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ORIGINAL PAPER

Development and characterization of biofilms on stainless steel and titanium in spent nuclear fuel pools

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Abstract The aim of the present research was to study the biofilms developed in a Spanish nuclear power plant and their ability to entrap radionuclides. In order to carry this out, a bioreactor, which was then submerged in a spent nuclear fuel pool, was designed. To characterise the biofilm on two different metallic materials (stainless steel and titanium), standard culture microbiological methods and molecular biology tools, as well as epifluorescence and scanning electron microscopy were used. The bacterial composition of the biofilm belongs to several phylogenetic groups (α, β, and γ-*Proteobacteria*, *Actinobacteridae*, and *Firmicutes)*. The radioactivity of the biofilms was measured by gamma-ray spectrometry. Biofilms were able to retain radionuclides from radioactive water, especially ^{60}Co. The potential use of these biofilms in bioremediation of radioactive water is discussed.

Keywords Biofilm · Radioactive water · Nuclear power plant · Spent nuclear fuel pool

Introduction

Although bioremediation processes have been used in the last few decades for environmental purposes, they had not been proposed to eliminate radionuclides found in radioactive water in the nuclear industry until now. Compared to the conventional method of ion exchange, bioremediation could be considered more acceptable for two reasons. First, it is more cost effective and, second, it is very useful for detoxification of even very dilute effluents. In order to do this, it was necessary to find materials that can support the growth of bacteria that can perform the bioremediation process. Even though many studies have been carried out about the formation of biofilm and the interaction between microorganisms and materials, the nuclear industry began to focus on this phenomenon only some years ago when the presence of microorganisms was detected in radioactive and oligotrophic water used as radioactive barriers in spent nuclear fuel (SNF) pools [4, 22], and in biofilms that developed on materials submerged in SNF pool water [5, 24, 29].

Microbial biomass has a high affinity for hydrocarbons, heavy metals, actinide elements and radionuclides, as observed both in laboratory settings and in the natural environments and as a result, has an enormous potential when it comes to removing pollutants [15, 18, 19, 23]. For this reason, the microbial bioremediation of radioactive waste is increasingly considered as a potential technique to improve the current system of removing radionuclides from contaminated sites.

In this study, a new approach to bioremediate radioactive water in nuclear power plants (NPPs) is presented.

Materials and methods

Spent nuclear fuel pool and water analyses

This study was conducted inside the SNF pool at the Cofrentes NPP in Valencia, Spain. The radioactive water

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filling the SNF pool is treated in a closed-loop system including filtration and demineralisation that ensures the high purity of the water.

Physical/chemical analysis of the water is carried out once a week in the NPP as part of the routine test for radiological security control. Conductivity, pH, temperature, and total organic carbon (TOC) were analysed according to AWWA-APHA-WEF [1]. The concentrations of nitrates, sulphates, and chlorides were measured by gradient ion chromatography (Model DX500, Dionex IC) with a microbore configuration, conductivity detection, and Peaknet 5.1 software.

Following the recommendations of Debertin and Helmer [8], radiochemical analyses were carried out using a Canberra gamma-ray spectrometer consisting of a High Purity p-type coaxial Germanium detector, Canberra-associated electronic equipment, and a Canberra Genie2k gamma-ray spectrometry software package.

Biofilms development

The study was carried out in a stainless steel bioreactor adapted to a submersible water pump and to a flow meter that permits the radioactive water to go through the bioreactor (Fig. 1a). The whole system was supported by a stainless steel perforated sheet basket and submerged at a depth of three meters in the SNF pool. The water pump was on for two hours and off for an hour. This "on-off" cycle was carried out for 365 days. The pump had a capacity of approximately 2.3 $m^3 h^{-1}$.

The tested materials were introduced in the bioreactor. In both cases, the selected materials are non-corrosive and non-degradable in this environment. The balls were degreased using acetone in an ultrasonic bath, and sterilised before being submerged. At first, stainless steel (≈UNS S44200) balls (weighing 20 g each) were introduced in the bioreactor. When these were extracted the second time, the titanium (99.9%) balls, made with shavings coming from the construction of the condenser of the NPP and braided wool and shaped into 5-g balls each, (Fig. 1b) were introduced in the same bioreactor, although separated from the other ones by a stainless steel sheet.

The stainless steel balls were extracted after 51, 80, 106, 142, 170, 254 and 365 days of immersion. The titanium balls were extracted after 26, 62, 90, 174 and 285 days of immersion.

The techniques used for both materials were the same. The purpose was to determine the biofilm formation, the bacteria growing on this adherent biofilm, and the radionuclides accumulated in the biofilm.

Microscopic analyses of biofilms

Biofilm formation on the stainless steel and titanium was observed directly by epifluorescence microscopy. The metallic balls were removed after exposure and were cut into fragments of less than 1 cm^2 (samples) and maintained in Milli-Q sterile water. Afterwards, the metal samples were stained using a commercially available fluorescent stain (LIVE/DEAD *Bac*Light Viability Kit L-7012, Molecular Probes Inc., Eugene, OR, USA) that distinguishes viable bacterial cells from dead ones on the basis of membrane integrity. The kit contains two fluorochromes: SYTO 9 and propidium iodide (PI). The green fluorescent SYTO 9 labels both live and dead bacteria. In contrast, PI penetrates only into the bacteria with damaged membranes and shows up red. Thus, live bacteria with intact membranes are green, while dead bacteria with damaged membranes are red. Each metal sample was covered with a 1:1 staining solution with final concentration of SYTO 9 and PI of 3.41 and 6.01 μM, respectively. The samples were incubated for 15 minutes in the dark at room temperature. The samples were then washed with Milli-Q sterile water and air-dried. Finally, the samples were placed with oil mounting (Molecular Probes, Inc. $\eta^{25} = 1.517 \pm 0.003$) and observed under the microscope.

The samples were visualised with an epifluorescence microscope (Axioskop 2, Zeiss, Germany) using an ×100 immersion oil objective and ×100 immersion oil (Immersol™

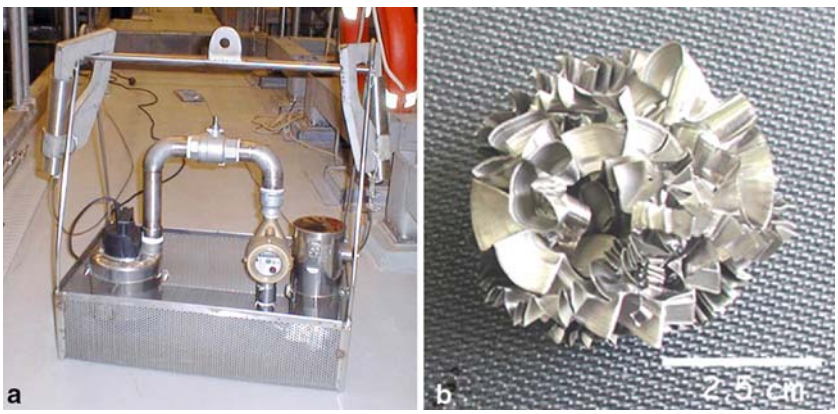

Fig. 1 **a** Bioreactor designed to carry on the tests of radionuclides bioremediation in the SNF pool in the NPP; **b** titanium ball introduced in the bioreactor

518N, $n_e = 1.518$ (23 °C). SYTO 9 and PI could be observed together by using a filter block (Zeiss 487709: excitation 450–490 nm, FT 510 nm, LP 515 nm). Microphotographs were obtained using an Olympus Digital Camera C-3030 Zoom.

The materials were also observed by scanning electron microscopy (SEM) using metal samples (approx. 1 cm^2), as described above. The metal samples were washed in a sodium cacodylate 0.01 M buffer, and were then fixed with 2.5% glutaraldehyde in sodium cacodylate 0.01 M at 4 °C for 2–3 h. After that, the samples were dehydrated with a series of acetone-water washes (20, 40, 60 and 80%) and submerged in these solutions for 30 min at 4 °C. Finally, the samples were maintained in a 100% acetone solution at 4 °C. The specimens were further processed applying critical point procedure (CPD 020, Balzers Union) sputtered with gold (SCD 004, Balzers Union), and observed under a scanning electron microscope (DSM 960, Zeiss) operated at 15 kV accelerating voltage.

Cultivation and DNA extraction

Biofilms were removed from the metal samples using an ultrasonic bath for 15 min in 250 ml of deionised sterile water. After sonication, 50 ml of a 1:10 dilution was filtered through 0.45 µm filters (HAWP04700, MF™ Membrane filters, Millipore), and then the filter was plated onto different solid media in order to isolate a high number of species. The culture media used were: NA (Nutrient Agar, Oxoid CM3), BHI (Brain Heart Infusion, Oxoid CM225; supplemented with agar (UPS), purissimum, Panreac), TSA (Triptone Soya Broth, Oxoid CM 129; supplemented with agar (UPS), purissimum, Panreac), STC (starch from potato soluble for analysis, Panreac, 10 g l^{-1}; casein hydrolysate (acid), Oxoid, 1 g l^{-1}; potassium phosphate monobasic, KH_2PO_4, Panreac, 0.5 g l^{-1}; agar (UPS), purissimum, Panreac, 20 g l^{-1}; pH = 7.2), R2A Medium Oligotrophic Agar (DMT 215, Microkit Iberica, S.L.).

Due to the high levels of radiation of the filters (up to 2,030 Bq cm^{-2}), they were incubated at 30 °C until colonies appeared inside the NPP. Colonies growing on the plates were picked in the NPP and purified by restreaking on the same culture media several times outside it. Genomic DNAs of the isolated bacteria were then extracted using PrepMan™ Ultra (PE Applied Biosystems), according to the protocol given by the manufacturer, or using freeze-thawed (−20 °C, +60 °C) cycles.

Polymerase chain reaction amplification, sequencing and phylogenetic identification

Polymerase chain reaction (PCR) amplification of 16S rDNA fragments was performed by a "touchdown" PCR using the universal primers of *Escherichia coli* 5F (5′-TGGAGAGTTTGATCCTGGCTCAG-3′) and 531R (5′-TACCGCGGCTGCTGGCAC-3′). The annealing temperature was lowered from 50 °C to 40 °C over 20 cycles and then another 20 cycles at 43 °C. The mixture was preincubated for 2 min at 94 °C. The temperature cycles were at follows: 45 s at 94 °C, 1 min at the appropriate annealing temperature, and 45 s at 72 °C. Following the final cycle, the reaction was extended for 7 min at 72 °C. PCRs were carried out in a GeneAmp PCR System 2400 (Perkin Elmer). In all cases, a PCR-master kit (Roche) was used. Amplification products were analysed by electrophoresis in 1% (wt/vol) agarose gels in TBE 1X with ethidium bromide (0.5 µg ml^{-1}).

Identification of bacteria was accomplished by sequencing 500 bp using the MicroSeq 500™ 16S rDNA or using the BigDye™ Terminator v1.1 Cycle Sequencing kit (L-7012, PE Applied Biosystems). Sequences were resolved in an ABI PRISM™ 310 Genetic Analyser following the manufacturer's instructions. The sequences obtained were compared directly to all the known sequences deposited in the NCBI (National Center for Biotechnology Information) databases using the basic local alignment search tool Blastn.

Sequences were aligned using ClustalX software version 1.81 [26]. Phylogenetic and molecular evolutionary analyses were conducted using molecular evolutionary genetics analysis (MEGA) version 2.1 [17]. Phylogenetic trees were constructed using the neighbor-joining method with the Jukes--Cantor model [14]. A total of 1,000 bootstrapped replicate resampling datasets were generated.

Accumulation of radionuclides in the biofilms

The presence of radionuclides in the biofilm was analysed by gamma-ray spectrometry previously described by Sarró et al. [24], and in accordance with Debertin and Helmer's [8] recommendations. The metallic balls were washed in sterilised water; they were directly introduced in the gamma-ray spectrometer. In case the detector was saturated, they were cut into two or more pieces. One of the pieces was analysed after it was weighed in order to obtain a measure of radioactivity.

Nucleotide sequence accession numbers

The DNA sequences were deposited in GenBank under the accession numbers AY791999 to AY792035.

Manipulation of samples and waste management

All the samples obtained in this study were collected and manipulated following the recommendations of the Nuclear

Security Council to prevent radiological contamination [7]. In all cases, the samples and filters were removed from the NPP only when the radioactivity level permitted it. Moreover, materials used in this research as well as all waste products generated were treated as radioactive waste.

Results

Water analyses

The concentrations of nitrates, sulphates, and chlorides in water treated in the closed-loop water system typically fell within the 0.45–0.59 (μg l^{-1} range; the water conductivity was 0.8–1.5 (μS cm^{-1}; pH values varied between 5.0 and 6.5; the temperature remained between 25 and 30 °C; and the TOC values ranged from 0.01 to 0.61 ppm. These data are in accordance with the recommended values given for the NPP, and indicate the oligotrophic character of the water.

The radiochemical analysis of the water carried out by gamma-ray spectrometry during the study is shown in Fig. 2. The high peak matches with the refuelling period. The most common radionuclides found in the water were ^{60}Co, ^{137}Cs, ^{134}Cs, ^{54}Mn, and ^{65}Zn. The presence of these radionuclides is usual in SNF pools of NPPs that operate with a boiling water reactor (BWR).

Microscopy analyses of biofilms

Epifluorescence microscopy studies of biofilms could not give cell densities due to the morphology of the biofilm but revealed a progressive colonisation on materials with time (Figs. 3, 4). The bacterial colonisation was quicker in titanium biofilms than in those of stainless steel. Filamentous bacteria and long rod-shaped bacteria were the most predominant in colonies formed on the surface of both materials. The highest number of dead cells was found in the oldest biofilms, whereas the living and metabolically active bacteria outnumbered the dead ones in spite of high levels of radiation.

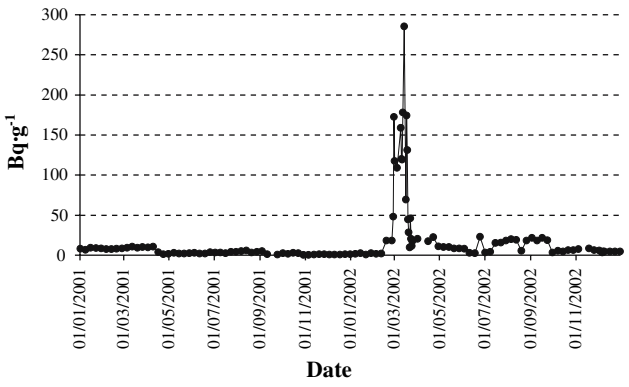

Fig. 2 Radiochemical SNF pool water analysis using gamma-ray spectrometry (Bq g^{-1}) during 2001–2002. The peaks observed in the figure agree with movements of the spent nuclear fuel for maintenance works or with periods after refuelling in the NPP

SEM confirmed the epifluorescence results of microbial colonisation and the diversity of bacteria within biofilms (Fig. 5). SEM suggested that a complex biofilm was formed on stainless steel after 51 immersion days and that there was a flux influence of the biofilm structure (Fig. 5a). After 365 immersion days, the extracellular polymeric substances (EPS) strands were evident as threads spread over the surface. On the titanium, the colonisation was apparent after 26 immersion days. Differences in the colonisation could be seen on the surface morphology of the titanium material depending on the advanced stages of immersion. On the rough or obverse side, the colonisation was most abundant, and even covered the surface of the material completely. This side had a few colonies that were very large (Fig. 6b, d), while the smooth or reverse side had more colonies that were smaller (Fig. 6a, c).

Bacterial isolation and identification

From the filters cultured on different solid media, 146 colonies were isolated; 88 from biofilms on stainless steel and 58 from biofilms on titanium. The best results were obtained with R2A (38%) and TSA (33%) media, NA and BHI were not good media to isolate bacteria in this environment. Their DNA was extracted and amplified using PCR methods. In order to identify them, a 500 bp fragment of the 16S rDNA was sequenced and compared to those deposited in the NCBI databases. Isolated bacteria in the biofilms belonging to five different phylogenetic groups (α, β and γ-*Proteobacteria, Actinobacteridae*, and *Firmicutes*) were present on both materials, but some species were only isolated from one of them (Fig. 7).

The percentages of Gram-positive and Gram-negative bacteria were similar on both materials, but the colonisation was different. The microbial diversity in the biofilm on stainless steel increased with time reaching the highest at 170 immersion days (6 genera). The diversity in the biofilm on titanium remained relatively constant with time with four of five genera per sample. The genera most present in the stainless steel biofilm at all times were *Bacillus* and *Stenotrophomonas*, whereas in the titanium biofilm *Ralstonia* and *Mycobacterium* were most present.

Biofilm radioactivity

To carry out the study, stainless steel and titanium balls were stored in the bioreactor submerged in the pool. The

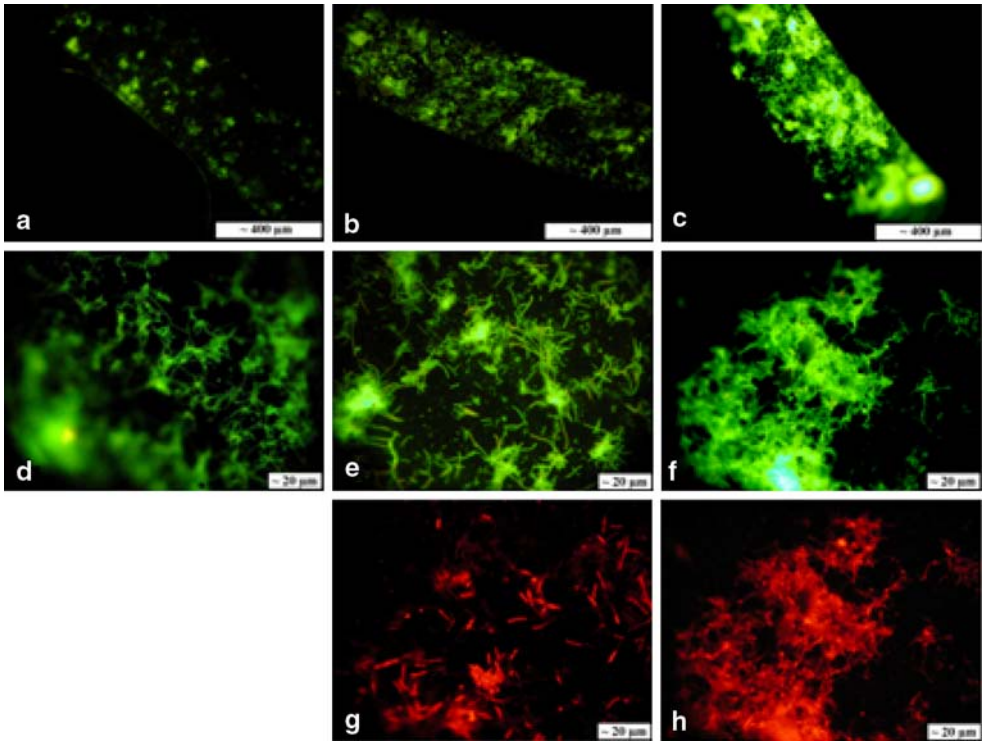

Fig. 3 Epifluorescence micrographs of stainless steel colonisation stained with SYTO 9 and PI. **a** after 142 immersion-days, **b** after 254 immersion-days **c** after 365 immersion-days **d** after 106 immersion-days, **e** after 254 immersion-days, **f** after 365 immersion-days, **g, h** same micrographs that (**e**) and (**f**) using the filter set for PI showing the dead cells in the biofilms

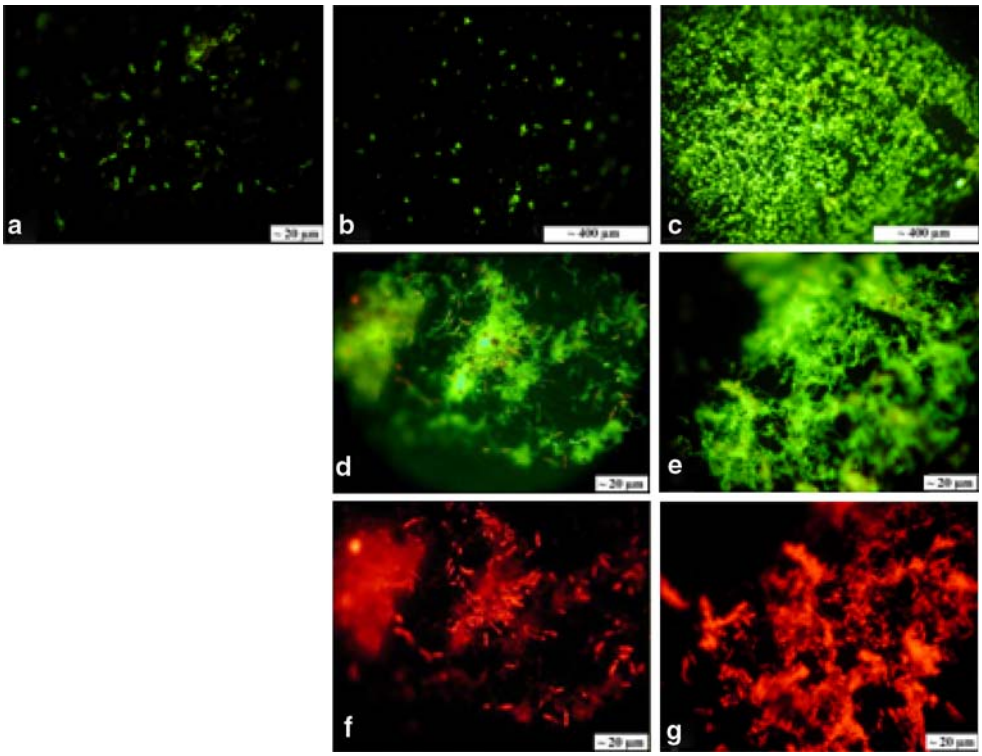

Fig. 4 Epifluorescence micrographs of titanium colonisation stained with SYTO 9 and PI. **a** after 62 immersion-days, **b** after 90 immersion-days **c** after 285 immersion-days **d** after 174 immersion-days, **e** after 285 immersion-days, **f, g** same micrographs that (**d**) and (**e**) using the filter set for PI showing the dead cells in the biofilms

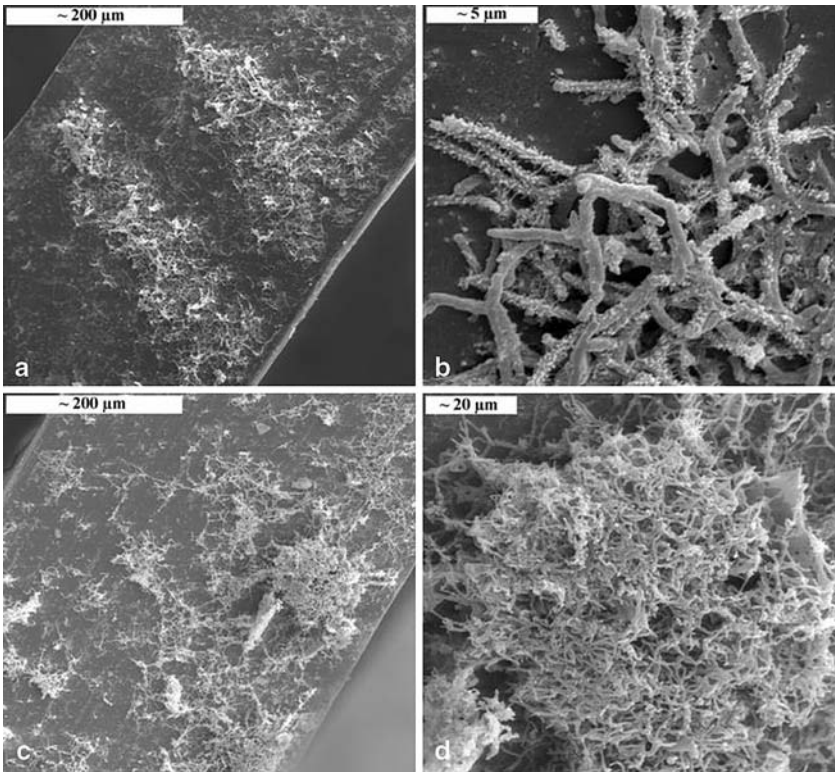

Fig. 5 Electron micrographs of stainless steel colonisation after different exposure times: **a** after 51 immersion-days, the biofilm formation is conditioned by the flux of water in the bioreactor; **b** after 170 immersion-days; **c, d** after 365 immersion-days a mature biofilm was formed on stainless steel

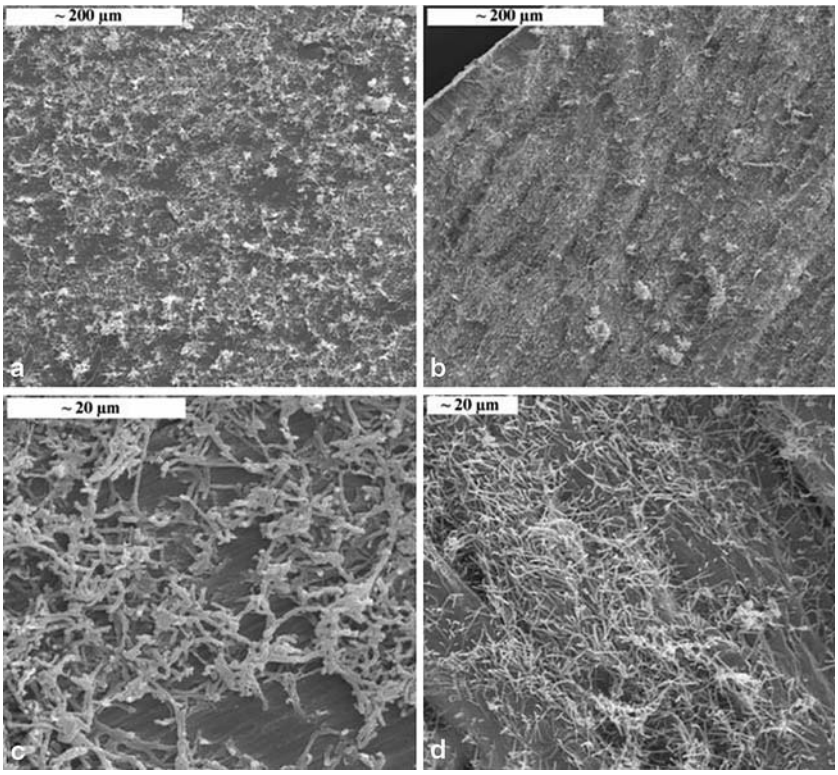

Fig. 6 Electron micrographs of titanium colonisation after different exposure times: **a** after 285 immersion-days in the smooth zone (reverse); **b** after 285 immersion-days in the roughness zone (obverse); **c, d** after 285 immersion-days in the reverse and obverse of the material

radiochemical analyses of these balls show that some radionuclides accumulated in the biofilm developed on the surface of the materials. The gamma-ray spectrometry analyses suggest that biofilm communities accumulated radionuclides from the water, although the degree of accumulation was different depending on the material (Fig. 8).

The main radionuclides accumulated in the biofilm on stainless steel were ^{60}Co, ^{65}Zn and ^{54}Mn. ^{60}Co was the most

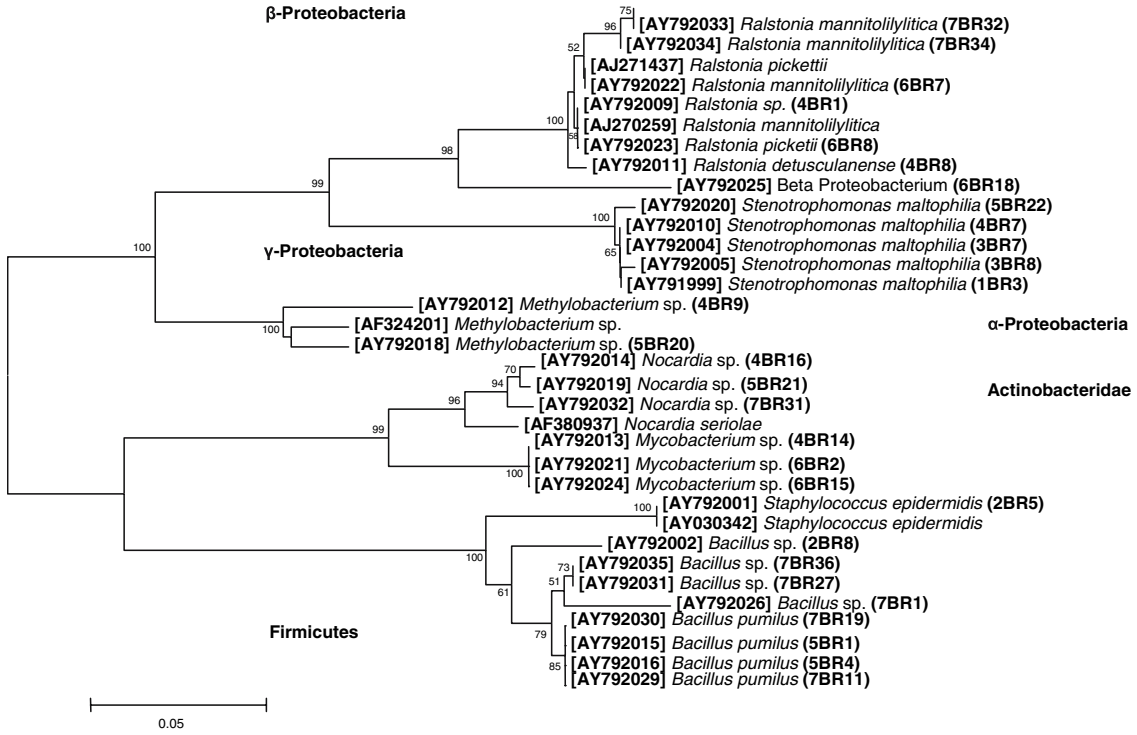

Fig. 7 Phylogenetical relationships (16 rDNA gene sequences, 462 bp) of strains isolated from the biofilms forming on stainless steel and titanium immersed during 365 and 285 days, respectively in the SNF pool's water. The distance tree was generated by the neighbor-joining method with the Jukes–Cantor model. Nodal support was assessed by using 1,000 bootstrap replicates. Only bootstrap values greater than 50% are shown

abundant radionuclide accumulated in the biofilm. When the immersion lasted from 106 up to 142 days, the radionuclides rose to 755 Bq·cm^{-2}. After this time, at 170 days, there was a slight decrease in number, but later this increased to 2,154 Bq·cm^{-2} when the immersion period extended to a maximum of 365 days. The behaviour of ^{65}Zn and of ^{54}Mn was similar, with the zinc always higher than that of manganese. The highest radionuclide-accumulation percentages in the biofilm correspond to ^{60}Co, which caused 85–90% of the total activity. The accumulation of ^{65}Zn varied between 6% and 10%, and the ^{54}Mn varied between 3–4%.

The main radionuclides accumulated in the biofilm on titanium were ^{60}Co, ^{59}Fe, ^{95}Nb, ^{65}Zn, and ^{54}Mn. As in the case of stainless steel, ^{60}Co was the most abundant with accumulation values of approximately 80%, except with two samplings where the levels decreased when other radionuclides appeared. An increase of ^{60}Co was observed from the beginning of the immersion and gradually rose to 1,075 Bq·cm^{-2} after being immersed for a maximum of 174 days. The accumulation of ^{59}Fe and ^{95}Nb were similar. The accumulation of these radionuclides was detected at 90 and 174 immersion days (25% and 13%, respectively), but was not detected in other samplings. The ^{65}Zn (2–5%) and ^{54}Mn (5–10%) were similar and showed low accumulation values in all samplings.

Discussion

This research shows that bacteria belonging to different phylogenetic groups and present in the SNF pool's water are viable and can be growth forming biofilms on stainless steel and titanium, in spite of the oligotrophic and radioactive environment. They may also retain radionuclides.

The increase of radioactivity in the biofilm of both materials (stainless steel and titanium) is mainly due to the accumulation of ^{60}Co. In stainless steel biofilms, the main radionuclides present are ^{60}Co, ^{65}Zn, and ^{54}Mn, while in those of titanium, they are ^{60}Co, ^{59}Fe and ^{95}Nb. These differences imply that there is a selective process of ^{59}Fe and ^{95}Nb accumulation in titanium biofilms at 90 and 174 days, as both radionuclides are less abundant in the water than ^{65}Zn and ^{54}Mn. On the other hand, the accumulation is quicker in titanium biofilms from the very start when compared to those of stainless steel, but in longer immersion periods, this tends to change and the radioactive accumulation on stainless steel rises.

Sequencing techniques provided measurements of the bacterial 16S rDNA gene composition present in the biofilms along time in titanium and stainless steel samples. The R2A media may give a good representation of bacteria

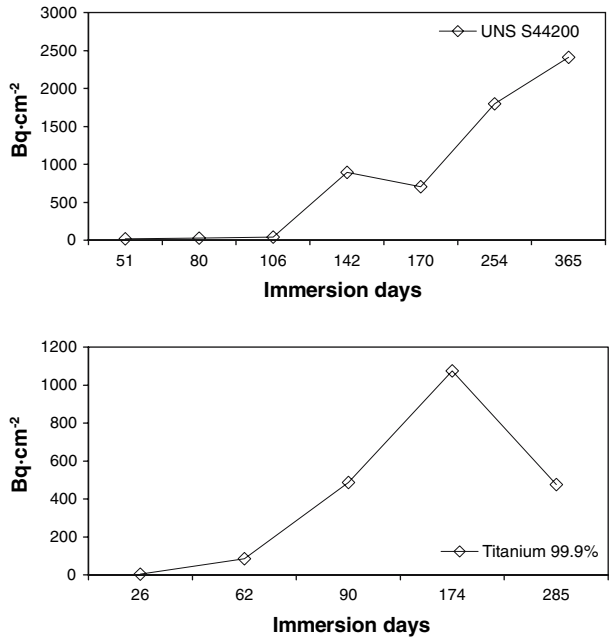

Fig. 8 Radionuclide accumulation with time in the biofilms forming on stainless steel and titanium (from August 2001 to August 2002)

present in this type of environment, in concordance with studies developed about the bacterial quality in deionised water (low TOC) [12], because a statistically greater number of bacteria was observed growing in this media. For best results R2A should be combined with TSA media, which is a good option to isolate microorganisms in this environment in accordance with the results of this study. The bacteria in the biofilms belong to five different phylogenetic groups (α, β and γ-*Proteobacteria, Actinobacteridae*, and *Firmicutes*) are present in both materials, but some species are only isolated from one of them. Among the isolated bacteria Gram-positive and Gram-negative were found. The percentages of Gram-positive and Gram-negative are similar in both materials, but there seems to be a greater number of Gram-negative bacteria in the biofilms and in the SNF pool's water [4] in contrast with other studies in radioactive environments [11]. The differences among studies could be due to the different culture conditions. Although the resistance of bacteria isolated in this research has not been tested, bacteria isolated from the same SNF pools and belonging to similar genera presented D10 values of about 1.4 kGy (data not published). Moreover, bacteria were growing in the culture media on filters with radioactivity levels of up to 2,030 Bq cm^{-2}.

The differences between both materials in the accumulation of radionuclides could be due to the composition of the micro-organisms' biofilm and the processes involved in the accumulation and to the increment of EPS in mature biofilms and other bio-products of microbial metabolism. The simple organic acids and alcohol compounds ranged in size and even become macromolecules such as polysaccharides and other components as humic and fulvic acids that can bind significant amounts of potentially toxic metals and radionuclides [6, 10].

In titanium biofilms, the high diversity of *Ralstonia* could be related to the rapid increase of radioactivity as a result of their capacity to produce polymers. This genus has been isolated previously forming biofilms in radioactive [5] and oligotrophic environments [16, 20]. Our research group had previously isolated *Burkholderia* from the water of SNF pools [5]. This genus is considered an ubiquitous genus of soil bacteria related to PAH-degrading [21], TCE-degrading [3] and herbicide degrading [13] processes. The Microbial Genomics Program, Department of Energy (DOE, USA) has classified both genera as potentially useful in removing different compounds and elements. In advanced stages of exposure, the most abundant genus on both materials is *Bacillus*, especially *Bacillus cereus*. This genus presents an impressive physiological diversity and their spores are highly resistant to unfavourable conditions: gamma radiation, UV, H_2O_2, and desiccation [11, 28]. It has also been related to the selective accumulation of Mn, Zn, Cs, U, and Co [25, 27, 28], among others. Moreover, some of these strains, and especially their spores, are able to irreversibly bind large amounts of metals [25], and the presence of spores on advanced stages of colonisation could explain why the radioactivity in the biofilm increases. Other genera predominantly present in the biofilm are *Stenotrophomonas* and *Mycobacterium.* In the former case, the genus was isolated when the biofilm was formed on stainless steel samples submerged in SNF pools [24]. In the latter case, the genus had never been previously documented in radioactive environments. In both cases, the genera have been related to the degrading processes of organic compounds [9] or hydrocarbons [2]. Other genera present on the biofilms are *Nocardia* and *Staphylococcus*. Both were isolated in the water [4] or in biofilms in these SNF pools [5, 24], as well as in other radioactive environments.

This research presents an initial characterization of biofilms in this hostile environment, and therefore indicates which species might be good candidates for future bioremediation studies. The dynamic system used for this research could be a new approach to microbial bioremediation and an alternative to the conventional organic ion-exchange based treatment in the closed-loop water system of NPPs and should be investigated in future studies. Although radioactive liquid volume reductions are achieved by trapping the metals in the ion-exchanger, the minimization of waste volume could not be carried out. This is possible when bacteria form biofilms on metallic balls, as those identified in this work. Further studies should focus on the evaluation of the efficiency of in situ bioremediation in NPPs using other culture-independent techniques to study

the complexity of the environmental microbial population and the mechanisms involved in bioremediation processes.

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