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ORIGINAL PAPER

Influence of americium-241 on the microbial population and biodegradation of organic waste

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Abstract The present study investigated the influence of ^{241}Am on microbial growth and the degradation of organic waste. Leachate samples collected in a lysimeter were periodically analyzed for bacterial growth, under both aerobic and anaerobic conditions. ^{241}Am inhibited bacterial growth, and the degradation of organic matter was delayed in comparison with the control. Minimal inhibitory concentration assays and survival curves revealed that it inhibits the growth of *Pseudomonas putida* F1. The assay also revealed that ^{241}Am is more toxic than ^{238}U, Zn^{2+} and Cd^{2+}. This study further led to the finding of four new radionuclide-tolerant bacterial strains: *Flavobacterium* spp., *Pseudomonas gladioli, Chryseobacterium indologenes and Ochrobactrum anthropi.* The survival curves of *P. gladioli, C. indologenes* revealed that these bacteria are resistant to metal as consortia.

Keywords Americium · Lightning rod · Organic waste · Bacteria · Degradation

Introduction

In 1989, the Brazilian National Authority suspended the licensing for use of radioactive sources in lightning rods manufactured in Brazil. However, only 20% of the estimated total number of installed rods was delivered to the Brazilian Nuclear Energy Commission. An assessment of the risk of contamination by ^{241}Am used in lightning rods discarded as domestic waste was recently published (Marumo et al. 2008). In that study, the radionuclide was placed in a lysimeter filled with organic waste from a restaurant. The leachate was periodically analyzed to determine environmental parameters such as pH, redox potential, solid content and concentration of radioactive material (Marumo et al. 2008). As the disposal of food waste mixed with general waste in open garbage dumps is still common in some parts of Brazil, this kind of waste was used in the present study.

It is also important to understand the mechanism of ^{241}Am release or retention in waste and its influence on decomposition processes involving microorganisms. Under appropriate conditions, a microorganism can convert the insoluble form of actinide into a soluble form of stable complexes with organic acids or chelates (Francis 1990). The metal can also be removed through sorption onto the cell surfaces of these microorganisms (Takenaka et al. 2007). In bioremediation processes, it is well known that toxicity from heavy metals can affect the biota (Mukherjee and Nuorteva 1994), inhibiting microbial growth (Bewley 1980) and affecting the performance of the microorganism as well as the biodegradation velocity.

Recently, Ruggiero et al. (2005) reported that the actinides Pu(IV), U(IV) and Np(V) are less toxic than most other metals (Cd, Pb, Co, Cr, Zn, Cu, As and Ni), indicating that growth inhibition is primarily chemical, not radiological. In order to understand all the processes involved in radionuclide removal, the impact of the heavy metal on the microorganism needs to be elucidated.

The aim of the present study was to investigate the influence of ^{241}Am on a microbial population in lysimeter leachate samples. Additional experiments on minimal

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inhibitory concentration (MIC) and survival curves, based on the microbial response to different soluble concentrations of americium (^{241}Am), uranium (^{238}U), zinc and cadmium, were also carried out, using consortia from the leachate samples and F1 strains of *Pseudomonas putida,* a well-known highly metal-tolerant bacterium (Hu and Zhao 2007).

Materials and methods

Sampling procedure

Three small-scale lysimeters (Lys 5, Lys 6 and Lys 8) and one control were assembled in tubes with a 10 cm diameter and a 50 cm length. Organic waste collected from a restaurant located at the Nuclear and Energy Research Institute (IPEN – CNEN/SP) was carefully transferred to the lysimeters in such a way as to prevent the occurrence of void spaces. Three ^{241}Am sources were placed in the middle of each tube (except the control) and covered with more waste. The americium sources consisted of a steel base with a fixed layer of AmO_2 and gold coating. The initial inventories of ^{241}Am were 19.1 (0.62), 18.0 (0.58) and 19.2 (0.63) MBq (10^{-6} mol), for Lys 5, Lys 6 and Lys 8, respectively. Distilled water was used as leachant, and the irrigation rate was based on the pluviometric data for the city of São Paulo (Brazil; Instituto de Pesquisas Espaciais 2006). Considering the surface area of the lysimeters, the required volume of leachant to be added was 30 ml per day.

Leachate samples were periodically collected in 220-ml flasks, to determine americium concentration and microbial growth. ^{241}Am concentration was determined by direct measurements using an HPGe detector (Canberra, model GX2518), and the results were expressed in Becquerels per milliliter (Bq ml^{-1}).

Microbial growth assay

Leachate samples generated in lysimeters were periodically analyzed for microbial growth from the 89th to the 370th day, by performing bacterial plate count. Microbial growth assay started at 89th day of the experiment when the lysimeters started to become well behaved in terms of color, pH and volume of the leachant.

Samples were collected in polyethylene flasks, and 5 ml of aliquots were transferred aseptically to tubes containing 45 ml of buffer solution [KH_2PO_4 0.25 M and $MgCl_2$ 0.4 M (pH = 7)] for serial dilution. Four agar count plates were prepared for each dilution—two for bacterial growth under aerobic and another two for growth under anaerobic conditions. The plates were incubated for 48 h at 37°C. Anaerobiosis was achieved with jars and CO_2 generators. Only sterile materials were used in this procedure.

Minimal inhibitory concentration (MIC)

Minimal inhibitory concentration was determined by using the broth-dilution method, according to the National Committee for Clinical Laboratories Standards (NCCLS 2003). The inocula were previously prepared in Luria broth with leachate samples collected from lysimeters on the 370th day and agitated (120 rpm) at 37°C for 24 h. MICs ranged from 0.005 (145) to 0.15 (4,640) μM (Bq ml^{-1}) $AmCl_3$, 0.50 (1.490) to 5.0 (14.898) mM (Bq ml^{-1}) $UO_2(NO_3)_2{\cdot}5H_2O$, 0.078 mM to 5.0 mM $CdCl_2$ and 0.078 mM to 5.0 mM $ZnSO_4{\cdot}7H_2O$ in mineral salt medium (Tris medium), respectively (Mergeay et al. 1985). Samples were analyzed after being kept for 3 days at 37°C. Cell concentration in the suspension was estimated by optical density at 600 nm. The lowest concentration of metal salts at which no colony-forming units were observed was considered to be the MIC. *Pseudomonas putida* F1 was analyzed under the same conditions.

Antimicrobial resistance assay

A radionuclide source consisting of a steel base and americium strips was placed aseptically on Tris medium agar containing leachate samples from the control lysimeter and kept for 48 h; the active face of the source was kept in contact with the agar surface. A metal base of the source without ^{241}Am was used as control.

Survival curves

Survival curves of *Pseudomonas putida*, *Pseudomonas gladioli* and consortia, from leachate samples generated in lysimeters on the 370th day, were obtained for the cations ^{241}Am, ^{238}U, Zn^{2+} and Cd^{2+} by counting the number of viable cells. Cells were cultivated in different cation concentrations for 24 h, and 1 ml aliquots from each solution was transferred aseptically to tubes containing 9 ml of buffer solution [KH_2PO_4 0.25 M and $MgCl_2$ 0.4 M (pH = 7)]. Serial dilutions (between 10^{-1} and 10^{-8}) were prepared and used to determine viable counts using plate-count agar (PCA). The plates were incubated at 35°C for 48 h, and colony-forming units (CFU ml^{-1}) were counted. All experiments were run in triplicate of each lysimeter. The average number of surviving viable cells in the samples was obtained and the survival rates were calculated.

Statistical

Linear regression was used to fit the experimental data showed in Figs. 5, 7 and 8. The points of the survival curves for initial bacterial count were omitted from the statistical calculation.

Bacteria identification

During this study, four bacterial strains were isolated form the Luria broth liquid medium and 0.11 μM of americium solution. They were identified at the Microbiology Laboratory of the Federal University of São Paulo (UNIFESP). Subcultures of the isolates were established on 5% sheep blood agar, incubated in a 5% CO_2 atmosphere for 24 h at 35°C, and then analyzed using a Phoenix automated Microbiology system (BD Diagnostics, USA).

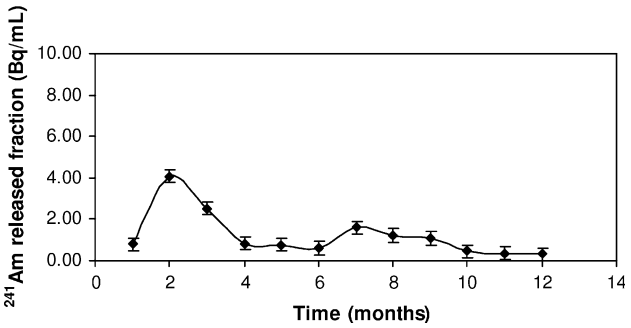


Fig. 1 ^{241}Am released fraction in function of time

Results and discussion

As reported previously (Marumo et al. 2008), the release of ^{241}Am was not constant, the initial amount of americium being relatively large, followed by a more constant long-term release. Figure 1 shows the average values obtained from the three lysimeters. Total solid content decreased over time, ranging from 4.5 to 0% within 300 days. The pH increased for 8 months in all lysimeters, reaching a maximum of approximately 7.5 during the 10th month. Samples collected from the control lysimeter exhibited a faster pH raise.

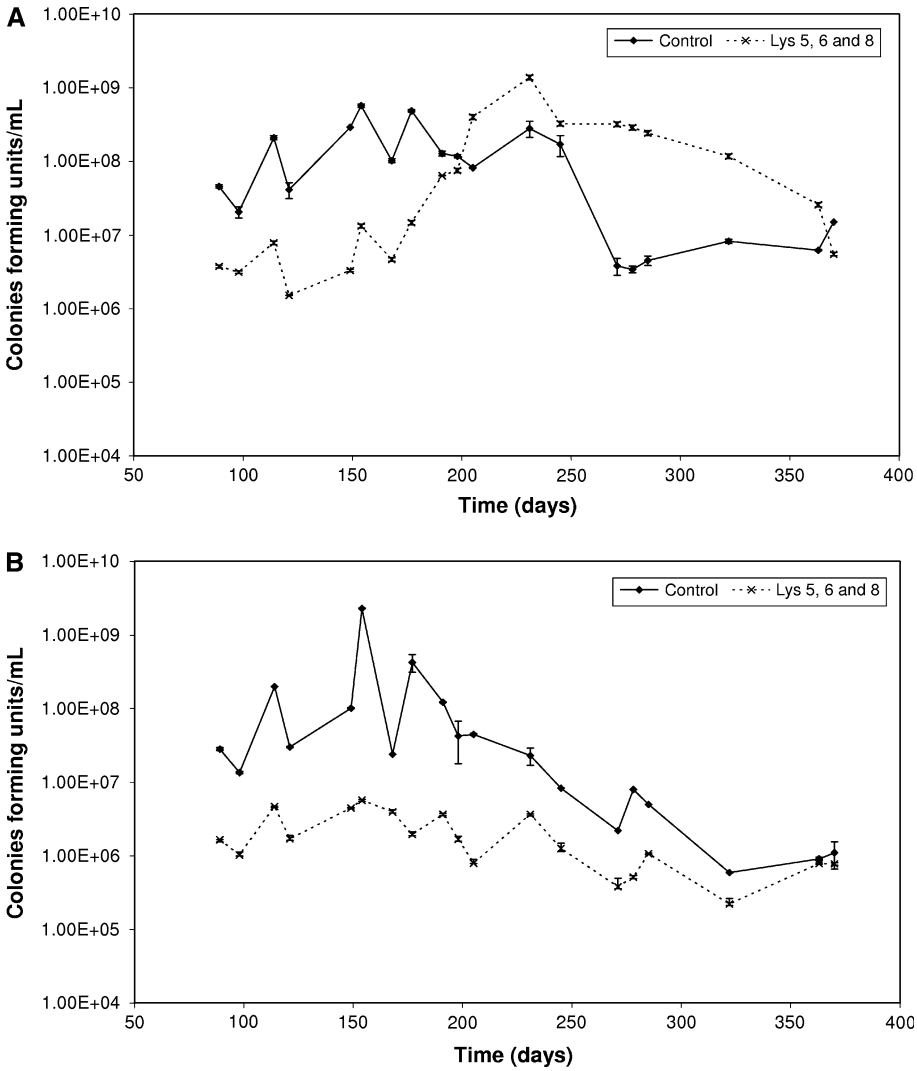


Fig. 2 Bacterial growth under aerobic (**a**) and anaerobic (**b**) conditions and their respective controls

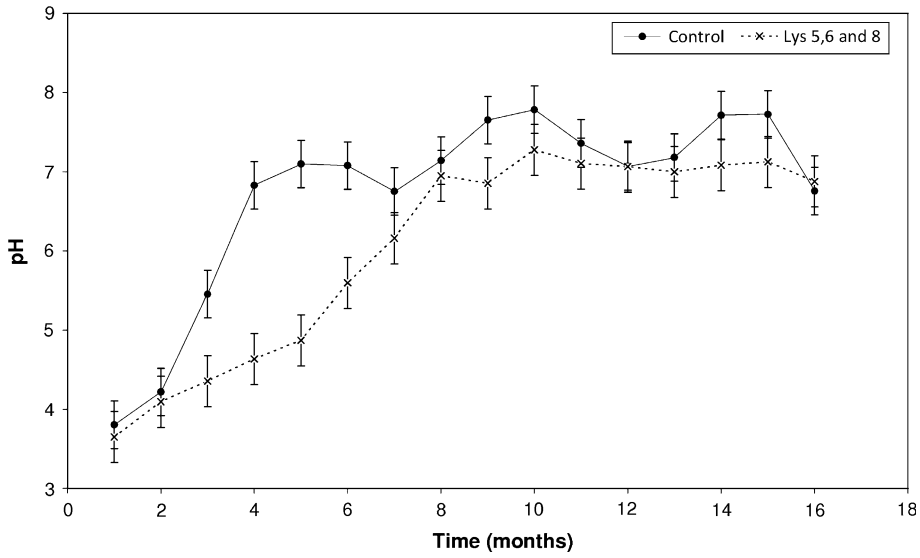


Fig. 3 Lysimeter leachate pH as a function of time

Figure 2 shows the analysis of the influence exerted by ^{241}Am on the bacterial cell count in the lysimeter. Each point represents the average count of three lysimeters.

Cell counts for bacteria grown under both conditions were compared. In the control sample, the microbial population decreased considerably on ca day 250. Under anaerobic conditions, the population remained small until the end of the experiment, whereas under aerobic conditions there was a delay in bacterial growth, and a tendency toward a decreasing cell count was observed from the 250th day onwards. The decrease in the bacterial count observed generally may be attributed to the reduction of substrate concentration.

The bacterial count increased in the acid phase, and began to decrease slowly at pH 7. Months later, still at the same pH, the bacterial cell counts were lower. This behavior occurred faster in the control when compared to the medium with ^{241}Am (Fig. 3).

According to O'Leary and Tchobanoglous (2002), biodegradation can be evaluated by the pH values of organic waste. The acid phase involves the degradation of the organic compounds into simple acid compounds, such as acetic acid and more complex organic acids. This is followed by the conversion of these compounds into CH_4 or CO_2 by anaerobic and aerobic bacteria, respectively. As a result, the pH in the solid content rises to neutral values. In the presence of organic acids, AmO_2 may be converted to its soluble form by complexation.

For further experiments, aerobic conditions were chosen, in view of the higher bacterial counts observed in comparison with anaerobic conditions (Fig. 4).

Aerobic growth was compared with the amount of americium released during the experiments and a linear regression curve was fitted, as shown in Fig. 5. The CFU data were standardized by the average amount of ^{241}Am released by the lysimeters in each period of time.

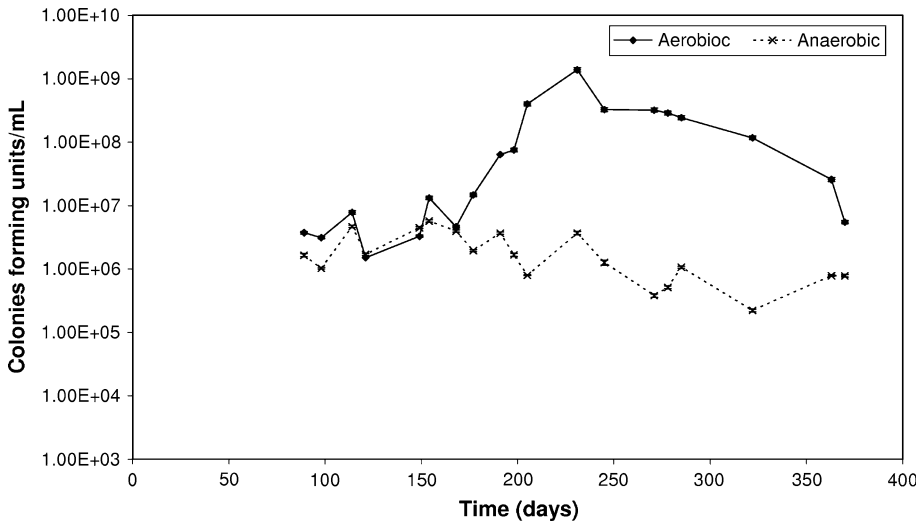
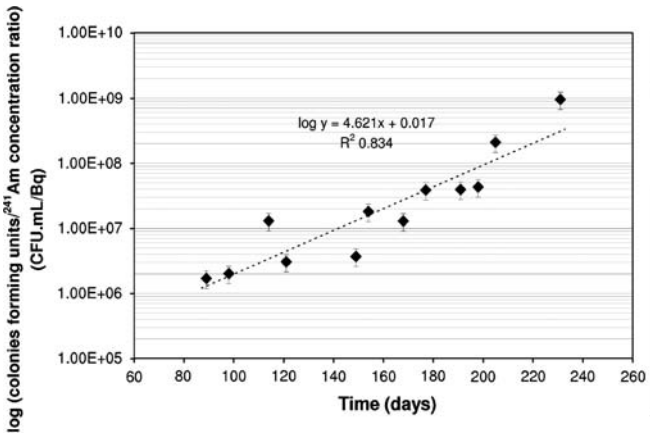


Fig. 4 Bacterial growth under aerobic and anaerobic conditions



Time (day)	^{241}Am released (Bq/mL)
89	2.20
98	1.55
114	0.60
121	0.49
149	0.89
154	0.73
168	0.36
177	0.38
191	1.62
198	1.74
205	1.91
231	1.45

Fig. 5 Colony-forming units to ^{241}Am ratio in function of time

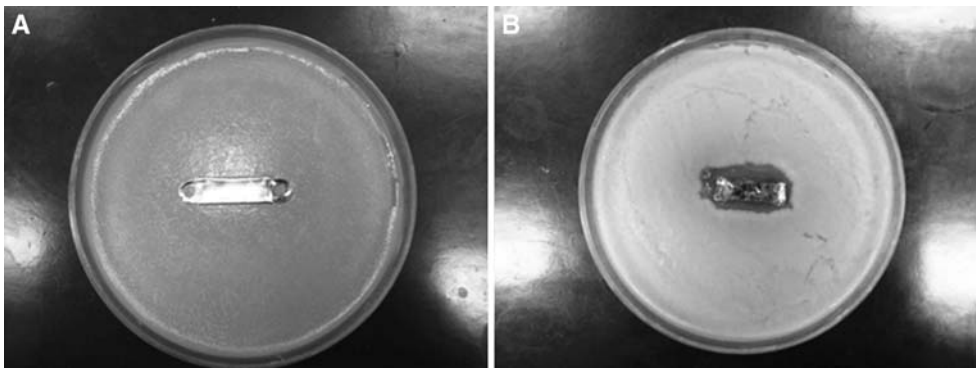


Fig. 6 Culture plates showing antimicrobial resistance to americium: **a** steel base without ^{241}Am; **b** ^{241}Am strip

Linear regression analysis indicated a positive correlation between the two parameters ($R^2 = 0.83$), suggesting that the bacterial growth until ca 230th day was influenced by ^{241}Am. This could indicate a bacteria adaptation period to ^{241}Am in which their activity was lower.

All data discussed and showed in Figs. 3, 4 and 5 indicate that ^{241}Am affects bacterial growth, delaying the degradation of the organic matter by at least 3 weeks, followed by a reduction in the bacterial count. Both aerobic and anaerobic growth conditions were affected by ^{241}Am and, consequently, the biodegradation of the organic waste in the lysimeter was not efficient.

In order to study the bacterial behavior in presence of ^{214}Am, antimicrobial resistance experiments were performed using a diffusion assay with americium source, and as control the experiment was run in absence of the radionuclide. As the base of the source was made of steel, no microorganism inhibition was observed (Fig. 6). Figure 6b shows an inhibition halo around the source with the ^{241}Am strips, which may be attributed to americium.

Bacterial tolerance levels to americium solutions were also studied. This is the first report of minimal inhibitory concentration (MIC) assays using different concentrations of americium. For all consortia from leachate samples, the MIC values ranged from 0.04 (1,161) to 0.09 (2,632) µM (Bq ml^{-1}). This finding suggests that these bacteria were adapted to high levels of this actinide, being four times more metal-tolerant regarding americium than *Pseudomonas putida* F1, whose MIC values range from 0.01 (290) to 0.02 (580) µM (Bq ml^{-1}).

In order to confirm the high ^{241}Am toxicity, MIC assays were performed and survival curves were established using also uranium, cadmium and zinc salts for both consortia from the leachate samples and *Pseudomonas putida* F1.

MIC assays (Table 1) revealed that the americium salt is more toxic than most other metals under both conditions. *P. putida* showed a growth inhibition concentration for $^{241}Am^{3+}$ that was 30,000 and 60,000 times higher than the MIC of Cd^{2+} and Zn^{2+}, respectively, and 100,000 times higher than that of $^{238}U^{6+}$. For all consortia, Cd^{2+} and

Table 1 Toxicities of Am^{3+}, Cd^{2+}, Zn^{2+}, U^{6+} to *P.putida* and consortia

Metal	*P. putida*	Consortia
$^{241}Am^{3+}$	0.01 ($\times 10^{-6}$ M)–0.02 ($\times 10^{-6}$ M)	0.04 ($\times 10^{-6}$ M)–0.08 ($\times 10^{-6}$ M)
Cd^{2+}	0.31 ($\times 10^{-3}$ M)–0.62 ($\times 10^{-3}$ M)	0.62 ($\times 10^{-3}$ M)–1.25 ($\times 10^{-3}$ M)
Zn^{2+}	0.62 ($\times 10^{-3}$ M)–1.25 ($\times 10^{-3}$ M)	1.25 ($\times 10^{-3}$ M)–2.5 ($\times 10^{-3}$ M)
$^{238}U^{6+}$	1.00 ($\times 10^{-3}$ M)–2.00 ($\times 10^{-3}$ M)	2.00 ($\times 10^{-3}$ M)–4.00 ($\times 10^{-3}$ M)

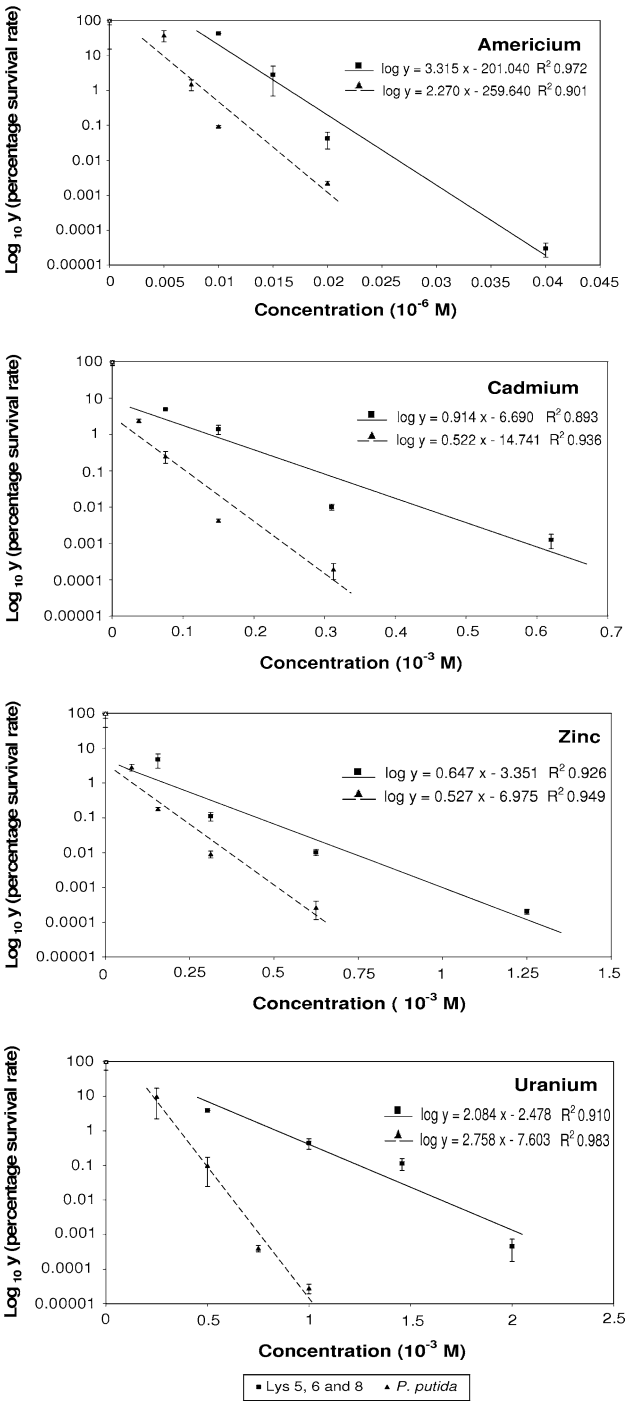


Fig. 7 Survival curve of *P. putida* and consortia on americium, cadmium, zinc and uranium

Zn^{2+} were less toxic, causing growth inhibition, respectively, at 0.62×10^{-3} M–1.25×10^{-3} M and 1.25×10^{-3} M–2.5×10^{-3} M, whereas U^{6+} was 3- and 1.5-times less toxic than Cd^{2+} and Zn^{2+}, respectively. $^{241}Am^{3+}$ caused growth inhibition of the consortia at 0.04×10^{-6} M–0.08×10^{-6} M, values which are 50,000 times higher than those observed for U^{6+}.

Table 2 Taxonomic characteristics of bacterial isolates

	O. anthropi	*A. species*	*P. gladioli*	*C. indologenes*
Gram	−	−	−	−
Enzymatic hydrolysis of amide OU glycosidic				
L-Phenylalanine-AMC	−	−	+	+
4MU-*N*-Acetyl-BD-Glucosaminide	−	−	−	−
L-Pyroglutamic acid-AMC	+	−	−	−
L-Tryptophan-AMC	−		+	+
L-Glutamic acid-AMC	+	−	−	+
L-Proline-AMC	−	−	+	
L-Arginine-AMC	−	−	−	+
Arginine-arginine-AMC	−	−	−	+
Glycine-AMC	+	−	−	+
L-Leucine-AMC	+	−	+	+
Lysine-alanine-AMC	−	−	−	+
Glutaryl-glycine-arginine-AMC	−	−	−	+
Glycine-proline-AMC	−	−	−	+
Resistance to the antimicrobial agent				
Colistin	−	−	+	+
Polymyxin B	+	−	−	+
Utilization of a carbon source				
D-mannitol	−	−	+	+
Citrate	+	+	−	
Acetate	−	+	−	+
Adonitol	−		−	+
Malonate	−	−	+	+
Alpha-ketoglutaric acid	+	+	−	+
Tiglic acid	−	−	−	−
Enzymatic hydrolysis of the colorless amide				
L-proline-NA	−	+	−	−
Gamma-L-glutamil-NA	−	+	−	−
Enzymatic hydrolysis of the aryl substituted glycoside				
Bis (PNP) phosphate	−	+	−	−
PNP-BD-glucoside	−	+	−	−
Utilization of carbohydrate			−	
Beta-allose	−	−	−	−
N-acetyl galactosamine	−	−	−	−
N-acetyl galactosamine	−	−	−	−
N-acetyl galactosamine	−	−	−	−
Sorbitol	−	−	−	−
Sucrose	−	−	−	−
Galacturonic acid	−	−	−	−
Maltulose	−	−	−	−
L-rhamnose	−	−	−	−
Beta-gentiobiose	−	−	−	−
Dextrose	−	−	−	−

Table 2 continued

	O. anthropi	*A. species*	*P. gladioli*	*C. indologenes*
D-galactose	–	–	–	–
D-fructose	–	–	–	–
D-gluconic acid	–	–	–	–
D-melibiose	–	–	–	–
L-arabinose	–	–	–	–
Methyl-B-glucoside	–	–	–	–
Utilization of ornithine				
Ornithine	–	–	–	+
Hydrolysis of urea				
Urea	–	–	–	+
Hydrolysis of esculin				
Esculin	–	+	–	–

Table 3 Toxicities of Am^{3+}, Cd^{2+}, Zn^{2+}, U^{6+} to *P. gladioli and C. indologenes*

Metal	*P. gladioli*	*C. indologenes*
$^{241}Am^{3+}$	0.04 ($\times 10^{-6}$ M)–0.08 ($\times 10^{-6}$ M)	0.04 ($\times 10^{-6}$ M)–0.08 ($\times 10^{-6}$ M)
Cd^{2+}	0.62 ($\times 10^{-3}$ M)–1.25 ($\times 10^{-3}$ M)	0.62 ($\times 10^{-3}$ M)–1.25 ($\times 10^{-3}$ M)
Zn^{2+}	1.25 ($\times 10^{-3}$ M)–2.5 ($\times 10^{-3}$ M)	1.25 ($\times 10^{-3}$ M)–2.5 ($\times 10^{-3}$ M)
$^{238}U^{6+}$	2.00 ($\times 10^{-3}$ M)–4 ($\times 10^{-3}$ M)	2.00 ($\times 10^{-3}$ M)–4 ($\times 10^{-3}$ M)

The survival curves were also established for Cd^{2+}, Zn^{2+}, U^{6+} and $^{241}Am^{3+}$, using *P. putida* and all consortia studied. The average results from Lys 5, 6 and 8 (Fig. 7), showed that, for these cations, the numbers of viable *Pseudomonas putida* cells were lower than those of other consortia and also that *P. putida* was the less resistant. Thus, $^{241}Am^{3+}$ may be considered more toxic than Cd^{2+}, Zn^{2+} and U^{6+}.

During this study, four bacteria from lysimeter leachate samples were isolated from Luria broth liquid medium and 0.11 μM of americium solution: *Flavobacterium* spp., *P. gladioli, Chryseobacterium indologenes* and *Ochrobactrum anthropi*. It is noteworthy that, although *Flavobacterium* spp., *P. gladioli* (Piotrowska-Seget et al. 2005) and *O. anthropi* (Ozdemir et al. 2004) have been previously described as metal-tolerant bacteria, none of them was tested for radionuclide resistance.

The taxonomic characteristics of each isolated bacterium are shown in Table 2.

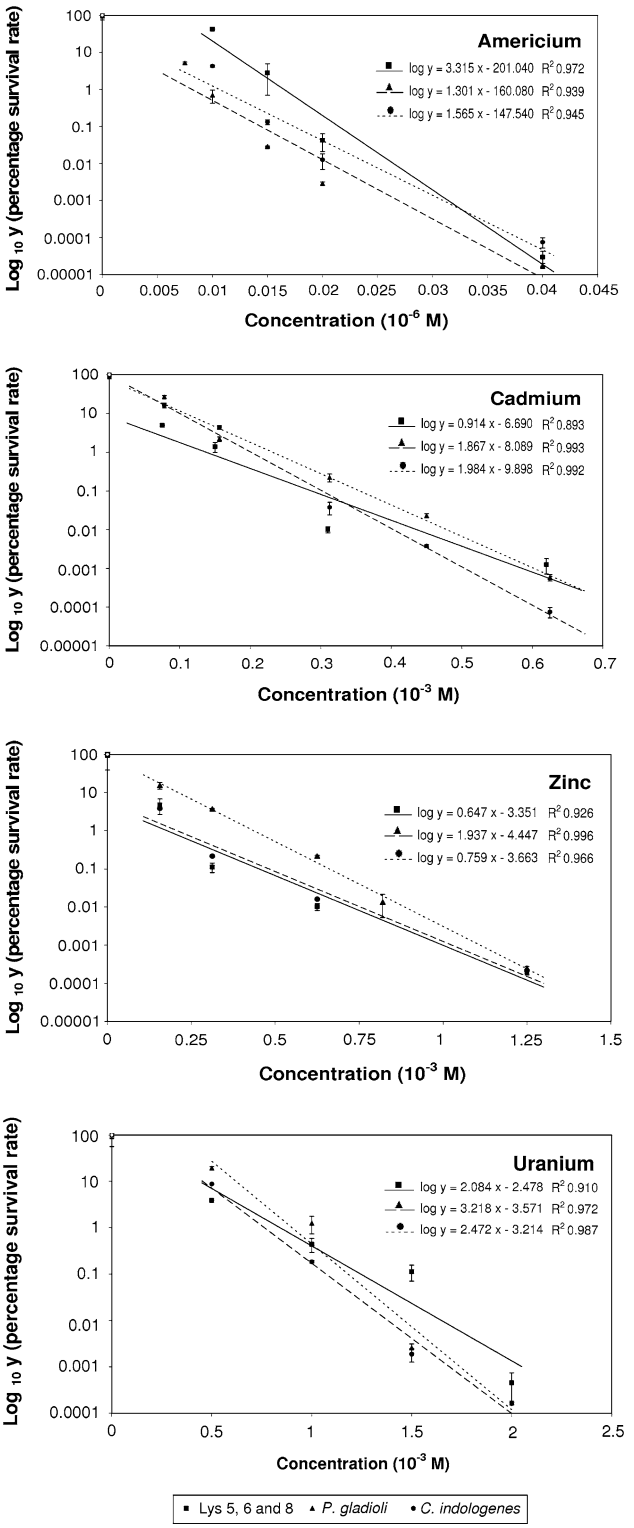


Fig. 8 Survival curve of *P. gladioli, C. indologenes* and consortia on americium, cadmium, zinc and uranium

As *Flavobacterium* spp. and *O. anthropi* are considered pathogenic bacteria further experiments were not performed with them.

The survival curves and MIC of *P. gladioli* and *C. indologenes* were obtained for Cd^{2+}, Zn^{2+}, U^{6+} and $^{241}Am^{3+}$. The MIC results revealed that these bacteria are resistant to metal as all consortia (Tables 1 and 3) and all survival curves showed similar behavior (Fig. 8)

Conclusion

The present study indicates that the presence of ^{241}Am has an influence on the microbial community responsible for the degradation processes of organic waste. The results revealed inhibitory effects on the growth of microorganism, with a reduction in the biodegradation process. Moreover, MIC assays revealed that $^{241}Am^{3+}$ appears to be more toxic than Cd^{2+}, Zn^{2+} and U^{6+}, and *P. putida* is less resistant to americium than all consortia studied. Among the new radionuclide-tolerant bacterial strains found during this study, *P. gladioli* and *C. indologenes* showed metal resistance similar to the consortia.

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