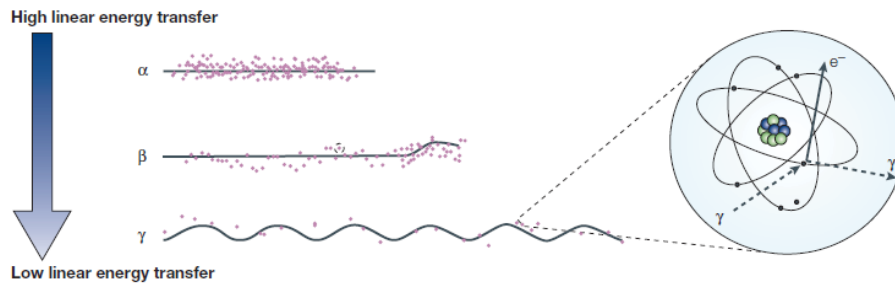


Figure 18. Overview of three different types of ionizing radiation ranked from high to low linear energy transfer (LET). Dots represent energy deposition events. On the right, the ejection of an electron is depicted through the collision with a photon (gamma-ray) via Compton scattering (Adapted from Cox & Battista 2005).

Radioactivity is measured in Becquerel (Bq), which expresses the number of atom disintegrations per second. The energy deposited by ionizing radiation in cells or tissue on the other hand is expressed in rads or Gray (100 rad = 1 Gy). Finally, the Sievert unit (Sv) is used to express the biological effects caused by ionizing radiation on the organism.

4.2 Effect on cells

Ionizing radiation directly or indirectly affects cellular structures such as nucleic acids, proteins and lipids (Halliwell & Gutteridge 1999). With regard to direct effects, α and β particles, as well as neutrons for example can interact with DNA molecules through collisions and disrupt the sugar backbone as well as the purine and pyrimidine bases, causing the DNA structure to unravel (Close *et al.* 2013). These direct interactions caused by ionizing radiation account for approximately 20% of the deleterious effects experienced by cells (Halliwell & Gutteridge 1999). Indirect effects on the other hand imply the intervention of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), dihydrogen (H_2), superoxide anions ($\text{O}_2^{\bullet-}$) and hydroxyl radicals ($\text{OH}^{\bullet}$) generated through water radiolysis.

These ROS can subsequently interact with cellular components such as proteins, lipids, nucleic acids and carbohydrates, resulting in impaired cell survival (Halliwell & Gutteridge 1999; Close *et al.* 2013). The indirect effects carried out through ROS account for the remaining 80% of DNA damage undergone by cells impacted by ionizing radiation (Kottemann *et al.* 2005). Both direct and indirect effects can cause chromosomal DNA lesions (Fig. 19) by introducing single-strand and double-strand breaks (SSBs and DSBs), which can result in genome instability and subsequent cell death if the DNA lesions are not adequately repaired (Rich *et al.* 2000; Hoeijmakers 2001).

Apart from their damaging effect on DNA, ROS can also impact proteins by cleaving their backbone, thereby inducing protein oxidation (Madian & Regnier 2010). In addition, ionizing radiation increases the occurrence of protein carbonylation, consisting of the post-translational addition of carbonyl groups to amino acid side chains, resulting in altered protein folding and eventually loss of protein function (Sukharev *et al.* 1997; Maisonneuve *et al.* 2009). If not adequately folded by chaperones or degraded by the proteasome, carbonylated proteins can have a cytotoxic effect (Dalle-Donne *et al.* 2006). Finally, oxidative damage carried out by ROS can significantly alter the transport properties of lipid bilayers as well as their transmembrane potential through lipid peroxidation. This causes cells to accumulate toxic products (Ziegler & Wessels 1998).

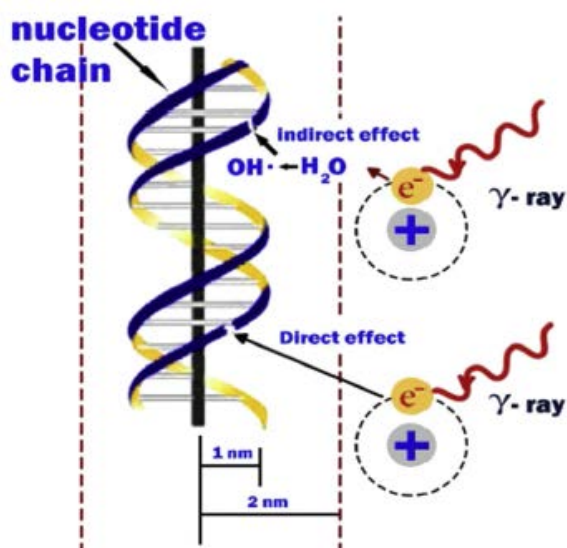


Figure 19. Direct and indirect effects of ionizing radiation on DNA (Adapted from Hall & Giaccia 2011).

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4.3 Cellular defense mechanisms

Microorganisms across the three domains of life (Bacteria, Archaea and Eukaryotes) employ a broad variety of strategies to mitigate the harmful effects of ionizing radiation on their cells. Although none of the implemented strategies appear to be universal, two cellular mechanisms can be broadly distinguished. The first represents the protection of cellular proteins and DNA from oxidative damage induced by ROS through enzymatic defense systems and antioxidant compounds. The second consists of the implementation of specialized DNA repair mechanisms adopted to functionally reconstruct the disrupted genome (Pavlopoulou *et al.* 2015). To a lesser extent, nucleoid condensation can also be utilized, which protects the DNA from oxidative damage and facilitates the DNA repair process.

4.3.1 Protection mechanisms against ROS

4.3.1.1 Enzymatic radio-protective processes

Cells can protect themselves against oxidative stress through antioxidant enzymes such as superoxide dismutases, catalases and peroxidases, which protect cellular components from the harmful effects of ROS. The role of superoxide dismutase is the catalysis of the conversion reaction of oxygen superoxide anions ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2). The latter is subsequently converted to H_2O by catalases and/or peroxidases. Peroxidases are in turn linked to redox pathways such as the ascorbate-glutathione-NADPH pathway (del Río *et al.* 2002).

With regard to Archaea, superoxide dismutase is mostly found in aerobic organisms such as in the *Halobacterium* genus, as well as in a small number of anaerobic organisms like *Methanosarcina barkeri* (Brioukhanov *et al.* 2006). In other anaerobic Archaea, the enzyme taking over this role is superoxide reductase (SOR) (Jenney *et al.* 1999; Weinberg *et al.* 2004; Grunden *et al.* 2005).

4.3.1.2 Non-enzymatic processes : radioprotective antioxidant compounds

Another mechanism for the protection against ROS-induced adverse effects lies in an increased $[Mn]/[Fe]$ ratio inside the cell. This prevents the generation of hydroxyl radicals through the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow 2 OH^{\bullet} + Fe^{3+}$) (Imlay 2003). Mn^{2+} ions can also act as ROS scavengers, conferring an additional protective effect (Archibald & Fridovich 2014).

Intracellular Mn^{2+} concentrations in the most radiation-resistant microorganisms are approximately 300 times higher and Fe^{2+} concentrations approximately three times lower than in the most radio-sensitive species, emphasizing the crucial role of these two elements in radiation protection mechanisms (Daly *et al.* 2007). In addition, high $[\text{Mn}]/[\text{Fe}]$ ratios correlate with low levels of protein oxidation (Daly *et al.* 2007). Next to bacteria, a high intracellular $[\text{Mn}]/[\text{Fe}]$ ratio was also detected in the highly radiation-resistant archaeon *Halobacterium* (Kish *et al.* 2009). Other ions can also protect cells against oxidative stress, such as bromine ions, which scavenge hydroxyl radicals and thereby reduce DNA and protein oxidation (Kish *et al.* 2009).

Next to ions, pigments such as the red carotenoid pigment are also effective in scavenging ROS such as peroxy radicals ($\text{ROO}\bullet$) (Tatsuzawa *et al.* 2000; Stahl & Sies 2003). Other pigments such as melanin, beta-carotene, phytoene and lycopene have also been found to play a role in radiation resistance (Tian *et al.* 2007). In addition, proteins with reductase activity such as thioredoxin and glutaredoxin are also known to have antioxidant and therefore radioprotective effects (Wang & Schellhorn 1995; Makarova *et al.* 2001). Other organic components such as lipoic acid and folates also act as antioxidants (Pote *et al.* 2006; Manda *et al.* 2007).

The protection of proteins is a crucial component of radiation resistance, as cells need functional proteins for DNA repair immediately after the irradiation event (Daly *et al.* 2007). Cell death indeed occurs primarily through protein oxidation, rather than DNA damage.

4.3.2 DNA repair mechanisms

DNA damage upon irradiation of microbial cells can occur either through single- or double-strand breaks (SSBs or DSBs). Out of the two, DSBs potentially have the most lethal effect on cells and present a greater challenge in terms of DNA repair mechanisms. Therefore, microorganisms have evolved a number of strategies to account for the efficient repair of DSBs, including homologous recombination (HR), single strand annealing (SSA), extended synthesis dependent strand annealing (ESDSA) and non-homologous end joining (NHEJ). Whereas the first three mechanisms require cells to contain at least one additional intact copy of the disrupted DNA, the latter does not share this requirement in order to join two separate fragments (Hefferin & Tomkinson 2005). Fig. 20 gives an overview of all the different mechanisms used to repair DSBs.

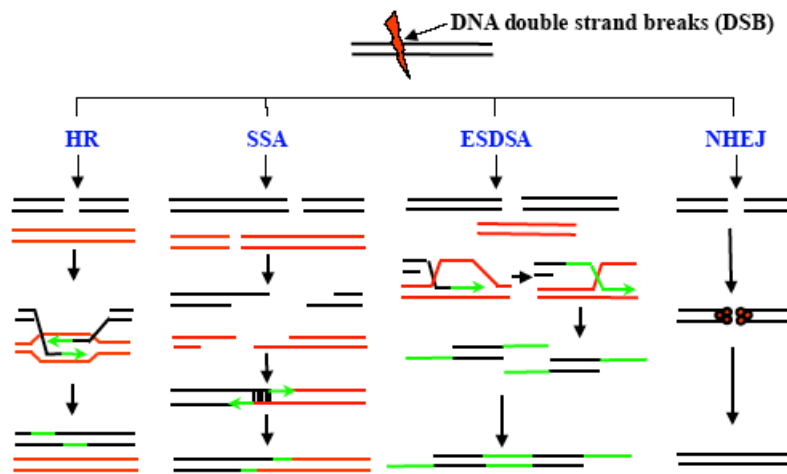


Figure 20. Overview of the different DNA double strand break (DSB) repair mechanisms. Newly synthesized DNA is represented in green. Distinct copies of the same chromosomal region are displayed in red and black. HR = Homologous Recombination, SSA = Single-Strand Annealing, ESDSA = Extended Synthesis-Dependent Strand Annealing, NHEJ = Non-Homologous End-Joining (Adapted from Blasius *et al.* 2008).

4.3.2.1 Homologous Recombination (HR)

HR is commonly accepted to be the primary mechanism for DSB repair in bacteria (Wyman & Kanaar 2006) as well as in the eukaryotic yeast model *Saccharomyces cerevisiae* (Pâques & Haber 1999). This mechanism involves the utilization of an intact homologous DNA molecule as a template to reestablish the correct DNA sequence in the disrupted region. First, single-stranded overhangs are created, on which a recombinase enzyme (RecA for bacteria, RadA for Archaea and Rad51 for eukaryotes) subsequently attaches itself, thereby creating a nucleoprotein structure (Seitz *et al.* 1998). Homologous overlapping fragments then invade the intact DNA template, DNA strands are exchanged and elongated by a DNA polymerase and finally the generated crossover structures are resolved.

4.3.2.2 Single-Strand Annealing (SSA)

If two DSBs are located in the same region of two identical chromosomes, SSA can take place. An exonuclease first cleaves off the DNA ends to be removed, thereby producing single-stranded overhangs. Annealing can occur if the overhangs are composed of complementary sequences.

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Single-stranded portions of DNA in the recombined chromosome are then extended by a DNA polymerase. This process does not require the intervention of a recombinase enzyme (Daly & Minton 1996).

4.3.2.3 Extended Synthesis-Dependent Strand Annealing (ESDSA)

In ESDSA, a single-stranded overhang is produced by DNA resection and subsequently invades a partially overlapping fragment of the same chromosome, after which DNA synthesis is initiated. However, contrary to the traditional DNA replication process of double-stranded DNA, new DNA strands are synthesized through displacement of the D-loop in a fashion resembling the transcription process where transcribed RNA is progressively released from the transcription machinery as the RNA polymerase advances on the DNA molecule. Single strand elongation can continue until the end of the DNA molecule. The generated DNA tails can subsequently anneal if they contain complementary sequences, thereby generating long intermediate double-stranded DNA molecules, which can subsequently recombine into a circular chromosome. Several DNA polymerases come into play for the large amount of required DNA synthesis (Slade *et al.* 2009). In addition, RecA plays a role in the invasion of homologous DNA in order to initiate DNA synthesis as well as in the recombination of linear intermediate DNA molecules into circular chromosomes. ESDSA is a major component of the radiation-resistance mechanism of *D. radiodurans*.

4.3.2.4 Non-Homologous End-Joining (NHEJ)

In eukaryotes, the primary DSB repair mechanism consists of NHEJ, although it has also been detected in bacteria (Weller *et al.* 2002; Shuman & Glickman 2007; Chayot *et al.* 2010). This mechanism involves the binding of certain proteins to DSBs in a DNA template, such as PprA specifically in *Deinococcaceae* (Tanaka *et al.* 2004), and the subsequent joining of DNA ends by DNA ligases (Blasius *et al.* 2007). NHEJ is another process that does not require recombination enzymes such as RecA.

4.3.3 Nucleoid condensation

Nucleoids are compact structures comprised of bacterial chromosomes associated with proteins. *D. radiodurans* is known to form nucleoids displaying high levels of genome condensation by maintaining a tight ring structure (Pavlopoulou *et al.* 2015). Even after high-dose gamma irradiation accompanied by DNA damage in the form of SSBs and DSBs, the nucleoid structures remains unaltered (Levin-Zaidman *et al.* 2003).

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This prevents the diffusion of DNA fragments over long distances, which would significantly hamper DNA repair mechanisms. However, this phenomenon is still considered somewhat controversial (Cox & Battista 2005). Nucleoid condensation would also protect microorganisms from additional DNA damage by lowering the access of ROS to chromosomes as well as histone-like proteins (Begusova *et al.* 2001; Anuchin *et al.* 2011; Spotheim-Maurizot & Davidkova 2011).

4.4 Radiation-resistant microorganisms

Microorganisms are classified as radiation-resistant if they do not possess the ability to form spores and if ionizing radiation doses of 1 kGy or more are required to reduce their initial cell viability by 90% ($D_{10} > 1$ kGy) (Sghaier *et al.* 2008). Ionizing radiation levels in natural environments are usually relatively low. Nonetheless, some microorganisms across all three domains of life (Archaea, bacteria and eukaryotes) were observed to exhibit high levels of radiation resistance (ranging from 1 to 10 kGy). This is in contrast to *Escherichia coli* for example, which displays a D_{10} of 0.2-0.7 kGy (Karam & Leslie 1999; Confalonieri & Sommer 2011). As a reference, humans have a D_{50} of 3 Gy. Different microorganisms across the tree of life employ a wide variety of radiation-resistant strategies, which sensibly differentiates them from their radiation-sensitive counterparts.

4.4.1 Bacteria

Among radiation-resistant bacteria, the most well-known and well-studied species is *D. radiodurans*, which is able to survive extremely high doses of gamma radiation ($D_{10} = 12$ kGy) (Daly 2012). This species also displays high levels of resistance to desiccation, UV-C radiation and oxidative stress (Slade & Radman 2011). Due to its extremely radioresistant phenotype, *D. radiodurans* is commonly used as a model for the unravelling of radiation-resistance mechanisms. It has evolved a variety of cellular defense mechanisms against the adverse effects of ionizing radiation, such as high antioxidant concentrations and enzymatic activities as well as specific DNA repair systems (Cox & Battista 2005; Daly 2009). Counterintuitively, the latter do not appear to be particularly different from the ones implemented by radiation-sensitive species. In fact, DNA repair mechanisms in *D. radiodurans* were shown to be relatively uncomplicated when compared to the ones used by *E. coli* and *Bacillus subtilis* (Makarova *et al.* 2001).

The most important one is the previously described ESDSA process, where newly synthesized, single-stranded DNA tails allow for full genome reconstruction from a small number of chromosomal sections fragmented by ionizing radiation (Zahradka *et al.* 2006). Next to DNA repair mechanisms, *D. radiodurans* also possesses efficient antioxidant defense systems, both enzymatic and non-enzymatic, in order to mitigate the adverse effects of radiation-induced ROS. As such, *D. radiodurans* harbors a unique carotenoid pigment, deinoxanthin, which has a high ROS scavenging capability and thereby protects DNA from oxidative damage with a higher efficiency than other pigments such as β -carotene or lycopene (Tian *et al.* 2007). Another component playing a role in the radiation resistance of this bacterium is pyrroloquinoline-quinone (PQQ), a redox cofactor associated with glucose dehydrogenases (Duine, 1990). Finally, the antioxidant defense mechanism that has been studied the most thoroughly in *D. radiodurans* is the system involving the Mn^{2+} complex. Indeed, this species contains much higher levels of manganese coupled with lower levels of iron than its radiation-sensitive counterparts, with $[Mn]/[Fe]$ ratios in *D. radiodurans* and *E. coli* amounting to 0.24 and 0.0072, respectively (Daly *et al.* 2004). Other radiation-resistant bacteria such as *Enterococcus*, *Lactobacillus* and cyanobacteria also display higher levels of intracellular Mn^{2+} , indicating the crucial role that this element plays in the bacterial radiation resistance phenotype (Daly *et al.* 2004). The accumulation of Mn^{2+} inside the cells acts on the level of preventing protein oxidation, rather than DNA damage resulting from ionizing radiation. Moreover, Mn^{2+} /orthophosphate complexes discovered in *D. radiodurans* are able to catalytically remove superoxide, thereby protecting cells from its deleterious effects (Barnese *et al.* 2008; Daly *et al.* 2010). In line with this, Mn^{2+} /peptide complexes were also observed to act as ROS scavengers (Berlett *et al.* 1990; Daly *et al.* 2010). Enzymatic antioxidant strategies, on the other hand, rely on the intervention of several enzymes such as catalase and superoxide dismutase in order to convert ROS into harmless molecules like H_2O . Although *D. radiodurans* and *E. coli* harbor approximately the same number of these enzymes, they are more active in the former rather than in the latter organism (Hua *et al.* 2003; Tian *et al.* 2004).

Another example of a radiation-resistant bacterium is *Kineococcus radiotolerans*, which was isolated from a radioactive area at the Savannah River Site, USA (Phillips *et al.* 2002). It displays a relatively high resistance to ionizing radiation ($D_{10} = 2$ kGy) in addition to being highly resistant to other stressors such as desiccation and oxidative stress.

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This organism relies on the use of divalent cations such as Cu^{2+} as a protective mechanism against radiation (Bagwell *et al.* 2008b). Similar to other radiation-resistant bacteria, *K. radiotolerans* also exhibits high $[\text{Mn}]/[\text{Fe}]$ ratios, although it contains lower intracellular concentrations of both ions overall (Bagwell *et al.* 2008b). Furthermore, DNA repair mechanisms are proposed to be of higher importance in the radiation-resistance phenotype of *K. radiotolerans* than ROS scavenging strategies. This is demonstrated by the fact that the majority of genes upregulated by exposure to ionizing radiation are involved in DNA replication, recombination and repair, whereas only one of the many ROS scavenging genes contained in the genome is upregulated under the same irradiation conditions (Bagwell *et al.* 2008a; Li *et al.* 2015).

Other bacteria displaying a high resistance to radiation are the thermophilic species *Rubrobacter radiotolerans* ($D_{10} = 11$ kGy) and *Rubrobacter xylanophilus* ($D_{10} = 5.5$ kGy), two members of the Actinobacteria isolated from hot water environments (Yoshinaka *et al.* 1973; Carreto *et al.* 1996; Ferreira *et al.* 1999). *R. radiotolerans* exhibits radiation resistance mechanisms that sensibly differ from the ones found in other radiation-resistant bacteria. Indeed, the number of DNA DSBs occurring after exposure to ionizing radiation is relatively lower in this species when compared to other radiation-resistant bacteria, with 2.03 and 7.5 DNA DSBs occurring per genome upon exposure to 1 kGy of gamma-radiation for *R. radiotolerans* and *D. radiodurans*, respectively (Kitayama & Matsuyama 1971; Terato *et al.* 1999). In addition, *R. radiotolerans* contains a specific carotenoid pigment, namely bacterioruberin, which also aids in its resistance to radiation (Saito *et al.* 1994).

4.4.2 Archaea

Archaea are microorganisms known to inhabit extreme environments such as waters with high saline concentrations and extremely high or low pH. One example of such an extremophile is *Thermococcus gammatolerans*, which was isolated from a deep-sea hydrothermal vent and is able to survive a dose of 3 kGy without significant loss of cell viability (Jolivet *et al.* 2003a). The radiation resistance of this species depends mostly on the availability of nutrients in the environment (Tapias *et al.* 2009). Contrary to other Archaea, *T. gammatolerans* does not harbor unique DNA repair genes, but instead expresses proteins involved in DNA repair mechanisms constitutively (Zivanovic *et al.* 2009).

Other hyperthermophiles such as *Pyrococcus furiosus* and *Pyrococcus abyssi* (D_{10} for both = 3 kGy) display levels of radiation resistance similar to *T. gammatolerans* (DiRuggiero *et al.* 1997; Gerard *et al.* 2001). The resistance to ionizing radiation displayed by these strains might be a byproduct of their resistance to extremely high temperatures (up to 100°C), as DNA damage induced by ionizing radiation is similar to damage induced by high temperatures. The radiation-resistance mechanisms of *Pyrococcus* strains appear to be similar to the ones exhibited by *T. gammatolerans* (Williams *et al.* 2007). Indeed, the majority of genes associated with DNA repair mechanisms and the genes implicated in ROS detoxification and homeostasis are constitutively expressed after exposure to gamma-radiation in these species (DiRuggiero *et al.* 1997; Jolivet *et al.* 2003b).

Halobacterium salinarum (D_{10} = 5 kGy) is a halophilic archaeon whose radiation-resistance mechanisms have been extensively studied (Kottemann *et al.* 2005). It displays high intracellular concentrations of inorganic cations such as K^+ as well as osmotic regulators as adaptive mechanisms to survive the extremely saline environment it inhabits (Kokoeva *et al.* 2002; Engel & Catchpole 2005). Next to osmotolerance, this species also displays high levels of resistance to desiccation, UV radiation, vacuum and most notably gamma-radiation (Baliga *et al.* 2004; Kottemann *et al.* 2005). High concentrations of intracellular salts such as KCl efficiently mitigate the deleterious effects of ROS on cellular structures, which renders this strain functionally resistant to gamma-radiation (Marguet & Forterre 1998; Shahmohammadi *et al.* 1998). In addition, *H. salinarum* exhibits polyploidy, defined as having multiple copies of its full chromosome set (Breuert *et al.* 2006). This might be an additional mechanism for radiation resistance as extra copies of the same gene might compensate for the loss of genetic information potentially occurring after exposure to ionizing radiation. Furthermore, this species also displays a high $[Mn]/[Fe]$ ratio of 0.27, equivalent to the one exhibited by *D. radiodurans* (Daly *et al.* 2007; Kish *et al.* 2009). It contains small molecules such as orthophosphate and peptides, which can associate with Mn^{2+} ions to form ROS-scavenging complexes, in higher concentrations than the ones observed in the radiosensitive *E. coli* (Robinson *et al.* 2011). Finally, *H. salinarum* also contains the carotenoid pigment bacterioruberin, which gives it its characteristic bright pink color and exhibits a ROS scavenging activity, thereby effectively protecting the DNA structure from the adverse effects of ionizing radiation (Saito *et al.* 1997).

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4.4.3 Eukaryotes

In general, eukaryotic organisms such as plants and animals typically display a higher sensitivity to ionizing radiation (mainly gamma-radiation) than their archaeal and bacterial counterparts. However, some organisms such as certain groups of fungi display a remarkably high resistance to radiation (Saleh *et al.* 1988).

Alternaria alternata for example is a filamentous fungus found in highly radioactive sites such as in the proximity of the Chernobyl nuclear power plant (Mironenko *et al.* 2000). The main mechanism supporting the radiation resistance in this species is the presence of the black pigment melanin, which accumulates in the fungal mycelium (Kimura & Tsuge 1993). Indeed, fungi containing melanin in their cells supplant other fungal species in radioactive areas at the Chernobyl power plant and are even found inhabiting the walls of the reactor (Vember & Zhdanova 2001; Zhdanova *et al.* 2004).

Another example of a radiation-resistant fungus is *Cryptococcus neoformans*, which also contains melanin. This pigment seems to mediate energy transduction upon exposure to ionizing radiation, as radiation alters its electron transfer properties (Dadachova *et al.* 2007). *C. neoformans* might therefore utilize gamma-radiation as an energy source through the melanin-mediated conversion of electromagnetic energy into chemical energy. Some fungi isolated from radioactive environments indeed exhibit a phenomenon called radiotropism, where they grow towards the radiation source rather than away from it (Zhdanova *et al.* 1991). Fungal melanin was therefore proposed to play a similar role as chlorophyll in phototrophic plants. In addition to this energy transduction effect, melanin also protects cells from various environmental stressors such as oxidative damage, heavy metals and UV radiation (Nosanchuk & Casadevall 2003). It plays a role in radiation resistance through the scattering of photons and electrons, thereby acting as a barrier against the direct effects of radiation, as well as through ROS scavenging, hence mitigating the indirect effects (Dadachova *et al.* 2008). Interestingly, *C. neoformans* can produce melanin only when the appropriate substrates are provided.

Finally, some microalgae such as *Coccomyxa actinabiotis* isolated from a nuclear reactor environment have also been shown to be extremely resistant to ionizing radiation, with a D₅₀ of 10 kGy (Rivasseau *et al.* 2010, 2013, 2016). It probably uses highly efficient protection and repair mechanisms to maintain its cell function, such as a high intracellular [Mn]/[Fe] ratio.

Chapter II – Objectives

Chapter II – Objectives

Nuclear reactors contain various watery environments, such as spent nuclear fuel pools for the intermediate storage of spent nuclear fuel underwater, the primary and secondary cooling circuits for the cooling of nuclear fuel in the reactor vessel and different ultrapure water tanks for the replenishing of evaporated water. These water systems are maintained at high purity levels through constant filtration and deionization in purification circuits, resulting in ultrapure waters with low conductivities and nutrient levels, occasionally exposed to high radiation levels when they come into close contact with nuclear fuel.

Despite the extremely oligotrophic conditions in these waters combined with the presence of radioactivity, microorganisms such as bacteria, fungi and eukaryotic microalgae have been previously detected. While most of the previous studies were performed on spent nuclear fuel pools, the aim of this work was to investigate the bacterial communities in different watery environments of the BR2 nuclear research reactor at SCK CEN in Mol, Belgium, with a particular focus on an open basin surrounding the reactor vessel.

We first wanted to investigate the viable microbial population in a range of interlinked watery environments, namely the basin surrounding the reactor core, two storage pools within it, the primary circuit, the spent nuclear fuel pool as well as an external storage and refill tank containing ultrapure water. For this purpose, we isolated individual strains on oligotrophic culture media and proceeded with a first characterization of the radiation susceptibility of some of the isolated strains.

Secondly, we aimed to further characterize the microbial community specifically inhabiting the previously described basin. As the BR2 reactor runs in successive cycles of operation and shutdown, this creates a highly dynamic environment with periodically shifting physico-chemical parameters such as temperature, radiation and flow rate. Therefore, we also wanted to investigate the effects of these changing conditions on the long-term dynamics of the bacterial community using a 16S rRNA amplicon sequencing approach.

Finally, we adopted a shotgun metagenomics sequencing approach to characterize the bacterial community inhabiting the basin more in depth, both on the taxonomic and functional level. To this end, a specialized filtration system was custom designed to allow for the collection of a sufficient amount of biological material needed for such analyses.

Chapter III – Results

Chapter III – Section 1.

Isolation and identification of bacteria from nuclear
reactor waters

So far, the microbial communities in the waters of the BR2 nuclear research reactor have remained largely unstudied. In this work, we aimed to characterize the bacterial diversity in different interlinked watery environments from this reactor through a cultivation-based approach.

Moreover, we also tested the radiation sensitivity of some of the isolated strains from the basin surrounding the reactor core and SNFP by irradiating them at various doses using ^{60}Co as a gamma-radiation source in a specialized irradiation facility located in the SNFP.

This section is presented as a manuscript in preparation.

Isolation and identification of bacteria from various nuclear reactor waters

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Abstract

In this study, we investigated the microbial diversity in a variety of interlinked watery environments from an active nuclear reactor using a culture-based approach. We managed to build and store an extensive strain collection consisting of 33 distinct species across all the different environments, which is the largest catalogue of isolates in such nuclear waters described so far. Some overlap in microbial composition was detected between the different environments, with most water systems containing members of the *Bradyrhizobium*, *Curvibacter* and *Pelomonas* genera. Other environments however displayed unique species compositions, such as the basin and primary circuit, containing species such as *Hydrobacter penzbergensis* and *Methylobacterium fujisawaense*, respectively. This is likely due to the distinct physico-chemical characteristics in these environments. Furthermore, the radiation resistance potential of some species from the basin water and spent nuclear fuel pool was characterized, resulting in the identification of *Sphingomonas melonis* as a radiation-resistant species, as it managed to survive a cumulative dose of 2.1 kGy. Further characterizations such as heavy metal and radionuclide uptake potential as well as whole genome sequencing will shed new light onto potential candidates for bioremediation applications of contaminated environments.

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Introduction

Watery environments in nuclear reactors are constantly maintained in an ultrapure state by means of filtering and deionization through specialized purification circuits, containing specific filters and ion exchangers. This ensures the removal of any remaining radionuclides, together with other impurities and ions that could become activated by the radiation present in these environments. Despite the rather challenging conditions, combining an extremely low ionic strength and nutrient availability with the presence of varying levels of radiation, some microbes have been previously detected in these extremely oligotrophic environments. Several studies have investigated the microbes in watery environments inside the reactor buildings and surrounding the active nuclear reactors (Rao *et al.* 2000, 2009; Balamurugan *et al.* 2011; Kéki *et al.* 2013, 2019; Props *et al.* 2016, 2019; Petit *et al.* 2020). Most studies however have been performed on spent nuclear fuel pools (SNFPs), both indoor and outdoor (Santo Domingo *et al.* 1998; Bruhn *et al.* 1999, 2009; Diosi *et al.* 2003; Sarró *et al.* 2003, 2005, 2007; Chicote *et al.* 2004, 2005; Galès *et al.* 2004; Masurat *et al.* 2005; Giacobone *et al.* 2011; Rivasseau *et al.* 2016; McGraw *et al.* 2018; Foster *et al.* 2020). These pools are used to temporarily store nuclear fuel that is no longer usable in underwater racks. The water in these pools serves as coolant as well as shielding against the residual radiation emanating from the spent fuel rods.

The microbial populations residing in these environments can be found in the form of planktonic cells or biofilms formed on metal surfaces or on the walls of SNFPs (Sarro *et al.* 2003, 2005, 2007; Tisakova *et al.* 2013; Dekker *et al.* 2014). One study focusing on the isolation and identification of bacteria in these environments found a rather large species diversity, with a total of 21 different strains belonging to several genera such as *Bradyrhizobium*, *Burkholderia*, *Chryseobacterium*, *Methylobacterium*, *Ralstonia*, *Sphingomonas*, and *Staphylococcus* (Chicote *et al.* 2005). Another study collected six isolates, among which *Chryseobacterium* and *Staphylococcus* strains (Karley *et al.* 2019), suggesting that there might be an overlap in the microbial community composition between similar environments. Although the cultivable microbial populations found in SNFPs were largely dominated by bacteria, some eukaryotes were also discovered, such as fungi (Chicote *et al.* 2004; Silva *et al.* 2018) and microalgae (Rivasseau *et al.* 2016; McGraw *et al.* 2018).

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It was shown that some biofilms and isolates were capable of accumulating specific radionuclides, such as ^{137}Cs and ^{60}Co (Tisakova *et al.* 2013; Dekker *et al.* 2014) as well as ^{238}U , $^{110\text{m}}\text{Ag}$ and ^{54}Mn (Rivasseau *et al.* 2013). In addition, it was found that some microbes were capable of withstanding higher doses of radiation (up to several kGy), both as pure cultures (Diosi *et al.* 2003; Rivasseau *et al.* 2016; Karley *et al.* 2018; Foster, Muhamadali, *et al.* 2020) and as biofilms (Bruhn *et al.* 2009). It is important to characterize and specifically to monitor the microbial communities in SNFPs, as potential blooms in these environments could impact visibility during operations and maintenance, or even present a real health hazard (e.g. due to the presence of *Legionella* strains). Such blooms have been previously reported in other similar environments such as SNFPs located at Sellafield, UK (McGraw *et al.* 2018; Foster *et al.* 2020).

Our work focused on the isolation and identification of bacterial strains from an indoor SNFP in a nuclear reactor, but also from the primary cooling water circuit passing directly through the reactor core vessel as well as a water basin closely surrounding this vessel. These are highly dynamic and extreme watery environments, as they undergo various consecutive cycles of operation and shutdown and are exposed to high radiation doses. In addition, we also focused on a buffer tank filled with demineralized ground water used to collect spillover water from the basin during maintenance periods (storage tank), and the demineralized water tank used to refill the primary circuit to account for evaporation (DW2 tank). Furthermore, this study also aims to characterize the radiation susceptibility of some of the isolated strains.

Materials and methods

Sampling sites

All water samples were taken from the Belgian Reactor 2 (BR2), located at the Belgian Nuclear Research Centre (SCK CEN) in Mol, Belgium. The main function of this reactor is the production of radio-isotopes for medical purposes, and doped silicon for the semi-conductor industry, but it can also be used to test the properties of different materials under various irradiation conditions. The reactor runs in successive cycles of approximately 30 days followed by shutdown periods of variable length to allow for general maintenance. It contains different aquatic environments (Fig. 1), such as the reactor core vessel with a volume of 35 m³, where the nuclear fuel is loaded and various targets are bombarded with high fluxes of neutrons.

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This vessel is filled with water from the primary circuit, a closed loop with a total volume of 150 m³ that cools down the nuclear fuel when the reactor is operational. During cycles, the primary circuit is continuously replenished with water from a tank located outside the reactor building containing demineralized water (DW2) at a rate of approximately 20 l/h to account for evaporation losses. The water passes through a purification circuit for filtration and deionization, resulting in purified water with a conductivity of approximately 0.6 µS/cm. The water temperature in this tank is approximately 20 °C.

The core vessel is directly surrounded by a large central basin of 870 m³ that is open to the ambient air. The water in this basin acts as a shield against the radiation emanating from the core and circulates through a cooling circuit where it can cool down before being pumped back again into the pool through an inlet in the reactor mantle. When it reaches the top of the reactor vessel, it flows over the outer wall back into the basin. During shutdowns, the upper water layer is pumped to a tank with a 1000 m³ capacity located outside the reactor building (storage tank) in order to allow technicians to access the lower areas of the basin for general maintenance purposes. Before the start of each new cycle, the water is pumped back into the basin. A summary of the physico-chemical parameters of the primary circuit and basin for both the shutdown and cycle conditions can be found in Table 1.

Table 1. Physico-chemical parameters for the primary circuit and basin water

Environment	Primary circuit		Basin	
Condition	Shutdown	Cycle	Shutdown	Cycle
Water Temperature (°C)	Room temperature (ca. 21 ± 2)	~40	Room temperature (ca. 21 ± 2)	31 - 37
Pressure (bar)	Atmospheric	12	Atmospheric	
Radiation dose rate (kGy/h)	Not measured	~40	0.001 (residual radiation)	0.755 deposited close to the reactor vessel
Gamma activity (Bq/l)	Depends on ²⁴ Na decay	5 x 10 ⁶	< 80	2 x 10 ³
Flow rate (m³/h)	20	7000	50	500
Conductivity (µS/cm)	0.1 – 0.5		1.0 – 1.2	
Chemistry (µg/l)	Not measured		Total inorganic carbon (TIC): < 500 Total organic carbon (TOC): < 700 Acetate: 67 ± 33	

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			Nitrate: 44 ± 22 Nitrite: < 10
pH	7 ± 0.4	6 ± 0.4	6 ± 0.4
H₂	Not measured		
O₂ (ppm)	Not measured		Estimated at 8

At the end of each cycle, the nuclear fuel that has been used in the core vessel is transferred to a smaller pool of 85 m³ located in the upper part of the basin, the Gamma Irradiation Facility (GIF, Fig. 1), that also serves as an intermediate storage facility for produced radio-isotopes. Another small pool with a volume of 108 m³ is located just across the GIF, namely the Core Mock-up Facility (CMF), that is used to store materials and components that have been tested for high level irradiation in the core vessel. These two pools are separated from the central basin via dams, which are regularly opened during cycles to allow for the transport of radio-isotopes and other materials in and out. During shutdowns the dams stay closed. The physico-chemical composition of the water in the GIF and CMF is the same as in the basin, with the exception that both pools are subjected to higher doses of radiation, as they come into direct contact with nuclear fuel and irradiated materials, respectively.

The basin is connected to an adjacent SNFP via a transfer shunt, through which radio-isotopes and spent fuel that is no longer usable can be transported. The SNFP is a large pool of 1100 m³ where spent fuel from the core vessel is stored intermediately in underwater racks before being disposed to its final location. Each transfer between the basin and the SNFP therefore causes a mixture of both water systems. Furthermore, the SNFP and basin share a common purification circuit, which causes additional mixing. The water in the SNFP is at room temperature (21 ± 2 °C). A summary of all environments can be found in Table 2.

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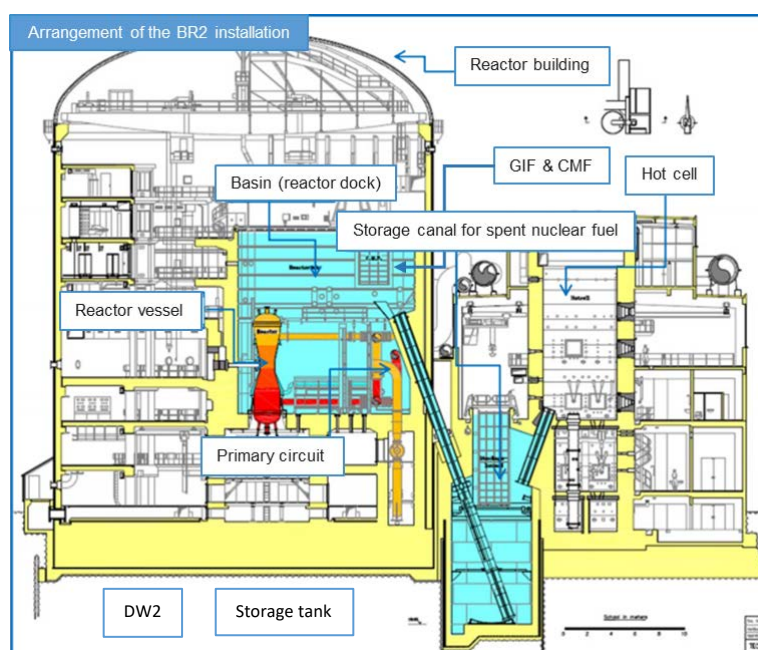


Figure 1. Overview of the watery environments of the BR2 reactor. All watery environments are represented in blue, orange and red. The reactor vessel system is color-coded in shades of orange and red in function of the water temperature in the primary circuit. The transfer shunt connects the basin with the SNFP (storage canal for spent nuclear fuel). The DW2 tank connected to the primary circuit and the storage tank connected to the basin are located outside of the reactor building.

Table 2. Summary and description of the watery environments of the BR2 reactor.

Environment	Description	Exposure to radiation
Primary circuit	Closed loop, cools down nuclear fuel in core vessel	Yes
Basin	Open pool surrounding core vessel	Yes
GIF	Small pool in basin, short-term storage of nuclear fuel and radio-isotopes	Yes
CMF	Small pool in basin, storage of irradiated materials	Yes
SNFP	Open pool, intermediate storage of spent nuclear fuel	Yes
Storage tank	Tank outside reactor building, buffer for basin water during shutdowns	No
DW2	Tank outside reactor building, refilling primary circuit during cycles	No

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Strain isolation

Strains from all previously described watery environments (primary circuit, basin, GIF, CMF, SNFP, storage tank and DW2 tank) were isolated by spreading water samples taken at different timepoints (February 2016, August 2016, December 2018, January 2019, February 2019) directly onto cultivation plates containing solid culture medium. Different culture media were tested in different dilutions and incubation temperatures, namely Luria-Bertani (LB) (Bertani 1952), Tryptic Soy Agar (TSA) (Difco 1998) and Reasoner's 2A (R2A) (Reasoner & Geldreich 1985). Since undiluted R2A incubated at room temperature yielded the best results (highest number of colony-forming units), this medium was selected for all further strain isolations. Briefly, 100 µl of collected water was spread onto R2A agar plates, which were incubated at room temperature (equivalent to the water temperature of all environments under shutdown conditions) until bacterial colonies appeared. The colony-forming units (CFUs) were subsequently enumerated and expressed as CFUs/ml. Different colonies were then selected based on differences in their morphologies such as shape, size, height or pigmentation. These colonies were then purified by restreaking them onto fresh R2A plates three consecutive times. As a side note, the isolation effort was not equal across all environments, resulting in a different number of isolates per environment.

16S rDNA amplification and Sanger sequencing

For the strains isolated in 2016, the 16S rRNA gene was amplified through colony PCR using the universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 926R (5'-CCGTCAATTCCTTTRAGTTT-3'). For each individual strain, one colony was suspended into 50 µl of milli-Q water and heated up at 95°C for five min. A master mix was then prepared for the PCR containing 2 µl of forward and reverse primers (0.4 µM), 25 µl DreamTaq Green PCR Master Mix (x2) (Thermo Fisher Scientific, Waltham, MA, USA), 20 µl milli-Q water and 1 µl template DNA from the heated cell suspension for a total volume of 50 µl per sample. 25 amplification cycles were applied, preceded by an initial denaturation step at 95 °C for 2 min. Each cycle consisted of a denaturation step at 95 °C for 30 sec, followed by annealing at 50 °C for 30 sec and extension at 72 °C for 1 min. After the last cycle, a final extension was performed at 72 °C for 10 min.

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The PCR products were then purified using a PCR clean-up kit (Promega, Madison, WI, USA) and run on an agarose gel (1% w/v) in TBE 1x buffer to visually check the inserts.

The PCR products were subsequently sent for Sanger sequencing to Eurofins Genomics (Ebersberg, Germany) at a concentration of 5 ng/μl.

For the strains isolated in 2018-2019, individual colonies from purified strains were grown in liquid R2A medium and sent directly to BaseClear (Leiden, the Netherlands) for genomic DNA isolation, amplification of the 16S rRNA gene using typical sequencing primers (XB16NOT (= W1 16SF) and 16S-870rev), PCR product purification, agarose gel analysis and Sanger sequencing.

For the species identification, the resulting merged forward and reverse reads were aligned with the NCBI Genbank database using the nucleotide Basic Local Alignment Search Tool (BLASTn) with a sequence similarity threshold of 97%. All sequences below that threshold were not taken into account for further analyses. They could nonetheless represent novel species that have not yet been included in the NCBI database.

Scanning Electron Microscopy (SEM)

An additional sample was taken from the SNFP in October 2020 at a depth of eight meters using a submersible pump located near the bottom of the pool. First, the pump was activated for one min at a high flow rate (20 liters per min), after which three glass bottles of one liter were filled. The samples were then checked by the radiation control department and subsequently transported to a laboratory where radioactive samples are allowed to be processed. They were filtered over 0.22 μm pore sized polyethersulfone (Supor®) filter membranes (Pall Corporation, Port Washington, NY, USA), of which a piece was cut out with sterile scissors to be prepared for the SEM analysis. The filter membrane piece was dehydrated by immersion in a series of solutions with ascending concentrations of ethanol (30, 50, 70, 90, 95 and 100% v/v). The final solution (100% ethanol) was renewed three times to ensure complete dehydration of the filtrate and the filter. It was then chemically dried by threefold immersion in pure hexamethyldisilazane (HMDS) for approximately 10 min. The resulting dried filter was mounted on an aluminum SEM stub using double-sided carbon tape to ensure electrical conductivity. It was sputter coated with gold for 120 sec at 700 V, with a current of 50 mA, using a gold sputter-coater (Scancoat six, UK).

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The material present on the surface of the filter membrane was visualized using a 5th generation Phenom-World Tabletop SEM (Thermo Fischer Scientific, Waltham, MA, USA) at a 6200x magnification. An Energy Dispersive X-ray (EDX) mapping analysis was also performed to check the elemental composition on the present material.

Irradiation experiment

To test the radiation susceptibility of some of the isolated strains, five strains from the basin and six strains from the SNFP were irradiated during a preliminary experiment using the RITA irradiation facility located in the SNFP of the BR2. This facility consists of a metal cylinder with a diameter of 38 cm and a height of 60 cm that can be lowered into the water between four rods arranged in a square shape, containing ⁶⁰Co as a gamma radiation source (see Fig. 2). The cylinder is kept under a pressure of 1.4 bar to prevent water from flowing into it. For the experiment, the ⁶⁰Co sources in the rods were positioned in such a way that the resulting dose rate amounted to 0.6 kGy/h. The 12 strains from the basin and SNFP combined were cultured in 5 ml liquid R2A, after which 200 µl was transferred to a 96-well plate. The plates were then secured into the metal cylinder of the RITA facility. Three different irradiation conditions were chosen, which were obtained by exposing the cultures to the ⁶⁰Co radiation for different amounts of time before lifting the cylinder out of the water. The first dose was 300 Gy, for which the cylinder was maintained under water for 30 min. The second dose was 2.1 kGy, for a total exposure time of 3h30 and the final dose of 5 kGy amounted to a total exposure time of 8h20. One non-irradiated control was also added per dose, which was kept next to the RITA facility in the dark for the same amount of time as the irradiated samples. Since this was a preliminary experiment, only one replicate was used per strain and per condition. After irradiation, serial dilutions were performed in the same 96-well plates, ranging from 10⁻¹ to 10⁻⁷. Next, viable cell counts were assessed by spotting 10 µl of each dilution onto R2A agar plates, which were subsequently incubated at room temperature on a laboratory bench under light conditions until colonies appeared after approximately two weeks. The CFUs in the countable dilutions (between 6 and 60 CFUs per spot) were enumerated, converted to CFUs/ml and compared with the control condition.

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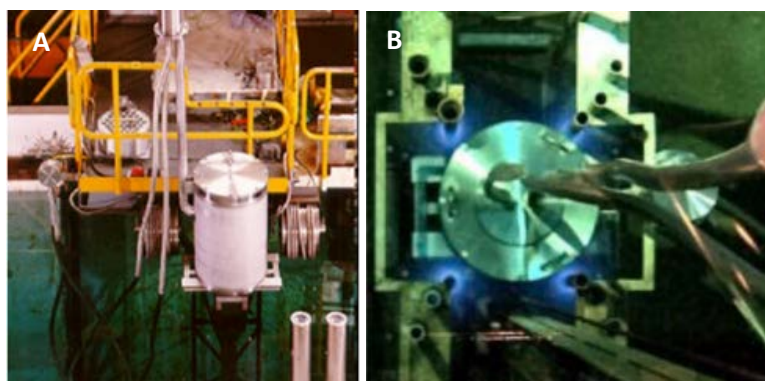


Figure 2. Gamma irradiation facility (RITA) in SNFP. A. Metal cylinder lifted out of the water. B. Metal cylinder lowered in the water in between the four ^{60}Co rods. Cherenkov radiation is visible under the form of a blue light emitted by the rods.

Results & discussion

Strain isolation

We managed to isolate a variety of strains from different watery environments (Table 3), during active cycles as well as during shutdowns of the nuclear reactor. From all the environments, a total of 33 distinct species were isolated and stored at $-80\text{ }^{\circ}\text{C}$ for future characterization. Most identified species are Gram-negative, aerobic heterotrophs. Some species are capable of growing on acetate (present in the basin water at a concentration of $67 \pm 33\text{ }\mu\text{g/l}$, see Table 1) as a sole carbon source, such as *Methylobacterium fujisawaense* and *Methylbium petroleiphilum* (Green *et al.* 1988; Nakatsu *et al.* 2006). However, some are also capable of hydrogeno-autotrophic growth, *i.e.* fixation of CO_2 while oxidizing hydrogen, such as *Pelomonas saccharophila* (Gomila *et al.* 2007). Moreover, some *Bradyrhizobium* strains are known to be photosynthetic (oxidation of H_2O) (Giraud *et al.* 2007; Giraud *et al.* 2013). The *Bradyrhizobium*, *Mesorhizobium* and *Methylobacterium* genera mostly found in the basin water and primary circuit are capable of nitrogen fixation, either in the symbiotic or free-living state (Reeve *et al.* 2013; Avontuur *et al.* 2019). This shows that different species have adopted a variety of strategies for their energy metabolism in these extremely oligotrophic watery environments.

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Some species found in our system such as *Hydrobacter penzbergensis*, *P. saccharophila* and *Ralstonia pickettii* as well as some *Bradyrhizobium*, *Mesorhizobium*, *Methylobacterium* and *Sphingomonas* species, were previously isolated from similar ultrapure water environments, namely the water purification system of a nuclear power plant and purified water systems from the pharmaceutical industry and semiconductor industry (Kéki *et al.* 2013; Eder *et al.* 2015; Mittelman & Jones 2018).

Among all the investigated water systems, those containing the least amount of bacteria in terms of CFU/ml were the primary circuit (during a cycle of operation), the Gamma Irradiation Facility (GIF) and the Core Mock-up Facility (CMF), with 1×10^1 , 1×10^1 and 3×10^1 CFU/ml, respectively. This can be explained by the fact that these environments are most exposed to ionizing radiation. Indeed, the radiation in the primary circuit during cycles is estimated at 40 kGy/h, due to the nuclear fission reaction taking place in the reactor core. The GIF and CMF are also exposed to high doses of radiation as they serve as intermediate storage facilities for nuclear fuel and irradiated materials, respectively. This suggests that bacteria are killed by the harsher conditions in these environments, which is also confirmed by comparing the CFU counts in the basin and primary circuit between the cycle and shutdown conditions (see Table 3), as radiation is much higher during cycles for both environments. All these bacterial density values are similar to those found in other nuclear reactor waters (values generally ranging between 10^1 and 10^4 CFU/ml), such as SNFPs (Chicote 2005; Tisakova *et al.* 2013; Karley *et al.* 2017, 2019) and other ultrapure water systems such as storage tanks and water purification circuits (Bohus *et al.* 2011; Kéki *et al.* 2019).

Although the primary circuit and the DW2 tank are connected to each other, only little overlap in species composition was detected between them. The physico-chemical characteristics of these two environments are indeed very different, since the primary circuit is a highly dynamic system with extreme conditions of high flow rate, pressure and radiation during cycles, followed by periods of shutdown where the conditions revert to baseline values (see Table 1). On the contrary, the DW2 tank is a rather static environment and is not exposed to radiation. The distinct selection pressures in both environments are therefore likely to lead to different microbial communities. A similar pattern could be observed for the basin and the storage tank. Although they are also in contact with each other, only some overlap was detected, with two out of the five identified species from the storage also detected in the basin water (*Mesorhizobium atlanticum* and *Sphingomonas echinoides*).

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Table 3. Bacterial strains isolated in the various environments.

Environment	BW_S	BW_C	PW_S	PW_C	SNFP	Storage tank	DW2	GIF	CMF
Sampling date	2/2016, 8/2016, 12/2018	2/2019	8/2016, 1/2019	2/2019	2/2016	12/2018	2/2019	2/2019	2/2019
CFU/ml	1 x 10 ³	4 x 10 ²	2 x 10 ²	1 x 10 ¹	1 x 10 ³	2 x 10 ³	2 x 10 ²	1 x 10 ¹	3 x 10 ¹
Bacterial species	Number of isolates/species/environment								
<i>Afipia broomeae</i>					1	1			
<i>Bacillus firmus</i>					1				
<i>Bradyrhizobium americanum</i>									1
<i>Bradyrhizobium centrosema</i>	2								
<i>Bradyrhizobium denitrificans</i>	9		39	3					
<i>Bradyrhizobium diazoefficiens</i>			2						
<i>Bradyrhizobium embrapense</i>			6						
<i>Bradyrhizobium erythrophlei</i>			7						
<i>Bradyrhizobium ganzhouense</i>			3						
<i>Bradyrhizobium liaoningense</i>			1						
<i>Bradyrhizobium oligotrophicum</i>	1								
<i>Bradyrhizobium paxllaeri</i>			1						
<i>Bradyrhizobium subterraneum</i>	1								
<i>Bradyrhizobium yuanmingense</i>	1								
<i>Brevibacillus centrosporus</i>			1						
<i>Brevibacillus nitrificans</i>		1							
<i>Curvibacter fontanus</i>	18		1	2			5		1
<i>Hydrobacter penzbergensis</i>	1								

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<i>Mesorhizobium atlanticum</i>	5	3				3			
<i>Mesorhizobium opportunistum</i>	11				2				
<i>Methylibium petroleiphilum</i>	5		1						
<i>Methylobacterium fujisawaense</i>			1						
<i>Mycolicibacterium aubagnense</i>					1				
<i>Pelomonas aquatica</i>	1		14		1				
<i>Pelomonas saccharophila</i>	5	1	15		1			1	1
<i>Ralstonia pickettii</i>	7						9		
<i>Siccirubricoccus deserti</i>						1			
<i>Sphingomonas echinoides</i>	2	1				2			
<i>Sphingomonas leidyi</i>						1			
<i>Sphingomonas melonis</i>					1				
<i>Staphylococcus hominis</i>			2						
<i>Staphylococcus warneri</i>			3						
<i>Vibrionimonas magnilacihabitans</i>			1						
Total species	14	4	16	2	7	5	2	1	3
Total isolates	69	6	98	5	8	8	14	1	3

BW = basin water, PW = primary water, S = shutdown, C = cycle, DW = demineralized water, GIF = gamma irradiation facility, CMF = core mock-up facility.

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Interestingly, *P. saccharophila* could be isolated from all environments exposed to ionizing radiation to varying degrees (primary circuit, basin, SNFP, GIF and CMF), but was absent from the two remaining environments not exposed to radiation (storage and DW2 tanks). A possible explanation for this could be that ionizing radiation causes water radiolysis, which results in the production of H₂, among other chemical species (Dzaugis *et al.* 2015). Since this *Pelomonas* species is capable of growing autotrophically on H₂, these environments could promote its survival and/or growth, whereas the ones that are not exposed to radiation could not provide the necessary H₂ supply. However, since we managed to isolate it on R2A in the absence of H₂, this strain is probably not strictly autotrophic. The presence of H₂ could nonetheless further promote its growth.

Some environments such as the SNFP also contain unique species not found in other environments, such as *Bacillus firmus*, *Mycolicibacterium aubagnense* and *Sphingomonas melonis*. In order to illustrate the microbial diversity of this environment, a Scanning Electron Microscopy (SEM) picture was taken (see Fig. 3). An EDX analysis was performed and revealed that the observed curved shapes consisted mainly of carbon, oxygen, nitrogen, sulfur and silicon, while phosphorus was just below the detection limit. Elemental hydrogen can technically not be detected with EDX, but is present as part of H₂O. This is the primary elemental composition of any life form, hence also microbes, confirming the biological nature of the visible curved shapes. Some microorganisms exhibited a slight constriction suggesting that they are in the process of cell division and are potentially actively growing.

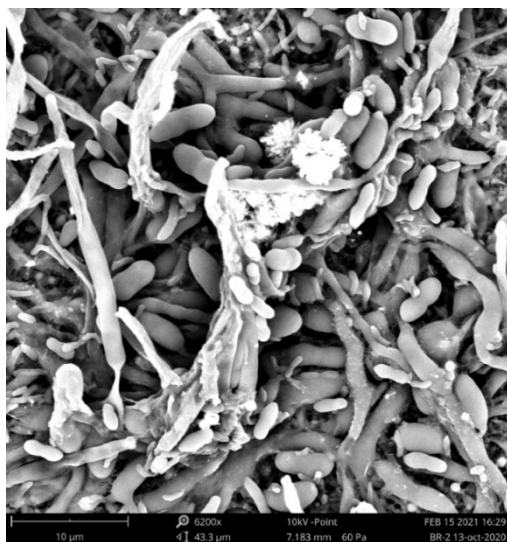


Figure 3. SEM picture of a Spent Nuclear Fuel Pool (SNFP) water sample. The sample was taken at eight meters of depth using a submersible pump. Microorganisms are visible as curved shapes of various sizes. Shapes with an indentation in the middle are most probably actively dividing. Used magnification: 6,200x.

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Irradiation experiment

A first characterization of the radiation resistance potential of some of the isolated strains was attempted through an irradiation experiment at the RITA facility in the SNFP of the BR2 reactor. Five strains from the basin and six strains from the SNFP were selected and irradiated at a dose rate of 600 Gy/h using ^{60}Co as a gamma radiation source. As can be seen in Fig. 4, all of the irradiated strains survived the dose of 300 Gy, with a reduction of 2-3 log CFU/ml. Only a single strain from the SNFP survived the 2.1 kGy dose, namely *S. melonis*, whereas none survived the 5 kGy dose. *S. melonis* is a Gram-negative, rod-shaped, non-motile and non-spore-forming bacterium with a respiratory metabolism forming yellow-pigmented colonies (Buonaurio *et al.* 2002). It was first isolated from Spanish melon fruits. Two other *Sphingomonas* species isolated from fresh water collected at a radioactive site in Japan were also shown to be slightly tolerant against gamma radiation, namely *Sphingomonas astaxanthinifaciens* and *Sphingomonas jaspsi* (Asker *et al.* 2007a, 2007b). These two species showed a 94% and 95% similarity with *S. melonis* when comparing their 16S rRNA gene sequences and had a 2.1% and 0.45% survival rate after exposure to a gamma radiation dose of 2.3 kGy originating from a ^{137}Co source, respectively. They were also characterized by their carotenoid production, resulting in their yellow-pigmented aspect. Carotenoids can serve as protectants against UV radiation and Reactive Oxygen Species (ROS).

Some *Bacillus* species also contain carotenoids, among which *B. firmus*, and these exhibit significantly higher levels of resistance to UV radiation than the non-carotenoid-containing species (Khaneja *et al.* 2010). Out of the other investigated species, some *R. pickettii* strains isolated from the Mars Odyssey Orbiter were also associated with a higher resistance to UV-C radiation (Mijnendonckx *et al.* 2013). Testing the radiation susceptibility as well as other relevant properties such as radionuclide and heavy metal uptake would be of particular interest for a larger selection of isolated strains in the future. Performing whole genome sequencing of some strains displaying high resistance to radiation and/or radionuclide uptake would also be a worthwhile endeavor in order to investigate the genomic adaptations responsible for this phenotype.

In addition, it could be interesting to test the radiation resistance of the isolated strains under more chronic irradiation conditions, which would more accurately reflect the conditions prevailing in the original environments.

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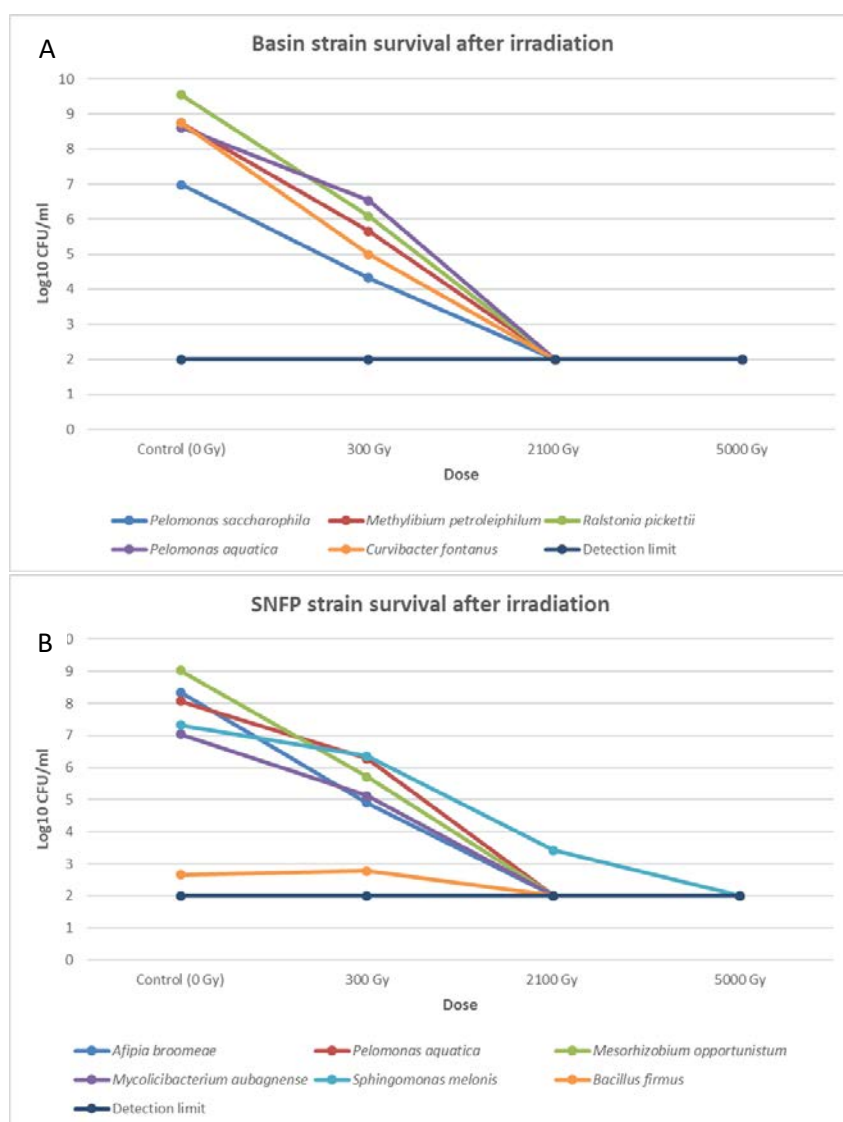


Figure 4. Basin and SNFP strain survival after irradiation. A. CFU/ml after irradiation for the basin strains and B. CFU/ml after irradiation for the SNFP strains.

Conclusion

We managed to isolate and store a strain collection consisting of 33 distinct species from a variety of interlinked watery environments of an active nuclear reactor, some of which are exposed to varying levels of ionizing radiation.

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Although there is some overlap in the microbial composition between the different environments, some clearly showed unique species compositions due to their distinctive physico-chemical characteristics causing different selection pressures on the communities. Some species were characterized for their radiation resistance potential, such as *S. melonis*, which survived a dose of 2.1 kGy. It could be of particular interest to test other species for this characteristic as well, together with their radionuclide accumulation potential. Specifically the strains isolated from the primary water could be worthwhile to investigate, as these are subjected to the highest doses of radiation when the reactor is operational. More intermediate doses below 2 kGy could be tested as well in order to obtain more accurate radiation resistance profiles. Whole genome sequencing will also provide relevant insights into their genomic and genetic adaptations to survive in these extreme environments. In addition, it could be of interest to investigate changes in pigmentation before and after irradiation, as this is known to be important in protection against ROS.

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Chapter III – Section 2.

Effect of cyclical patterns on the microbial dynamics
in the basin water of the BR2 reactor

The basin surrounding the core vessel of the BR2 nuclear research reactor undergoes periodic shifts in physico-chemical parameters such as temperature, flow rate and radiation. This creates a highly dynamic and challenging environment for the residing microbial community.

This section focuses on the characterization of the microbial community inhabiting this extreme environment using a 16S rRNA amplicon sequencing approach. Two sampling campaigns were performed in order to assess the long-term effect of the shifting conditions on the microbial dynamics.

This section is ready for publication in an appropriate scientific journal.

Cyclical patterns affect microbial dynamics in the water basin of a nuclear research reactor

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Running title: Microbial dynamics in a nuclear reactor

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Originality and significance of the work

The microbial communities in spent nuclear fuel pools, where spent fuel is stored underwater prior to long-term disposal, have been investigated in several studies before. However, our work focuses on a similar but much more extreme and dynamic environment, namely a basin surrounding the core vessel of an active nuclear reactor, with high levels of radiation and flow rates as well as periodic shifts in physico-chemical parameters. The microbial communities in these types of environments have rarely been studied. This pioneering study resulted in a first characterization of the inhabiting microbial community and its dynamics over different operational conditions and a time span of several months, using a 16S rRNA amplicon sequencing approach. We showed that the microbial community is very resilient over time despite the shifting external conditions. This novel study serves as a base for the further investigation of these types of extreme man-made environments, and a better understanding of the strategies used by microbes to survive in these extremely oligotrophic conditions, combining radiation and cyclical stress patterns.

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Abstract

The BR2 nuclear research reactor runs in successive phases of operation (cycle) and shutdown, whereby a water basin surrounding the reactor vessel undergoes periodic changes in physico-chemical parameters such as flow rate, temperature and radiation. The aim of this pioneering study is to explore the microbial community in this unique environment and to investigate its long-term dynamics using a 16S rRNA amplicon sequencing approach. Results from two sampling campaigns spanning several months showed a clear shift in community profiles: cycles were mostly dominated by two OTUs assigned to an unclassified *Gammaproteobacterium* and *Pelomonas*, whereas shutdowns were dominated by an OTU assigned to *Methylobacterium*. Although one year apart, both campaigns showed similar results, indicating that the system remained stable over this two-year period. The community shifts were linked with changes in flow rate, temperature and radiation by NMDS and correlation analyses. In addition, radiation was shown to cause a decrease in cell number, whereas temperature had the opposite effect. Chemoautotrophic use of H₂ and dead cell recycling are proposed to be used as a strategies for nutrient retrieval in this extremely oligotrophic environment.

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Introduction

Waters of nuclear facilities are constantly filtered and deionized to remove dissolved radionuclides, as well as impurities and ions that could become activated. They are also exposed to varying levels of ionizing radiation. These ultrapure waters thus represent a rather inhospitable environment for microorganisms. Yet, bacteria, fungi and microalgae have previously been detected in cooling waters of such facilities, namely in SNFPs, where spent nuclear fuel is stored in racks under water in order to cool down before being disposed (Santo Domingo *et al.* 1998; Chicote *et al.* 2005; Masurat *et al.* 2005; Rivasseau *et al.* 2016). Microbes can be present in the form of planktonic populations or biofilms, which can adhere to metal surfaces in SNFPs, potentially leading to microbiologically influenced corrosion (MIC) (Zhang *et al.* 1999; Giacobone *et al.* 2011; Smart *et al.* 2014). Although this phenomenon has been investigated, together with the radionuclide bioaccumulation potential of some bacterial strains (Jolley 2002; Tisakova *et al.* 2013), these studies mainly focused on cultivation-based approaches at a single point in time to identify and characterize the detected microorganisms. However, only a small percentage of environmental bacteria can be cultivated under laboratory conditions (Whitman *et al.* 1998). This fundamentally limits the potential to investigate the dynamics of the community as a whole. Conversely, with the advent of the next-generation sequencing (NGS) platform and –omics techniques, it has become possible to analyze the DNA from all the members of a microbial population, hereby gaining insights into the entire community. As such, those techniques are starting to get implemented in the study of microbial communities within SNFPs and other watery environments in nuclear reactors (Bagwell *et al.* 2018; McGraw *et al.* 2018; Petit *et al.* 2020; Ruiz-Lopez *et al.* 2020; Foster *et al.* 2020). Nevertheless, knowledge on the evolution of these communities over time is currently lacking.

Instead of the previously described SNFP microbiota, this work focuses on a similar but more extreme (higher radioactivity) environment consisting of a water basin directly surrounding the vessel of a nuclear reactor. By using a 16S rRNA amplicon sequencing approach, it was possible to identify bacterial taxa that went undetected before.

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Moreover, instead of sampling a single time point, our sampling campaign covering several months included a time series spanning both active and inactive periods of reactor operation, allowing us to investigate the dynamics of the microbial population over time. As the reactor is running in different phases, where an active period (cycle) is interspersed with periods of shutdown for reactor maintenance, these data shed a new light on the microbial dynamics within this unique environment. Since the transition between cycle and shutdown phases is accompanied by a shift in physical parameters such as temperature and radiation, this sudden change in external conditions is hypothesized to influence the community composition and cell density.

Results

In order to study the dynamics of the bacterial community in the basin water, two sampling campaigns (separated by a one-year interval) spanning multiple cycle and shutdown periods were performed using a 16S rRNA amplicon sequencing approach. The physico-chemical parameters of the water were monitored across both campaigns, whereas the cell number was assessed only during the second campaign. Finally, an experiment was designed to assess the effect of temperature on the basin water cell number.

Physical parameters

During this study, four relevant physical parameters were monitored in order to potentially correlate those with the microbial community. The first parameter was the flow rate of the basin water cooling circuit (Fig. 1A). During shutdown periods, the standard flow rate was 50 m³/h while during operation it increased to 500 m³/h to cool down the basin water. A second parameter was the global gamma radioactivity in the basin water (Fig. 1B). As expected, the activity during operation significantly increased (up to a maximum of 9500 cps) in comparison to shutdown periods (with a minimum of 3500 cps) because of the nuclear fission reactions taking place in the reactor core. When the reactor is running, the radiation is deposited in the pool at a calculated dose rate of 755 Gy/h, 98% of which is deposited in a zone within 5 cm around the core and 100 cm above and below the central plane. During shutdown periods, the dose rate lowers to 1 Gy/h.

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The third parameter was the temperature of the basin water (Fig. 1C), which followed the same trend as observed for the other parameters: a significant increase during cycles (up to a maximum of 35°C), followed by a decrease to previous levels during shutdowns (with a minimum of 15°C). Lastly, the conductivity of the basin water also showed some variation across the timeline, although to a much smaller extent than the other parameters, with values ranging from 0.96 to 1.25 $\mu\text{S}/\text{cm}$ (Fig. 1D). As a side note, the flow rate in the purification circuit where deionization happens through mixed bed ion exchange resins remains constant throughout cycles and shutdown periods with a value of 30 m^3/h .

Chemical analysis

The results of the chemical analysis performed on a selection of cycle and shutdown samples from campaign 1 can be found in Table 1. The concentrations of acetate and nitrate were extremely low, while the values for nitrite, phosphate, formate, TIC and TOC were below the detection limit of the measuring instruments (10 $\mu\text{g}/\text{l}$ for ion chromatography and 500 – 700 $\mu\text{g}/\text{l}$ for TIC-TOC analysis, respectively). Furthermore, no significant difference between cycle and shutdown values could be detected. Other elements such as Zn, Pb, Co, Ni, Be, Fe, Mg, Cu, Al, Ba, Li and F were also measured, but values were all below 20 $\mu\text{g}/\text{l}$. pH was maintained at an average value of 6 ± 0.4 . Finally, the total gamma activity across campaign 1 and 2 amounted to 1.9×10^3 Bq/l on average during cycles and <80 Bq/l during shutdowns. 95% of the activity during cycles originates from Na-24, a radioisotope with a 15-hour half-life. The remainder comes from other radioisotopes such as Cr-51, Sb-124, Re-186, Np-239, W-188 and Co-60.

Table 1. Chemical analysis performed on ecologically relevant compounds, during both cycle and shutdown periods. Values represent mean $\pm$ standard deviation.

Component	Cycle ($\mu\text{g}/\text{l}$)	n	Shutdown ($\mu\text{g}/\text{l}$)	n
Acetate	58 ± 16	5	67 ± 33	6
Nitrate	35 ± 3	5	44 ± 22	6
Nitrite	< 10	5	< 10	6
Phosphate	< 10	5	< 10	6
Formate	< 10	5	< 10	6
TIC	< 500	3	< 500	3
TOC	< 700	3	< 700	3

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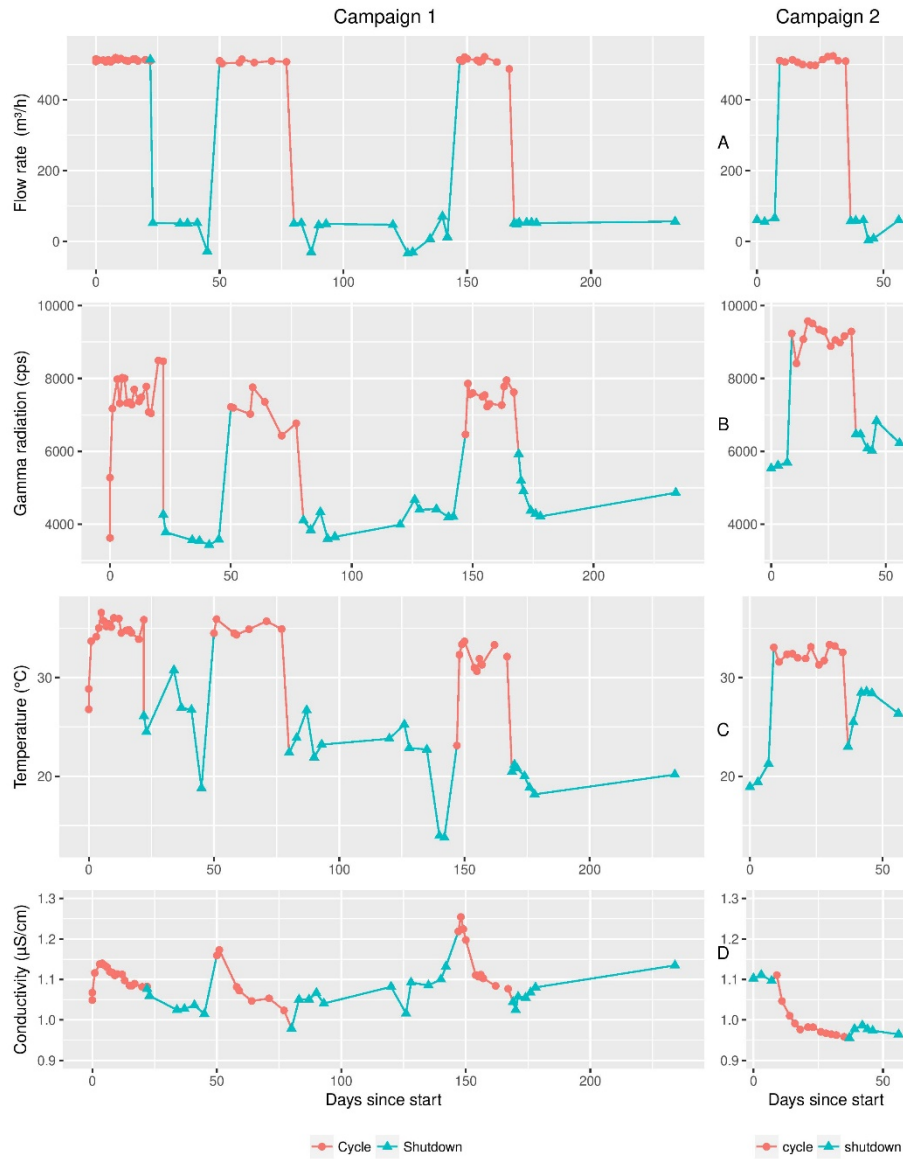


Figure 1. Four physical parameters monitored during the first and second sampling campaign. A: flow rate in the basin cooling circuit (m^3/h), B: gamma radiation in the basin water (cps), C: temperature in the basin water ($^{\circ}\text{C}$) and D: conductivity of the basin water ($\mu\text{S}/\text{cm}$). Cycle and shutdown periods are indicated by red circles and blue triangles, respectively.

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Cell counting

In order to explore the effect of the changing physical conditions on the cell number of the bacterial population, heterotrophic plate count was used during the second sampling campaign (Fig. 2). The data for this method follow a clear pattern: a steep decline in cell number of $1.2 \log_{10}$ at the start of the cycle, followed by a stabilization and slow increase of the population by $0.5 \log_{10}$ during the cycle and finally a recovery to previous cell density levels (up to $3.3 \log_{10}$ CFU/ml) after the cycle.

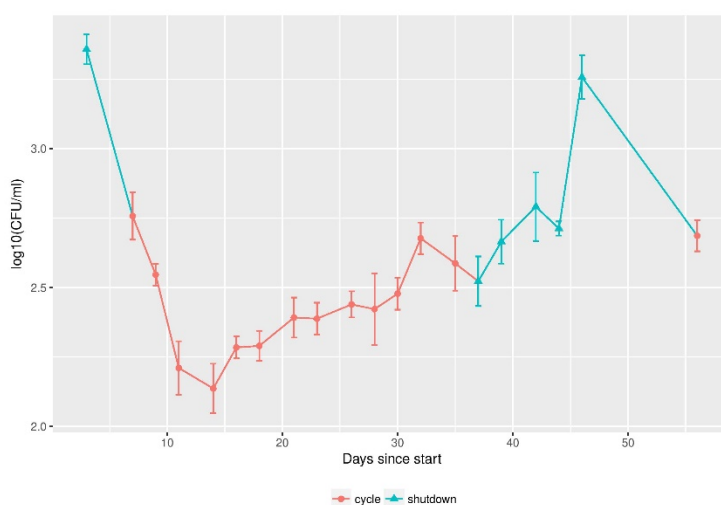


Figure 2. Cell counting during the second sampling campaign. Error bars represent standard deviation, $n = 3$. Cycle and shutdown periods are indicated by red circles and blue triangles, respectively.

The observed pattern of cell number can be correlated with the change in physico-chemical parameters described previously. The main factor causing a decrease in cell number during the cycle is most probably the exposure to radiation. During a cycle, the flow rate of the cooling circuit for the basin water goes up from 50 to 500 m^3/h . After passing through this circuit, the water is transferred back into the basin via a conduit following the reactor wall. At this point, the water is exposed to high levels of radiation originating from the nuclear fission taking place in the reactor core (gamma heating of $0,83 \text{ W/g} = 830 \text{ Gy/s}$ for a representative cycle of operation). Due to the high flow rate of the cooling circuit during a cycle, the water is frequently exposed to this radiation, with a turnover time of ca. 104 min.

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Bacterial community structure

The data analysis for the basin water samples from both sampling campaigns was performed using the OCToPUS pipeline. A summary of the results can be found in Table 2. It must be noted that the average number of OTUs for both campaigns did not significantly differ across cycles and shutdowns. Rarefaction curves for all samples are shown in Supplementary Figure S1.

Table 2. Summary of the data analysis performed for the first and second sampling campaigns.

	Campaign 1	Campaign 2
Sampling dates	06/09/2016 - 28/04/2017	16/04/2018 - 11/06/2018
Number of samples	61	21
Total number of reads	6,127,782	420,191
Average number of reads per sample	100,455	20,009
Minimum	55,044	11,683
Maximum	150,145	28,867
Standard deviation	18,990	3,611
Total number of OTUs	4,017	864
Average number of OTUs per sample	225	172

Fig. 3 shows the 10 most abundant OTUs in the basin water, representing 90% and 76% of the total reads for campaign 1 and 2, respectively. A clear shift in the community profile across the different cycle and shutdown periods is apparent. During the first sampling campaign, the most abundant OTU in the first two cycles was assigned to an unclassified *Gammaproteobacterium* (OTU5), accounting for 28% of total amplicon reads. For the third cycle, an OTU assigned to *Pelomonas* (OTU2) became predominant at 43%, although it could already be observed in lower proportions, at 5%, during the previous cycles. Interestingly, during the shutdown periods in between the cycles, the community profile shifted drastically and became dominated by an OTU assigned to *Methylobacterium* (OTU3) at 41%, while the previously mentioned OTUs (OTU5 and OTU2) were detected in much lower proportions (3% and 7%, respectively). This change can probably be assigned to the shift in physico-chemical parameters that occurs with the transition from a cycle to a shutdown period.

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Although the second sampling campaign took place one year after the first one, the same pattern could be observed, indicating that the system is rather stable. Nevertheless, a difference can be noted for the unclassified *Gammaproteobacterium* and *Pelomonas* observed in approximately equal proportions (13% and 10%, respectively), whereas the community was either dominated by one or the other during the first sampling campaign.

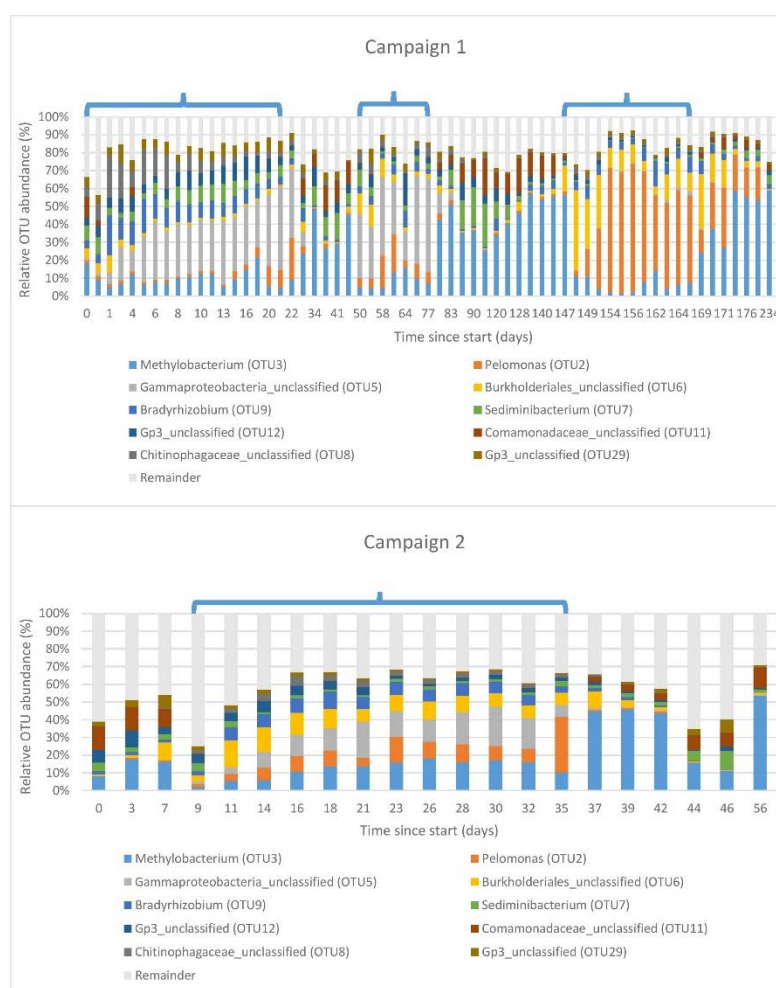


Figure 3. Bacterial community structure of the basin water environment. 10 most abundant OTUs displayed in relative abundances. Each OTU is taxonomically assigned at the genus level. Cycles are indicated by brackets, interspersed with shutdown periods. The horizontal axis indicates the number of days since the sampling start. Top: sampling campaign 1 (start-end: 06/09/2016-28/04/2017), bottom: sampling campaign 2 (start-end: 16/04/2018-11/06/2018).

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Temperature effect on cell number

To assess the influence of temperature, another critical parameter, on the community, an experiment was designed to test its effect on cell number (Fig. 4). Briefly, basin water was collected from the BR2 reactor during a shutdown period in an autoclaved glass container and incubated at 35°C (approximately the same temperature as observed during a cycle) for a period of 24 h. As a control, the same volume of water was kept at room temperature next to the incubator. After 24 h, a fresh sample was collected from the BR2 reactor in order to be compared with the control. Cell numbers for all three conditions were measured using the previously described flow cytometry procedure. As can be observed in Fig. 4, the cell number significantly increased when exposed to a higher temperature, indicating that temperature has an opposite effect to radiation. Since the overall cell number decreases during a cycle, this shows that the effect of radiation exposure largely outperforms the effect of temperature.

Effect of temperature on basin water cell number

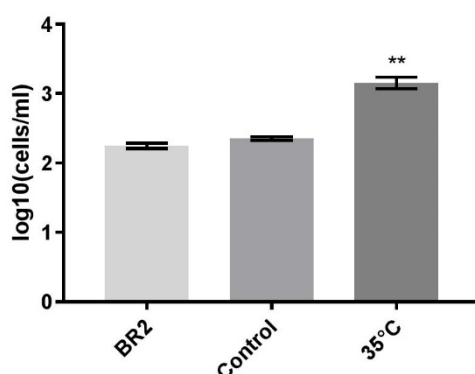


Figure 4. Effect of 24-h temperature increase on basin water cell number. Error bars represent standard error of the mean. Groups were compared using a non-parametric Kruskal-Wallis test. P-value = 0.0024, n = 4.

Alpha and beta diversity analyses

The alpha diversity was calculated in the form of the inversed Simpson diversity index, which takes both richness and evenness into account. Fig. 5 shows the data for both sampling campaigns. Interestingly, a peak in diversity can be observed at the start of each cycle, followed by a subsequent stabilization. This is true for both sampling campaigns, which further confirms the robustness of the system under study.

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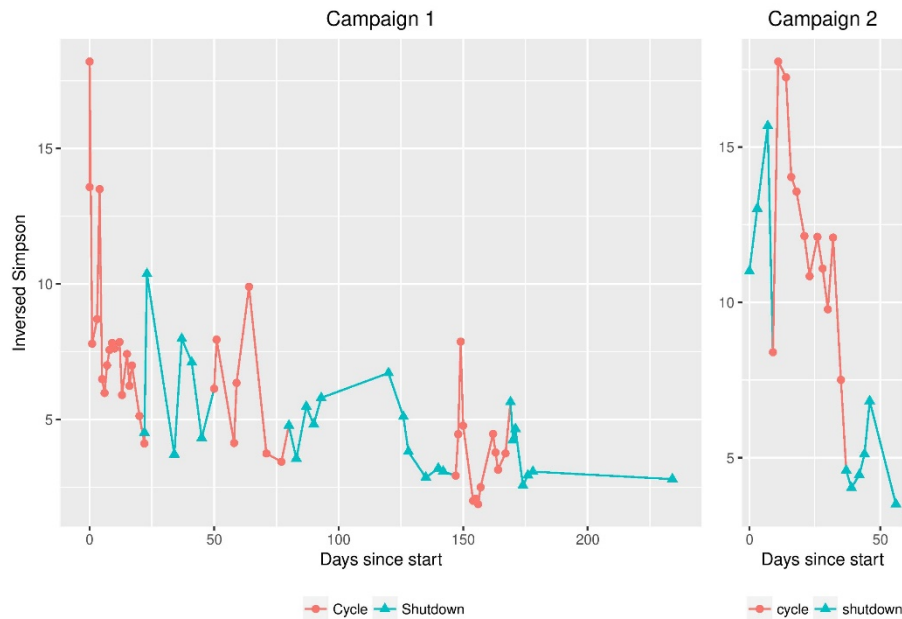


Figure 5. Alpha diversity plots (inversed Simpson diversity index). Left: Campaign 1, right: Campaign 2. The horizontal axis represents the number of days since the sampling start. Cycle and shutdown periods are indicated by red circles and blue triangles, respectively.

For the beta diversity analysis, an NMDS was performed on all the samples using the mothur program. As can be observed in Fig. 6, all shutdown samples from the first campaign clustered together, indicating that the community returns to an equilibrium after each cycle. Cycles 1 and 2 also clustered together in the top right quadrant, whereas cycle 3 was clustered separately in the bottom right quadrant, indicating a shift in the community at this point in time. This was also confirmed in Fig. 3. Furthermore, the data points for the second campaign can be observed to blend in with the data points from campaign 1. Taking into consideration that the sampling campaigns were approximately one year apart, this further corroborates the fact that the system is very stable. Indeed, the shutdown data points for both campaigns were clustered together in the two left quadrants, whereas the cycle data points were clustered in the two right quadrants. Interestingly, the cycle from campaign 2 intercalates in between the cycles from the previous campaign, which supports a model where the bacterial community oscillates between different states.

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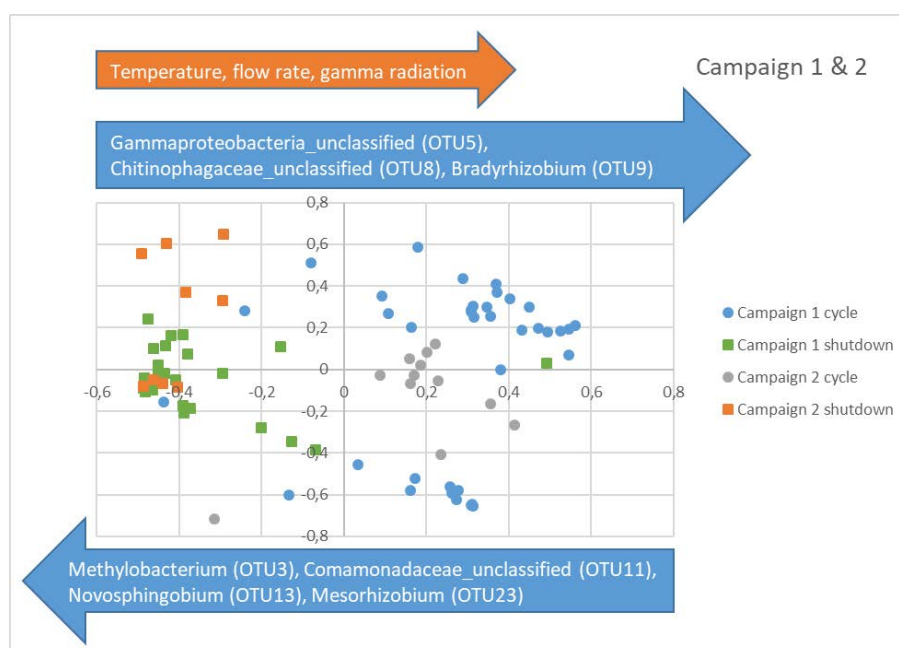


Figure 6. NMDS for campaign 1 and 2 combined. The blue and orange arrows respectively represent the OTUs and physico-chemical parameters showing a significant correlation ($r > 0.6$, $p < 0.05$) with the represented NMDS axes.

In order to evaluate which parameters were responsible for the community shifts, a Spearman rank correlation analysis was performed between the data points on the NMDS axes and the observed OTUs and physico-chemical parameters. The OTUs with the largest impact on the separation between cycle and shutdown samples are represented as blue arrows in Fig. 6. Regarding the physico-chemical parameters, it was observed that gamma radiation, flow rate and temperature showed a significant correlation with the horizontal axis, whereas conductivity and acetate and nitrate concentrations did not. This suggests that radiation exposure, flow rate and temperature could be important factors in the community shift observed across cycles and shutdown periods. Correlation coefficients and p-values for the described OTUs and physico-chemical parameters can be found in Table 3.

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Table 3. Summary of Spearman rank correlation analysis performed on OTUs and physico-chemical parameters represented on the NMDS plot.

OTU	Average abundance	r	p-value	Parameter	r	p-value
OTU5	11.39%	0.77	0	Temperature	0.71	0
OTU8	3.40%	0.70	0	Flow rate	0.63	0
OTU9	5.53%	0.62	0	Gamma radiation	0.62	0
OTU3	21.20%	-0.80	0	Conductivity	0.22	0.05
OTU11	3.76%	-0.84	0	Acetate	0.50	0.16
OTU13	2.06%	-0.69	0	Nitrate	0.32	0.37
OTU23	1.04%	-0.81	0			

Discussion

The aim of the present study was to explore the bacterial community dynamics in the basin surrounding the core vessel of a nuclear research reactor. The 16S rRNA amplicon sequencing results were processed with our in-house OCToPUS pipeline to identify OTUs and calculate the alpha and beta diversity. In addition, we monitored the physico-chemical parameters of the water to reveal correlations with the observed shifts in the community.

A first interesting observation was that the communities in the basin water underwent an apparent shift across cycle and shutdown periods. This was clearly observed in the community profiles and was further confirmed by the NMDS analysis, in which shutdown and cycle samples clustered separately. This shift correlated with a change in physico-chemical parameters such as temperature, flow rate and gamma radiation, whereas conductivity, nitrate and acetate showed no significant correlation. These individual parameters (or their combination) probably had an impact on the community shift from a population dominated by a member of the *Gammaproteobacteria* and *Pelomonas* during cycles to one dominated by *Methylobacterium* during shutdown periods. This pattern was consecutively observed for both sampling campaigns, indicating that this periodicity is very stable. However, additional research is needed to elucidate the role of each individual parameter on the community, since all three of them follow the same pattern (high values during cycles and low values during shutdowns).

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As such, temperature and radiation are believed to have the largest impact on the community, whereas the effect of flow rate could be somewhat smaller. The observed shift during a transition from a shutdown period to a cycle could also be facilitated by the effect of stress on the community. Indeed, it has been previously described that the level of invasion of new species in communities with identical evenness depends strictly on the presence of stress (De Roy *et al.* 2013), here in the form of radiation exposure. In the absence of stress, communities tend to be more resistant to invasion.

Interestingly, the most abundant OTU during the third cycle of the first sampling campaign was *Pelomonas*, as opposed to the previous cycles that were mostly dominated by an unclassified *Gammaproteobacterium*. However, the cycle monitored during the second sampling campaign showed an approximately equal prevalence of both the unclassified *Gammaproteobacterium* and *Pelomonas*. This is reflected in the NMDS analysis, where the data points of the campaign 2 cycle intercalate in the middle of the other cycle data points with little overlap. This suggests that the community during cycles can fluctuate between different states, either equally dominated by the two main OTUs or only dominated by one of them. Taking into consideration that the physico-chemical parameters across different cycles remain constant, this phenomenon can probably be attributed to stochastic effects. Indeed, it is known that the mechanisms governing community assembly can be both deterministic and stochastic in nature (Sloan *et al.* 2006; Nemergut *et al.* 2013; Evans *et al.* 2017; Zhou & Ning 2017). As such, next to deterministic factors like species traits, interspecies interactions and environmental conditions, stochastic processes in the form of random birth and death events, colonization and extinction are also known to play a role in this regard. Furthermore, it has been shown that a single source community can, under identical environmental conditions, generate drastically different communities with little overlap in composition or even global function (Zhou *et al.* 2013).

The basin water shared some bacterial taxa with different spent nuclear fuel pools and other cooling basins such as *Meiothermus*, *Methylobacterium*, *Bradyrhizobium*, *Pelomonas* and *Sediminibacterium* (Bagwell *et al.* 2018; McGraw *et al.* 2018; Petit 2018; Petit *et al.* 2020).

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Next to these recent studies, others mainly focused on the isolation and characterization of culturable strains from water (Tisakova *et al.* 2013; Dekker *et al.* 2014; Karley *et al.* 2018) or from biofilms (Sarro *et al.* 2003; Chicote *et al.* 2004; Sarro *et al.* 2005; Bruhn *et al.* 2009). Some of the genera found in the basin water were also identified in those studies (mainly *Meiothermus* and *Bradyrhizobium*). Thus, the community observed in our environment shows some overlap with the ones found in different spent nuclear fuel pools. This can probably be attributed to the fact that the physico-chemical parameters in both of these environments are quite similar. Indeed, the water in SNFPs is also very oligotrophic due to constant deionization via anion and cation exchangers and is exposed to residual levels of radiation originating from the spent fuel elements stored underwater. In contrast, the bacterial community in the secondary cooling water of the BR2 reactor analyzed by Props *et al.* (Props *et al.* 2016) showed no overlap with the one in the basin water due to its different environmental conditions (data not shown). Indeed, the secondary cooling system never comes into contact with radiation, most probably does not contain any H₂ and the conductivity is significantly higher, with values reaching up to 7 µS/cm.

The chemical analysis of the water samples showed that all of the measured components (nitrite, phosphate, formate, TIC and TOC as well as other chemical elements) except for acetate and nitrate were below the detection limit of the measuring instruments. This is due to the fact that the water is constantly being deionized through ion exchangers, resulting in a very low conductivity of approximately 1 µS/cm and an extremely oligotrophic environment. However, even in ultrapure waters, it is impossible to eliminate all impurities. In environments such as the one monitored in our study, these impurities mainly originate from metallic pipings in the cooling and purification circuits, dust that falls into the basin and the air in contact with the pool surface. Typical dominant impurities include Al³⁺, Fe²⁺, NO₃⁻, SO₄²⁻ and Cl⁻ (International Atomic Energy Agency 2011). These can in turn be utilized by bacterial communities. Furthermore, the basin of the BR2 research reactor is regularly emptied during shutdown periods for maintenance purposes, allowing technicians to access areas that would normally be submerged. This could periodically introduce organic material in the system, which could be used as an energy source by heterotrophic bacteria.

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Another source of organic material could be the ion exchange resins used in the purification circuit. These are mixed bed resins composed of polystyrene beads that absorb cations and anions on their surface. Due to radiation, these resins slowly degrade over time (approximately five years), hereby releasing small quantities of organic material into the water, which can then be used by bacteria. In addition, they could also be a substrate for bacterial growth, as has been demonstrated by one particular research group (Kéki *et al.* 2019).

The basin is an oxic environment, since it is an open system with high water circulation (50 – 500 m³/h) which allows for a homogeneous oxygen distribution. This is also evidenced by the fact that most identified genera based on the 16S rRNA amplicon sequencing data are strictly or facultatively aerobic. In addition, the presence of ionizing radiation causes water molecules to dissociate into several chemical species such as H₂, H₂O₂ and some radicals (OH[•], H[•], HO₂[•]), a phenomenon known as water radiolysis (Dzaugis *et al.* 2015). These characteristics combined with the low nutrient availability result in some unique conditions for which different bacteria have adopted different strategies. For example, it has been previously demonstrated that bacteria in SNFPs can oxidize H₂ from water radiolysis as an energy source, using O₂ as electron acceptor and CO₂ as carbon source (Galès *et al.* 2004). As such, the *Pelomonas* and *Bradyrhizobium* genera as well as the *Comamonadaceae* family detected in our system have been associated with chemoautotrophic growth on H₂ (Willems *et al.* 1991; Gomila *et al.* 2007; Franck *et al.* 2008). On the other hand, *Methylobacterium* is a facultative methylotroph known to be able to grow on C₂ compounds such as acetate as a sole carbon source (Smejkalova *et al.* 2010; Green & Ardley 2018). Furthermore, a large proportion of the identified taxa such as *Pelomonas*, *Novosphingobium*, *Meiothermus*, some members of the *Acidobacteria* subdivision 3 (Gp3), *Comamonadaceae* and *Chitinophagaceae* are able to reduce nitrate (Willems *et al.* 1991; Takeuchi *et al.* 2001; Gomila *et al.* 2007; Ward *et al.* 2009; Kampfer *et al.* 2011; Mori *et al.* 2012), which can also occur under oxic conditions (Carter *et al.* 1995; Ji *et al.* 2015). *Bradyrhizobium* and *Mesorhizobium* on the other hand are capable of N₂ fixation (Black *et al.* 2012). It must also be noted that bacteria in oligotrophic environments can rely on mixotrophy as a survival strategy, since this provides them with a greater nutritional flexibility (Mittelman & Jones 2018).

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In line with this, an aerobic heterotrophic member of the *Acidobacteria* has been shown to survive through periods of starvation where organic electron donors are scarce by relying on H₂ oxidation (Greening *et al.* 2015). Other heterotrophic taxa identified in our system could potentially rely on the same mechanism, as H₂ is more readily available here due to water radiolysis.

In addition, we propose that the established community is able to recycle dead cell material in order to survive long periods of nutrient deprivation. Indeed, it has been previously demonstrated that bacteria can survive for extended periods of time under conditions of starvation, and that this phenomenon is inversely proportional to cell density (Phaiboun *et al.* 2015). Moreover, bacteria in their natural environments are believed to exist in conditions resembling those of long-term stationary phase cultures, where stress-response genes and alternative metabolic pathways are essential for survival (Finkel 2006). It has also been shown that cell death and recycling were essential processes for cell survival during starvation, where survivors were able to utilize nutrients from dead cells parsimoniously as a strategy for long-term persistence (Takano *et al.* 2017). In our system, the cell number rapidly decreases with a magnitude of more than one log during the transition from a shutdown period to a cycle (Fig. 2). As previously described, this cell number decrease could be assigned to the water being exposed to high levels of radiation originating from the reactor core. We therefore propose that cells that have been killed off by radiation are rapidly being recycled by the surviving population for nutrient retrieval. This phenomenon could also partially explain the peak in diversity observed at the start of each cycle (Fig. 5). Indeed, the temporary availability in nutrients could allow other species to emerge, resulting in a brief increase in both richness and evenness before the community reverts towards an equilibrium state characterized by a lower diversity.

In conclusion, we managed to characterize the bacterial community in the unique environment of a basin surrounding the core vessel of a nuclear research reactor using a 16S rRNA amplicon sequencing approach. In addition, we studied the dynamics of the community over time and managed to correlate those with the physico-chemical parameters observed in this environment.

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As such, temperature, flow rate and radiation were shown to be correlated with the observed community shifts as well as the overall community composition. Furthermore, the system was shown to be very consistent over time, even when taking stochastic factors in community assembly into consideration. Finally, we managed to shed some light on the ecological mechanisms underpinning the survival of the community in this tremendously challenging environment, where the use of H₂ from water radiolysis as an energy source and dead cell recycling are believed to be of paramount importance. In this study, we mainly focused on the characterization of the bacterial community, but it could also be of interest to investigate the presence of eukaryotes such as micro-algae and fungi through 18S and ITS amplicon sequencing. Our study could also be complemented with additional research investigating the radiation resistance of individual strains isolated from this environment or of the entire community as a whole. In addition, it would be of interest to perform a deeper analysis on the community using next-generation sequencing techniques such as shotgun metagenomics and metaproteomics. This would allow for the development of a deeper insight into the specific genes and proteins responsible for the behavior of the community in this unique environment.

Experimental procedures

Sampling site and sample collection

The study was conducted at the BR2 nuclear reactor at the Belgian Nuclear Research Centre (SCK CEN) in Mol, Belgium. The sampling environment consisted of an open basin surrounding the reactor vessel (Fig. 7). The reactor successively goes through cycles of operation (e.g. production of radioisotopes for medical and industrial use) and shutdown of approximately 30 days each, which are associated with changes in physico-chemical parameters of the water such as flow rate, temperature and radioactivity. For this study, two sampling campaigns were performed: in the first one, the basin water was studied for three consecutive cycles interspersed with shutdown periods over a period of eight months (from 06/09/2016 to 28/04/2017). The second one was conducted one year later (from 16/04/2018 to 11/06/2018) where it was only monitored for one cycle. In this case, the cell density was also assessed for each sample.

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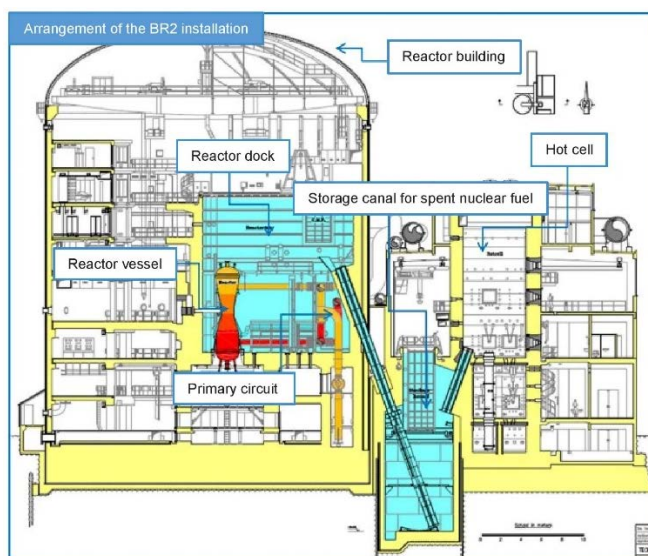


Figure 7. Schematic overview of the BR2 facility. Watery environments are represented in blue, orange and red. Reactor vessel color-coded in shades of orange and red in function of the temperature in the primary circuit water.

The study of the reactor basin environment represents a challenge in terms of its low bacterial load combined with the presence of radioactivity. For sample collection, a filtration system was therefore designed to minimize the risk of radioactivity exposure and contamination during sampling in accordance with the ALARA principle (As Low As Reasonably Achievable) and implemented in a sampling glove box, routinely used to take samples from the basin and other watery environments (e.g. the primary and secondary cooling circuits). This also ensured that enough cell material could be collected for downstream analyses. Of note, filtration was chosen as a preferred method over e.g. centrifugation, as this resulted in a more effective and safer sample collection.

The system was composed of a stainless steel filter holder containing a 0,2 μm pore sized polyethersulfone (Supor®) filter membrane (Pall Corporation, Port Washington, NY, USA), connected to the corresponding tap on one side and a flow meter on the other side in order to measure the filtered volume (Fig. 8). Ten liters of water were filtered per sample, after which the filter membrane was retrieved from the system and stored at -20°C in a controlled area before DNA extraction to allow for radioactive decay.

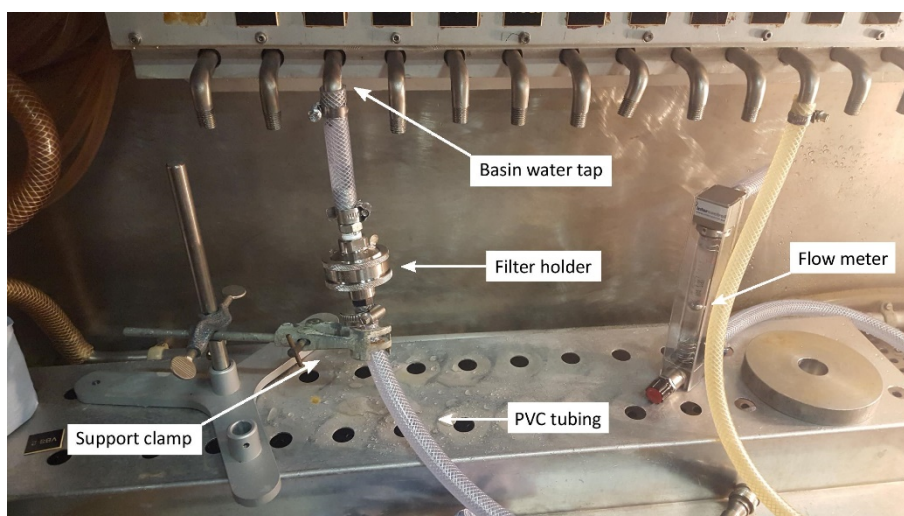


Figure 8. Filtration system implemented in a sampling glove box in the BR2 reactor. Individual components are indicated by arrows.

DNA extraction and 16S rRNA amplicon sequencing

DNA was extracted from the filter membranes following a previously described protocol (Vilchez-Vargas *et al.* 2013) and subsequently purified using Amicon® 30 kDa filter cartridges (Merck, Darmstadt, Germany). DNA concentrations were measured using a Quantus™ fluorometer (Promega, Madison, WI, USA). For the 16S rRNA amplicon sequencing, the hypervariable V3-V4 region was chosen to specifically target bacteria (as opposed to fungi and/or Archaea) and the resulting amplicons were sequenced in two runs using V3 chemistry (2 x 300 bp) with the Illumina MiSeq platform. Positive (*Cupriavidus metallidurans* CH34 cultures filtered over the same filter membranes as previously mentioned) and negative controls (blank filter membranes and demineralized water) were also added as part of the experiment. They underwent the same DNA extraction procedure as the other samples.

Sequencing Data analysis

The data generated by the sequencing platform were demultiplexed and the datasets consisted of two separate fastq files (forward and reversed). The sequences were subsequently analyzed through the OCToPUS pipeline as described in Mysara *et al.* (Mysara *et al.* 2017).

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In short, reads went successively through pre-assembly denoising (Bankevich *et al.* 2012), contig assembly, quality filtering using mothur (Schloss *et al.* 2009), denoising using IPED (Mysara *et al.* 2016), chimera removal using CATCH (Mysara *et al.* 2015) and finally OTU clustering with 97% cut-off using UPARSE (Edgar 2013). All resulting OTUs were taken into account for further analyses without applying a minimum abundance threshold.

The OTUs were then taxonomically assigned using the mothur *classify.seqs* command with the RDP dataset (version 16) as reference. In order to properly compare the samples from both campaigns combined, they were rarefied to the same read count as the smallest sample (11,683 reads), using the mothur *sub.sample* command. Although a large number of reads is typically removed during this process, this does not significantly affect the subsequent alpha and beta diversity analyses. Rarefaction curves were obtained by using the mothur *rarefaction.single* command and subsequently plotting the data in the R software. Sample metadata are included in Supplementary Table S1. The entire 16S rRNA amplicon sequencing dataset is available at the Sequence Read Archive (SRA) repository of NCBI under the BioProject accession number PRJNA725077.

Alpha (Inversed Simpson index) and beta (Theta-YC distance calculation followed by non-metric multi-dimensional scaling (NMDS)) diversity analyses were also performed in mothur using the *summary.single*, *dist.shared*, and *nmds* commands. In order to correlate the OTUs and metadata with the NMDS dimensions, a Spearman rank correlation analysis was performed using the mothur *corr.axes* command. OTUs and metadata that were significantly correlated ($r > |0.6|$, $p < 0.05$) with the represented NMDS axes are displayed on the NMDS plots. It must be noted that the structure of our data did not allow for the use of mixed models.

Physical characterization of the basin environment

The physical parameters of the BR2 waters are constantly monitored using on-line measuring instruments. Relevant data for the bacterial community (conductivity, temperature, radiation and flow rate) were retrieved from an in-house software (BIDASSE) database and organized according to the sampling time points.

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Chemical analysis

Water samples from both cycles and shutdown periods from campaign 1 were collected to perform a chemical analysis of ecologically relevant compounds such as nitrate, phosphate and carbon.

The ions (nitrite, nitrate, phosphate and acetate) were quantified through ion chromatography using a 930 Compact IC Flex (Metrohm, Herisau, Switzerland) with a Metrosep A Supp 16-250/4.0 column and a Metrosep A Supp 16 guard/4.0 column, using an eluent solution of 7.5 mM Na₂CO₃ and 0.75 mM NaOH. TIC (total inorganic carbon) and TOC (total organic carbon) were measured using a FormacsHT-I TOC analyzer (Skalar Analytical, Breda, The Netherlands). O₂, H₂ and ROS were not measured in our system.

Cell counting

During the second sampling campaign, a cell counting method was used to study the population dynamics of the basin water across the two reactor operation conditions (cycle vs. shutdown). The method consisted of heterotrophic plate count, where 100 µl of basin water was directly spread onto R2A agar (Reasoner & Geldreich 1985) plates in triplicates. The plates were incubated for two weeks at room temperature on a laboratory bench before colony counting. Flow cytometry was also considered as a cell counting method, but proved to be unreliable as the cell numbers during cycles dropped significantly, thereby making it impossible to distinguish between bacterial cells and instrument background. This meant that relative OTU abundances could not be corrected for absolute cell numbers. In contrast, for the experiment assessing the effect of temperature on the basin water cell number, a procedure using flow cytometry could be adopted: 100 ml of basin water collected during shutdown was centrifuged at 17 000 x g for 30 min, after which the supernatant was discarded and the (invisible) cell pellet re-suspended in 1 ml of Evian water filtered over a 0.2 µm Acrodisc syringe filter (Pall corporation). The solution was then stained with SYBR® green (Life Technologies, Carlsbad, CA, USA), incubated for 20 min at 37 °C and run on an Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA, USA) in four replicates of 200 µl. Cells were manually gated on green fluorescence (FL1) vs. size (FSC) plots based on a negative control (0.2 µm filtered Evian water).

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Abbreviations

SNFP: Spent Nuclear Fuel Pool

ALARA: As Low As Reasonably Achievable

OTU: Operational Taxonomic Unit

NMDS: Non-metric Multidimensional Scaling

Authors' contributions

V. Van Eesbeeck performed the sampling, laboratory work and data analysis and wrote the manuscript. R. Props provided advice on the research design and flow cytometry. M. Mysara contributed to the 16S rRNA sequencing data processing. P. Monsieurs provided advice on the research design and data interpretation. All authors critically reviewed the manuscript.

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Supporting information

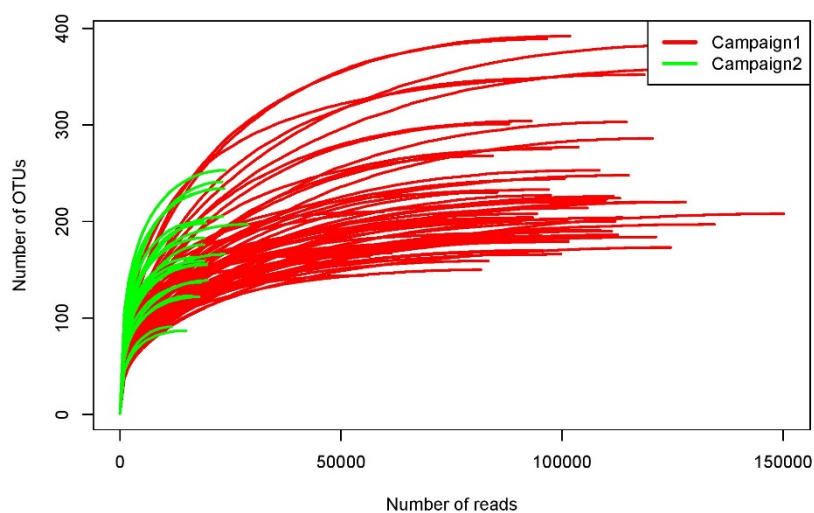


Figure S1. Rarefaction curves for all samples of campaign 1 and 2.

Table S1. Sample metadata for campaign 1 and 2.

Sample ID	Campaign	Sampling date	Days since start	Operational status
BW7_1	1	2016-09-06	0	Cycle
BW8_1	1	2016-09-06	0	Cycle
BW9_1	1	2016-09-07	1	Cycle
BW10_1	1	2016-09-09	3	Cycle
BW11_1	1	2016-09-10	4	Cycle
BW12_1	1	2016-09-11	5	Cycle
BW13_1	1	2016-09-12	6	Cycle
BW14_1	1	2016-09-13	7	Cycle
BW15_1	1	2016-09-14	8	Cycle
BW16_1	1	2016-09-15	9	Cycle
BW17_1	1	2016-09-16	10	Cycle
BW18_1	1	2016-09-18	12	Cycle
BW19_1	1	2016-09-19	13	Cycle
BW21_1	1	2016-09-21	15	Cycle

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BW22_1	1	2016-09-22	16	Cycle
BW23_1	1	2016-09-23	17	Cycle
BW24_1	1	2016-09-26	20	Cycle
BW25_1	1	2016-09-28	22	Cycle
BW26_1	1	2016-09-28	22	Shutdown
BW27_1	1	2016-09-29	23	Shutdown
BW29_1	1	2016-10-10	34	Shutdown
BW30_1	1	2016-10-13	37	Shutdown
BW31_1	1	2016-10-17	41	Shutdown
BW32_1	1	2016-10-21	45	Shutdown
BW33_1	1	2016-10-26	50	Cycle
BW34_1	1	2016-10-27	51	Cycle
BW35_1	1	2016-11-03	58	Cycle
BW36_1	1	2016-11-04	59	Cycle
BW37_1	1	2016-11-09	64	Cycle
BW38_1	1	2016-11-16	71	Cycle
BW39_1	1	2016-11-22	77	Cycle
BW40_1	1	2016-11-25	80	Shutdown
BW41_1	1	2016-11-28	83	Shutdown
BW42_1	1	2016-12-02	87	Shutdown
BW43_1	1	2016-12-05	90	Shutdown
BW44_1	1	2016-12-08	93	Shutdown
BW45_1	1	2017-01-04	120	Shutdown
BW46_1	1	2017-01-10	126	Shutdown
BW47_1	1	2017-01-12	128	Shutdown
BW49_1	1	2017-01-19	135	Shutdown
BW50_1	1	2017-01-24	140	Shutdown
BW51_1	1	2017-01-26	142	Shutdown
BW52_1	1	2017-01-31	147	Cycle
BW53_1	1	2017-02-01	148	Cycle
BW54_1	1	2017-02-02	149	Cycle
BW55_1	1	2017-02-03	150	Cycle
BW56_1	1	2017-02-07	154	Cycle
BW57_1	1	2017-02-08	155	Cycle
BW58_1	1	2017-02-09	156	Cycle
BW59_1	1	2017-02-10	157	Cycle
BW60_1	1	2017-02-15	162	Cycle
BW61_1	1	2017-02-16	163	Cycle

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BW62_1	1	2017-02-17	164	Cycle
BW63_1	1	2017-02-20	167	Cycle
BW64_1	1	2017-02-22	169	Shutdown
BW65_1	1	2017-02-23	170	Shutdown
BW66_1	1	2017-02-24	171	Shutdown
BW67_1	1	2017-02-27	174	Shutdown
BW68_1	1	2017-03-01	176	Shutdown
BW69_1	1	2017-03-03	178	Shutdown
BW71_1	1	2017-04-28	234	Shutdown
BW0_2	2	2018-04-16	0	Shutdown
BW1_2	2	2018-04-19	3	Shutdown
BW2_2	2	2018-04-23	7	Cycle
BW3_2	2	2018-04-25	9	Cycle
BW4_2	2	2018-04-27	11	Cycle
BW5_2	2	2018-04-30	14	Cycle
BW6_2	2	2018-05-02	16	Cycle
BW7_2	2	2018-05-04	18	Cycle
BW8_2	2	2018-05-07	21	Cycle
BW9_2	2	2018-05-09	23	Cycle
BW10_2	2	2018-05-12	26	Cycle
BW11_2	2	2018-05-14	28	Cycle
BW12_2	2	2018-05-16	30	Cycle
BW13_2	2	2018-05-18	32	Cycle
BW14_2	2	2018-05-21	35	Cycle
BW15_2	2	2018-05-23	37	Shutdown
BW16_2	2	2018-05-25	39	Shutdown
BW17_2	2	2018-05-28	42	Shutdown
BW18_2	2	2018-05-30	44	Shutdown
BW19_2	2	2018-06-01	46	Shutdown
BW20_2	2	2018-06-11	56	Shutdown

Chapter III – Section 3.

Studying the microbial dynamics in the water basin
of the BR2 reactor through shotgun metagenomics

In order to dig deeper into the microbial community dynamics occurring in the basin surrounding the core vessel of the BR2 reactor, a shotgun metagenomics approach was adopted. A sampling campaign was conducted using a custom designed filtration system in order to collect a sufficient amount of biological material for analysis.

The shotgun metagenomics data were combined with the 16S rRNA amplicon sequencing data obtained from two previous sampling campaigns (see Chapter III – Section 2.) for added statistical confidence. The microbial population was characterized both on the taxonomic and functional level.

This section is ready for publication in an appropriate scientific journal.

Microbial dynamics in the water basin surrounding a nuclear reactor in operation: a metagenomic approach

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Key words: BR2 nuclear reactor, microbiome, radiation, metagenome analysis

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Abstract

This paper describes, for the first time, the microbial dynamics in the water basin of a nuclear research reactor in operation, using a shotgun metagenomics approach. We aimed to characterize the dynamics of the microbial population living in this extreme man-made environment, i.e. an ultrapure deionized water basin, containing a range of radionuclides and exposed to high doses of radiation. Samples were taken at different time points across shutdowns and cycles of operation using a custom designed filtration system, which to our knowledge is the first study using this type of research design. A shotgun metagenomics approach was adopted, in combination with 16S rDNA amplicon sequencing, to characterize the microbial population both on the taxonomic and functional level.

The periodic shifts in physico-chemical parameters linked with the operational status of the reactor were shown to significantly affect the microbial community in the basin water. *Methylobacterium* seems to be a key player in the community, as it is the most abundant taxon overall. Moreover, it was significantly more abundant during shutdown, whereas an unclassified Gammaproteobacterium had a higher relative abundance during cycles. On the functional level, some pathways were more highly represented during shutdowns, namely pyruvate, glycerophospholipid and purine metabolisms, as well as biosynthesis of valine, leucine and isoleucine. These pathways indicate a role in cell function recovery after irradiation.

In addition, two genomes were almost entirely reconstructed from the metagenome. They corresponded to *Bradyrhizobium* sp. BTAi1 and *Methylobacterium* sp. UNC378MF, with a genome completeness of 97% and 84%, respectively. These genomes displayed significant adaptations that allowed them to thrive under the harsh conditions found in our basin water system.

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Introduction

Aquatic environments in nuclear facilities are maintained in an ultrapure state by constant filtering and deionization, thereby ensuring that dissolved radionuclides are reduced to minimal levels and preventing the corrosion of measuring instruments. In addition, these waters can be subjected to varying levels of ionizing radiation if they come into close contact with nuclear fuel, in the reactor core. Despite these harsh and extremely oligotrophic conditions, microbial life has been previously detected in these environments, such as in indoor pools and outdoor ponds containing spent nuclear fuel (SNFPs) (Santo Domingo *et al.* 1998; Chicote *et al.* 2005), where spent fuel can cool down in racks under water before being disposed to its final location. Among the detected microorganisms in SNFPs, some eukaryotes were found such as fungi (Chicote *et al.* 2004; Silva *et al.* 2018) and microalgae (Rivasseau *et al.* 2016; McGraw *et al.* 2018; Foster *et al.* 2020), but the majority belongs to the domain of the bacteria. Most of these studies were using culture-based approaches, hereby missing part of the potential biodiversity in these environments. Only recently, a small number of studies have started implementing next-generation sequencing (NGS) techniques such as 16S rDNA amplicon sequencing to better characterize the entire community (Bagwell *et al.* 2018; McGraw *et al.* 2018; Foster *et al.* 2020; Petit *et al.* 2020; Ruiz-Lopez *et al.* 2020). These studies found a much larger microbial diversity than could be observed through culture-dependent techniques alone, emphasizing the need for DNA-based approaches in these environments.

The genetic and functional traits of these communities making them capable of living in these unusual waters, are still largely unknown. Some isolates from SNFPs were tested for their radionuclide bioaccumulation and resistance potential (Tisakova *et al.* 2013; Dekker *et al.* 2014) or their capacity to form biofilms, which could potentially lead to microbial induced corrosion (MIC) (Bruhn *et al.* 2009; Giacobone *et al.* 2011; Karley *et al.* 2019). Only one study implemented a shotgun sequencing approach to assess both the diversity and biological function of the microbial population (Silva *et al.* 2018). This study revealed the unexpected presence of fungi next to bacteria in the system as well as a higher relative proportion of functions related to respiration and protein metabolism.

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So far, almost all of these studies were conducted on SNFPs, which are relatively static environments, where conditions remain quite stable over time. The present work however focuses on a highly dynamic nuclear aquatic environment, i.e. the water in the basin directly surrounding the core vessel of a nuclear research reactor, more precisely the Belgian Reactor 2 (BR2) located at the Belgian nuclear research center (SCK CEN) in Mol. The primary purpose of this reactor is the production of radio-isotopes for medical and industrial use. This happens in successive cycles lasting approximately 30 days, each followed by a shutdown period for maintenance purposes. This creates a set of interesting conditions where parameters such as flow rate, temperature and radioactivity periodically undergo large shifts before reverting to their baseline, i.e. the shutdown periods where the reactor is not operational. We therefore hypothesize that these periodic shifts greatly affect the microbial community inhabiting this extreme environment, both on the taxonomic and the functional level. This was assessed through a shotgun metagenomics approach in combination with 16S rRNA gene (rDNA) amplicon sequencing. The samples were collected during active cycles as well as shutdowns in order to comparatively analyze the microbiome in both conditions.

Materials and methods

Study site

Our study was conducted at the Belgian Reactor 2 (BR2) located at the Belgian Nuclear Research Centre (SCK CEN) in Mol, Belgium. When the reactor is operational, nuclear fuel is loaded into the core vessel and various targets are bombarded with neutrons in order to produce radio-isotopes and doped silicon or test the properties of various materials. This occurs in cycles of approximately 30 days followed by shutdown periods to allow for general maintenance. The reactor vessel itself, with a volume of 35 m³, is a closed system filled with water from the primary cooling water circuit, for a total volume of 150 m³. This reactor vessel is in turn directly surrounded by an open basin filled with 870 m³ of water acting as a shield against the radiation emanating from the core (Fig. 1). The water of this basin circulates through a cooling circuit before being pumped back into the basin through an inlet at the reactor mantle, so that it flows over the outer wall of the reactor vessel.

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After each cycle, the nuclear fuel is removed from the reactor vessel and temporarily stored in a small fuel storage pool incorporated in the basin, before being reused during the next cycle.

When the fuel is no longer usable, it is transferred to the adjacent spent nuclear fuel storage canal via a transfer shunt, hereby causing a mixture of both water systems.

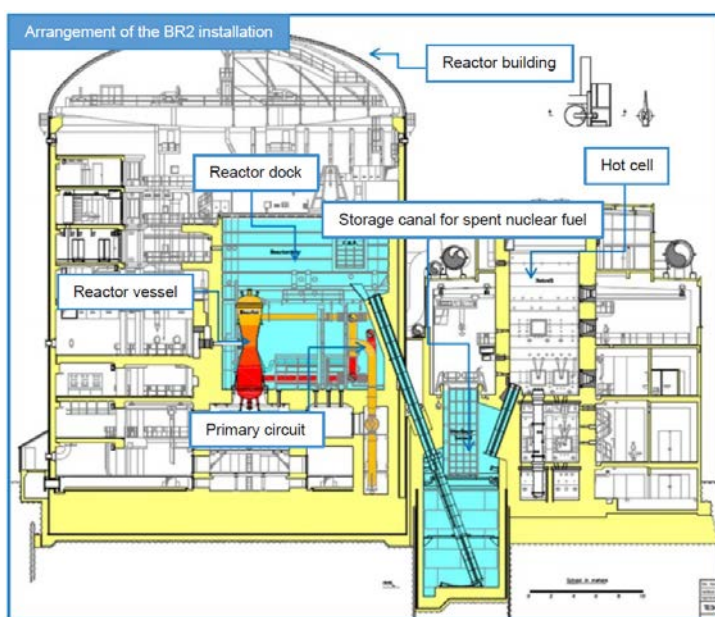


Figure 1. Schematic overview of the BR2 reactor. Watery environments are represented in blue, orange and red. The reactor vessel system is color-coded in shades of orange and red in function of the water temperature in the primary circuit which is fully closed and separate from the basin water. The basin surrounding the reactor vessel system includes a small fuel storage pool and is occasionally opened to the storage canal for SNF displacement through the transfer shunt.

The basin water is constantly pumped around and circulated over (1) a cooling system containing three identical heat exchangers in parallel and (2) a separate purification system containing a UV filter and mixed bed ion exchange resins (cation + anion) consisting of polystyrene beads with negatively and positively charged functional groups, respectively (AMBERSEP® 252 H and AMBERSEP® 900 OH, Rohm and Haas, Pennsylvania, USA). Parameters such as flow rate, temperature and radiation undergo periodic shifts associated with the operational status of the reactor.

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During shutdowns (32 days on average), the flow rate of the water passing through the cooling circuit is maintained at 50 m³/h, the water is at room temperature (ca. 21 °C ± 2 °C) and the dose rate of the residual radiation in the basin is 1 Gy/h.

During cycles (25 days on average), the flow rate through the cooling circuit increases 10-fold, to 500 m³/h, in order to adequately cool down the water. Indeed, the nuclear fission reaction taking place in the reactor vessel core produces heat that is partly absorbed by the basin water. The overall temperature rises to levels between 31°C and 37°C. Finally, the radiation is deposited in the water at a calculated dose rate of 755 Gy/h, 98% of which is deposited within 5 cm around the reactor vessel and within 100 cm above and below the central plane. Due to the high flow rate, the water is frequently exposed to this radiation, with a calculated turnover time of 104 min. The total gamma activity ranges from less than 80 Bq/l during shutdowns to 1.9 x 10³ Bq/l on average during cycles, 95% of which originates from Na-24, a radioisotope with a 15-h half-life. In the purification circuit, the flow rate of the water is continuously maintained at all times at 30 m³/h.

The conductivity of the basin water is constantly kept at very low levels - between 1 and 1.2 µS/cm – in order to maintain the purity of the water. Due to the oligotrophic nature of the water, the concentration of microbiologically relevant compounds is very low irrespective of the operational status of the reactor. Total inorganic and organic carbon (TIC and TOC) are lower than 500 and 700 µg/l, respectively. Other compounds measured through ion chromatography such as nitrite and phosphate are below the detection limit of 10 µg/l, whereas nitrate and acetate have slightly higher concentrations of 44 ± 22 µg/l and 67 ± 33 µg/l, respectively. H₂ and O₂ concentrations were not measured, but since the basin is an open system and the water constantly circulates through the cooling and purification circuits, the water is estimated to be saturated with O₂ (8 ppm). The pH of the water displays an average value of 6 ± 0.4. A summary of the physico-chemical parameters of the basin water can be found in Table 1.

Table 1. Basin water conditions and characteristics

	Shutdown	Cycle
Water Temperature	Room temperature (ca. 21 °C ± 2 °C)	31 °C – 37 °C

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Radiation in the basin water	1 Gy/h of residual radiation	755 Gy/h deposited in the basin water close to the reactor vessel
Gamma activity	< 80 Bq/l	1.9×10^3 Bq/l on average
Flow rate in the cooling water circuit	50 m ³ /h	500 m ³ /h
Flow rate in the purification circuit	30 m ³ /h	
Conductivity in the basin water	between 1.0 and 1.2 μ S/cm	
Chemistry in the basin water	Total inorganic carbon (TIC): < 500 μ g/l Total organic carbon (TOC): < 700 μ g/l Acetate: 67 ± 33 μ g/l Nitrate: 44 ± 22 μ g/l Nitrite: < 10 μ g/l	
pH	6 ± 0.4	
Light conditions	Continuous illumination via TL lamps	
H₂	Not measured	
O₂	Estimated at 8 ppm	

Sample collection

Because of its low biomass and the presence of radioactivity, the basin water represents a challenging environment in terms of biological sample collection. Therefore, a new filtration system was designed to minimize the risk of radioactivity exposure and contamination during sampling in accordance with the ALARA principle (As Low As Reasonably Achievable) and to ensure that enough cell material could be collected for downstream analyses. Over time, the initial sampling system was upgraded to enable the sampling of larger volumes.

The initial sampling set-up was implemented in an existing sampling glove box routinely used to take samples from the basin water, and the primary and secondary cooling circuits, for chemical water analysis. It was composed of a stainless steel filter holder containing a 0,2 μ m pore sized polyethersulfone (Supor®) filter membrane (Pall Corporation, Port Washington, NY, USA), connected to the corresponding tap on one side and a flow meter on the other side in order to measure the filtered volume.

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Ten liters of water were filtered per sample, after which the filter membrane was retrieved from the system and stored at -20 °C in a controlled area (at least one week) to allow for radioactive decay before DNA extraction.

This set-up was only used for the 16S rDNA amplicon sequencing approach, where two sampling campaigns were performed (Van Eesbeeck *et al.* 2021) in order to collect a sufficient amount of samples to establish a baseline of the microbial community and its dynamics over time. The first one (61 samples) was conducted over a period of eight months encompassing three consecutive cycles (24 days on average) interspersed with three shutdown periods (39 days on average). The second one (21 samples) took place one year later and only covered a single cycle (29 days) and shutdown period (20 days).

Because of the larger amount of DNA necessary for shotgun metagenomics sequencing, a more elaborate filtration system was custom designed to enable the sampling of larger volumes. The water filtration system was implemented in a sampling glove box, allowing sampling of the basin water as well as from the primary cooling water (Fig. 2). It consisted of a stainless steel filter holder, opened and closed using clamps, that contained a 0,2 µm pore sized polyethersulfone (Supor®) filter membrane. Water branching off from the basin cooling circuit flowed through the system via a metal conduit, after which the waste was collected in an underground tank for disposal. The amount of water that passes through the filter was measured via a water meter (DHV1300, DH Metering Europe, Belgium) incorporated outside the glove box upstream of the filtration system. During cycles, a volume of 1000 l were filtered per sample over several days, whereas during shutdowns 500 l were filtered (a smaller volume was sufficient due to a higher microbial load), to ensure that a sufficient number of cells could be filtered and DNA could be extracted.

Eight samples were collected from the basin using this sampling set-up at different time points during cycles and shutdowns. After sampling, the filter membranes were retrieved from the filter holder and stored at -20°C in a controlled area before DNA extraction to allow for radioactive decay (varying between one week and four months).

All samples were processed at the same time for DNA extraction. These samples were used for shotgun metagenomics in order to characterize the microbial community more in depth and investigate its functional dynamics.

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Eventually, due to low DNA quantity, only four samples were sequenced adequately (two from one single cycle and two from two different shutdowns). The analysis was proceeded with these remaining four samples. An overview of the sample information can be found in Table 2.

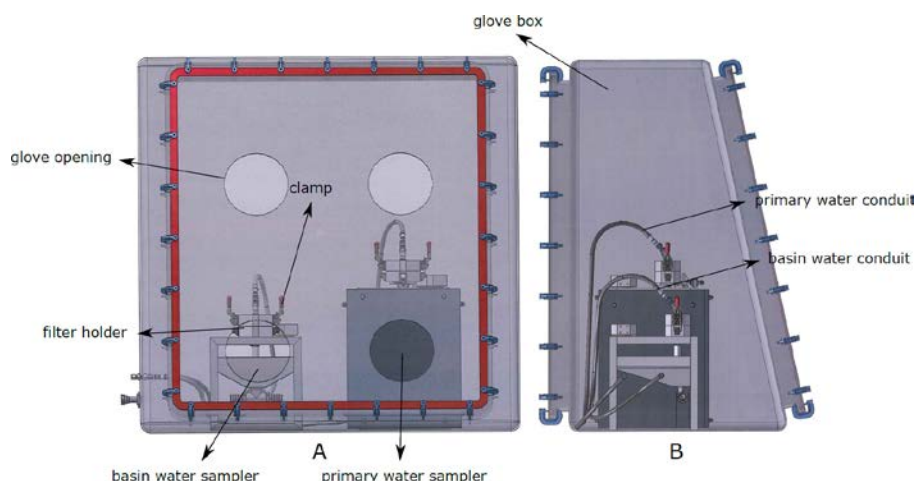


Figure 2. Schematic representation of the final custom made filtration system suitable for biological analysis. A: front view, B: side view. Individual components are indicated by arrows.

Table 2. Sample Inventory

Sampling system	Cycle	Shutdown
1 -16S rDNA amplicon study		
Volume	10 liters/sample	10 liters/sample
Samples – 1st campaign	Cycle 1 – 3 samples (C1 – C3)	Shutdown 1 – 3 samples (S1 – S3)
Description	61 samples collected over a period of eight months (September 2016 – April 2017) at a rate of one sample per weekday to two samples per week	
Number of days	24 on average	39 on average
Samples – 2nd campaign	Cycle 4 samples (C4)	Shutdown 4 samples (S4)
Description	21 samples collected one year later (April – June 2018) at a rate of three samples per week	
Number of days	29	20

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Sampling system 2 - Metagenome study	Cycle	Shutdown
Volume	1000 liters/sample	500 liters/sample
Samples	Cycle 5 (C5), of which 2 successful	Shutdown 5 (S5), of which 2 successful
Sample IDs	BW2 & BW3	BW11 & BW17
Description	8 samples of which 4 were successfully sequenced, collected immediately after the C4 – S4 sampling campaign (August 2018 – January 2019)	

DNA extraction and sequencing

DNA was extracted from the filter membranes following a previously described protocol (Vilchez-Vargas *et al.* 2013) and subsequently purified using Amicon® 30 kDa filter cartridges (Merck, Darmstadt, Germany). DNA concentrations were measured using a Quantus™ fluorometer (Promega, Madison, WI, USA).

For the 16S rDNA amplicon sequencing performed on the samples obtained with sampling system 1, the hypervariable V3-V4 region was targeted and resulting amplicons were sequenced in two runs using V3 chemistry (2 x 300 bp) with the Illumina MiSeq platform (sequencing performed at Eurofins Genomics in 2017). Positive and negative controls (extracted DNA from *Cupriavidus metallidurans* CH34 cultures filtered over 0.2 µm filter membranes, blank filter membranes and milli-Q water, respectively) were also added as part of the experiment (Van Eesbeeck *et al.* 2021).

For the metagenomics sequencing performed on the samples from system 2, a shotgun approach was applied with the Illumina HiSeq X Ten platform using 150 bp paired-end reads (sequencing performed at BGI Genomics in 2019).

Sequencing data analysis

16S rDNA amplicon sequencing data analysis

The data generated previously by the sequencing platform were demultiplexed and the datasets consisted of two separate fastq files (forward and reverse). The raw 16S rDNA amplicon sequencing data are submitted into the publicly available Sequence Read Archive (SRA) database of NCBI, under the BioProject accession number PRJNA725077.

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The sequences were subsequently analyzed through the OCToPUS pipeline as described in Mysara *et al.* (Mysara *et al.* 2017). In short, reads went successively through pre-assembly denoising (Bankevich *et al.* 2012), contig assembly, quality filtering using mothur (Schloss *et al.* 2009), denoising using IPED (Mysara *et al.* 2016), chimera removal using CATCh (Mysara *et al.* 2015) and finally OTU clustering with 97% cut-off using UPARSE (Edgar 2013). The OTUs were then taxonomically assigned using the mothur *classify.seqs* command with the RDP dataset (version 16) as reference. In order to adequately compare all the samples combined, they were rarefied to the same read count as the smallest sample (11,683 reads), using the mothur *sub.sample* command.

Functional prediction was performed using PICRUST (v.1.1.2) (Langille *et al.* 2013) in conjunction with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (v.58.1) (Kanehisa & Goto 2000). KEGG Orthology (KO) groups were compared between different conditions using STAMP (v.2.1.3) (Parks *et al.* 2014) and statistical significance was assessed through Welch's t-test for unequal variances. Raw p-values were adjusted for the False Discovery Rate (FDR) using the Benjamini-Hochberg correction (Benjamini & Hochberg 1995).

Shotgun metagenomics

The raw metagenome sequencing data were submitted into the European Nucleotide Archive (ENA) database, under accession number PRJEB44319/ERP128357. Individual reads generated by the sequencing platform were assembled into contigs with MetaSPAdes (Nurk *et al.* 2017). The contigs were then aligned using a Basic Local Alignment Search Tool (BLAST) against an in-house made database containing one randomly selected genome per bacterial species (selected by name) for a total of approximately 17,000 genomes (downloaded from NCBI in 04/2018). All the contigs with at least 50% of their sequence aligned to a certain genome were assigned entirely to that particular genome. The resulting metagenome-assembled genomes (MAGs) were annotated for genes using prokka (v.1.14.1) (Seemann 2014). Functional prediction was conducted with the Metagenomics Rapid Annotation using Subsystems Technology (MG-RAST) server based on the raw reads (Meyer *et al.* 2008), in conjunction with the KEGG database (v.58.1). An overview of the shotgun metagenomics data can be found in Table 3.

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Table 3. Summary of the shotgun metagenomics sequencing data

Sample	BW2	BW3	BW11	BW17
Phase	Cycle	Cycle	Shutdown	Shutdown
No. of total reads	37,325,420	37,858,962	41,383,562	41,026,228
No. of reads after QC	29,714,954	30,106,669	30,096,747	33,083,513
Proportion of reads assigned to Bacteria	99.1%	98.8%	98.8%	98.6%
Proportion of reads assigned to Eukaryotes	0.7%	0.9%	0.9%	1.1%
Proportion of reads assigned to Archaea	0.2%	0.3%	0.3%	0.3%
No. of contigs	259,571	234,570	515,857	313,066
No. of contigs > 1 kb	26,184	17,068	39,754	28,170
Average contig length > 1 kb	4,449	7,298	4,698	5,608
Min. contig length (bp)	55	56	55	56
Max. contig length (bp)	591,365	757,608	681,621	1,107,160

Comparison between both datasets

In order to statistically validate trends that were observed in the shotgun metagenomics data, the four successfully sequenced samples (two from a shutdown, two from a cycle) were complemented with 16S rDNA amplicon sequencing data from previous sampling campaigns monitoring four different cycles and shutdowns (Van Eesbeeck et al., unpublished). To be able to compare both datasets taxonomically, all 16S rRNA gene sequences were extracted from the metagenome using the Bacterial Ribosomal RNA Predictor (Barrnap v.0.9) software and subsequently trimmed to the V3-V4 region targeted for amplicon sequencing. The trimmed reads were then aligned to all OTUs with an average abundance > 0.1% from the 16S rDNA amplicon sequencing dataset. Principal Coordinates Analysis (PCoA) was performed in mothur (v.1.44.3) (Schloss *et al.* 2009) with Theta-YC distance calculation using the *pcoa* and *dist.shared* commands, respectively (supplemental fig. S1). For the comparison of the functional predictions of both datasets, KO groups obtained through PICRUST and MG-Rast were combined and analyzed in STAMP (v.2.1.3).

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Only KO groups with a statistically significant difference between the cycle and shutdown condition based on the 16S rDNA amplicon sequencing dataset (adjusted p-value < 0.05 after applying the Benjamini-Hochberg FDR correction) were taken into account. Out of those, only the ones that showed a consistent difference in average between cycle and shutdown samples (either positive or negative) across both datasets were selected.

Results

In order to test our hypothesis that the periodic shifts in physico-chemical parameters linked with the operational status of the reactor significantly affect the microbial community composition and functional traits in the basin water, samples were taken at different time points across shutdowns and cycles using our custom designed filtration system. To validate this hypothesis we used a shotgun metagenomics approach, and combined these metagenome data with the 16S rDNA amplicon sequencing formerly obtained from the basin water from a previous sampling campaign.

DNA extraction and shotgun metagenomics sequencing data

Although a larger volume of water was filtered during cycles (1000 liters/sample) than during shutdowns (500 liter/sample), about 15 times more DNA was extracted from the shutdown samples than the cycle samples (15 vs. 1 ng/μl on average per 100 liters of water, respectively), suggesting a larger microbial load during shutdowns than during cycles.

From the eight samples collected, only four could be successfully sequenced (due to the lack of enough high-quality DNA) and subsequently analyzed. The first two samples originated from one single cycle and the other two from two different shutdowns. The data show that 99% of all reads were assigned to bacteria, while only 1% was assigned to eukaryotes and archaea, regardless of cycle or shutdown condition.

Taxonomic community dynamics during cycle vs. shutdown

For the taxonomic analysis of the metagenome data set, the 16S rRNA gene sequences were extracted from the metagenome and were aligned to all OTUs exceeding 0.1% average abundance from the previously obtained 16S rDNA amplicon sequencing dataset.

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Based on a PCoA comparing all samples (supplemental Fig. S1), the 16S rDNA amplicon sequencing data of the samples from cycle and shutdown number four (last cycle and shutdown of the 16S rDNA sampling campaign) were selected for further analyses as those displayed the highest similarity with the shotgun metagenomics samples.

Fig. 3 shows the ten most abundant OTUs across both datasets. As can be observed from the bar plot, there is a clear difference in the community profile between shutdown and cycle samples. The difference appears to be consistent across both datasets (16S rDNA amplicon and metagenome sequencing campaign), which further confirms the comparability of both approaches. The OTU classified as *Methylobacterium* (OTU3) seems to be a key player in the community, both during shutdown and cycle, as it is the most abundant OTU overall. Moreover, it is significantly more abundant during shutdown, whereas an unclassified *Gammaproteobacterium* (OTU5) has a higher relative abundance during cycles.

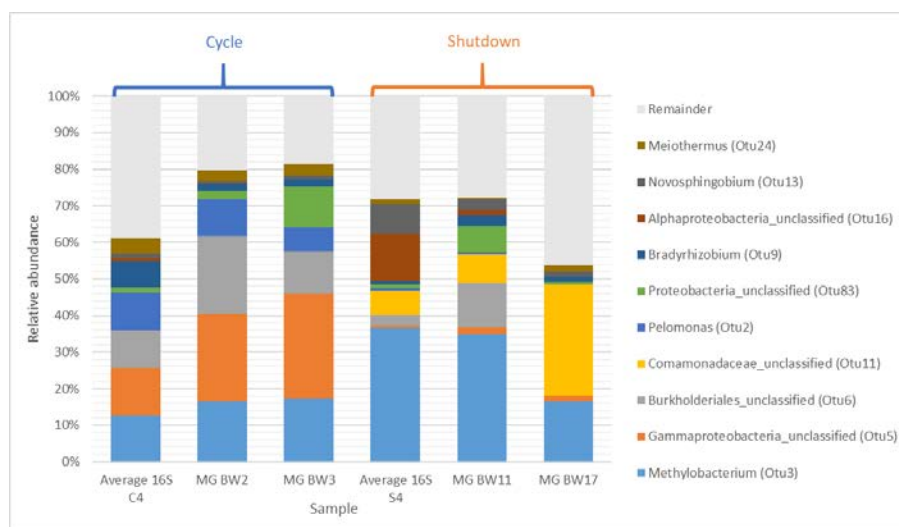


Figure 3. Bacterial community structure of the basin water based on shotgun metagenomics data, compared with previous 16S rDNA amplicon sequencing data. Only the ten most abundant OTUs are displayed for both datasets. Each OTU is classified at the genus level. The three bars on the left represent cycle samples, the three bars on the right represent shutdown samples. The first bar of each group represents the average of the 16S rDNA amplicon sequencing samples (n=12 for C4 and n=6 for S4) obtained from a previous sampling campaign (see Table 2, C4 – S4), added as a reference for the shotgun metagenomics samples.

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Functional community dynamics during reactor cycle vs. shutdown

Due to the limited number of samples, the functional comparison between cycle and shutdown based on the metagenomics dataset was complemented with the functional database created from the previously existing 16S rDNA amplicon dataset, i.e. the data from shutdown and cycle four (see Table 2) which was demonstrated to be taxonomically comparable. The results from the analysis based on the metagenomics dataset, and validated by additional data included from the 16S rDNA amplicon dataset, can be found in Fig. 4. KO groups from the shotgun metagenomics dataset were checked for differences between the cycle and shutdown condition. If the same difference could be found in the KO groups from the 16S rDNA amplicon sequencing dataset, those pathways were selected and p-values were subsequently calculated. Only pathways with a corrected p-value < 0.05 were taken into account. Regarding the functional comparison, out of 279 pathways for both datasets combined, there were 151 overlapping pathways (54% of the total number), which were subsequently selected for the functional analysis of the community dynamics comparing cycle and shutdown conditions. Only the pathways displaying the same shift between shutdown and cycle (either positive or negative) across both datasets were selected for further discussion. The pathways are ranked from highly significant to significant differences between the shutdown and cycle condition. Some pathways such as pyruvate metabolism, glycerophospholipid metabolism and purine metabolism are more highly represented during shutdown, whereas others such as histidine metabolism were less represented or reduced during shutdown. This means that the DNA extracted from the microbial community through both the 16S rDNA amplicon sequencing and metagenomics approach contained relatively more genes from those pathways during shutdowns than during cycles. Out of all the displayed pathways, the ones that had the most reads assigned to them were valine, leucine and isoleucine biosynthesis and pyruvate metabolism, with average mean proportions of 2.05% and 1.25% of reads assigned, respectively. Some pathways such as alzheimer's disease and apoptosis might contain enzymes or other proteins associated with these pathways, but which might have other functions in bacteria than in humans.

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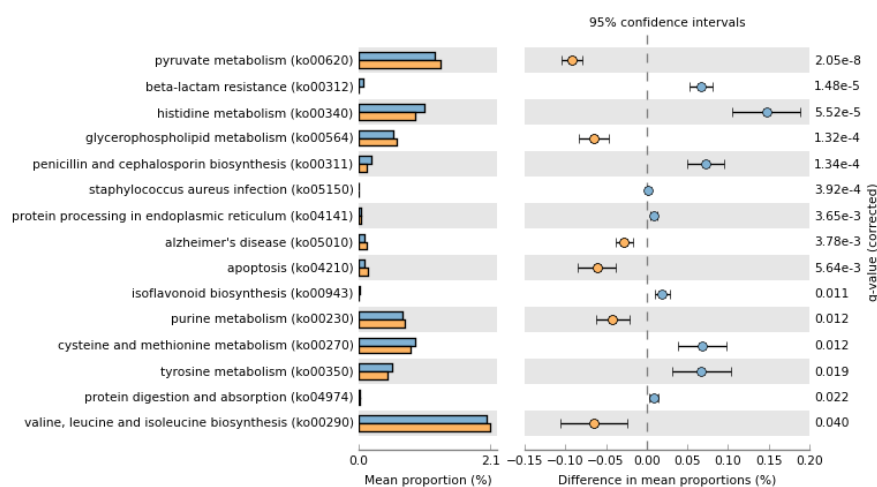


Figure 4. Differentially abundant KEGG pathways for cycle vs. shutdown. Orange = shutdown, Blue = cycle. Only the pathways from the 16S rDNA amplicon sequencing dataset with a corrected p-value < 0.05, are shown. Mean proportions for shutdown (n=6) and cycle (n=12) conditions for each pathway are displayed as percentages of reads assigned to that particular pathway divided by the total number of reads assigned to all other pathways. Pathways are ranked from top to bottom according to increasing corrected p-values, thus from highly significant to significant.

Metagenome assembly

Out of the assembled metagenome from all four samples, the genomes of two different species, MAG1 and MAG2 were almost entirely reconstructed, corresponding most highly with two strains from our database: *Methylobacterium* sp. UNC378MF (matches *Methylobacterium* OTU3) and *Bradyrhizobium* sp. BTAi1 (matches *Bradyrhizobium* OTU9), with 84% and 97% of their genome completed, respectively. The MAG1 *Methylobacterium* species amounted to 5.8 Mb with an average coverage (depth of sequencing) of 165, and the MAG2 *Bradyrhizobium* species to 8.7 Mb with an average coverage of 19. A summary of the MAG properties for both MAGs can be found in Table 4.

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Table 4. Metagenome-assembled genome (MAG) properties for MAG1 and MAG2.

N50 = shortest contig length needed to cover 50% of the genome. L50 = smallest number of contigs whose length sum makes up half of the genome. Average Nucleotide Identity (ANI) = similarity index between the MAGs and reference strains. A cutoff score of > 95% indicates that they belong to the same species.

MAG property	<i>Methylobacterium</i> sp. MAG1	<i>Bradyrhizobium</i> sp. MAG2
Total length (Mb)	5.8	8.7
Shortest sequence (bp)	509	503
Longest sequence (bp)	351,404	319,132
Number of sequences	90	226
Genome completeness (%)	84	97
Genome coverage	165	19
GC content (%)	70	65
N50 (bp)	121,665	109,168
L50	15	27
No. of genes	2,460	3,520
Reference strain	<i>Methylobacterium</i> sp. UNC378MF	<i>Bradyrhizobium</i> sp. BTAi1
ANI similarity (%)	99.1	99.9

Discussion

It was hypothesized that the periodic shifts in physico-chemical parameters linked with the operational status of the BR2 reactor significantly affect the microbial community dynamics in the basin water, both on the taxonomic and functional level. In order to test this hypothesis, a shotgun metagenomics approach was performed in combination with 16S rDNA amplicon sequencing results from a previous sampling campaign, on a selected number of samples taken during cycle and shutdown periods, using a custom designed filtration system.

A first important finding is that a microbial community can indeed be found in the nuclear reactor basin water, throughout both the shutdown and the active period (cycle).

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Based on the amount of intact DNA that could be extracted per liter of water however, it seems that during shutdowns a higher load of microbes/DNA was present, compared to during cycles. It must be stressed that the environment under study is much more stringent than the previously studied SNFPs, with much higher flow rates (up to 500 m³/h) and radiation (755 Gy/h deposited in the pool close to the reactor vessel and 1.9 x 10³ Bq/l gamma activity during cycles) than the more static SNFP environments. So the strong flow rate and/or irradiation conditions during cycles have a detrimental effect on the sample quality and/or microbial community, which can restore during shutdowns.

At least part of the microbial community was confirmed to be viable during both cycle and shutdown conditions, as some isolates were grown and purified on R2A medium from both conditions (data not shown). As the basin in our study is an open system that is regularly accessed for maintenance during shutdown periods (after pumping the upper water volume to an underground buffer tank), additional microbes to the ones already present in the demineralized ground water used to replenish the basin might enter the system via dust particles falling in the pool, through maintenance operations carried out by technicians and/or through mixing with the water in the buffer tank that also contains bacteria. This could also introduce additional nutrients to be used by the microbial community.

The metagenomics dataset revealed that the basin environment was dominated by bacteria (99% of all reads), with only 1% of eukaryotes and archaea, regardless of cycle or shutdown condition. This is consistent with other similar environments such as cooling pools of nuclear reactors and spent nuclear fuel pools, where the microbiome was recently investigated and only bacteria could be detected, even with protein-based techniques such as proteotyping (Petit *et al.* 2020; Ruiz-Lopez *et al.* 2020). In contrast, another study investigating the microbial composition of the biofilms on the walls of a spent nuclear fuel pool using a shotgun metagenomics approach found a high proportion of fungi (Silva *et al.* 2018), whereas yet another one found the presence of a green microalga in a similar environment (Rivasseau *et al.* 2016). This suggests that equivalent environments might still harbor significant differences in their microbiome.

The four metagenomics samples analyzed here showed a high degree of taxonomic similarity with data of a previous 16S rDNA amplicon sequencing of the same basin water, one year earlier.

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The highest level of similarity was observed for cycle and shutdown four (C4 and S4), which were also temporally closest to the metagenomics samples. Next to the taxonomic composition, a significant number of overlapping pathways were found between the metagenomics dataset and the predicted functions based on the 16S DNA taxons found in the C4 and S4 samples (151 in total). It was therefore concluded that both datasets are comparable, both taxonomically and functionally, and could be used to complement each other.

Regarding the taxonomic analysis of the metagenome dataset, Fig. 3 highlights a clear difference in OTU composition between the shutdown and cycle condition, which was consistent with the previous 16S rDNA amplicon sequencing datasets (Van Eesbeeck *et al.* 2021). As can be observed, OTU3, classified as a *Methylobacterium*, is the most abundant OTU overall in the metagenome dataset and 16S rDNA dataset, indicating that it is best adapted to the oligotrophic conditions observed in the environment. Furthermore, it had a consistently higher relative abundance during shutdown compared to the cycle condition, together with an unclassified member of the *Comamonadaceae* family (OTU11). In contrast, an unclassified *Gammaproteobacterium* (OTU5) was more relatively abundant during cycles of operation. *Methylobacterium* is an aerobic facultative methylotroph that can use one-carbon compounds such as methanol, but also two-carbon compounds like acetate, as a sole carbon and energy source (Smejkalova *et al.* 2010). The acetate detected in our system might be coming from the organic ion exchange resins consisting of polystyrene beads, which slowly degrade over time due to the ambient radiation and are therefore replaced every five years. Interestingly, *Methylobacterium* was also found to be the most abundant genus in the cooling pool of the French nuclear reactor Osiris during shutdown periods (Petit *et al.* 2020). Additionally, this genus has been identified in similar environments such as SNFPs in Spain and in the USA (Chicote *et al.* 2005; Bagwell *et al.* 2018). Finally, *Methylobacterium* was also described to be strongly resistant to gamma radiation (Nogueira *et al.* 1998; Kang & Srinivasan 2018; Kim *et al.* 2020). These data indicate that its presence is probably not facility- or operation-dependent, but rather environment-dependent and that this bacterium might be well adapted to life in the extreme conditions of a nuclear reactor basin combining low nutrient levels with high levels of radiation.

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Furthermore, it has been previously demonstrated that some bacteria in SNFPs can utilize H₂ originating from water radiolysis as an energy source (Galès *et al.* 2004). OTU11 was classified as a member of the Comamonadaceae family, which has been previously associated with chemoautotrophic growth on H₂ (Willems *et al.* 1991). Some genera from this family, such as *Hydrogenophaga* and *Variovorax* known to be able to grow on H₂, have been found in SNFP environments as well as the cooling pool of the Osiris reactor (Ruiz-Lopez *et al.* 2020; Petit *et al.* 2020). The OTU associated with this bacterial family might therefore be well suited to our basin environment, where residual radiation might be causing radiolysis during shutdown periods, thereby producing a steady stream of H₂ to be used as energy source by these bacteria.

Regarding the functional analysis, some predicted pathways are proportionally more represented during shutdown periods than during cycles (Fig. 4). Most of these pathways, namely pyruvate, glycerophospholipid and purine metabolisms and the biosynthesis of valine, leucine and isoleucine, suggest a role in cell function recovery after ionizing radiation.

The pyruvate metabolism is of crucial importance during post-irradiation recovery, as it improves the energy supply of the cells. Pyruvate is the end product of glycolysis and is involved in the tricarboxylic acid cycle (TCA). Glycolysis and the TCA cycle were found to be progressively induced in the late phase of recovery after acute irradiation of *Deinococcus radiodurans* R1 (Liu *et al.* 2003). In another study investigating the proteomics changes in a radiation-resistant *Micrococcus luteus* strain after gamma irradiation, several proteins were found to be differentially expressed upon 2-kGy irradiation, among which a major component of the pyruvate dehydrogenase complex involved in the TCA cycle (Deng *et al.* 2016).

Ionizing radiation is known to damage the cell membrane lipid bilayer, mostly indirectly through oxidation by Reactive Oxygen Species (ROS) produced by water radiolysis (Benderitter *et al.* 2003). In order to repair this damage, a cell needs to upregulate the glycerophospholipid metabolism. It was demonstrated that *E. coli* was indeed capable of removing membrane lesions through an enzymatic repair process after being exposed to ionizing radiation (Gillies *et al.* 1984).

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Furthermore, some bacteria such as *Pantoea agglomerans* and *Salmonella enterica* have been shown to change their membrane fatty acid composition (saturated to unsaturated ratio) as a mechanism for protection upon gamma irradiation (Dussault *et al.* 2009; Lagha *et al.* 2015) by controlling membrane fluidity (Denich *et al.* 2003).

Another site of ionizing radiation damage is DNA itself. This can occur directly through the disruption of the sugar backbone and the purine/pyrimidine base (Close *et al.* 2013) and indirectly through oxidation by ROS (Jung *et al.* 2017). This can result in single-strand breaks (SSBs) and double-strand breaks (DSBs), potentially causing genome instability and cell death if not appropriately repaired (Azzam *et al.* 2012; Terato *et al.* 1999). Fig. 4 shows that the predicted purine metabolism pathway is more represented in the data during shutdowns, suggesting a role in DNA repair after cell irradiation during cycles. In an *E. coli* strain, transcriptomic and proteomic approaches indeed found that several pathways, such as base- and nucleotide-excision repair, were upregulated as stress response to gamma irradiation (Gaougaou *et al.* 2020). Interestingly, the same study also showed a dramatic reduction in histidine biosynthesis after irradiation, which corresponds to the pattern for histidine biosynthesis observed in our own data. This could be explained by the fact that histidine enhances oxidative DNA damage to *E. coli* cells by generating OH• radicals in the presence of H₂O₂ (Nagao *et al.* 2018).

Finally, ionizing radiation also has an impact on proteins, causing oxidative damage and potential cell death (Daly 2012). A recent study investigating proteomic oxidation in *E. coli* using mass spectrometry found that leucine was one of the amino acids that was significantly impacted by ionizing radiation, displaying multiple oxidative modifications (Bruckbauer *et al.* 2020). Therefore it is not surprising that the valine, leucine and isoleucine biosynthesis pathway was more highly represented in our data during shutdowns, indicating a role in protein repair after irradiation. Furthermore, irradiation of a highly radiation-resistant micro-alga isolated from a nuclear reactor resulted in the cellular increase of these same three amino acids (Rivasseau *et al.* 2010).

With respect to the metagenome assembly, the genomes of two different species could be almost entirely reconstructed, which corresponded with two strains from our database, namely *Bradyrhizobium* sp. BTAi1 and *Methylobacterium* sp. UNC378MF with a genome completeness of 97% and 84%, respectively.

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To our knowledge, this is the first time this has ever been described for bacteria living in these types of dynamic environments that combine extremely oligotrophic conditions with high flow rates and high radiation doses.

Bradyrhizobium sp. BTAi1 is a symbiotic, photosynthetic strain that was isolated from an aquatic plant species (*Aeschynomene indica*) in North America. It must be noted that its photosynthetic capacity is a rare trait among rhizobia (Giraud *et al.* 2013). The involved genes are clustered in one single region on the genome, namely the photosynthesis gene cluster (PGC), also found in purple photosynthetic bacteria (Giraud *et al.* 2007). It contains a single, circular chromosome of 7.5 Mb as well as a 229 kb-plasmid, pBTAi1. The genome of this strain is highly plastic, with 29 horizontally acquired genomic islands (HAIs), which contain genes encoding important metabolic functions such as the previously mentioned PCG encompassing the ribulose-1,5-biphosphate carboxylase-oxygenase enzyme (RuBisCo), as well as enzymes involved in nitrogen fixation and a chemotaxis operon. In addition, the genome also contains a hydrogenase gene cluster containing uptake hydrogenase complexes. The pBTAi1 plasmid also contains genes encoding heavy metal resistance-related proteins (Cytryn *et al.* 2008).

These bacteria are also capable of nitrogen fixation not only in the symbiotic, but also in the free-living state (Alazard 1990). Our *Bradyrhizobium* MAG also contained several genes involved in photosynthesis (*cbbL*, *cbbS* and *cbbT*), nitrogen fixation (*nif* and *fix*), chemotaxis (*che*) and *hup* genes encoding hydrogenases. It also contained one gene encoding a heavy metal exporter (*atm1_2*). The strain found in our basin water system might thus be well adapted to life in these extremely oligotrophic conditions where essential nutrients such as carbon and nitrogen are scarce and residual radionuclides reside. It might also be using the artificial lighting present in the BR2 reactor as a light source for photosynthesis.

Methylobacterium sp. UNC378MF is an unclassified *Methylobacterium* strain whose full genome was deposited in the NCBI database in the form of 77 contigs, with a genome coverage of 324x. Our *Methylobacterium* sp. MAG2 contained several genes related to DNA repair processes, such as *RecB*, *RecA* and *Ssb*, which are vital in the radiation resistance mechanism. These genes were also found in a radiation-resistant *Methylobacterium* strain isolated from gamma-irradiated soil in South Korea (Kang & Srinivasan 2018).

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Furthermore, several genes related to the acetate metabolism, such as *poxB*, *ackA*, *actP_2* and *actP_3*, were also detected. The former two are involved in the acetate generation from pyruvate or acetyl-CoA, whereas the latter two encode symporters responsible for acetate uptake across the cell membrane (Pinhal *et al.* 2019). Similarly, a *Methylobacterium* strain was shown to better express its acetate transporter (*actP*) during growth on acetate (Schneider *et al.* 2012). Finally, two genes related to nitrogen fixation (*fixK_1* and *fixK_2*) were also observed in the genome of the *Methylobacterium* sp. MAG2. These data suggest that the *Methylobacterium* strain found in our basin water might be able to thrive under the harsh conditions characterizing this system, combining varying levels of radiation and radionuclides, low nutrient availability and the presence of residual levels of acetate.

Conclusion

In conclusion, we managed to characterize the microbial community found in the basin water of a nuclear reactor, which combines high levels of radiation and high flow rates with low nutrient availability, using a metagenomics approach in combination with 16S rDNA amplicon sequencing. Our results confirmed our main hypothesis stating that the periodic shifts in physico-chemical parameters affect the microbial community, both on the taxonomic and functional level. Indeed, the community was dominated by a *Methylobacterium* (OTU3) and a member of the Comamonadaceae family (OTU11) during shutdowns, whereas a member of the Gammaproteobacteria (OTU5) was relatively more abundant during cycles. On the functional level, most of the pathways that were more highly represented during shutdowns suggested a role in cell function recovery after irradiation. Furthermore, the two MAGs that were almost entirely reconstructed from the metagenome (most similar to *Bradyrhizobium* sp. BTAi1 and *Methylobacterium* sp. UNC378MF) displayed adaptations in their genomes that allowed them to thrive under the harsh conditions found in our basin water system, where vital nutrients are scarce and varying levels of radiation and radionuclides prevail.

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Supplementary data

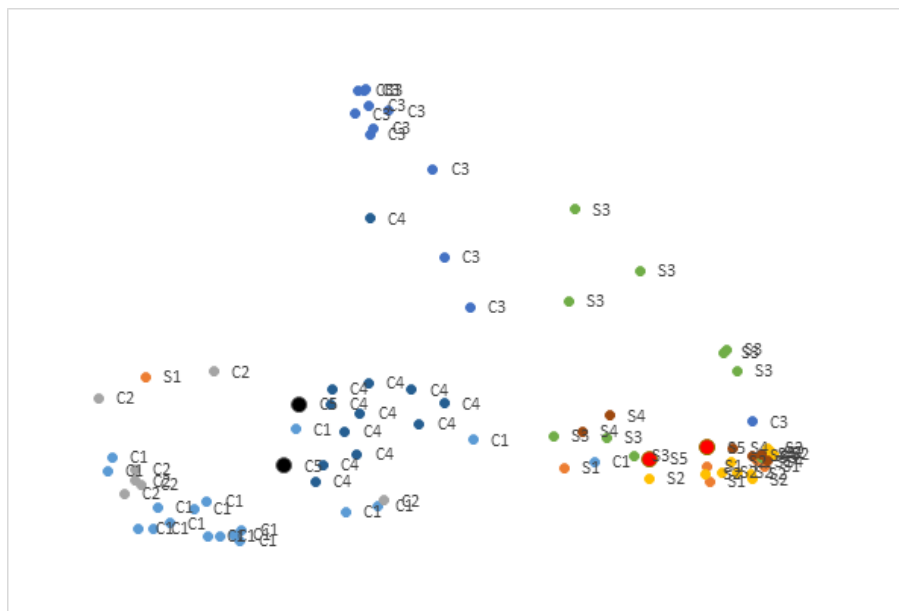


Figure S1. Comparison of the 16S rRNA gene sequences extracted from the shotgun metagenomics, with the previously obtained 16S rDNA amplicon sequencing data of the basin water. 16S rRNA gene sequences extracted from the shotgun metagenomics dataset and trimmed to the V3-V4 region were compared to OTUs from the 16S rDNA amplicon sequencing dataset, via Principle Component Analysis (PCoA). C = cycle, S = shutdown. The C1 to C4 and S1 to S4 dots represent all the 16S rDNA amplicon sequencing samples. The C1 to S3 samples were collected consecutively in time over a period of eight months at a rate of one sample per weekday to two samples per week. The C4 to S4 samples were collected one year later at a rate of three samples per week. The two large black dots (C5) and the two large red dots (S5) represent the shotgun metagenomics samples, which were collected right after the S4 samples.

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1. Microbial diversity in the watery environments of the BR2

1.1 Microbial genera found across three different approaches

In order to investigate the microbial communities in the various watery environments of the BR2, three different approaches were implemented: cultivation of individual strains on oligotrophic media, 16S rRNA amplicon sequencing and shotgun metagenomics. For the cultivation approach, strains were isolated from a variety of different environments, namely the primary circuit, the basin surrounding the reactor core vessel, the GIF and CMF pools, the DW2 tank used to replenish water in the primary circuit during reactor operation (cycles) and the storage tank used to transfer the upper water layer of the basin during shutdowns. On the other hand, 16S rRNA amplicon sequencing and shotgun metagenomics were used to specifically study the microbial dynamics in the basin surrounding the reactor vessel, as this is a very dynamic environment characterized by periodic shifts in physico-chemical parameters such as temperature, radiation and flow rate, in accordance with the operational status of the reactor (cycle or shutdown). When comparing the microorganisms detected through all three approaches by comparing their respective DNA sequences, several genera were commonly found in the basin, namely *Bradyrhizobium*, *Mesorhizobium*, *Pelomonas* and *Sphingomonas*. In addition, two strains from the *Methylobacterium* and *Vibrionimonas* genera isolated from the primary circuit were also detected in the basin through the 16S rRNA amplicon sequencing and shotgun metagenomics approaches. When comparing the unique species isolated from the basin with the OTUs from the 16S rRNA amplicon sequencing dataset, we found a fraction of 0.3% that could be cultivated.

Bradyrhizobia are described as Gram-negative, rod-shaped, aerobic, motile and non-spore forming bacteria capable of nitrogen gas (N₂) fixation. (Jordan, 1982). Some strains are also capable of CO₂ fixation through photosynthesis (Giraud *et al.* 2007) and contain photosynthetic pigments such as bacteriochlorophyll a and carotenoids (Molouba *et al.* 1999). This could grant them additional protection against ROS. The *Bradyrhizobium* genus was detected in several indoor SNFPs in the Cofrentes nuclear power plant in Spain (Sarró *et al.* 2003, 2005, 2007; Chicote *et al.* 2005) and at the Savannah River Site in the USA (Bagwell *et al.* 2018),

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as well as in the storage tank and water purification system of a Hungarian power plant (Bohus *et al.* 2011; Kéki *et al.* 2013, 2019). In addition, it was detected in other ultrapure water systems employed in various industries such as the semiconductor industry (Kulakov *et al.* 2002). Thus it appears to thrive in oligotrophic water systems, both in the presence and absence of ionizing radiation. As previously discussed in Chapter III – Section 3, the *Bradyrhizobium* sp. MAG1 strain detected in the basin (most similar to *Bradyrhizobium* sp. BTai1) harbors genes related to both nitrogen fixation and photosynthesis, which could also occur under the artificial light present in the BR2 reactor. The latter is a rare trait among rhizobia and could be used as an efficient survival strategy to cope with the extremely oligotrophic conditions prevailing in our system.

Species in the *Mesorhizobium* genus are Gram-negative, rod-shaped, aerobic, non-spore forming, motile bacteria that are also capable of nitrogen (N_2) fixation (Chen *et al.* 2015). They generally grow at slower rates than other rhizobia. Similar to *Bradyrhizobium*, this genus was commonly detected in various SNFPs in Spain, Argentina and Brazil (Sarró *et al.* 2003, 2005, 2007; Giacobone *et al.* 2011; Silva *et al.* 2018). In addition, it was also isolated from the storage tank and water purification system of a Hungarian power plant (Bohus *et al.* 2011; Kéki *et al.* 2013, 2019).

The *Pelomonas* genus is characterized by Gram-negative, rod-shaped, aerobic, non-spore forming, motile bacteria that are associated with autotrophic growth (CO_2 fixation) on H_2 (Gomila *et al.* 2007). Furthermore, some species such as *P. saccharophila* have been demonstrated to be capable of N_2 fixation (Barraquio *et al.* 1986). The *Pelomonas* genus was previously detected in a SNFP at the Savannah River Site in the USA (Bagwell *et al.* 2018) and in the cooling pool of the Osiris nuclear reactor in France (Petit *et al.* 2020). It was also retrieved from the water purification system of a Hungarian power plant (Kéki *et al.* 2013, 2019). Specifically, *Pelomonas saccharophila* (formerly known as *Pseudomonas saccharophila*) was isolated from various UPW systems implemented in the semiconductor industry and from an SNFP in Spain (Kulakov *et al.* 2002; Chicote *et al.* 2005). In our study, *P. saccharophila* was also isolated from the primary circuit, basin and SNFP of the BR2 reactor.

Members of the *Sphingomonas* genus are described as Gram-negative, non-spore forming, rod-shaped, chemo-heterotrophic, strictly aerobic bacteria that typically produce yellow-pigmented colonies (White *et al.* 1996).

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Although not a common trait, some strains have been associated with nitrogen fixation (Videira *et al.* 2009).

This genus was commonly observed in various SNFPs in Spain, USA, UK and Brazil (Chicote *et al.* 2005; Bruhn *et al.* 2009; McGraw *et al.* 2018; Silva *et al.* 2018; Ruiz-Lopez *et al.* 2020) and in the cooling pool of the Osiris nuclear reactor in France (Petit *et al.* 2020). It was also detected in the storage tank and water purification system of a Hungarian power plant (Bohus *et al.* 2011; Kéki *et al.* 2013, 2019), in various UPW systems used in the semiconductor industry (Kulakov *et al.* 2002) and in a radioactive site in Japan (Asker *et al.* 2007c).

The *Sphingomonas* strain isolated from the SNFP of the BR2 (*S. melonis*) was shown to be slightly resistant to radiation, as it was able to survive an acute γ -irradiation dose of 2.1 kGy originating from ^{60}Co sources. It has been reported that the possession of carotenoid pigments, like the ones produced by this yellow *Sphingomonas* strain, can have an antioxidant effect and provide radiation protection.

Species from the *Methylobacterium* genus are strictly aerobic, non-spore forming, facultatively methylotrophic, slow-growing bacteria that form pink-pigmented colonies. They are rod-shaped and have one polar flagellum for motility (Patt *et al.* 1976). They are ubiquitously found in a variety of natural environments such as soil, dust, leaf surfaces and fresh water (Vanechoutte *et al.* 2011; Sanders *et al.* 2000). Some strains are also known to be capable of nitrogen fixation (Sy *et al.* 2001). Similar to the other described genera, *Methylobacterium* was commonly detected in different SNFPs in Spain (Sarró *et al.* 2003, 2005, 2007; Chicote *et al.* 2005) and the USA (Bagwell *et al.* 2018), in the cooling pool of the Osiris reactor in France (Petit *et al.* 2020), in the water purification system of a Hungarian power plant (Kéki *et al.* 2013, 2019), in ultrapure water used in hospital environments and in the pharmaceutical industry (Kressel & Kidd 2001; Kawai *et al.* 2004) and in the ISS (Bijlani *et al.* 2021). The *Methylobacterium* strain detected in the basin of the BR2 might be able to grow heterotrophically on acetate, which is present in low concentrations in the water. Indeed, several genes related to acetate metabolism were found in the *Methylobacterium* MAG strain whose genome was obtained through shotgun metagenomics. In addition, it also contained several genes involved in nitrogen fixation and DNA repair, a crucial component of radiation resistance.

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Finally, the recently described *Vibrionimonas* genus is characterized by Gram-negative, aerobic, curved rod-shaped bacteria with a single known species – *V. magnilacihabitans* – isolated from lake water (Albert *et al.* 2014). This genus was not previously found in SNFPs or any other waters associated with nuclear reactors. Therefore it could be interesting to further investigate its metabolism and susceptibility to ionizing radiation.

As can be noted, most of the identified genera share the common characteristics of being Gram-negative, aerobic, motile and non-spore forming, which seem to be evolutionary adaptations to the conditions found in the waters of the BR2. However, a few exceptions were also detected, such as the Gram-positive *Bacillus*, *Brevibacillus* and *Staphylococcus* genera isolated from the SNFP, basin and primary circuit, respectively.

1.2 Survival in nuclear reactor waters: quite a challenge !

As we have seen, the different described genera have adopted a variety of strategies for survival in the extremely challenging conditions prevailing in the watery environments of the BR2, specifically the basin surrounding the reactor core, combining low nutrient availability with the presence of ionizing radiation.

Some detected bacteria could be capable of autotrophic fixation of CO₂ through photosynthesis (*Bradyrhizobium*) or by using H₂ as an energy source (*Pelomonas* and other members of the *Comamonadaceae* family). Several bacterial genera were also capable of nitrogen fixation (*Bradyrhizobium*, *Mesorhizobium* and potentially *Methylobacterium*, *Pelomonas* and *Sphingomonas*). These are important traits enabling the bacteria to survive in the extremely oligotrophic conditions prevailing in the waters of the BR2, as they are no longer dependent on the presence of organic nutrients, but can produce their own organic molecules from inorganic C and N sources in the air. These are homogeneously mixed with the water by constant circulation in the various cooling and purification circuits through pumping. This also explains why most of the described strains are aerobic.

The heterotrophic bacteria detected in our study (such as *Sphingomonas* and *Methylobacterium*) are likely relying on mixotrophy and/or nutritional flexibility as a coping mechanism for the extremely oligotrophic conditions.

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In one particular study, bacteria isolated from an UPW system in a semiconductor manufacturing plant were classified into two groups: oligotrophic and copiotrophic (found in nutrient-rich environments), according to their capacity to grow on oligotrophic media (Kim *et al.* 1997). When compared to the copiotrophic bacteria, the oligotrophic bacteria were demonstrated to be capable of utilizing a significantly larger range of organic substrates, confirming that nutritional flexibility is an adaptive mechanism specific for oligotrophic bacteria. Along the same line, the *R. pickettii* strain isolated by McAlister *et al.* (2002) was also observed to grow on a wide range of organic substrates, corroborating the previous findings. The bacteria retrieved in our basin water system are therefore believed to use whatever substrate becomes available to them as an efficient survival strategy.

Indeed, bacteria in these waters could also rely on so-called “cryptic growth”, a phenomenon where lysis products from dead biomass can be used as carbon and energy sources by viable cells (Ryan 1959). Bacteria in oligotrophic environments are believed to make particular use of this strategy in order to avoid starvation. The impact of dead bacterial cells in ultrapure water has been somewhat underestimated. One specific study investigating the bacteria in an UPW system using fluorescence microscopy found that a significant percentage (50-90%) of the bacterial population in this system was actually nonviable (McAlister *et al.* 2001). Another study showed that some bacterial strains isolated from UPW systems were indeed capable of cryptic growth when supplemented with heat-killed cells (McAlister *et al.* 2002). One of these isolates was a *Ralstonia pickettii* strain, a species also isolated from the BR2 basin in our study. Cryptic growth is also known to occur in biofilms, which can be several cell layers thick in UPW systems (Henley 1992; McFeters *et al.* 1993). Accumulating dead cells in these biofilms can be used as carbon and energy sources by successive generations of bacteria (Roszak & Colwell 1987). This is also believed to occur in the various water systems of the BR2, as we never detected high concentrations of TOC in suspension.

The formation of biofilms is also a mechanism enabling bacteria to better cope with the presence of ionizing radiation. Bacteria in various SNFPs and other ultrapure water environments are indeed known to form biofilms as a survival strategy by adhering to inner piping and other surfaces (Sarró *et al.* 2003, 2005, 2007; Chicote *et al.* 2005; Mittelman & Jones 2018).

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The EPS matrix in which the microbes of these biofilms are embedded form a protective layer against ionizing radiation, allowing bacteria that might otherwise be more susceptible to radiation to better tolerate this additional environmental stressor. Biofilms were indeed also observed (macroscopically) on the wall surfaces and underwater racks used for spent fuel storage in the SNFP of the BR2, as well as under the form of biological aggregates in the GIF and CMF pools located in the basin (data not shown). Unfortunately the sampled biofilms could not be further analyzed in this study due to their high radioactivity load and residual dose rate. And while not directly observed, they are also believed to be present in the piping of the various cooling and purification circuits of the reactor. Biofilms in these circuits are not directly exposed to ionizing radiation, which would allow bacteria inhabiting this ecological niche to escape direct contact with radiation. The population of planktonic bacteria studied in this work might originate directly from these biofilms through detachment caused by circulating water and/or the release of planktonic cells. This could also explain why most of the tested bacterial strains isolated from the basin and SNFP were not observed to be particularly resistant to gamma radiation.

Another mechanism by which bacteria could cope with the presence of ionizing radiation is the production of pigments harboring antioxidant and thereby radio-protective activities. This is seen in several genera such as the photosynthetic bradyrhizobia, *Methylobacterium* and *Sphingomonas*, associated with radiation resistance.

2. Complementarity of sequencing and –omics technologies

In this work, we mainly focused on the study of bacteria (and to a lesser extent Archaea) through the targeting of the 16S rRNA gene (Sanger and amplicon sequencing). For a more complete picture of the entire microbial community, we could also have investigated the presence of eukaryotes through 18S rRNA and ITS sequencing. The latter is used in particular for the detection of fungi, which have previously been found in a few SNFPs in Spain (Chicote *et al.* 2004) and Brazil (Silva *et al.* 2018), as well as in the cooling pool of a nuclear reactor in France (Petit 2018). The presence of fungi in those pools could be explained by the fact that these organisms can form spores which allow them to temporarily survive the extreme conditions found in these environments until more suitable conditions for growth become available (e.g. in biofilms). Other eukaryotes such as micro-algae were also detected through targeting the 18S rRNA gene in two SNFPs in France and the UK (Rivasseau *et al.* 2016; McGraw *et al.* 2018). Moreover, some algal species were also found in the cooling pool of a nuclear reactor in France (Petit *et al.* 2018). However, as revealed by the shotgun metagenomics sequencing approach applied to the basin water studied in this work, the microbial community in our system was shown to be mostly dominated by bacteria, with only *ca.* 1% of the reads belonging to eukaryotes, archaea and viruses. It must be noted that this could be due to a bias in the used DNA extraction methods, where bacterial DNA could be more easily extracted than eukaryotic DNA for example. Nonetheless, it is unlikely that these organisms contribute much to the ecological network in this system. Moreover, the presence of archaea was specifically investigated in the basin through the use of specific primers, but this approach did not yield any detectable signal (data not shown). Although they were not detected in our system, archaea and viruses could be a promising topic for further research in other watery environments associated with nuclear reactors, as these are not yet described in the scientific literature. Viruses such as bacteriophages could also be considered as an option for biocontrol in these environments, as they would potentially be able to further reduce the bacterial load in order to minimize contamination.

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In addition to 16S rRNA amplicon and shotgun metagenomics sequencing through the Illumina platform, which typically yields short read lengths of up to 300 bp, long-read sequencing technologies such as the SMRT technology implemented by PacBio could be used as complementary approaches to determine the microbial community composition in the watery environments of the BR2 more accurately. The PacBio platform can be used for high-throughput, full-length 16S rRNA amplicon sequencing, allowing for better taxonomic classification of bacterial populations at the species or even strain-level (Callahan *et al.* 2019). In addition, this technology could also be used for a more efficient characterization of the entire microbial community, as it can reliably generate highly accurate long reads (HiFi reads) of 10 kb, which allow for optimal metagenome profiling and assembly (Beaulaurier *et al.* 2018). However, due to the high error rate, this technology might still need to be combined with Illumina sequencing. The portable MinION device developed by Oxford Nanopore Technologies can also be used for whole genome metagenomics, with the added benefit of accurate species identification in real time (Leggett *et al.* 2020). The implementation of these technologies could further improve the taxonomic and functional resolution of the microbial communities detected in the waters of the BR2. So far, these techniques have not yet been applied to SNFPs and other watery environments of nuclear reactors around the world.

Next to DNA sequencing techniques, another complementary approach could be the implementation of metaproteomics in order to validate the functional predictions – obtained through DNA analysis – in the entire microbial proteome. In one particular study, the microbial community in the cooling pool of the Osiris nuclear reactor in France was investigated through two different approaches, namely 16S rRNA amplicon sequencing and proteotyping through the analysis of metaproteomics data obtained via tandem mass spectrometry (Petit *et al.* 2020). The latter approach provides additional taxonomic information on the microbial community and allows for the estimation of biomass contributions for each identified taxon (Kleiner *et al.* 2017; Grenga *et al.* 2019). It is therefore complementary to 16S rRNA amplicon sequencing, since this approach is more qualitative than quantitative due to various biases related to DNA extraction, PCR amplification, the size of the fragment to be amplified or the chosen primers (Brooks *et al.* 2015). Metatranscriptomics could also be attempted, but the timeframe necessary to allow for radioactive decay after sampling would complicate the procedure, as this would alter the transcriptome.

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3. Considerations on isolated microbial strains

The viability of the microbial population in the different watery environments of the BR2 was confirmed through strain isolation on specific culture media. Different media such as TSA, LB and R2A were tested at different dilutions and incubation temperatures (room temperature, 30 °C and 37 °C). The best results (highest number of CFU/ml) were obtained with undiluted R2A medium at room temperature. This can be explained by the fact that this medium best reflects the oligotrophic conditions found in the various water bodies, where bacteria mainly reside at room temperature.

For the viable bacterial cell enumerations, plate counts were used and cell concentrations were found to range between $10^1 - 10^3$ CFU/ml depending on the environment. ATP measurements, epifluorescence microscopy and flow cytometry (even after a concentration step) were also attempted as methods for cell enumeration, but proved to be unreliable due to the low microbial cell densities. Unsurprisingly, the environments displaying the lowest bacterial cell numbers were the ones most exposed to ionizing radiation, such as the primary circuit and the basin during cycles as well as the GIF and CMF pools ($10^1 - 10^2$ CFU/ml). This further confirms the adverse effect of radiation on overall cell densities.

Viable cell concentrations in the SNFP were found to be slightly higher (10^3 CFU/ml). Although radiation is also present in this environment, it is relatively localized in the area around the spent fuel elements stored at the bottom of the pool. Bacteria inhabiting this environment could therefore have developed specific niches, either dwelling as planktonic organisms in the upper water layers not exposed to radiation or as biofilms on the pool walls and piping of the cooling and purification circuits. This would allow them to escape the additional radiation pressure and enable their proliferation to somewhat higher cell densities. The water samples taken during this study from the SNFP for microbial strain isolations were only collected from the surface of the pool. The microbial community might therefore still harbor taxonomic variations according to the sampling depth.

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As previously discussed, a large proportion of the microbes found in these waters are believed to reside in the VBNC state, which does not allow for their isolation as CFUs under laboratory conditions. This is due to the fact that essential aspects of their natural environment (e.g. pH, nutrient composition, temperature and osmotic conditions) fail to be replicated (Stewart 2012).

In addition, some microbes are thought to live in strong metabolic cooperations in their natural habitat and might need to be co-cultured with other bacteria originating from the same environment in order to grow successfully. As an example, the widespread marine autotrophic bacterial genus *Prochlorococcus* is known to grow in culture media only when in the presence of heterotrophic “helper” bacteria (Morris *et al.* 2008). In one particular study investigating the bacterial population in the water purification system of a Hungarian power plant, several new media were developed to maximize the capacity for isolation of individual strains (Kéki *et al.* 2013). The designed media displayed a very oligotrophic nutrient composition as they attempted to better reproduce the “natural” conditions prevailing in this water system. In addition, this research group used refined saltless water from a tank implemented in the purification circuit instead of the generally used distilled water for the completion of the media. Moreover, bacterial “extract” originating from autoclaved bacterial strains previously isolated from this system was also added. As a large proportion of bacterial cells in UPW systems were demonstrated to be present as dead biomass (McAlister *et al.* 2001), this could aid to better mimic the conditions found in these systems, where cryptic growth using lysed cell components is believed to play a role in the survival of the viable bacterial population. This approach could also be applied to enhance the strain isolation efficiency in the watery environments of the BR2. Media could be supplemented with water originating directly from these environments (in accordance with the As Low As Reasonably Achievable – ALARA – principle) and previously isolated, heat-killed bacterial strains could be added as a way to better reproduce the conditions found in these waters.

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The microbial communities inhabiting various watery environments of nuclear reactors have been investigated across the world, although the scientific literature describing these microorganisms is still relatively scarce. Most of the available studies described the presence of microbes in spent nuclear fuel pools using cultivation-based approaches (Sarró *et al.* 2003, 2005, 2007; Chicote *et al.* 2004, 2005; Galès *et al.* 2004; Masurat *et al.* 2005; Bruhn *et al.* 2009; Giacobone *et al.* 2011; Tisakova *et al.* 2013; Dekker *et al.* 2014; Rivasseau *et al.* 2016; Pipiska *et al.* 2018). Other research groups focused on water systems not exposed to ionizing radiation, such as cooling and purification circuits, as well as water tanks implemented therein (Rao *et al.* 2000, 2009; Balamurugan *et al.* 2011; Bohus *et al.* 2011; Kéki *et al.* 2013, 2019). Later on, as high-throughput sequencing techniques became more readily available, other studies started exploring the non-cultivable fraction of the microbial communities in these environments through techniques such as 16S and 18S rRNA amplicon and shotgun metagenomics sequencing (Props *et al.* 2016, 2019; Bagwell *et al.* 2018; McGraw *et al.* 2018; Silva *et al.* 2018; Foster *et al.* 2020; Petit *et al.* 2020; Ruiz-Lopez *et al.* 2020).

These waters are typically maintained in an ultrapure state by constant filtering and deionization, hereby preventing the corrosion of measuring instruments, wall and pipe surfaces as well as removing any dissolved radionuclides. This results in extremely low conductivities, typically ranging between $< 1 \mu\text{S}/\text{cm}$ to $10 \mu\text{S}/\text{cm}$. In addition, these waters can be subjected to varying levels of ionizing radiation if they come into close contact with nuclear fuel. Such is the case for the primary circuit, cooling pools surrounding the reactor core and SNFPs, where spent fuel is intermediately stored in underwater racks in order to cool down before being safely disposed to its final location. Microbes inhabiting these extreme environments must therefore face a range of challenging conditions including low nutrient availability as well as coping with the presence of ionizing radiation and residual levels of dissolved radionuclides.

In this work, we first wanted to investigate the viable microbial population in a range of interlinked watery environments of the BR2 reactor located at SCK CEN in Mol, Belgium, using a cultivation-based approach. To this end, we isolated a variety of strains on oligotrophic culture media (R2A) from various water bodies, namely the primary circuit, the basin surrounding the reactor core, two storage pools implemented within it, the spent nuclear fuel pool as well as an external storage and refill tank containing ultrapure water.

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A significant bacterial diversity could be observed across all the different environments, with individual strains belonging to 33 distinct bacterial species. This is the largest catalogue of isolates described so far in a single study for these types of environments. Some overlap in microbial composition could be detected across all the studied environments, with most of these waters harboring members of the *Bradyrhizobium*, *Curvibacter* and *Pelomonas* genera. Other environments such as the SNFP contained unique genera including *Bacillus*, *Mycolicibacterium* and *Sphingomonas*. This could be explained by the fact that, although these environments are connected to each other, they each display their own set of unique physico-chemical characteristics, which may lead to distinct selection pressures and therefore different microbial communities. Furthermore, we attempted to characterize the susceptibility to ionizing radiation of some of the isolated strains, which resulted in the identification of *Sphingomonas melonis* as the most radio-resistant species, since it managed to survive an acute irradiation dose of 2.1 kGy.

With regard to this work, it would be of interest to further characterize the radiation susceptibility of other isolated strains as well as to investigate their potential for heavy metal and radionuclide uptake. Whole genome sequencing of interesting isolates could also be performed in order to identify genomic adaptations allowing these strains to survive in these extremely challenging environments. This would allow for the identification of potential candidates for bioremediation applications of contaminated environments. One example of such a species is *Coccomyxa actinabiotis*, a green autotrophic microalga isolated from a nuclear facility in France (Rivasseau *et al.* 2013). It was demonstrated to be capable of withstanding ionizing radiation doses of up to 20 kGy and was able to strongly accumulate radionuclides such as ^{238}U , ^{137}Cs , $^{110\text{m}}\text{Ag}$ and ^{60}Co . It proved to be as effective for the purification of nuclear effluents as the conventionally used ion exchangers. This organism was successfully implemented for the real-scale radionuclide bioremediation of one specific SNFP.

Secondly, while most of the previous studies investigating microbial communities in the watery environments of nuclear reactors were performed on SNFPs, another aim of this work was to study the microorganisms in the basin surrounding the reactor core using a 16S rRNA amplicon sequencing approach.

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In addition, we used this method to investigate the long-term dynamics of the microbial population in this specific environment, as it is periodically subjected to shifts in physico-chemical parameters such as temperature, radiation and flow rate according to the operational status of the reactor (cycle or shutdown). To this aim, two sampling campaigns spanning several months and separated by a one-year-interval were performed, which resulted in the characterization of a diverse bacterial population displaying clear shifts in community profiles: the microbial population during cycles was mostly dominated by two OTUs assigned to an unclassified *Gammaproteobacterium* and *Pelomonas*, whereas these were replaced by an OTU assigned to *Methylobacterium* during shutdowns. Both campaigns displayed similar taxonomic profiles and shifts, suggesting that the system is quite stable over time. The observed shifts in community profiles were linked with changes in physico-chemical parameters such as flow rate, temperature and radiation through NMDS and correlation analyses. Radiation was shown to cause a significant decrease in cell densities, whereas the effect of temperature was opposite to that of radiation.

Finally, in order to dig deeper into the taxonomic and functional characteristics of the microbial community in the basin and characterize its dynamics more in depth, we adopted a shotgun metagenomics sequencing approach and combined it with the previously obtained 16S rRNA amplicon sequencing data for added statistical power. To this aim, we designed a specialized filtration system in order to be able to collect a sufficient amount of cell material needed for this approach. This system was permanently implemented in the BR2 and could be used in the future for further samplings. With regard to the functional characterization of the community, several pathways believed to play a role in cell function recovery after irradiation were more highly represented during shutdowns, namely the pyruvate, glycerophospholipid and purine metabolisms, as well as the biosynthesis of valine, leucine and isoleucine. Furthermore, we managed to almost entirely reconstruct two MAGs from the metagenome, corresponding to *Bradyrhizobium* sp. BTAi1 and *Methylobacterium* sp. UNC378MF. These strains harbored significant adaptations in their genome allowing them to cope with the extremely challenging conditions prevailing in the basin.

For future research, the shotgun metagenomics sequencing data could be complemented with a metaproteomics approach, in order to study how the predicted pathways detected in the DNA sequencing dataset are expressed in the proteome.

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The previously designed filtration system could be used for the collection of biological material, as these approaches require large amounts of cells for the extraction of a sufficient amount of RNA and proteins, respectively. In addition, it would be interesting to test the photosynthetic capacity of some isolated *Bradyrhizobium* strains, as the reconstructed *Bradyrhizobium* MAG1 was predicted to harbor this characteristic. Nitrogen fixation could also be tested in these strains, as well as in some other species predicted to possess this trait, such as *Mesorhizobium* and potentially *Methylobacterium*, *Sphingomonas* and *Pelomonas*. The capacity for *Methylobacterium* to grow on acetate could also be investigated, as well as the chemo-autotrophic growth of some *Pelomonas* isolates and other isolated strains on H₂ in the presence of CO₂ and O₂. This could be tested by adding these gases to closed culture chambers using solid or liquid culture media without added carbon sources.

In conclusion, we showed that microbes (mainly bacteria) are present in all the investigated water environments, even the primary circuit, but at very low cell densities, which makes them difficult to detect and study. The observed microbes comprise rather common Gram-negative bacteria that are nonetheless very well adapted to oligotrophic environments, in the presence or absence of ionizing radiation. CO₂ and N₂ fixation, as well as organic nutrient versatility and biofilm formation seem to be important traits for survival in these systems. We established an extensive collection of isolates which can be used for further metabolic studies to unravel these survival strategies more in depth. In addition, their sensitivity to particular antimicrobial agents could be tested, which could be utilized for maintaining the water quality in nuclear reactors. We also managed to assemble two MAG genomes, the first to be obtained for these types of environments, and tried to go beyond DNA technologies with metaproteomics, although unsuccessfully so far. Therefore, more optimization is still needed in that respect to be able to work with very low cell densities.

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Appendix A.

Microbial life in an operating nuclear reactor

So far, most of the studies investigating the microbial communities in nuclear facilities have focused on SNFPs used to store spent nuclear fuel underwater. However, to date, nothing is known about microbial life in pools that directly cool nuclear reactor cores.

In this work, we investigated the microbial communities inhabiting the cooling pool of the Osiris nuclear reactor in France using direct meta- omics analyses, namely DNA metabarcoding and proteotyping through 16S rRNA gene sequencing and peptide analysis, respectively.

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Direct Meta-Analyses Reveal Unexpected Microbial Life in the Highly Radioactive Water of an Operating Nuclear Reactor Core

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Abstract

The pools of nuclear reactor facilities constitute harsh environments for life, bathed with ionizing radiation, filled with demineralized water and containing toxic radioactive elements. The very few studies published to date have explored water pools used to store spent nuclear fuels. Due to access restrictions and strong handling constraints related to the high radioactivity level, nothing is presently known about life in water pools that directly cool nuclear cores. In this work, we investigated the microbial communities in the cooling pool of the French Osiris nuclear reactor using direct meta-omics approaches, namely, DNA metabarcoding and proteotyping based on 16S ribosomal RNA gene sequencing and on peptide analysis, respectively. We identified 25 genera in the highly radioactive core water supply during operation with radionuclide activity higher than 3×10^9 Bq/m³. The prevailing genera *Variovorax* and *Sphingomonas* at operation were supplanted by *Methylobacterium*, *Asanoa*, and *Streptomyces* during shutdown. *Variovorax* might use dihydrogen produced by water radiolysis as an energy source.

Keywords: environmental microbiome; nuclear reactor; irradiation; metabarcoding; 16S rRNA amplicon sequencing; proteotyping

1. Introduction

Nuclear reactor facilities constitute extreme environments for life and little information is available on the species capable of surviving in there. The few studies published to date on this type of environment have focused on pools used to store spent nuclear fuels once the fuel has been used for energy production. These water coolant pools sustain high levels of ionizing radiation originating from the cooling fuels, contain radioactive elements in solution including toxic metals, and are filled with demineralized water. The very few studies investigating the microbial diversity in such environments highlighted the presence of Proteobacteria, Firmicutes, Actinobacteria, Deinococcus-Thermus, and also a Chlorophyta [1,2,3,4]. These organisms represent a unique opportunity to study the mechanisms of radioresistance and radionuclide accumulation and to develop new decontamination biotechnologies [5]. Nuclear reactor installations also primarily house the reactor core, which is bathed by the primary cooling circuit. Due to the access restrictions and the difficulty in handling such samples, nothing is currently known about life in water used to cool nuclear reactor cores. Such an environment is subject to even more severe conditions than spent nuclear fuel pools (SNFPs) since the water directly flows around the fuel rods, sustaining enormous amounts of radiation and exhibiting extremely large radionuclide concentrations during reactor operation. In this work, we investigated an open-core reactor, the French Osiris reactor, in which the reactor block is immersed in a pool of water and in communication with it. We explored the microbial communities present in the Osiris reactor core coolant water during operation and compared them with those present at shutdown.

Most studies focusing on spent nuclear fuel storage pools have identified microorganisms after cultivation [1,2,3,4], which results in an important loss in diversity since only 0.1 to 10% of the microorganisms present in water are culturable [6]. Direct meta-analysis based on 16S ribosomal RNA (rRNA) gene sequencing (metabarcoding) is a powerful tool to explore microbial community composition in the environment [7]; however, several sources of biases, including, among others, DNA extraction, PCR amplification, the choice of the 16S rRNA primers and the size of the fragment to sequence, make this approach more qualitative than quantitative [8].

Metaproteomics based on peptide analysis by tandem mass spectrometry has been used to assess functional aspects of communities in their environment. Such analysis also provides taxonomical information on microbial communities, based on proteotyping [9], and estimates biomass contributions of each taxon with relatively good confidence [10], but has never been applied to samples from nuclear facilities. In this work, we investigated the microbial population present in the reactor's pool using complementary direct approaches of metabarcoding and proteotyping.

2. Materials and Methods

2.1. Water Samples from the Cooling Pool of the Nuclear Reactor Core

The facility studied is the French 70 MW nuclear reactor Osiris located in CEA Saclay. We investigated the cooling pool containing light water in direct contact with the reactor core. The reactor contains uranium sources inside the core unit which helps to position the fuel elements and channels the cooling water (**Figure 1a**). The pool is 11 m deep, 7.5 m long, and 6.5 m wide. Water samples were collected at three points of the basin: location A (Loc. A, bottom of the pool, 2 m away from the core), location B (Loc. B, inside the core unit), and location C (Loc. C, above the core unit). While the reactor was in operation, 2 L of water were collected at Loc. A on December 2015 using a permanently installed pipe extensively purged before sampling. The samples were collected directly into a sterile plastic container connected to the pipe. When the reactor was shut down, 8 L of water were collected on March 2017 at Loc. A using the same device, and at Loc. B and Loc. C using a sterile sampling vial (Wildco, Yulee, FL, USA) attached to a pole which was filled underwater at the sampling point. Comparison of the microbial composition of samples collected by both methods validated the sampling methodology and brought out that either sampling mode did not induce unexpected contamination.

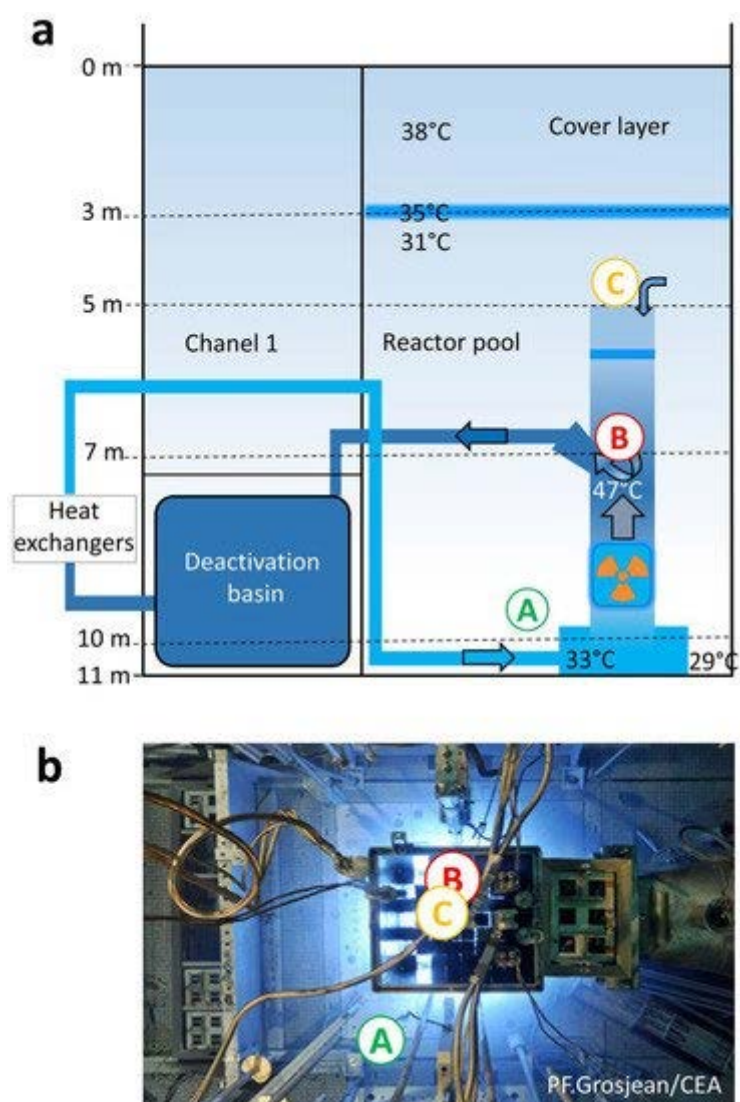


Figure 1. Configuration of the Osiris reactor's pool (adapted from [11]) and sampling points A (pool's bottom), B (inside the core unit), and C (above the core unit). (a) Schematic view, (b) top view. At shutdown, the radiation dose rate reached 25 Gy/h at point B, 15 μ Gy/h at point C, and was below 0.1 μ Gy/h at point A.

While in operation, the pool's demineralized water, pH 7.0, conductivity 0.5 $\mu\text{S}/\text{cm}$, maintained at 30 ± 1 °C below a 3.5 m depth, contained the β -emitter ^3H ($2.9 \times 10^8 \text{ Bq}/\text{m}^3$) and various γ -emitter radionuclides detailed in **Table S1** yielding a total gamma activity of $3.2 \times 10^9 \text{ Bq}/\text{m}^3$. During the shutdown, the pool's demineralized water, pH 5.4, conductivity 0.5 $\mu\text{S}/\text{cm}$, contained the β -emitter ^3H ($2.5 \times 10^8 \text{ Bq}/\text{m}^3$) and various γ -emitters detailed in **Table S1** yielding a total gamma activity of $3.3 \times 10^6 \text{ Bq}/\text{m}^3$. During the cycle, the water was continuously filtered at 500 m^3/h and purified on ion exchange resins at 30 m^3/h , with a turnover of 18 h. The 500 m^3/h filtration operation was maintained at shutdown. Microbial cell density was assessed using a Malassez cell after water centrifugation (50 mL) at 20,000 $\times g$ for 40 min.

Samples collected during the reactor operation were stored in the sterile containers for three days at 4 °C before handling to reduce their activity by radioactive decay of ^{24}Na (half-life 15 h) to 24 $\mu\text{S}/\text{h}$ in contact with water. Microorganisms were then concentrated and harvested either by centrifugation at 16,000 $\times g$ for 20 min at 10 °C or by filtration on 0.22 μm sterile polyethersulfone filters (MicroFunnel, Pall Corporation, Saint-Germain-en-Laye, France) in a sterile environment. Samples harvested during shutdown were processed immediately after collection. Each sample was divided into three 1 L aliquots for metabarcoding and 4.5 L for proteotyping analysis. They were then centrifuged or filtered as above. During this pre-treatment step by centrifugation or filtration, contamination controls were carried out by using tubes containing liquid culture media (LB, BHI, and TSB diluted ten times, R2A and NB diluted twice) which remained negative. The way potential contamination was challenged throughout sample treatment is detailed in **Supplementary materials Table S4**. The sample concentration step has been introduced to ensure that biomass in all subsequent sample treatment steps was sufficient for an exhaustive analysis of the population and to avoid issues arising with low-biomass samples [12,13]. These concentrated biomass samples, which contain of the order of 10^6 cells (see the Results and Discussion section for the cell density values), were then treated for DNA extraction and sequencing and for protein extraction and peptide analysis.

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2.2. DNA Extraction, Illumina Sequencing, and Sequence Data Analysis by Metabarcoding

Three methods combining different concentration protocols and DNA extraction methods were used to treat the 1 L water samples prior to amplicon sequencing. The use of different protocols enabled us to assess potential exogenous contamination originating from processing methods or DNA reagents. These three methods included centrifugation at 16,000× *g* for 20 min followed by phenol–chloroform–isoamyl alcohol DNA extraction, adapted from Vilchez-Vargas [14] (**Supplementary method**) (Method 1), filtration on 0.22 µm filter followed by phenol–chloroform–isoamyl alcohol DNA extraction (Method 2), and filtration on 0.22 µm filter followed by DNA extraction using the DNeasy PowerWater kit (Qiagen, Hilden, Germany) (Method 3). Methods 1 and 2 were used in the 2015 campaign. The three methods were used in the 2017 campaign.

Control samples and samples of different origin were processed alongside the Osiris samples through pretreatment, DNA extraction, amplification, library preparation, sequencing, and data treatment. A mock ZymoBIOMICS (Zymo Research, Irvine, CA, USA) DNA standard was also processed concomitantly from the amplification stage (**Table S4**).

The V4V5 region of the 16S rRNA gene was amplified by LGC Genomics, UK, using the primers pair 515YF-926R (5'-GTGYCAGCMGCCGCGGTAA-3' and 5'-CCGYCAATTYMTTTRAGTTT-3') [15] and the resulting amplicons were sequenced on an Illumina MiSeq platform using V3 chemistry (2 × 300 bp) (**Supplementary method**). Sequencing data were processed through the OCToPUS (version 1) pipeline (Belgian nuclear research center/Vrije Universiteit Brussel/KULeuven, Mol, Brussels, Leuven, Belgium) [16] for contig assembly, quality filtering, de-noising, chimera removal, and operational taxonomic unit (OTU) clustering at 97% cutoff. Each OTU was taxonomically assigned at 80% confidence score against the Ribosomal Database Project database (version 16) using mothur (version 1.39) software (Michigan University, Ann Arbor, MI, USA) [17]. To improve taxonomic assignment, the main OTUs unidentified at the genus level were blasted against the nucleotide collection database using Megablast in the Nucleotide BLAST (version 2.8.1) tool from NCBI (National Center for Biotechnology Information/National Institutes of Health, Rockville Pike, Bethesda, MD,

USA). Based on the ZymoBIOMICS standard analysis, only OTUs whose abundance exceeded a cut-off threshold of 0.016% were considered, to minimize false-positives rate (**Table S5**). Alpha diversity was calculated using *mothur*. For sample comparison and mean value calculation, sequencing results were subsampled at the second lowest read count (28,977 reads) using *mothur*.

2.3. Protein Extraction, Peptide Analysis Using nanoLC–MS/MS, and Taxonomic Analysis by Proteotyping

Following filtration, microorganisms were resuspended in 15 mL of sterile MilliQ water, centrifuged at 20,000× *g* for 20 min at 10 °C, and processed as described [18]. After addition of 50 µL of Laemmli buffer LDS1X (Invitrogen, Illkirch, France) and bead-beating, protein samples were denatured for 5 min at 99 °C and purified by electrophoresis on 4–12% gradient NuPAGE gels (Invitrogen). The whole proteome band was recovered, reduced with dithiothreitol, treated with iodoacetamide, and proteolyzed with trypsin (Gold Mass Spectrometry Grade, Promega, Walldorf, Germany) in the presence of 0.01% ProteaseMAX (Promega) [19]. Peptides were analyzed using a Q Exactive HF mass spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with an ultra-high field Orbitrap analyzer and coupled to an Ultimate 3000 176 RSL Nano LC System (Thermo). They were separated on an Acclaim PepMap 100 C18 column using a 60 min gradient of CH₃CN in 0.1% formic acid [20]. The Q Exactive HF instrument was operated with a Top20 strategy with MS/MS on ions potentially charged +2 or +3 and a 10 s dynamic exclusion. MS/MS spectra were interpreted with the National Center for Biotechnology Information non-redundant fasta file (NCBI-nr) database with the Mascot (version 2.6) search engine (Matrix Science, London, UK). Peptide to MS/MS spectrum assignation was performed with full trypsin specificity, a maximum of one missed cleavage, mass tolerances of 5 ppm on the parent ion and 0.02 Da on the MS/MS, static modification of carboxyamidomethylated cysteine (+57.0215), and oxidized methionine (+15.9949) as dynamic modification. Mascot data files were post-processed to obtain taxonomical proteotyping based on peptide-to-taxa assignments. A positive control consisting in a Zymobiomics mixture of ten microorganisms was treated in similar conditions and the results have been recently published [21].

3. Results and Discussion

During the reactor working cycle in December 2015, the core's pool water was highly radioactive, with a tritium activity reaching 2.9×10^8 Bq/m³ and a gamma activity reaching 3.3×10^9 Bq/m³, mainly due to radioactive sodium (²⁴Na, **Table S1**). The concentration of gamma-emitters was 1000 to 10,000 times higher than that prevailing in the SNFPs, such as in the one previously investigated by our team [5]. For security reasons, water was only sampled at the bottom of the pool (Loc. A) and not in the core unit (**Figure 1a,b**). The samples were stored for three days to reduce their radioactivity to a level enabling their handling in controlled conditions. Metabarcoding analyses yielded more than 50,000 raw reads (**Table S2**). The microbial cell density was below 0.5×10^3 cells/mL which is consistent with the quantity of DNA extracted. The rarefaction curves reached an asymptote, indicating sufficient sequencing depth (**Figure S1**). The sample alpha diversity quantified by the Shannon index, which combines species richness and evenness, was below 1. A single OTU, classified as Comamonadaceae family and attributed to *Variovorax* sp. using BLASTn (top hits at 100% similarity), dominated the population. As shown in **Figure 2a**, Proteobacteria were largely represented (95% of the sequences) with 16 out of the 25 identified genera (**Figure S2**). The two most represented genera were *Variovorax* (75%) and *Sphingomonas* (18%) (**Figure 2b**).

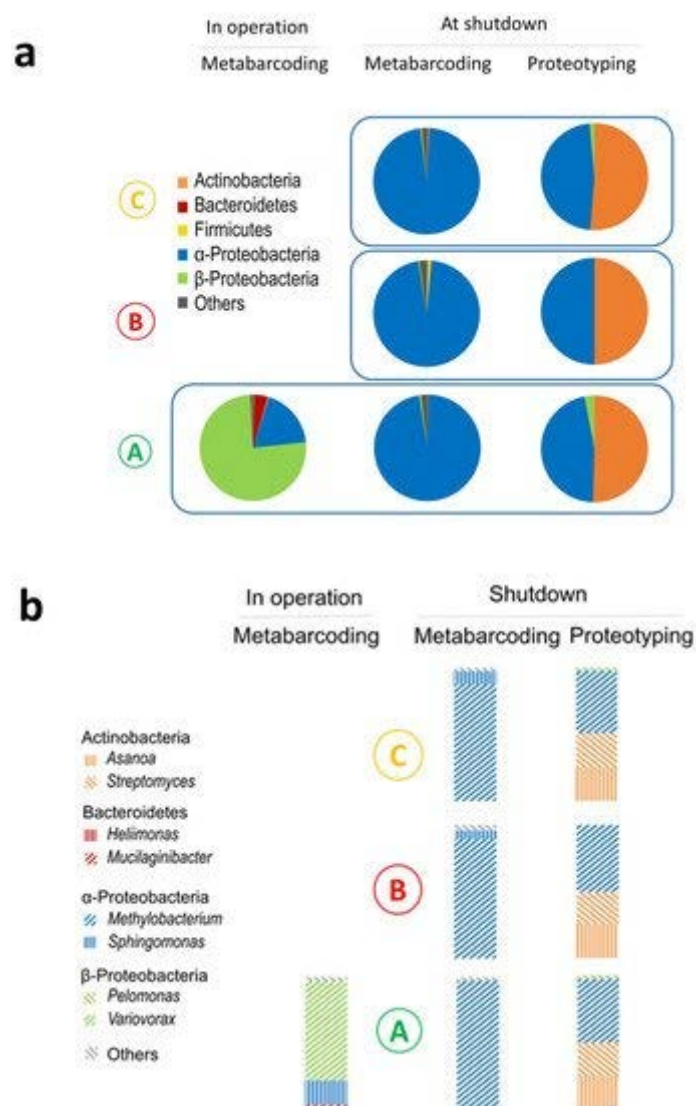


Figure 2. Microbial communities present in the cooling pool of the Osiris nuclear reactor core. **(a)** Main phyla (class for Proteobacteria) and **(b)** main genera (abundance above 1%) detected in operation and at shutdown using metabarcoding and proteotyping. Mean taxon abundance obtained by the different methods (see Materials and Methods Section).

During the reactor shutdown in March 2017, the tritium activity was near to that in operation whereas the gamma activity was 1000 times lower.

Water could be sampled inside and above the core unit (Loc. B and Loc. C, **Figure 1a**) where the radiation dose rate reached 25 Gy/h and 15 $\mu\text{Gy/h}$, respectively, as well as from Loc. A where the dose rate was below 0.1 $\mu\text{Gy/h}$. Sampling at three different locations corresponding to very different irradiation dose rates, by a factor of one hundred million, and different water circulation areas enabled us to test the homogeneity of the microbial composition and concentration in the pool. The microbial cell density reached $2.7 \pm 0.6 \times 10^3$ cells/mL, $0.5 \pm 0.2 \times 10^3$ cells/mL, and $1.4 \pm 0.2 \times 10^3$ cells/mL at Loc. A, B, and C, respectively. The continuous water filtering and cleaning at a high flow rate, the low conductivity, and the high radiation dose rate maintained a low microbial density, similar to that reported in some SNFPs [2,3]. Though less dense, bacteria were not so rare at Loc. B where they sustained 25 Gy/h. With a single exception, 29,000 to 127,000 sequences were obtained. Rarefaction curves reached an asymptote. The alpha diversity was still low (Shannon index 0.2–1.2) (**Table S2, Figure S1**). Overall, 90 OTUs were identified. This is considerably below the several thousands of OTUs detected using metabarcoding in an SNFP where much less stringent conditions yielded higher microbial density and diversity (1.8×10^3 to 2.8×10^5 cell/mL, Shannon index of 5) [22]. Proteobacteria were predominant (97% of the sequences) (**Figure 2a**), comprising the genera *Methylobacterium* prevailing in all locations (one OTU, 87–97% depending on the sampling point) and *Sphingomonas* mostly present at Loc. B and Loc. C (0.1–9%) (**Figure 2b, Figures S3–S5**). Major OTUs representing 98.8% of the sequences were common to the three locations (**Figure S6**).

The microbial composition of the samples obtained at shutdown, which were rich in biological material, could be determined by proteotyping by tandem mass spectrometry (**Table S3**). Proteotyping data highlighted the predominance of two phyla in equal proportion irrespective of the sampling point, namely, Proteobacteria with the genus *Methylobacterium* (50% of the taxon-spectrum matches) and Actinobacteria with two genera *Asanoa* (25%) and *Streptomyces* (25%) (**Figure 2a,b**). The main genera were common to all locations likely due to the continuous and intense water circulation despite the stronger selection pressure at Loc. B.

The DNA metabarcoding and proteotyping approaches were highly complementary and both highlighted *Methylobacterium* as the most abundant genus. By contrast, the other two main genera of the Actinobacteria phylum were uniquely detected by proteotyping. Proteotyping can be applied on any biological material, dead or alive [23,24]. The protocol used does not introduce specific bias depending on the Gram staining. Conversely, the metabarcoding approach suffers from non-quantitative representativeness, particularly for Gram-positive bacteria. Most classical DNA extraction protocols considerably underestimate this fraction, likely due to the higher mechanical resistance of the bacterial cell walls [25]. Proteotyping with shotgun tandem mass spectrometry data probably gives a more accurate assessment of the biomass contributions of the main members of the consortia, despite biases linked to protein extraction, cell size, and a less extensive database [10]. Nonetheless, the higher sensitivity of metabarcoding brought to light low abundance species and diversity.

It should be highlighted that the species detected here are only prokaryotes. Although analysis of the 18S rRNA gene sequence was not performed in the present study, which focuses on the identification of bacteria, proteotyping could have allowed the detection of eukaryotic microbes. As recently shown, this approach can actually detect in the same run prokaryotes and eukaryotes such as fungi and microalgae [23]. In the present results, however, no specific peptides were found for any eukaryotic unicellular organisms.

Our data show that life was present in the cooling pool of an operational nuclear reactor, albeit with a restricted number of bacteria prevailing in this environment. The surface of the pool is open to the ambient air, and loading and unloading operations are carried out within the pool. Consequently, these environmental bacteria might have gained entry to the pool through airborne or equipment contamination owing to the traffic in and out of the reactor and to the operations of maintenance and exploitation. The strong selection pressure implies that the microorganisms detected in this pool might have been specifically selected for.

It is likely that at least part of the microbial community was alive in the reactor pool water because it was actually able to multiply in growth conditions. Indeed, aliquots of water samples cultured in different media showed biomass growth after seven days [26]. Proteotyping analysis of the consortia in the culture highlighted the presence of genera identified by the direct approaches described here, including notably *Variovorax*, *Sphingomonas*, and *Methylobacterium* [26]. Another point supporting the hypothesis of the survival of these bacteria in the radiative environment is the presence of the same genera in both campaigns. This could imply that these microorganisms can be metabolically active under such conditions.

Their presence in suspension in water despite the continuous circulation and filtering either may be linked to the heterogeneity of water mixing which, in certain areas, could allow space and time for the microbial population to survive and replicate, or may be due to the formation of biofilms attached to the walls or to material which provides some protection against the harsh environmental conditions.

The presence of a *Variovorax* strain in abundance during the reactor operation might be related to its ability to utilize dihydrogen produced during the working cycle. High energy radiations emitted by the burning fuel generate enormous amounts of dihydrogen through water radiolysis. Some strains of *Variovorax* (biotype I) are lithoautotrophic and are capable of growth using dihydrogen as an energy source [27,28]. The advantage conferred to *Variovorax* by this capacity might explain its prevalence over other genera unable to use dihydrogen energy. Noteworthy, the genus *Variovorax* has been detected in radiative environments, such as in a uranium contaminated soil in China [29] and in the spent fuel pool of the Cofrentes nuclear power plant, Spain, where a *Variovorax paradoxus* strain has been identified after a cultivation step [2]. To counter the radiative and metallic stress, *Variovorax* has been shown to possess multiple metal resistance elements as well as elements involved in antioxidant response [30]. All these specificities, combined to its capacity to use dihydrogen, makes this genus well equipped to survive in the radiative, oligotrophic and metallic conditions which prevail in nuclear pools.

Although the *Variovorax* genus has great adaptability and survival capabilities to various environmental conditions, when the reactor shuts down, the dihydrogen levels dropped, and other genera not sufficiently competitive during the reactor operation might have been better adapted to the new conditions. This may explain the disappearance of *Variovorax* to the benefit of one *Methylobacterium* species. In further experiments, it would be interesting to test the ability of *Methylobacterium* to grow with *Variovorax* when co-cultured and describe their relationships.

The second main genus during operation, *Sphingomonas*, remained present at shutdown essentially at points of higher dose rates. This species might be less competitive in an oligotrophic environment when the gamma radiation pressure alleviates. The dramatic alterations in the microbial profile depending on the functioning mode (**Figure 3**) is likely due mainly to variation in radiation doses and in water characteristics, including dihydrogen and radionuclide concentration. Going forward, it will be worthwhile to characterize how these microbial communities function by means of metaproteomics. In the present work, tandem mass spectrometry data were used for the identification of the taxa but the number of peptides detected were lower than in a metaproteomics classical approach where the biological material is more abundant. In order to exploit the data at protein level, it will be necessary to increase the quantity of biological material to analyze, and thus increase the volume to be sampled while maintaining the safety conditions associated with the handling of radioactive samples. Of note, the genera *Methylobacterium*, *Sphingomonas*, and *Variovorax* have been identified in SNFPs in Spain and in the USA [1,2,22] and some *Methylobacterium* and *Streptomyces* were found in soils contaminated by radionuclides [31,32]. Several species of these last two genera exhibit strong gamma-radiation resistance [32,33].

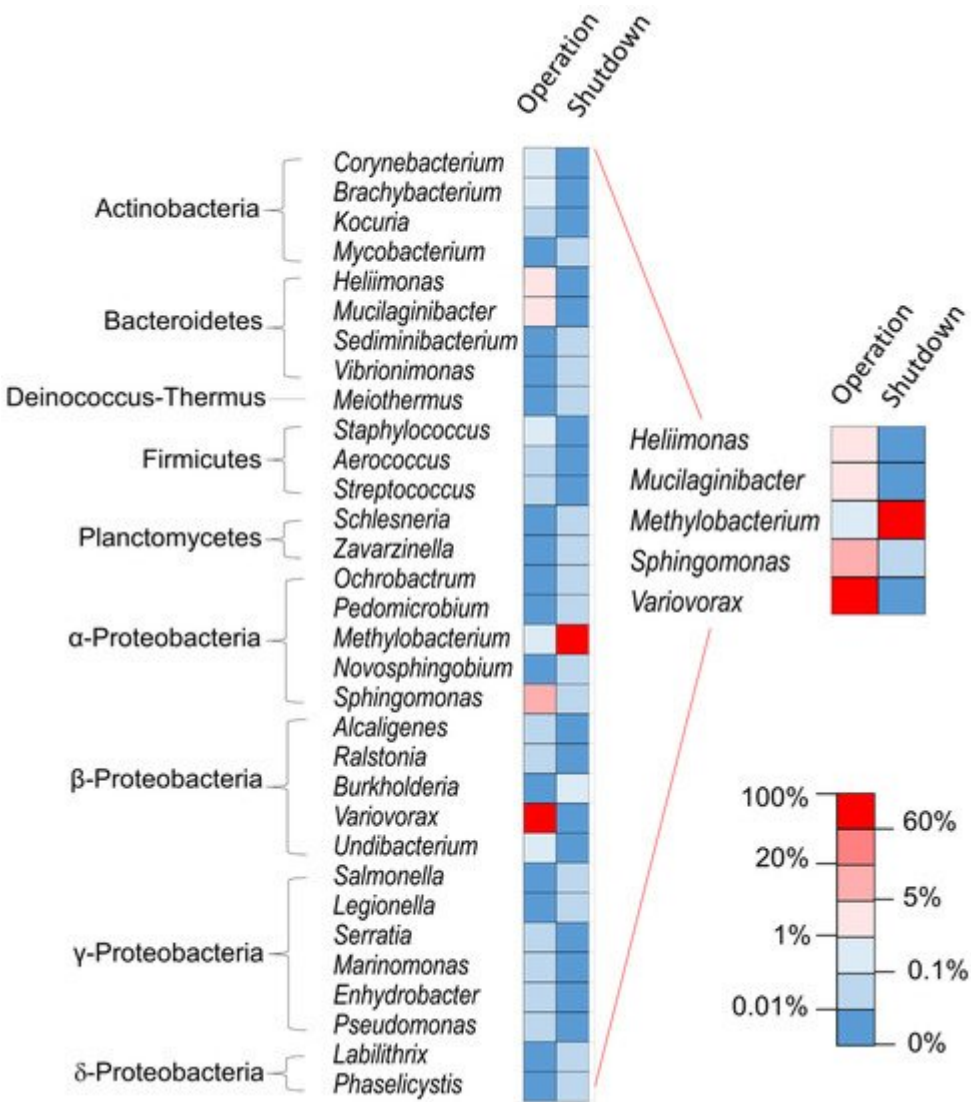


Figure 3. Comparison of the microbial profile at the taxonomic level genus at point A depending on the functioning mode, analyzed by metabarcoding. Mean taxon abundance obtained by the different methods (see Materials and Methods Section).

4. Conclusions

Against all odds, we detected the presence of diverse microorganisms in the extreme environment of a working nuclear core pool. This finding breaks new ground in the discovery of radioresistant species and in the understanding of the radiotolerance mechanisms that could be exploited in medicine, in radionuclide bioremediation in the environment or in nuclear installations, and in space programs. The identification of the dominant genus *Variovorax* during operation calls for the search for other hydrogen-metabolizing microorganisms in functioning nuclear facilities. Finally, it should be noted that the strategy combining proteotyping by tandem mass spectrometry and 16S rRNA amplicon sequencing proved worthy to decipher the microbiota composition in such samples and could be more largely exploited in environmental microbiome analysis.

Supplementary Materials

The following are available online at <https://www.mdpi.com/2076-2607/8/12/1857/s1>, Table S1: Content in gamma-emitter radionuclides in the reactor water; Table S2: Analysis of the sequencing data obtained for the metabarcoding analysis of water sampled from the reactor's cooling pool based on 16S rRNA amplicon sequencing, Figure S1: Rarefaction curves; Figure S2: Microbial communities present in the cooling pool of the reactor's core during operation analyzed at Loc. A, analyzed by 16S rRNA amplicon sequencing; Figure S3: Microbial communities present in the cooling pool of the reactor's core during shutdown at Loc. A, Figure S4: at Loc. B, and Figure S5: at Loc. C, analyzed by 16S rRNA amplicon sequencing; Figure S6: Comparison of microbial communities present in the pool water at shutdown depending on the sampling point; Table S3: Microbial communities determined by direct proteotyping; Table S4: Challenging potential contamination during sample pretreatment and 16S rRNA amplicon sequencing analyses and validation of taxa genuine presence; Table S5: Determination of a cut-off threshold for the analysis of Illumina MiSeq sequencing data using a ZymoBIOMICS microbial community DNA standard; Supplementary methods.

Author Contributions

Conceptualization, C.R. and J.A.; methodology, P.C.M.P., C.R. and G.S.; software, V.V.E., M.M., P.M. and O.P.; validation, C.R., J.A., P.M., M.M. and C.A.; investigation, P.C.M.P., C.R. and G.S.; formal analysis, V.V.E., M.M., P.M., O.P. and C.A.; resources, C.R., J.A., P.M., data curation, C.R. and J.A.; writing—original draft preparation, P.C.M.P., C.R. and J.A.; writing—review and editing, C.R. and J.A.; supervision, C.R., P.M. and J.A.; project administration, C.R. and J.A., funding acquisition, C.R., P.M. and J.A. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

Sequence data generated in this study has been made available at the Sequence Read Archive (SRA) on NCBI under project number PRJNA679180.

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Appendix B.
Scientific communications

Oral presentations

- Van Eesbeeck, V., Monsieurs, P., Mahillon, J. and Leys, N. (2017). Bacterial communities in the cooling waters of the BR2 nuclear research reactor. Topical day on microbes and ionizing radiation at the Belgian Nuclear Research Centre (SCK CEN), 21 April 2017, Mol (Belgium).
- Van Eesbeeck, V., Monsieurs, P., Mahillon, J. and Leys, N. (2017). Bacterial communities in the cooling waters of the BR2 nuclear research reactor. European Nuclear Young Generation Forum (ENYGF) conference, 11-16 June 2017, Manchester (UK).
- Van Eesbeeck, V., Monsieurs, P., Mysara, M., Mahillon, J. and Leys, N. (2018). Bacterial communities in the cooling waters of the BR2 nuclear research reactor. National Symposium for Applied Biological Sciences (NSABS), 8 February 2018, Brussels (Belgium).
- Van Eesbeeck, V., Monsieurs, P., Mysara, M., Mahillon, J. and Leys, N. (2019). Effect of temperature and radiation on the microbial community in the cooling water of a nuclear reactor. 7th international conference on radiation in various fields of research (RAD), 10-14 June 2019, Herceg Novi (Montenegro).
- Van Eesbeeck, V., Monsieurs, P., Mysara, M., Goussarov, G., Mahillon, J. and Leys, N. (2019). Bacterial communities in the cooling waters of the BR2 nuclear research reactor. Topical day: Ionizing radiation – how do bacteria cope and what can we learn? (SCK CEN), 12 September 2019, Brussels (Belgium).

Posters

- Van Eesbeeck, V., Monsieurs, P., Mahillon, J. and Leys, N. (2016). Microbiological analysis of spent nuclear fuel pools: towards the identification of radiation-resistant bacteria. Belgian Interdisciplinary Biofilm Research (BIBR) meeting, 8 September 2016, Liège (Belgium).
- Van Eesbeeck, V., Monsieurs, P., Mahillon, J. and Leys, N. (2016). Microbiological analysis of spent nuclear fuel pools: towards the identification of radiation-resistant bacteria. Belgian Society for Microbiology (BSM) meeting, 28 October 2016, Brussels (Belgium).
- Van Eesbeeck, V., Mysara, M., Monsieurs, P., Mahillon, J. and Leys, N. (2017). Microbiological analysis of spent nuclear fuel pools: towards the identification of radiation-resistant bacteria. 7th congress of European Microbiologists (FEMS), 9-13 July 2017, Valencia (Spain).
- Van Eesbeeck, V., Mysara, M., Monsieurs, P., Mahillon, J. and Leys, N. (2017). Microbiological analysis of the cooling waters of a nuclear research reactor. BSM meeting, 20 October 2017, Brussels (Belgium).
 - Best poster award
- Van Eesbeeck, V., Mysara, M., Monsieurs, P., Mahillon, J. and Leys, N. (2018). Microbiological analysis of the cooling waters of a nuclear research reactor. Knowledge for growth conference, 17 May 2018, Ghent (Belgium).
- Van Eesbeeck, V., Mysara, M., Monsieurs, P., Mahillon, J. and Leys, N. (2018). Microbiological analysis of the cooling waters of a nuclear research reactor. 5th International Radiation Protection Association (IRPA) congress, 4-8 June 2018, The Hague (the Netherlands).
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