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Study of the radiation effect of $^{99}Mo/^{99m}Tc$ generator on Bacillus subtilis and Bacillus pumilus species

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HIGHLIGHTS

- Microorganisms in radionuclide generator may impair the quality of the product.
- Killing of *Bacillus pumilus* was not complete even after 20 days of exposition.
- Alumina column was a physical barrier for the microbial recovery.
- An alternative biological indicator based on *B. pumilus* spores is proposed.

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ABSTRACT

In this work, molybdenum-99 loaded columns were challenged with *Bacillus subtilis* vegetative cells and *Bacillus pumilus* spores inside and outside the alumina column, and microbial recovery and radiation effect were assessed. Alumina was a barrier for the passage of microorganisms regardless the species, whilst spores were more retained than vegetative cells with a lower microbial recovery, without significant differences between 9.25 and 74 GBq generators. *Bacillus pumilus* biological indicator showed lower recoveries, suggesting a radiation inactivating effect on microorganisms.

1. Introduction

Radionuclide generators were developed for medical applications for convenience in obtaining radionuclides of short half-life in clinics and hospitals. In principle, a parent radionuclide with a longer physical half-life generates a daughter radionuclide with a shorter half-life by radioactive decay. Provided they are different elements, the daughter radionuclide can be separated by a simple chemical process, usually by liquid elution from a chromatographic column (Zolle, 2007).

The $^{99}Mo/^{99m}Tc$ generator consists of a glass column containing alumina (Al_2O_3) and adsorbed ^{99}Mo. The parent nuclide with a 66 h physical half-life decays to ^{99m}Tc with a 6 h physical half-life. As the affinity of $^{99m}TcO_4^-$ is small, it may easily be eluted using a sterile saline solution. The eluate injection is a clear, colorless, sterile, isotonic solution that can be used in patients directly or after the preparation of ^{99m}Tc radiopharmaceuticals with different compounds (Zolle, 2007).

Due to the complexity of the assembly of $^{99}Mo/^{99m}Tc$ generator and high level of human intervention along the production, the manufacturing process can exert great influence on the microbiological contamination level in the finished product. It is essential to avoid the introduction of microbial contaminants, that may be present in raw materials, equipment and production environment. Invariably, microorganisms can be transferred to the product when there are no properly controlled conditions or the production personnel is not properly skilled and trained (Clontz, 2009).

Current Good Manufacturing Practices require that injectable radiopharmaceuticals, in this case the $^{99m}TcO_4^-$ eluate, must be sterile and prepared under conditions which minimize risks of

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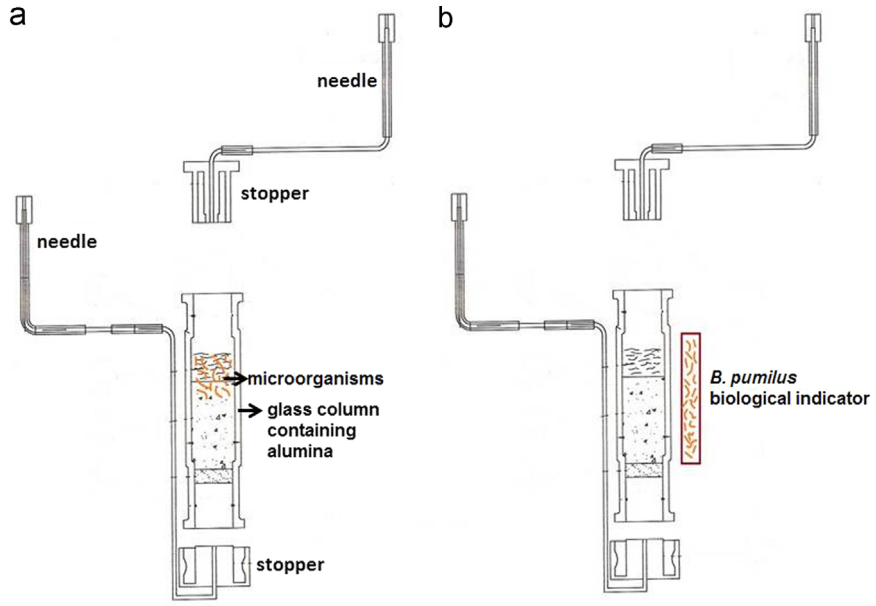

Fig. 1. Side view of alumina columns inoculated with microorganisms (a) inside and (b) outside the column.

contamination by particulate matter, pyrogens and microorganisms (EudraLex, 2008). The sterilization of generator components and ^{99}Mo solution as a raw material also play an important role in ensuring the final product to be sterile (Zolle, 2007; Hammad, 2008). The effectiveness of sterilization by the intrinsic radioactivity of radiopharmaceuticals has been barely described (Sorensen et al., 1977; Allwood and McCarthy, 1980, Petrik et al., 2012). Sorensen et al. (1977) disassembled decayed generators previously used for clinical routine, autoclaved alumina columns, infected them with different strains of microorganisms and assessed microbial recovery. As the number of microorganisms was reduced by a factor of 10^4–10^6, they found that it would be acceptable from a microbiological point of view to omit autoclaving and membrane filtration of the final product since proper aseptic techniques were applied. Later, Allwood and McCarthy (1980) recovered *Escherichia coli* and found pyrogen in the eluates of different generator columns: columns without the addition of ammonium molybdate, columns with less than 37 Bq of ^{99}Mo residual activity and columns with at least 3.7 GBq of ^{99}Mo residual activity. Low bacterial recoveries led to the following conclusions: entrapment and filtration of cells, toxicity of aluminum and molybdenum, generators are hostile environment for microorganisms but not self-sterilizing. In both studies, the activities of ^{99}Mo/^{99m}Tc generators was either low (3.7 GBq) or almost zero. Petrik et al. (2012) have studied the survival of different strains of microorganisms in non-radioactive eluates from ^{68}Ge/^{68}Ga generators and concluded that the conditions are highly unfavorable to survival or growth of microorganisms. In these works, the authors have used various bacterial and fungal species, but not spores from *Bacillus* genus. That genus comprises Gram positive bacteria that often produce spores while in harsh growth conditions (Pelczar et al., 1993), as it is the environment inside a radionuclide generator. *B. pumilus* is a known radiation resistant species whose spores were evaluated for radioresistance in Trypticase Soy Agar (TSA) plate for 10 days at 35 °C, resulting in more than 90% sporulation. The D10 value for *B. pumilus* spores was found to be 1.7–1.8 kGy (Prince, 1976). Parisi and Antoine (1975) prepared *B. pumilus* spores as biological indicators by inoculating open strips sutures with a spore suspension. They have also studied a spore concentration gradient on Whatman 1 paper, by means of biological indicators inoculated with 0.1 mL of varying amounts of *B. pumilus*.

The aim of this work was to study the effect of both the ionizing radiation from ^{99}Mo/^{99m}Tc generator and the alumina bed on *Bacillus subtilis* vegetative cells and *B. pumilus* spores, as well as to characterize the differences between the two species regarding microbial recovery. We tested the extreme values of ^{99m}Tc activity (9.25 and 74 GBq) from generators produced in Brazil.

2. Material and methods

^{99}Mo/^{99m}Tc generators were produced at the Radiopharmacy Center of the Nuclear Energy and Research Institute in São Paulo, Brazil, IPEN-CNEN/SP, BRAZIL; *B. pumilus* ATCC® 14,884 strains were purchased from Microbiologics®, USA, and *B. subtilis* NCTC® 10400 from Biomérieux®, France; the Culture media Trypticase Soy Broth (TSB), Plate Count Agar (PCA) and Trypticase Soy Agar (TSA) plate were purchased from Plastlabor®, Brazil.

All accessories used to assemble the generators including alumina columns, stoppers, hoses and filters were sterilized in the Multipurpose ^{60}Co Gamma Irradiator from the Radiation Technology Center at IPEN-CNEN/SP. For minimum (9.25 GBq) and maximum (74 GBq) activity generators, *B. pumilus* or *B. subtilis* quantified microorganisms were inoculated directly into the alumina columns (Fig. 1a) before loading with different activities of ^{99}Mo. For *B. pumilus* spores, a biological indicator was positioned in the outer region of the column, but inside the generator lead shielding (Fig. 1b). In each case, the microbial recovery was evaluated.

2.1. Preparation and quantification of B. subtilis suspension and B. pumilus spores

A *B. subtilis* suspension containing 10^5–10^6 colony forming units (CFU) per mL was prepared by transferring a small portion of a *B. subtilis* NCTC 10400 mother culture with a bacteriological loop to a TSA plate to be incubated at 32 °C for 24 h. The microorganisms were extracted with two 4 mL washes of 0.9% sodium chloride sterile solution. Serial dilutions of 10^{-2}–10^{-6} were performed and 1 mL of each dilution was dispensed in Petri dishes in duplicate and then 15–20 mL of Plate Count Agar medium were added for quantification by pour plate. After 3 days of incubation

at 32 °C, the CFU were counted.

A *B. pumilus* spore suspension containing 10^8–10^9 CFU mL^{-1} was prepared by transferring a small portion of a *B. pumilus* ATCC 14844 mother culture with a bacteriological loop to a TSA plate to be incubated at 37 °C for 10–12 days. The bacterial content was extracted from the plate with three washes of 10 mL sterile water using a glass rod and transferred into a 15 mL Falcon tube. After each wash and transfer the material was centrifuged at 1500 g for 20 min at 8 °C and the supernatant was discarded. The formed pellet was subjected to thermal shock in a water bath at 70 °C for 15 min and cooled immediately in an ice bath. 10 mL of sterile water were added to the Falcon tube containing the pellet, which was then subjected to vortexing. Serial dilutions of 10^{-5}–10^{-10} were performed and 1 mL of each dilution was dispensed in Petri dishes in duplicate for quantification by pour plate.

A count range of 30-300 CFU per plate was considered for the quantification calculation (Norrell and Messley, 1997).

The standard curve to quantify the microorganisms was constructed using a spectrophotometer by measuring absorbance at 580 nm of successive two-fold dilutions from the undiluted suspension, i.e., 1:2; 1:4; 1:8; 1:16; 1:32; 1:64; 1:128. For *B. pumilus*, Wirtz–Conklin staining was performed to verify the percentage of sporulation.

2.2. Column assembly with ^{99}Mo (nominal activity of 9.25 GBq and 74 GBq of ^{99m}Tc) and addition of microorganisms

For *B. subtilis* and *B. pumilus*, a generator system was set up separately without the sterilizing filter in order to evaluate the recovery of microorganisms. Suspension of *B. subtilis* vegetative cells or *B. pumilus* spores in the range 10^5–10^8 CFU mL^{-1} were inoculated into an alumina column separately, followed by loading with about 9.25 GBq or 74 GBq of $^{99}MoO_4^{-2}$. For each experiment, one column was prepared without radioactive material and named as control. The *B. subtilis* and *B. pumilus* suspension concentrations were obtained by interpolating the absorbance value in the calibration curves.

Elutions were performed daily for five days with 6 mL of 0.9% sodium chloride sterile solution. On the fifth day, immediately after the daily elution, ten more successive elutions were performed; from each vial, one milliliter was taken and pooled into a single vial (this pool was named as 'exhaustive eluate'). Samples of the single eluates of the generators, obtained during the 5-days elution, and the exhaustive eluate were diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. One milliliter of the undiluted sample and each subsequent dilution were dispensed in Petri dishes in duplicate. Then 15–20 mL of Plate Count Agar medium were added for quantification by pour plate. After 3 days of incubation at 32 °C, the number of colonies was manually counted.

Recovery was calculated considering the average count of duplicate (in the 30–300 CFU working range) multiplied by the dilution factor and the elution volume (6 mL for daily eluates and 60 mL for the exhaustive eluate), as follows:

% Recovery = {[total CFU (five days + exhaustive elution)] × 100}/number of UFC in the inoculum.

2.3. Preparation of B. pumilus biological indicator

A 4.7 × 0.5 cm^2 aluminum silica gel strip, commercially known as TLC-SG (Thin Layer Chromatography–Silica Gel) was divided in four segments of 1 cm each and used as support for the biological indicator (Fig. 2).

A suspension of *B. pumilus* spores of approximately 10^8–10^9 CFU mL^{-1} was subjected to centrifugation at 1500 g for 20 min at 8 °C. The supernatant was discarded and 1 mL of sterile 70% alcohol was added to the pellet. The mixture was homogenized and 10 μL of the suspension was dispensed on each segment of the biological indicator strip. After five days in a desiccator, the strip was cut out and each segment immersed in 1 mL 0.9% sodium chloride sterile solution. The silica gel was scraped with the aid of a bacteriological loop to extract the microorganisms and 1 mL of the undiluted suspension and 10^{-4} dilution were pipetted in Petri dishes in duplicate and then 15–20 mL of Plate Count Agar medium were added for quantification by pour plate. After 3 days of incubation at 32 °C, the CFU were counted. The CFU of each segment was expressed considering the 10^{-4} and 10^{-5} dilutions and the mean count (CFU mL^{-1}) was used to quantify and calculate the microbial recovery.

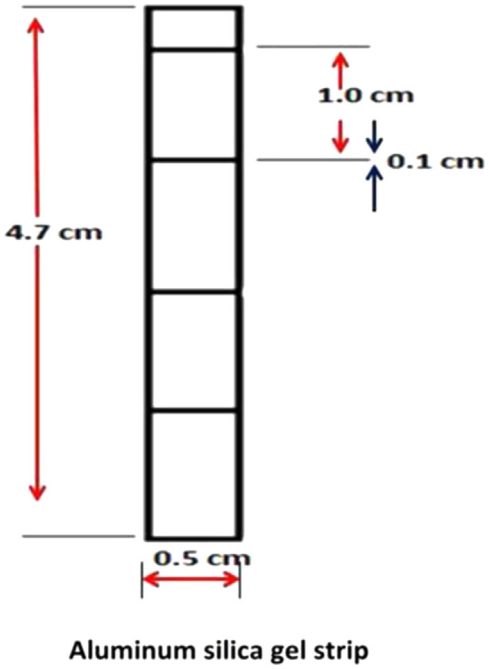

Fig. 2. Scheme of the *B. pumilus* biological indicator.

2.4. Assembly of 9.25 GBq and 74 GBq ^{99}Mo/^{99m}Tc generators with B. pumilus biological indicator

Two *B. pumilus* spore indicators with approximately 10^8–10^9 CFU mL^{-1} were prepared and wrapped with parchment paper, one for control and the other for the 9.25 GBq loaded generator (Fig. 1b). The same procedure was used for a 74 GBq loaded generator. Daily elutions with 6 mL of a 0.9% sodium chloride sterile solution were performed for five days in the loaded generator and on the 6th day both biological indicators were removed and unwrapped. Each segment was cut and extracted with 1 mL of 0.9% sodium chloride sterile solution. The 10^{-5} dilution was used to calculate the microbial recovery.

2.5. Dosimetric calculations

Dosimetric calculations were performed for 9.25 and 74 GBq generators simulating the worst condition-that is, the point of the highest concentration of ^{99}Mo and ^{99m}Tc at the center of the upper region of the alumina column, prior to the elution of the generators. Similar calculations were made considering microorganisms at the border of the segment bearing the highest radioactive concentration and also at the bottom of the alumina column.

3. Results and discussion

In this work, we propose quantification by spectrophotometry and pour plate technique of microorganisms in $^{99}Mo/^{99m}Tc$ generator eluates, and suggest a simple calculation for recovery of microorganisms from generator columns. For low microbial charges, the pour plate technique is adequate as it allows bacterial growth both on the surface and within the agar plate. The raw counts were described in the tables as observed in the plates, although only counts inside the working range were used to estimate the bacterial concentration. The recovery percentage expressed the proportion of microorganisms present in the eluates to those present in the initial inocula.

3.1. Quantification of *B. subtilis* and *B. pumilus* suspensions

The bacterial concentration estimated from 10^{-4}–10^{-6} serial dilutions of *B. subtilis* suspension is shown in Table 1. Dilutions of 10^{-2}–10^{-4} and 10^{-6} presented counts outside the working range and were not considered. According to Norrell and Messley (1997), the reason for counting plates that have between 30 and 300 colonies is that the most representative samples are obtained when the number of colonies falls within that range. Less than 30 colonies is not considered a representative sample (too few organisms). More than 300 colonies are similarly considered non-representative because the overcrowding of the colonies will result in poor growth of the bacteria, and some colonies may not have grown to visible size.

Table 2 shows the bacterial concentration estimated from *B. pumilus* serial dilutions.

The undiluted spore suspension yielded more than 90% of sporulation by Wirtz–Conklin staining. Successive twofold dilutions of the initial *B. subtilis* vegetative cells and *B. pumilus* spore concentrations and their respective absorbances at 580 nm were used to obtain the standard curves (Fig. 3). The inserted graph represents the linear range for both microorganisms.

In the linear range, the Equations to determine the concentration of *B. subtilis* and *B. pumilus* by spectrophotometric method were, respectively:

$$A = 1.0 \times 10^{-8}[B.\ subtilis] + 0.035\left(\text{range: } 7.7 \times 10^{4} \text{ to } 9.8 \times 10^{6} \text{ CFU mL}^{-1}\right) \quad (1)$$

$$A = 5.0 \times 10^{-9}[B.\ pumilus] + 0.030(\text{range} : 1.0 \times 10^{7} \text{ to } 1.5 \times 10^{8} \text{ CFU mL}^{-1}) \quad (2)$$

where A is the absorbance and $[\times]$ is the estimated bacterial concentration.

B. pumilus spores have a standard curve that is linear in a higher concentration than *B. subtilis* but in a reduced range. *B. subtilis* standard curve showed a very different profile from *B. pumilus* curve, with bacterial count in the order of 10^6 and spanning a longer linear range wherein all the points were considered in the construction of the straight line; at the same time, the linear range for the *B. pumilus* spores was narrower. It should be stressed that the photometric method usually measures mass and not the number of cells, and it allows accurate measurement of cell number only when the cell size is uniform (Norrell and Messley, 1997). The different colonies morphologies between species may also have contributed to the differences in the accuracy of the counts, because while *B. subtilis* has well-defined colonies, *B. pumilus* colonies present varying sizes-some with undefined edges and more extensive surface, which may cover up surrounding colonies and make quantification tough.

Table 1
Microbial counts per plate and estimation of the bacterial concentration of undiluted *B. subtilis*.

Dilution	Raw count (CFU)	Mean count (CFU)	Bacterial concentration (CFU mL^{-1})
10^{-4}	2636 2216	NA	NA
10^{-5}	96 100	98	98×10^5
10^{-6}	23 14	NA	NA
Mean concentration:			9.8×10^6 CFU mL^{-1}

CFU: colony-forming unit; NA: not applicable (outside the working range – from 30 to 300 CFU)

Table 2
Microbial counts per plate and estimation of the bacterial concentration of undiluted *B. pumilus*.

Dilution	Raw count (CFU)	Mean Count (CFU)	Bacterial concentration (CFU mL^{-1})
10^{-6}	1040 1048	NA	NA
10^{-7}	125 123	124	124×10^7
10^{-8}	15 14	NA	NA
10^{-9}	1 2	NA	NA
10^{-10}	No growth No growth	NA	NA
Mean concentration			1.2×10^9 CFU mL^{-1}

CFU: colony-forming unit; NA: not applicable (outside the working range – from 30 to 300 CFU)

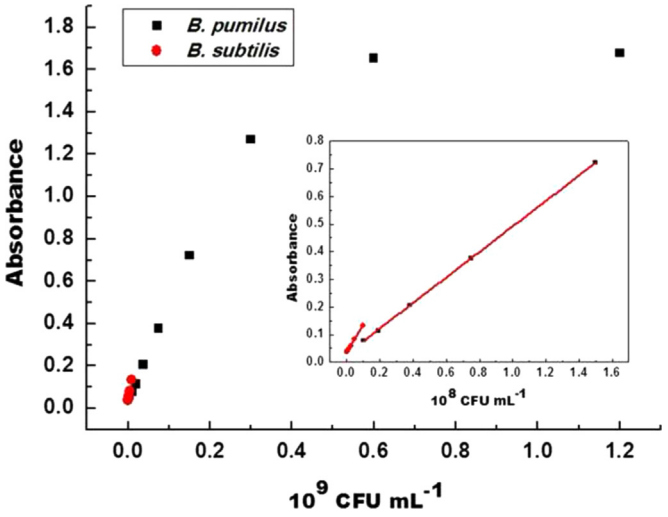

Fig. 3. Standard curves for determination of *B. subtilis* and *B. pumilus* concentrations.

3.2. Column assembly with ^{99}Mo and microorganisms-microbial recovery in $^{99}Mo/^{99m}Tc$-generator eluates

From a *B. subtilis* mother suspension a 1:8 dilution was made and absorbance was read at 580 nm to determine the concentration using Eq. (1) and correcting for dilution; the bacterial concentration in the mother suspension was 7.2×10^6 CFU mL^{-1}. 500 microliters of the mother suspension (equivalent to 3.6×10^6 CFU) were then applied in both a control generator (not radioactive) and a 9.25 GBq loaded generator. Table 3 shows the recovery of *B.*

Table 3
Microbial counts per plate and estimation of the bacterial concentration of *B. subtilis* for control and 9.25 GBq generator eluates.

Elution (day)	Control generator[a] Dilution										9.25 GBq generator[a] Dilution									
	Undil[b]		10^{-1}		10^{-2}		10^{-3}		10^{-4}		Undil[b]		10^{-1}		10^{-2}		10^{-3}		10^{-4}	
1	14	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
5	70	60	8	7	3	1	1	0	0	0	4	6	0	0	0	0	0	0	0	0
5 (Exhaustive)	49	18	56	1	2	0	0	0	1	0	20	2	0	0	50	10	69	51	20	0
Total CFU	2.2×10^3										1.9×10^5									

[a] The amount of *B. subtilis* inoculated into the columns was 3.6×10^6 CFU.
[b] Undil: eluate without dilution. The counts below 30 CFU were not considered; all the plate counts were expressed in Colony-Forming Units (CFU).

subtilis in both cases, during five days of elution and ten successive elutions at the fifth day ('exhaustive' elution).

After 20 days, the control column was immersed in 200 mL of phosphate buffer pH 7.2 and an aliquot was submitted to membrane filtration; the membrane was incubated in TSA plate for seven days at 32 °C. No bacterial growth was observed. The procedure was not performed with the column loaded with ^{99}Mo because of the high residual activity.

Table 3 shows that even for the undiluted sample, the CFU counts were higher in almost all eluates from the control generator than the 9.25 GBq loaded generator. A higher amount of microorganisms was found in the fifth and in the exhaustive eluates-indicating that it is necessary a greater volume of elution to extract the microorganisms from the column, since the alumina column should work as a physical barrier. As the initial amount of *B. subtilis* into the column was 3.6×10^6 CFU, the recoveries were 0.06% and 5.4% for the control and 9.25 GBq generators, respectively. As it was expected to observe lower recovery in the 9.25 GBq loaded generator, variations in the results may be attributed to preparation and quantification of inoculum, dilution of samples including pipetting and homogenization, CFU counts and operator's experience and skill, no automated process for preparing alumina column subjected to varying conditions in material handling and differences in the compression of alumina column.

Table 4 and Table 5 show the recoveries of *B. pumilus* during five daily elutions and 'exhaustive' elution of a control generator and 9.25 and 74 GBq generators inoculated with a spore suspension containing 5.4×10^7 CFU. The procedure of quantification and contamination of column was similar to that executed with *B. subtilis*.

Table 4 and Table 5 showed that six milliliters in daily elutions during five days were not enough to extract the spores of *B. pumilus*, probably because of the alumina physical barrier and loss of spore viability. Differently from *B. subtilis*, the spores were more retained in the column and even the exhaustive elution with 60 mL was not sufficient to extract a considerable amount of microorganisms. Even though the initial concentration of *B. pumilus* was 15 times higher than the concentration of *B. subtilis*, the recoveries of the spores for the control and 9.25 and 74 GBq generators were very low, below the counting range.

After 20 days, both 9.25 and 74 GBq control columns were immersed separately in 200 mL of phosphate buffer pH 7.2 and aliquots were submitted to membrane filtration; the membranes were incubated in TSA plate for seven days at 32 °C. The microbial recovery for the 9.25 GBq control column was 147 CFU and for the 74 GBq control column was more than 300 CFU (outside the working range) showing that a significant fraction of *B. pumilus* remained in the column. According to Bower et al. (1996), spores generally bind to surfaces at a higher rate than vegetative cells due to the relatively high hydrophobicity of spores and the presence of some structures, which cover the surface of the spore walls.

The recovery results confirmed the findings of Sorensen et al. and Allwood and McCarthy with respect to the alumina column which actually constituted a barrier to the passage of microorganisms, especially spores, possibly because of the entrapment of the bacterial cells in the alumina bed with or without radioactivity.

The eluates were analysed for bacterial endotoxins using the *in vitro* LAL test which detects lipopolysaccharides from the outer cell membrane of Gram negative bacteria. Negative results were obtained as expected because *B. pumilus* and *B. subtilis* are Gram positive species.

3.3. Microbial recovery of B. pumilus from biological indicators

The microbial counts for the biological indicator (CFU mL^{-1}) are related in Table 6.

Table 4
Microbial counts per plate and estimation of the bacterial concentration of *B. pumilus* for control and 9.25 GBq generator eluates.

Elution (day)	Control generator[a] Dilution										9.25 GBq generator[a] Dilution									
	Undil[b]		10^{-1}		10^{-2}		10^{-3}		10^{-4}		Undil[b]		10^{-1}		10^{-2}		10^{-3}		10^{-4}	
1	0	1	0	0	0	2	0	0	0	0	0	0	2	0	1	0	0	0	0	0
2	31	1	0	0	2	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0
3	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5 Exhaustive	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total CFU	31										NA									

[a] The amount of *B. pumilus* inoculated into the columns was 5.4×10^7 CFU
[b] Undil: eluate without dilution. The counts below 30 CFU were not considered; NA: not applicable; all the plate counts were expressed in Colony-Forming Units (CFU).

Table 5
Microbial counts per plate and estimation of the bacterial concentration of *B. pumilus* for control and 74 GBq generator eluates.

Elution (day)	Control generator[a] Dilution										74 GBq generator[a] Dilution									
	Undil[b]		10^{-1}		10^{-2}		10^{-3}		10^{-4}		Undil[b]		10^{-1}		10^{-2}		10^{-3}		10^{-4}	
1	0	0	0	7	0	0	1	11	2	4	0	22	0	0	0	0	0	0	0	1
2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	3	0	0
3	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
4	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
5 Exhaustive	7	12	0	3	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Total CFU	NA										NA									

[a] The amount of *B. pumilus* inoculated into the columns was 5.4×10^7 CFU
[b] Undil: eluate without dilution. The counts below 30 CFU were not considered; NA: not applicable; all the plate counts were expressed in Colony-Forming Units (CFU).

Table 6
Microbial counts per plate and estimation of the bacterial concentration of *B. pumilus* along the segments of a biological indicator. Each segment received 10 μL of a 1.6×10^9 CFU mL^{-1} suspension of *B. pumilus*.

Segment[a]	Undiluted	10^{-5} Dilution	Mean CFU
1	NA	53	7.2×10^6
2	NA	95	
3	NA	78	
4	NA	62	

[a] The amount of *B. pumilus* inoculated in each segment was 1.6×10^7 CFU; NA: not applicable (above 300 CFU); all the plate counts were expressed in Colony-Forming Units (CFU).

The recovery of 45% indicated that silica gel surface of TLC strip was less aggressive to the microorganisms than the alumina column.

3.4. Assembly of 9.25 GBq and 74 GBq generators with B. pumilus-based biological indicator

Table 7 and Table 8 show the microbial recovery of *B. pumilus* from biological indicators exposed to 9.25 GBq and 74 GBq generators, respectively.

The counts in the segments showed no significant differences among them. The biological indicator of the control column had significantly higher CFU counts (recovery of 44–300%) than the column with radioactive material (the recoveries were 25% and 5.7% for 9.25 GBq and 74 GBq generators, respectively, considering the inoculated quantity). This experiment with biological indicator minimized the interference of the alumina column although the effect of silica gel on the microorganisms should be considered.

The differences among the recoveries in the experiments with microorganisms inoculated into *e* outside the column with ^{99}Mo can also be explained by the displacement of ^{99}Mo and ^{99m}Tc along the alumina column by daily elutions causing irradiation of microorganisms and suggesting a probable microbial inactivation.

Table 7
Microbial counts per plate and estimation of the bacterial concentration of *B. pumilus* along segments of a biological indicator exposed to a 9.25 GBq generator.

Segment[a]	Control generator Dilution		9.25 GBq generator Dilution	
	10^{-4}	10^{-5}	10^{-4}	10^{-5}
1	1068	52	314	39
2	306	205	351	31
3	456	87	329	42
4	744	137	610	58
CFU	NA	4.8×10^7	NA	4.2×10^6

[a] The amount of *B. pumilus* inoculated in each segment was 1.6×10^7 CFU; NA: not applicable (all counts per segment exceeded 300 CFU); all the plate counts were expressed in Colony-Forming Units (CFU).

Table 8
Microbial counts per plate and estimation of the bacterial concentration of *B. pumilus* along segments of a biological indicator exposed to a 74 GBq generator.

Segment[a]	Control generator Dilution		74 GBq generator Dilution	
	10^{-4}	10^{-5}	10^{-4}	10^{-5}
1	652	91	98	9
2	382	88	84	6
3	428	51	89	5
4	327	53	91	12
CFU	NA	7.1×10^6	9.1×10^5	NA

[a] The amount of *B. pumilus* inoculated in each segment was 1.6×10^7 CFU; NA: not applicable (all counts per segment were outside the working range of 30-300 CFU); all the plate counts were expressed in Colony-Forming Units (CFU).

3.5. Dosimetric calculations

The total absorbed dose calculated to the point of the highest ^{99}Mo and ^{99m}Tc radiation exposure at the center of the upper region of the alumina column, prior to the elution of the generators, was 78.9 kGy and 631.1 kGy for 9.25 and 74 GBq generators, respectively, indicating very high doses indeed. Dosimetric calculations at the border of the segment bearing the highest radioactive concentration and also at the bottom of the alumina column showed the values of 3728.3 and 193.3 Gy for 9.25 GBq, and 29,828.2 and 1546.1 Gy for 74 GBq generators respectively.

The absorbed dose for a microorganism in the region next to ^{99}Mo concentration area is significantly higher than at the bottom of the column. The greater the distance from the radioactive source, the lower the absorbed dose and, therefore, the radiation exposure of a microorganism is lower, increasing the survival chances.

4. Conclusion

For all ^{99}Mo/^{99m}Tc and control generators studied, though the number of generators used was limited, alumina column decreased the microbial recovery in the eluate, working as a physical barrier. During five days of elution more *B. subtilis* were recovered than *B. pumilus*. We conclude that alumina behaved like a barrier for the passage of microorganisms regardless the species, whilst spores were more retained than vegetative cells with a lower microbial recovery without significant differences between 9.25 or

74 GBq generators. Despite the $^{99}Mo/^{99m}Tc$ generator column being hostile environment as postulated by Allwood and McCarthy (1980), killing of *Bacillus pumilus* was not complete even after 20 days of exposition as significant amounts were recovered for 9.25 and 74 GBq generators. *B. pumilus* biological indicator positioned outside the column showed low recovery of microorganisms after extraction, suggesting a partial radiation inactivating effect on microorganisms.

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