microbial biotechnology

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Minireview

Microbial interaction with and tolerance of radionuclides: underlying mechanisms and biotechnological applications

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Summary

Radionuclides (RNs) generated by nuclear and civil industries are released in natural ecosystems and may have a hazardous impact on human health and the environment. RN-polluted environments harbour different microbial species that become highly tolerant of these elements through mechanisms including biosorption, biotransformation, biomineralization and intracellular accumulation. Such microbial–RN interaction processes hold biotechnological potential for the design of bioremediation strategies to deal with several contamination problems. This paper, with its multidisciplinary approach, provides a state-of-the-art review of most research endeavours aimed to elucidate how microbes deal with radionuclides and how they tolerate ionizing radiations. In addition, the most recent findings related to new biotechnological applications of microbes in the bioremediation of

radionuclides and in the long-term disposal of nuclear wastes are described and discussed.

Introduction: Radionuclides: natural/anthropogenic sources and environmental impact

Anthropogenic activities related to mining of uranium (U), atmospheric nuclear weapon tests and nuclear industry are the main sources of radionuclides in the environment (Hain *et al.*, 2020). Radionuclides such as uranium (U), plutonium (Pu) or curium (Cm) have an adverse impact on human health and the environment. These effects are closely tied to their mobility and bioavailability, which in turn strongly depend on their speciation and physico-chemical form.

Uranium is a naturally occurring radionuclide whose levels of contamination are associated with mining activities, the weathering of uranium-containing minerals or accidental local release (Lloyd and Macaskie, 2000; Meinrath *et al.*, 2003). The environmental impact of U is related to its chemistry (Burns 1999). Uranium occurs in four oxidation states: U(III), U(IV), U(V) and U(VI). While U(VI), the soluble, mobile and toxic U species, occurs mainly under oxic conditions, U(IV), which is insoluble, immobile and a less toxic U species, is distributed mainly under reducing conditions (Burns 1999). Plutonium is the naturally occurring chemical element with the highest atomic number (Burns 1999). Trace amounts of natural Pu are present in natural ^{238}U deposits. Yet, the main anthropogenic sources of Pu include nuclear weapon testing (Sholkovitz 1983), accidental release (Zheng *et al.*, 2012) and discharges from nuclear fuel reprocessing sites or nuclear power plants (Dai *et al.*, 2005). Anthropogenic radionuclides ^{239}Pu and ^{240}Pu have high radiological toxicity and can persist for a long time in the environment. Curium and americium are trivalent actinides, considered as minor actinides due to their low environmental concentration, and are produced by bombarding ^{239}Pu with neutrons and alpha particles in nuclear plants. Cm(III) can be used as a molecular probe to mimic the chemical speciation and behaviour of

Received 27 June, 2020; revised 9 November, 2020; accepted 12 November, 2020.
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Microbial Biotechnology (2020) **0**(0), 1–19
doi:10.1111/1751-7915.13718
Funding Information This work was supported by the ERDF-financed grant RTI2018-101548-B-I00 (80% funding by FEDER) (Ministerio de Ciencia e Innovación, Spain).

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trivial actinides such as [241]Am due to its excellent fluorescence properties (Edelstein *et al.*, 2006). [241]Am concentrations in contaminated areas range from 4 to 10.004 Bq/kg soil (Thakur and Ward, 2019). These concentrations correspond to 1.3×10^{-7} to 3.3×10^{-4} μM Am[III].

The Chernobyl nuclear power plant accident in 1986 was responsible for an environmental accumulation of about 1.5×10^{14} Bq of [241]Am through the decay of approximately 6×10^{15} Bq of [241]Pu released from this plant (Thakur and Ward, 2019). This corresponds to 1181 g of [241]Am and illustrates the dire need for knowledge of the behaviour of trivalent actinides in biological systems.

In the last decade, different studies were focused in analysing the structure and composition of microbial populations in radionuclide-containing environments and to elucidate the mechanisms by which these microbes interact with these elements. In addition, the application of these studies in the field of bioremediation was largely investigated. However, few works addressed the new applications of these mechanisms in the field of nuclear industry (e.g. safety of future deep geological disposal of radioactive wastes) and in bioelectrochemical device-based bioremediation. The present review provides insights on microbial diversity of radionuclide-polluted environments, the mechanisms involved in microbial tolerance to radiation and interaction with radionuclides. In addition, new application of microbe–radionuclide interactions is highlighted.

Microbial diversity and activity in radionuclide-contaminated sites

The microbial community is a sensitive indicator of environmental stress, reflecting even small changes in the geochemical composition of their microhabitat due to anthropogenic activities (Li *et al.*, 2017; Guillot *et al.*, 2019; Hallsworth 2019; Xiao *et al.*, 2019). Microorganisms play a major role in element cycling as well as the weathering of rocks and sediments (Bennett *et al.*, 2001; Jaswal et al., 2019a). They may also affect the geochemical properties of groundwater by modifying the transport of organic and inorganic contaminants (Stegen *et al.*, 2016; Jaswal *et al.*, 2019b).

Interest in the biodiversity and activity of microorganisms inhabiting radionuclide-contaminated sites has increased significantly over the past 25 years. Such settings are dominated by diverse groups of microorganisms whose structure and function determine their dynamics and interactions with biotic and abiotic components (Narendrula-Kotha and Nkongolo, 2017; Xiao *et al.*, 2019). Culture-independent studies provide new insights into the microbiology of radionuclides (Carlson

et al., 2019; Xiao *et al.*, 2019). To date, numerous metagenomic studies have demonstrated that contamination – including that by radionuclides – heavily impacts the structure and functions of bacterial communities (Fan *et al.*, 2018; Hemme *et al.*, 2010, 2015; Narendrula-Kotha and Nkongolo, 2017; Xiao *et al.*, 2019, Yan *et al.*, 2016). Because radionuclides can have complex impacts on the microbial community structure, understanding the mechanisms underlying their impact will lead to a better management of microorganisms for bioremediation.

In the past few decades, the microbial diversity and activity in radionuclide-contaminated sites have been studied exploring mine tailings, mine wastes, mining-impacted sites and other contaminated environments containing radionuclides (Islam and Sar, 2011; Kumar *et al.*, 2013; Radeva *et al.*, 2013; Yan *et al.*, 2016; Narendrula-Kotha and Nkongolo, 2017); the composition of the bacterial communities is now known to be site-specific and connected to concentration levels and/or different geological and physiological environmental conditions. For instance, *Acidithiobacillus*, *Pseudomonas*, *Acinetobacter* and *Nitrosomonas* were found to be abundant in various U mine waste sites in Germany (Radeva and Selenska-Pobell, 2005; Yi and Yao, 2012), while highly abundant *Sphingomonas*, *Acidovorax*, *Acinetobacter* and *Ralstonia* may be linked to U-contaminated radioactive waste (Akob *et al.*, 2007; Yan *et al.*, 2016, Jaswal et al., 2019a). Abundant and active Proteobacteria (Alpha-, Beta-, Delta- and Gammaproteobacteria), Acidobacteria, Actinobacteria, Bacteroidetes and Firmicutes have been frequently identified in radionuclide-contaminated environments (Hemme *et al.*, 2010; Kumar *et al.*, 2013; Suriya *et al.*, 2017; Jaswal *et al.*, 2019a,2019b).

Fungi are also known to possess the metabolic capacity to overcome high environmental stresses (Rummel *et al.*, 2014; Stevenson *et al.*, 2017), including high uranium concentrations. However, little is known to date about fungal diversity in radionuclide-contaminated environments and how they may tolerate such toxicity (Mumtaz *et al.*, 2013, Narendrula-Kotha and Nkongolo, 2017).

As an example, Carlson *et al.* (2019) focused on microbial communities in a contaminated aquifer in Oak Ridge, Tennessee (USA). The conditions at this site involve large gradients of pH in addition to widely varying concentrations of uranium, nitrate and many other inorganic ions including Mn^{2+}, Al^{3+}, Cd^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} (Brooks 2001; Moon *et al.*, 2006; Carlson *et al.*, 2019). In general, taxa frequently associated with contaminated environments can (i) reduce nitrate and heavy metals, (ii) be resistant to heavy metal toxicity and (iii) degrade polyaromatic compounds and polychlorinated biphenyls (Akob *et al.*, 2007). Phylotypes such as *Rhodanobacter*, *Methylobacterium*, *Caulobacter*, *Sphingomonas*, *Acidovorax*, *Acinetobacter* and *Ralstonia* are

nitrate-reducing bacteria that tolerate acidic and low-nutrient environments, and are highly resistant to metals. They tend to be plentiful in the microbial communities of such contaminated sites (Spain and Krumholz, 2011; Thorgersen *et al.*, 2019), and it may be that toxic compounds select taxa typically resistant to the high concentrations of radionuclides in contaminated environments such as those reported in the RN-contaminated aquifer in the Oak Ridge (Carlson *et al.*, 2019).

Likewise, Hoyos-Hernandez *et al* (2019) studied the bacterial community structure and functional genes in RN-contaminated soils at Chernobyl and Fukushima. They explored the ability of microorganisms to survive in these contaminated environments and identified Proteobacteria, Acidobacteria and Actinobacteria as the most common phyla in all studied soils. In addition, the main detected taxa were those tolerant to extreme environmental conditions such as Rhodospirillales, Acetobacteraceae and Candidatus *Solibacter*, as well as to metals such as Acidimicrobiales (Gołębiewski *et al.*, 2014). Verrumicrobia were also detected and previously isolated from uranium deposits (Mondani *et al.*, 2011; Theodorakopoulos *et al.*, 2017). Again, these authors concluded that the presence of these microorganisms would suggest some form of selection for tolerant microorganisms to uranium and heavy metal contamination.

Similarly, the highly contaminated (with uranium and other metals) sediments of the Cauvery river (India) were found to contain abundant Proteobacteria (Gamma, Alpha, Delta, Epsilon and Beta) followed by Bacteroidetes and Firmicutes (Suriya *et al.*, 2017). In addition to confirming the impact of uranium and metal accumulation on the bacterial communities in RN-contaminated sites, these authors highlighted specific species as bioindicators of contamination and as candidates for bioremediation strategies.

In general, bacteria are sensitive to environmental contamination, and their response to heavy metals varies. Proteobacteria, the predominant phylum common to all RN-contaminated sites, harbour many U(VI)-reducing genera and play a crucial role in decreasing U toxicity and bioavailability (Barns *et al.*, 2007; Rastogi *et al.*, 2010; Merroun *et al.*, 2011; Kolhe *et al.*, 2018). They can survive in oligotrophic environments, function at low water activity, adapt to metal reduction and metal resistance, and play a very important role in U immobilization in biostimulated U-contaminated sediments or groundwater (Suzuki *et al.*, 2003; North *et al.*, 2004; Li *et al.*, 2017). Members of the genera *Thiobacillus* (beta), *Ferribacterium* (beta), *Pantoea* (gamma), *Geobacter* (delta) and *Pseudomonas* (gamma), to name a few, are frequently detected. Others, such as *Desulfomicrobium*, *Desulfotomaculum*, *Desulfovibrio*, *Acidovorax* and *Shewanella*, known to reduce or immobilize U, have been detected in many RN-contaminated sediments/soils (Rastogi *et al.*, 2010; Spain and Krumholz, 2011). Belonging to Proteobacteria, members of the families Geobacteraceae and Shewanellaceae are able to reduce U(VI), iron(III), nitrate and elemental sulfur with electron donors such as alcohol, acetate and aromatic compounds (Richter *et al.*, 2012; Lovley 2013). Geobacteraceae became numerically prevalent over other groups as U reduction proceeded in uranium-contaminated sites (Lovley *et al.*, 2011). Sulfate-reducing bacteria such as *Desulfomicrobium*, *Desulfotomaculum* and *Desulfovibrio* are known to reduce U to a less soluble uraninite. The nearly ubiquitous *Desulfovibrio* involves the reduction in many electron acceptors (such as sulfate, nitrate and nitrite) for growth and respiration with the enzymatic reduction in chromium(VI), manganese(IV), iron(III), U(VI) and technetium(VII) (Wall and Krumholz, 2006; Zeng *et al.*, 2019).

Acidobacteria are also frequently retrieved from radionuclide-contaminated soils (Barns *et al.*, 2007; Mumtaz *et al.*, 2018). They are known to tolerate extreme conditions, including metal contamination, acidic pH and limited nutrients (Kielak *et al.*, 2016). Among the known genera of this phylum, *Geothix*, capable of nitrate reduction, were found to be largely associated with Fe(III) and indirectly to U(VI) reduction under field conditions (Cardenas *et al.*, 2008; Li *et al.*, 2018). Bacteroidetes are known for their ecological importance as primary metabolizers of dead organic matter (Thomas *et al.*, 2011). Their resistance to high levels of radionuclide contamination may be due to the involvement of classes such as Sphingobacteria that produce sphingolipids, signalling molecules that protect cell surfaces and functions in the face of a variety of environmental stresses (Hannun and Obeid, 2008; Mumtaz *et al.*, 2018).

Actinobacteria also exist in uranium-contaminated sediments and soils (Akob *et al.*, 2007; Akob *et al.*, 2011, Jaswal *et al.*, 2019a); among them, the family Cellulomonadaceae (*Cellulomonas*) is known to reduce/immobilize U(VI) by means of phosphate precipitation and reduction based on environmental conditions (Sivaswamy *et al.*, 2011). *Arthrobacter* and *Microbacterium* have likewise been reported in many radionuclide-contaminated areas and are known to remove high amounts of U in heavy metal-enriched environments (Islam and Sar, 2016). Moreover, *Arthrobacter* is well adapted to nutrient-deprived (oligotrophic) conditions (Osman *et al.*, 2008). Besides being one of the candidates used to resolve the molecular basis for resistance against uranium (Chauhan *et al.*, 2018), *Arthrobacter* can account for about a third of the total soil cultivable bacterial community in highly radioactive sediments (Fredrickson *et al.*, 2004).

Tolerance of microbes to radiation

In addition to their chemical toxicity, active species of radionuclides such as Pu, Np and Cm exhibit also radiotoxicity, which should be considered as one of the main criteria for screening of microbes with bioremediation potential of aqueous radioactive wastes generated by nuclear fuel reprocessing.

It is well documented that ionizing radiations lead to degradation of DNA through radiolysis or loss of electron from its structure (Ravanat and Douki, 2016) with consequent inhibition of microbe reproduction (IAEA 2017). Some microbes inhabiting radionuclides impacted environments developed different mechanisms for their survival and tolerance to ionizing radiation. For instance, *Deinococcus radiodurans*, the most radiation-resistant microbes presently known, tolerate up to 15 ,000 Gy of acute ionizing radiation and 60 Gy h^{-1} of chronic radiation (Daly 2006). The highly efficient radiation–protection mechanisms of *Deinococcus* are mediated by a combination of passive and active defence processes, (i) efficient repair of disintegrated DNA including self-repair of DNA damage (Krisko and Radman, 2013), (ii) efficient cellular damage clearance mechanisms through hydrolysis of damaged proteins, overexpression of repair proteins, etc., and (iii) effective elimination of reactive oxygen species (ROS) (Jin *et al.*, 2019).

Paterson-Beedle *et al.* (2006) reported that members of genus *Serratia* tolerate high levels of Cs^{137}, Sr^{90} and Co^{60}. In addition, cells of *Serratia* sp. were described for their ability to precipitate high levels of hydrogen uranyl phosphate (HUP) via the activity of a highly radiostable phosphatase enzyme. The radiostability of this enzyme was increased to 100% by the incorporation of radioprotectant such as mercaptoethanol (Paterson-Beedle *et al.*, 2012). Malo and Ddachova (2019) reported that some fungi used melanin as radioprotector for their survival in extreme environments with high levels of ionizing radiation such as the damaged nuclear reactor at Chernobyl. In addition, these Chernobyl-associated fungi are able to harvest usable energy from forms of ionizing radiation, phenomenon termed as radiotrophism (Dadachova and Casadevall, 2008).

Microbial interactions with radionuclides

Microorganisms are able to carry out several metabolic processes influencing the cycling of organic and inorganic species (Falkowski *et al.*, 2008). Under certain conditions, microbially driven processes are used for biotechnological applications (Lloyd 2003). The different microbial–radionuclide interaction mechanisms include biomineralization, biosorption, biotransformation and intracellular accumulation. However, it is necessary to point out that radionuclides exhibit highly microbial species-specific behaviour in their interactions with microorganisms (Brookshaw *et al.*, 2012).

Biomineralization is the process by which microorganisms transform ions in solution into solid minerals as the result of cellular activities (Simkiss and Wilbur, 2012). This process is an efficient way to sequester radionuclides within relatively stable solid phases. Three different types of biomineralization processes have been described: biologically induced biomineralization, which results from indirect modification of chemical conditions such as a pH shift or redox transformations in the environment; biologically influenced biomineralization, defined as passive mineral precipitation in the presence of organic matter, such as cell surfaces or extracellular polymeric substances, whose properties influence crystal morphology and composition; and biologically controlled biomineralization, specific cellular activity that directs the nucleation, growth, morphology and final location of a mineral (Benzerara *et al.*, 2011). Oxides, phosphates, sulfides and oxalates are the most commonly precipitated biominerals, having high metal sorption capacities and redox catalysis due to their chemical properties (Gadd and Pan, 2016), while radionuclide carbonates and hydroxides are precipitated by localized alkalinization at the cell surface (Newsome *et al.*, 2014). U biomineralization processes have been extensively studied. They are generally based on passive U sorption by the negatively charged cell wall extracellular polymers and active secretion of phosphate groups owing to phosphatase activity, under acidic and neutral conditions (Martinez *et al.*, 2007; Merroun and Selenska-Pobell, 2008). Some examples of bacterial U biomineralization include *Pseudomonas aeruginosa* (Choudhary and Sar, 2011), *Bacillus* and *Sphingomonas* (Merroun *et al.*, 2011; Lopez-Fernandez *et al.*, 2014), *Gallionella* (Krawczyk-Bärsch *et al.*, 2020) or *Staphylococcus aureus* (Shukla *et al.*, 2020). Moreover, the bacterial strain *Stenotrophomonas* sp. Br8 mediates U(VI) immobilization under changing environmental conditions due to phosphatase enzymes (Sánchez-Castro *et al.*, 2020; Fig. 1). In the case of *Serratia* sp., biomineralization entails the activity of both alkaline and acidic phosphatases (Newsome *et al.*, 2015; Chandwadkar *et al.*, 2018), as well as an indigenous bentonite microbial population (Povedano-Priego *et al.*, 2019). Moreover, fungal U biomineralization has also been described by *Aspergillus niger* and *Paecilomyces javanicus* (Liang *et al.*, 2015) or *Saccharomyces cerevisiae* (Zheng *et al.*, 2018). In addition, phosphate mineral formation by several yeast strains may involve an organic source of phosphorus such as glycerol 2-phosphate or phytic acid, in the presence of soluble U (Liang *et al.*, 2016). Biomineralization processes with other radionuclides, such as caesium,
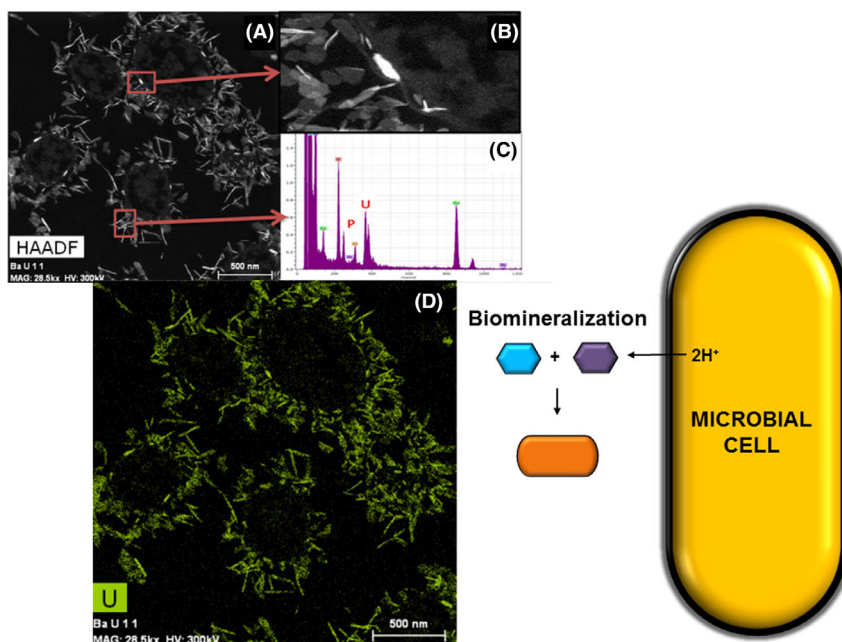
Fig. 1. Example of uranium precipitates (1 mM) around *Stenotrophomonas* sp. Br8 cells from Sánchez-Castro *et al.* (2020).A. HAADF-STEM micrograph, B. zoomed-in view and C. EDXS spectrum of the points marked with a red square in A, and D. EDX element distribution map for U, plus schematic representation of the bioprecipitation mechanism.

strontium, technetium, plutonium or neptunium, have also been described (Macaskie *et al*., 1994; Macaskie and Basnakova, 1998; Hallberg and Ferris, 2004; Mitchell and Ferris, 2005; Thorpe *et al*., 2016, Cleary *et al*., 2019).

Biosorption describes the metabolism-independent sorption of radionuclides to biomass (Newsome *et al*., 2014). It is a complicated process influenced by parameters such as the status of the biomass (living or not living), properties of radionuclide solution chemistry or pH (Das 2012). Concretely, the biosorption of metal ions by living cells is a two-step process. First, radionuclides accumulate at the cell surface through physical adsorption or ion exchange, and then penetrate the cell membrane of atoms or molecules of one phase, forming a solution with the second phase, mainly through chemical functional groups such as carboxyl, amine, hydroxyl, phosphate and sulfhydryl groups (Lloyd and Macaskie, 2000; Goyal *et al*., 2003; Newsome *et al*., 2014). Differences in cell wall composition among microorganisms lead to significant differences in the type and amount of metal ion binding to them (Goyal *et al*., 2003). U biosorption has been extensively studied in bacteria (Hu *et al*., 1996; Nakajima and Tsuruta, 2004; Merroun *et al*., 2005), fungi (Khani et al. 2005; Lopez-Fernandez *et al*., 2018; Chen *et al*., 2020) and even in lichens (Purvis *et al*., 2004). An early study reported U uptake from aqueous solutions by *Rhizopus arrhizus* (Tsezos and Volesky, 1982). Further findings entail plutonium,

americium and cerium sorption from aqueous solutions at pH 1-2 by immobilized *Saccharomyces cerevisiae* (Kedari *et al*., 2001); the reversible and pH-dependent biosorption of curium by yeast *Rhodotorula mucilaginosa* (Lopez-Fernandez *et al*., 2019, Fig. 2); or strontium biosorption from aqueous solutions by several microorganisms (Ilyas *et al*., 2020). Moreover, caesium sorption has been described in conjunction with various microorganisms. The native and chemically modified biomass of marine algae *Sargassum glaucescens* and *Cystoseira indica* might be used as caesium biosorbents (Jalali-Rad *et al*., 2004; Dabbagh *et al*., 2008), while *Pseudomonas fluorescens* and *Bacillus cereus* have caesium biosorption capacity (Mao *et al*., 2011; Kim *et al*., 2016). However, a recent review addressing the radiocaesium soil-to-plant transfer indicates that although mycorrhizal fungi can play a role in the biosorption of radiocaesium, they may not significantly contribute to its uptake by plants (Almahayni *et al*., 2019). The marine actinobacterial *Nocardiopsis* sp. and immobilized *Aspergillus niger* have been described as strontium biosorbent (Pan *et al*., 2009; Sivaperumal *et al*., 2018), and strontium can be biosorbed from aqueous solution by *Bacillus cereus* (Long *et al*., 2017). Thorium biosorption in living and dead cells of *Streptomyces sporoverrucosus* is apparently dependent on pH and ionic strength (Ding *et al*., 2014), while neptunium can be biosorbed by whole cells, the cell wall and extracellular polymeric substances of *Shewanella* algae (Deo *et al*., 2010).
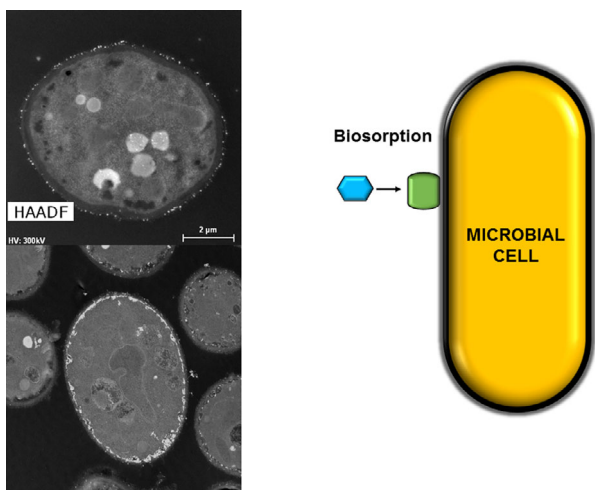
Fig. 2. Example of curium biosorption by *Rhodotorula mucilaginosa*, using europium (1 mM Eu(III)) as inactive analogue of curium. High-angle annular dark-field images (scale bars: 2 μm) and schematic representation of the biosorption mechanism.

Fig. 3. Example of selenium biotransformation by *Stenotrophomonas bentonitica* (2 mM Se(IV)) under anaerobic conditions.A. VP-SEM image showing the Se nanoparticles (scale bar: 200 nm), B. HAADF-STEM micrograph and element distribution map of Se nanoparticles (scale bar: 500 nm) and schematic representation of the biotransformation mechanism.

Biotransformations are microbial structural modifications in a chemical compound by organisms/enzyme systems that lead to the formation of molecules with relatively greater polarity (Smitha *et al.*, 2017). These transformations, including reduction and oxidation, are useful in a wide range of biotechnological processes (Cresnar *et al.*, 2011). A wide diversity of prokaryotes can enzymatically reduce U (Williams *et al.*, 2013). The first case of bioreduction was described for iron reducers *Geobacter metallireducens* and *Shewanella oneidensis*, coupling their growth to the dissimilatory reduction in U (Lovley *et al.*, 1991). Microbial cells therefore use different mechanisms to transfer electrons from the electron donor to an electron acceptor, such as c-type cytochrome, pili, flagella or extracellular carriers to bioreduce U (Newsome *et al.*, 2014). But not only bioreduction in this radionuclide has been described. Lloyd (2003) reviewed the microbial reduction in selenium, neptunium, plutonium and technetium. A mix of technetium and uranium was reduced by the indigenous microbial community of a nitrate-contaminated aquifer (Istok *et al.*, 2004). Different bacterial strains have also shown their plutonium biosorption capability, for example *Sporomusa* sp. cells enhanced the removal of plutonium from solution by biosorption and bioreduction (Moll *et al.*, 2017), and the extracellular polymeric substances of *Pseudomonas* sp. are an effective reductant and sorbent for plutonium, influencing its redox cycling and mobility in the environment (Boggs *et al.*, 2016). Plutonium polymers were found to bind to *Desulfovibrio äspöensis* biomass during the first 24 h of contact time; yet, most of the reduced plutonium dissolved from the cell envelope went back to the aqueous solution due to the weak complexing
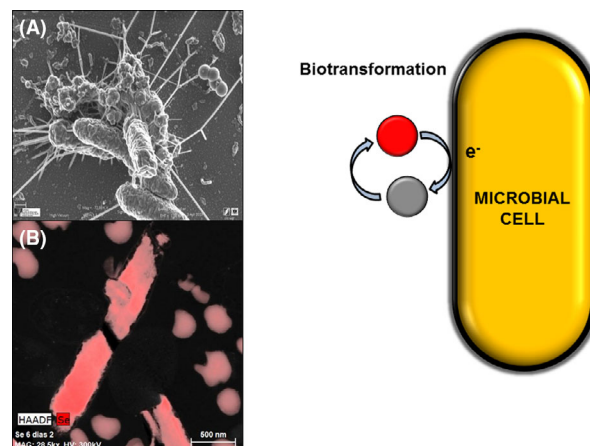
properties of this plutonium oxidation state (Moll *et al.*, 2006). Finally, the mobility of selenium within the deep geological radioactive waste repositories was also studied, relating the bioreduction in this element in anaerobic bentonite samples using acetate as electron donor (Ruiz-Fresneda *et al.*, 2019, Fig. 3) and the biotransformation of amorphous selenium nanospheres to trigonal 1D nanostructures (Ruiz Fresneda *et al.*, 2018).

Intracellular accumulation is considered to be a metabolism-independent process. Microbial cells are able to immobilize radionuclides via different bioaccumulation mechanisms (Merroun and Selenska-Pobell, 2008). This uptake may occur because the transported metals are similar to essential elements needed for cell functioning, so are actively taken up into the cell (Newsome *et al.*, 2014); or due to increased membrane permeability caused for example by U toxicity (Suzuki and Banfield, 1999). Several studies describe the intracellular accumulation of U by bacteria and fungi (Fomina *et al.*, 2007; Choudhary and Sar, 2011; Lee and Hur, 2014; Zhang *et al.*, 2014; Gerber *et al.*, 2018, Fig. 4). One in-depth proteogenomic study revealed the complex cellular response to uranium in *Microbacterium oleivorans*, perturbing the phosphate and iron metabolic pathways by means of several transporters specifically associated with uranyl stress (Gallois *et al.*, 2018). In addition, caesium accumulation has been demonstrated at the cell surface, and in cytoplasm and mycelia (Kato *et al.*, 2000; Kuwahara *et al.*, 2011; Dräxl *et al.*, 2013; Sivaperumal *et al.*, 2018). Further examples are the accumulation of strontium into biogenic carbonate minerals by *Bacillus* sp. (Horiike *et al.*, 2017) or into calcium carbonates by cyanobacteria (Mehta *et al.*, 2019). The bacterial
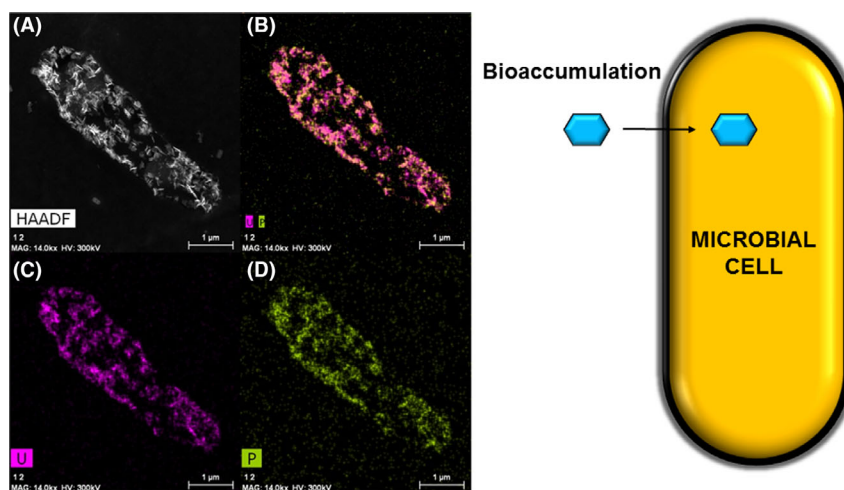
Fig. 4. Example of intracellular accumulation of uranium (0.1 mM U(VI)) by *Rhodosporidium toruloides* as needle-like structure localized at the inner cytoplasm membrane.A–D. Micrograph and distribution analysis of uranium (purple) and phosphorus (green) from Gerber *et al.* (2018) and schematic representation of the bioaccumulation mechanism

accumulation of technetium (Sierra *et al.*, 2008) or plutonium by the iron reducers *Geobacter sulfurreducens* and *Shewanella oneidensis* (Renshaw *et al.*, 2009) has been documented, as well as the sorption of plutonium by bacteria and fungi, accumulating this radionuclide intracellularly (Lujaniene et al., 2017).

Microscopic/spectroscopic/omics-based methodologies used to study the interaction of radionuclides with microbes

A wide variety of methodologies are usually employed to characterize the physicochemical properties – morphology, size, structure, local coordination, elemental composition, chemical speciation, oxidation state, etc. – of microbial interaction products with radionuclides. The application of complementary techniques including microscopy, spectroscopy and synchrotron-based techniques may successfully determine the microbial processes involved in these interactions. In recent years, powerful new omic-based methodologies are revolutionizing the nature of biomolecular studies in the field of microbial–radionuclide interactions.

Fourier transform infrared (FTIR) spectroscopy

Infrared (IR) spectroscopy uses the absorption of IR radiation by the molecular bonds to identify the bond types that can absorb energy by oscillating, vibrating and rotating (Mattox 2010). An IR spectrum of a sample is generated by scanning the intensity of IR radiation before and after passage of the IR beam through the sample. The IR spectra of most materials comprise a large number of absorption bands, which originate from the interaction

between discrete light quanta and mechanical motions (vibrational and rotational modes) of the molecules that are excited by the absorption of IR radiation (Naumann 2006). In FTIR spectrometers, the interference patterns of the modulated signals from interferograms are amplified, digitized, computer-stored and transformed into a spectrum by the fast Fourier transform (FFT) algorithm (Naumann 2006; Beekes *et al.*, 2007).

This technique is usually employed in microbiology to study the nature of functional groups from bacterial cells involved in the interaction with radionuclides and other toxic elements. IR absorption bands observed in the region between approximately 800 and 4000 cm^{-1} can often be assigned to particular functional groups (Naumann 2006). These bands are sensitive to molecular-level and structural changes (molecular interactions, membrane constitution, lipid–protein interaction, etc.) in the composition of the cellular macromolecules involved. Carboxyl, amide, hydroxyl and phosphoryl groups from *Pantoea* sp. TW18 and *Nocardiopsis* sp. 13H reportedly play an important role in the accumulation and adsorption of U(VI) and strontium (Sr^+) respectively (Sivaperumal *et al.*, 2018; Zhang *et al.*, 2018a). In contrast, yet diffuse reflectance FTIR spectroscopy did not detect structural changes in the cellular macrocomponents of the interaction between [241]Am and *Photobacterium phosphoreum* (Kamnev *et al.*, 2013). This technique may also be used to investigate the nature of organic matter (proteins, lipids, polysaccharides, etc.) attached to biogenic nanoparticles of interest (Eswayah *et al.*, 2019; Fischer *et al.*, 2020; Ruiz-Fresneda *et al.*, 2020), which could affect their interactions and hence their environmental fate. For instance, the presence of absorption bands at 1659 and 1538 cm^{-1} corresponding to C-O, N-

H and C-N vibrations attributed to amide I and amide II groups in purified selenium nanoparticles from *Stenotrophomonas bentonitica* suggested the presence of proteins surrounding them (Ruiz-Fresneda *et al.*, 2020).

X-ray absorption spectroscopy (XAS)

XAS is a well-established synchrotron-based analytical technique used extensively to characterize the local chemical structure of diverse materials, including semiconductors in solid or liquid, crystalline or amorphous, bulk or nanoscale form (Schnohr and Ridgway, 2015). Under this technique, a sample of interest is bombarded with X-rays of a given energy. Some of the X-rays are absorbed by the atoms in the sample, inducing the excitation of a core electron (Calvin 2013). By stepping through a range of energies in this way, a spectrum is created. The sharp rise typical of XAS spectrum is generally called the edge. At energies below the edge, incident X-rays do not have energy enough to excite electrons, while above the edge they do (Calvin 2013). The XAS spectrum is commonly divided into the extended X-ray absorption fine structure (EXAFS) and the X-ray absorption near-edge structure (XANES) region. The EXAFS region comprises the oscillations above the edge and provides reliable structural information concerning the local, short-range coordination environment and the chemical and electronic structure of specific sites within materials, including the number, chemical nature and distance of neighbouring atoms from the atomic site of interest (Ormerod, 2001). The XANES region includes peaks and other features near or on the edge, and basically provides information about the oxidation state of the atoms.

XAS revealed the impact of the bacterial strain *Sporomusa* sp. MT-2.99, isolated from Mont Terri Opalinus Clay (selected as a reference material for engineered barriers in the deep geological repository system of radioactive waste), on Pu speciation through a reduction pathway (Moll *et al.*, 2017). Since Pu can be released from nuclear waste disposal sites, the characterization of complex Pu–bacteria interactions is key for a better understanding of the fate of such toxic elements. The structural parameters of plutonium (number and chemical identities of near neighbour atoms and the average interatomic distances) helped to determine the crystalline and non-crystalline nature of selenium nanoparticles produced by *Methylococcus capsulatus* and *Methylosinus trichosporium* (Eswayah *et al.*, 2017). The first Se-Se coordination shell at a bond distance of 2.35 Å reflected an amorphous nature of the nanoparticles, while the second Se-Se shell at 3.69 Å suggested their crystalline structure. Similar results were obtained for selenium nanoparticles produced by *S. bentonitica* (Ruiz-Fresneda

et al., 2020). They found two Se-Se coordination shell that would correspond to amorphous and trigonal Se, and suggested, with the help of other techniques, a transformation process from amorphous to trigonal selenium. In addition, the XANES region indicated the zerovalent oxidation state of the nanoparticles and confirmed they are produced by a reduction process from Se (+IV). Meanwhile, EXAFS analysis of U-bacteria interactions showed that *Desulfovibrio vulgaris* is able to reduce U (VI) to non-crystalline U(IV) associated with cell-bound phosphate groups, most probably from bacterial extracellular polymeric substances (EPS) (Stylo *et al.*, 2015).

Other synchrotron-based techniques such as X-ray fluorescence (XRF) imaging have been employed to study the accumulation of radionuclides by plants through arbuscular mycorrhizal (AM) fungi. Several studies indicated the role of AM in the translocation of ^{238}U, Cd and Zn into root tissues and in their limitation into other tissues of the plant as a defensive mechanism (Rufyikiri *et al.*, 2002, 2003; Chen *et al.*, 2008; Nayuki *et al.*, 2014; Davies *et al.*, 2015). Phytoaccumulation of the toxic metals Cd and Zn in plant and fungal structures has been demonstrated by XRF imaging analysis of mycorrhizal root sections of *Lotus japonicum* and *Rhizophagus irregularis* (Nayuki *et al.*, 2014). The former work indicates the potential of synchrotron-based imaging methodologies, in combination with XAS spectroscopy, for the analysis of other elements such as U in AM fungal–plant tissues.

Time-resolved laser-induced fluorescence spectroscopy (TRLFS) analysis

TRLFS is a useful technique for the identification of certain actinide (An) or lanthanide (Ln) species resulting from various biogeochemical processes (Collins *et al.*, 2011). Fluorescent metal ions emit fluorescence emission spectra and decay lifetimes when irradiated with laser pulses. This phenomenon occurs as a consequence of their transition from electronically excited states to the ground state. Analysis of the emission spectra and lifetimes allows different chemical species produced by a fluorescent metal ion to be identified (Collins *et al.*, 2011; Saito *et al.*, 2015). TRLFS can be applied to studies of actinide speciation in aqueous and solid phases, or the coordination of metal ions adsorbed to mineral and bacterial surfaces (Collins *et al.*, 2011).

TRLFS measurements can help determine the microbial functional groups involved in An/Ln–microorganism interaction. The complexation of Cm(III) with carboxylic functional groups from the archaeon *Halobacterium noricense* DSM15987[T] was observed using TRLFS (Bader *et al.*, 2019). This technique also allowed identifying the association of phosphate species from this halophilic

archaeon with Eu(III), the inactive analogue of Cm(III). Moll *et al*. (2020) demonstrated a stable interaction of Cm(III) with the protein S-layer of *Lysinibacillus sphaericus* JG-A12 by means of TRLFS. They indicated that S-layer protein carboxyl sites could be involved in Cm(III) binding. The importance of investigating not only the interaction between cellular surfaces and actinides, but also cellular protein layers with these elements relies on the fact that proteins can highly influence their migration behaviour in the environment through the formation of colloids (Moll *et al*., 2020).

Microscopic techniques

The morphology and cellular location of interaction products can be analysed by means of transmission or scanning electron microscopy (TEM/SEM), equipped with energy dispersive X-ray (EDX) for elemental composition analysis. Several high-resolution microscopes – e.g. the high-angle annular dark-field scanning transmission electron microscope (HAADF-STEM) – allow for a structural characterization of biologically and chemically produced materials by using selected-area electron diffraction (SAED) and high-resolution TEM combined with fast Fourier transform (FFT). Another useful system is variable pressure field emission scanning electron microscopy (VP-FESEM), equipped with a Raman X-ray detector, which made possible an in situ 3D structural and elemental characterization. Field emission gun environmental scanning electron microscopy (FEG-ESEM), equipped with secondary and circular backscatter electron detectors such as ETD (Everhart-Thornley detector) and CBS (concentric backscatter detector), provides high-resolution imaging of surface morphology and topography, and elemental composition as a function of image contrast (Vernon-Parry 2000; Zhang *et al*., 2016).

In the study of Boggs *et al*. (2016), Pu(V) and Pu(IV) were shown by TEM analysis to be associated with the cell walls of *Pseudomonas* sp. strain EPS-1W. Wild-type cells with bound extracellular polymeric substances (EPS) were found to sorb more efficiently than cells without EPS in the case of Pu(V). Another example is the sorption of U(VI) by microorganisms, reported widely owing to electron microscopy (Bader *et al*., 2018; Povedano-Priego *et al*., 2019; Kolhe *et al*., 2020). SEM images of the yeast *Yarrowia lipolytica* exposed to uranyl carbonate clearly showed the apparition of uranyl deposits attached to the cell surfaces, due to biosorption and biomineralization processes (Kolhe *et al*., 2020). In summary, microscopic techniques provide visual evidences of the specific mechanisms involved in the microbe–radionuclide interaction, which may be very useful and improve the quality of the works in the field.

Multi-omics

The development of multiple omic techniques – including genomics, proteomics, metabolomics and transcriptomics – is becoming a very important tool for biomolecular studies in many different fields. These high-throughput techniques can provide a comprehensive analysis of the total pool of molecules of the same type (DNA, proteins and other metabolites) from a biological sample. Specifically, multi-omics is leading the way towards more proficient studies and approaches in the field of bioremediation.

Genomic approaches including genome sequencing, and comparative genomics have proven useful for the identification of essential genes (key players) encoding biomolecules, mainly proteins, involved in radionuclide and heavy metal detoxification (Yan *et al*., 2016; Hemmat-Jou *et al*., 2018, Jaswal *et al*., 2019a). For instance, metagenomic-based taxonomic studies performed by Jaswal *et al*. (2019a) from contaminated ecosystems led to the identification and characterization of U-resistant microorganisms and fungi belonging to *Burkholderia* and *Penicillium* species and having environmentally relevant functions. The genome is usually stable, whereas the protein expression level by the genome depends on the environmental conditions. Qualitative proteomics can systematically identify the types of proteins from a biological sample, while quantitative proteomics can determine the amount of a specific protein expressed under different environmental conditions. Proteomics therefore serves to determine the type and abundance of proteins and enzymes synthesized as a consequence of radionuclide stress response, which contributes to our understanding of the specific peptides involved in the interaction process. It is well known that c-type cytochromes in *Geobacter* species promote the reduction of U(VI) to poorly soluble U(IV) as a strategy to prevent its migration through contaminated environments (Lovley *et al*., 2011). Recently, a c-type cytochrome (GscA) from *Geobacter* sp. M18 was determined by proteomic studies to play an important role during uranium bioremediation (Yun *et al*., 2016).

Transcriptomics is acquiring a crucial role in bioremediation studies through the analysis of gene expression and regulation. For instance, a targeted gene expression analysis indicated the transcriptional induction of arsenate reductase genes, among others related to the *ars* gene cluster (*arsR, arsA, acr3* and *arsS*) by the cells of *Rhodococcus aetherivorans* BCP1 when grown in the presence of As(V) at sublethal concentrations (Firrincieli *et al*., 2019). The latter work revealed that unique metabolic mechanisms of a *Rhodococcus* strain could tolerate this toxic metal. Transcriptional studies of the response of the strain *Cupriavidus metallidurans* CH34 to heavy

metals were performed as well (Monsieurs *et al.*, 2011). Similar expression profiles were found when the cells were exposed to different heavy metals, suggesting a complex transcriptional-level interaction between the different metal responses.

Biotechnological applications of radionuclide microbe interactions: Bioremediation and deep geological repository

The different processes by which microbes interact with radionuclides, as described in this review, give rise to diverse biotechnological applications in the field of bioremediating radionuclide-contaminated sites and in the implementation of deep geological disposal of radioactive wastes.

Bioremediation of radionuclide-polluted environments

Most works conducted on bioremediation of radionuclide-polluted environments have been focused on uranium, attempting to overcome the legacies of past U mining around the world. Western countries including France and German, having ceased their U mining activities decades ago, nowadays focus on developing effective technology for the remediation of U pollution. Conventional remediation techniques are based on the use of chemical and physical agents, entailing high costs and low contaminant specificity (Gavrilescu *et al.*, 2009; Selvakumar *et al.*, 2018; Zhang *et al.*, 2018). In contrast, remediation using microorganisms, known as bioremediation, is gaining importance as a feasible and ecofriendly *in situ* technology for the clean-up of environmental pollutants (Pollmann *et al.*, 2006). Bioremediation is a process based on reducing the solubility, mobility, bioavailability and toxicity of contaminants. In the case of U, the toxicity is intimately related to its chemical speciation, which in turn depends on its oxidation state. U(IV), a highly insoluble and less toxic form of U, is present in the precipitated form as mineral uraninite (UO_2) under anaerobic conditions (Nymans et al. 2006). In contrast, U(VI) species are much more soluble and toxic, and occur predominantly as mobile aqueous species under oxic conditions (Burns 1999). Under aerobic conditions, biomineralization of U(VI) phosphates is upheld as an efficient long-term strategy to remediate U. These processes are less prone to U re-oxidation in contrast to the product of U bioreduction, uraninite, which tends to re-oxidize back to more soluble U (Kulkarni *et al.*, 2013). The biomineralization of U has proven successful when using bacterial strains able to produce phosphatase (acid, neutral and alkaline phosphatase) (Hu *et al.*, 2005; Beazley *et al.*, 2009; Sánchez-Castro *et al.*, 2020; Shukla *et al.*, 2020). Phosphatases release

orthophosphates from organic phosphate substrates, leading to the formation of different U(VI)-phosphate minerals, mainly autunite [$Ca(UO_2)_2(PO_4)_2 \cdot 10\text{-}12H_2O$], although chernikovite [$(H_3O)_2(UO_2)_2(PO_4)_2 \cdot 6H_2O$] or ankoleite [$K_2(UO_2)_2(PO_4)_2.6(H2O)$] has been also observed (Merroun and Selenska-Pobell, 2008; Beazley *et al.*, 2009; Salome *et al.*, 2017). Bacteria and fungi were documented for their ability to express phosphatase activity in the presence of U to biomineralize radionuclide. The process was first reported for *Citrobacter* sp. by Macaskie *et al.* (2000), and thereafter described in several bacterial strains, including *Pseudomonas*, *Caulobacter crescentus, Deinococcus radiodurans, Serratia*, *Sphingomonas* and *Bacillus*, as well as in fungi including *Aspergillus*, *Paecilomyces* species or archaea *Halobacteriium noricense* DSM 15987 (Hu *et al.*, 2005; Merroun *et al.*, 2011; Liang *et al.*, 2016; Tu *et al.*, 2019). In addition, the potential utility of genetically engineered phosphatase for the bioprecipitation of uranium from alkaline solutions has been demonstrated by Nilgiriwala *et al.* (2008). This work reports on the cloning and overexpression of alkaline phosphatase PhoK from *Sphingomonas* sp. strain BSAR-1 in *E. coli* strain EK4 for bioprecipitation of uranium from alkaline solutions.

In addition to the great impact of microbial phosphatase in the biomineralization of U described above, the economic costs of such bioremediation-based technology should be taken into account. Certain strategies may reduce the costs of industrial upscaling U bioremediation technology: i) use of cheap organic phosphate substrates (e.g. organic phosphate-containing wastes such as manure, crop residues and sludge from different treatments in sustainable waste handling systems) and ii) immobilization of phosphatase-producing microbes on inorganic substrates (e.g. alginates).

Several international experiments were undertaken to remove U from U mining wastes in Germany, France and the USA. Recent studies have aimed to design U bioremediation technologies based on U phosphate biomineralization of contaminated former U mining sites in France (Sánchez-Castro *et al.*, 2017, 2020). Over 100 bacterial strains were isolated from U mill tailings pore waters in France and screened for U bioremediation potential (Sánchez-Castro *et al.*, 2017). The strains had to fulfil the following properties to be further investigated and applied at large scale: (i) high tolerance to U and other heavy metals (e.g. Cd, Se, Pb), (ii) high phosphatase activity and (iii) U immobilization potential through U(VI) phosphate biomineralization. A highly U-tolerant *Stenotrophomonas* sp. Br8 strain – isolated from U mill tailing pore waters with high phosphatase activity – was described for its U phosphate biomineralization (Sánchez-Castro *et al.*, 2020).

In Germany, ongoing investigations are dedicated to remediating U-contaminated sites in Saxony and Thuringia after U mining activities ceased. Recently, Krawczyk-Bärsch *et al.* (2018) explored the U(VI) phosphate biomineralization potential of *Acidovorax facilis*. Species of the genus *Acidovorax* were widely found in U-contaminated sites (e.g. U mine tailings) (Bondici *et al.*, 2013).

Recently, new bioremediation technologies based on the use of redox behaviour of some microbes through exchange of electrons form cell compartments such as proteins and pili to metals and electrodes have been developed. This bioelectrochemical device-based bioremediation technology referred to microbial fuel cell (MFC) exhibits excellent abilities to remove pollutants along with electrical power generation within the concept of circular economy (Fang and Achal, 2019). MFCs consist of a two-chamber system where the anode chamber is occupied by bacterial cells separated from the cathode chamber by a polymeric proton exchange membrane.

Most MFCs use aqueous cathodes where water is bubbled with air to provide dissolved oxygen to electrode (Das 2020). MFCs are constructed to remove radionuclides such as uranium through the cathodic reduction reaction, while organic substrates are oxidized and serve as the carbon and electron donor at the anode (Das 2020). Electrochemically active bacteria used in the bioremediation of U through this innovative technique include *Shewanella putrefaciens* and *Geobacter sp.*, directly transfer electrons from the microbe to the electrode through reduction of U(VI) to U(IV) (Kato 2015).

Impact of microbial processes in the deep geological disposal of radioactive wastes

In the past decade, a number of studies have aimed to study the impact of microbial processes on the safety of future deep geological repositories (DGR) to dispose radioactive wastes generated by the nuclear industry, U mining activity, etc. DGR is the safest option for the disposal of radioactive waste when encapsulated in metal containers surrounded by artificial (e.g. bentonite) and natural barriers (clays, granite and salt formations) (Alexander and McKinley, 2011). The microbial populations of these barriers, described as abundant, can affect the safety of the disposal system through different microbial processes: (i) transformation of clay mineralogy; (ii) corrosion of metal canisters; and (iii) mobility of radionuclides (e.g. U, Se, Cm, Pu, Am, Np) (Anderson *et al.*, 2011).

Several studies report on the impact of microbial processes in the mobility of radionuclides in terms of DGR safety (Povedano-Priego *et al.*, 2019). In Spain, research has looked into the microbial processes occurring at the bentonite/radionuclide interface that may be involved in the mobility of U, Cm and Se. In the case of uranium, Lopez-Fernandez *et al.* (2018) put forth a multidisciplinary approach combining X-ray absorption and IR spectroscopy; the cells of the yeast *Rhodotorula mucilaginosa* BII-R8, isolated from Spanish bentonites, sorbed mobile uranium species (U-hydroxides and U-hydroxo-carbonates) from solution via a time-dependent process initiated by the adsorption of uranium species to carboxyl groups, and then by organic phosphate groups, forming uranium complexes with a structure similar to that of meta-autunite. This yeast strain was reported to bind Cm(III), another critical radionuclide, through phosphoryl and carboxyl groups present in bacterial cell envelopes (Lopez-Fernandez *et al.*, 2019). Povedano-Priego *et al.* (2019) documented the immobilization of U through natural U phosphate biomineralization by bentonite bacteria in microcosms amended with glycerol-2-phosphate as an electron donor and organic phosphate source. In the case of selenium, a bentonite isolate from the genus *Stenotrophomonas* was studied to determine its impact on the mobility of this element under DGR-relevant conditions (Ruiz Fresneda *et al.* (2018; Ruiz-Fresneda 2019, 2020). Reduction of Se(IV) to Se(0) nanoparticles under aerobic and anaerobic conditions was reported. HRTEM and electron diffraction studies revealed that the structure of the Se nanoparticles depends upon their shape, while Se nanospheres are amorphous, and (a-Se) nanowires and hexagons exhibited trigonal structures (t-Se) due to the presence of two distinct lattice spacings of 0.37 and 0.29 nm, respectively, corresponding to the (100) and (101) planes of t-Se. Given the low solubility of t-Se nanostructures compared with that of a-Se nanospheres and Se(IV), the mobility of selenium in the environment may be significantly reduced. All these studies are needed to enhance understanding of how microbial processes can influence the safety and performance of future repositories in Europe and around the world.

The above summarized laboratory-scale radionuclide microbe interaction is the basis for future large-scale application. However, these field bioremediation applications are still in their infancy and several challenges related to the biotic and abiotic complexity of contaminated environments (pH, presence of cations, anions, etc.) should be broadly investigated. In addition, other parameters should be taken into account in assessing field bioremediation technologies including systematic life cycle analysis take into account technical, economic and sustainability issues (Gong et al. 2018).

Conclusions and future perspectives

Despite the substantial amount of basic research and remarkable advances in microbial–radionuclide

interactions summarized in this review, biotechnological applications based on such knowledge are still limited. Largely to blame is the restricted access to such contaminated environments, along with their high geochemical and biological complexity. Therefore, the application of key technologies could best be ensured through collaborative work, involving interdisciplinary teams of geologists, geochemists and microbiologists, adopting multidisciplinary approaches combining microbial ecology, radiochemistry, geochemistry, mineralogy, microscopy and spectroscopy, plus the emerging techniques based on omics, for instance. New developments are required to gain a sound understanding of the impact of microbial processes on the geochemical cycle of plutonium, neptunium, curium and radon, and subsequently design bioremediation technologies for these actinides. Another emerging application of microbes in the framework of energy and chemical wastes that calls for further study is metal cycling. There is a lack of balance between the available resources of U (natural resources; U-containing minerals) and the industrial demand for this radionuclide. The fact that U-tolerant microbes are able to recover radionuclides (including U) from radioactive wastes means that waste can take on an active role as raw material under the concept of circular economy. In addition, from an educational point of view, microbiologists should consider radionuclide-contaminated environments as didactic tools and materials to attract the attention of primary and secondary scholars through the organization of excursions, seminars, etc. (McGenity *et al.*, 2020).

Acknowledgments

Conflict of interest

None declared.

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