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ORIGINAL ARTICLE

Effects of gamma radiation on wastewater microbiota

Sandra Cabo Verde[1] · Telma Silva[1] · Paula Matos[2]

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Abstract Wastewater treatment by gamma radiation is a promising technology, with the capacity to reduce the impact of chemical and biological pollution of effluents in the environment. The aim of this study was to find out the effect of gamma radiation on the inactivation response of wastewater microorganisms. Wastewater samples were irradiated at a Co-60 facility, at different dose rates and at sublethal doses. The D10-values of total coliforms and mesophilic microbiota were determined for each sample and dose rate. Radio-resistant microorganisms in wastewater samples were isolated and their growth and inactivation kinetics in different composition substrates were determined, to find out the capacity of these bacteria to biodegrade the organic content of the wastewater. The results obtained suggest that irradiation substrate and dose rate influence the response of microorganisms to gamma radiation and could be also important factors for bioremediation.

Keywords Wastewater · Gamma irradiation · Microbial inactivation · Coliforms · Dose rate effect · Substrate effect

✉ Sandra Cabo Verde
sandracv@ctn.ist.utl.pt

1 Centro de Ciências e Tecnologias Nucleares, Instituto Superior Técnico, Universidade de Lisboa, Estrada Nacional 10, km 139, 7, 2695-066 Bobadela LRS, Portugal

2 Laboratório de Aceleradores e Tecnologias de Radiação, Instituto Superior Técnico, Universidade Técnica de Lisboa, Estrada Nacional 10 km 139, 7, 2695-066 Bobadela LRS, Portugal

Introduction

The contamination of water resources by pollutants from industry, municipal and agriculture discharges is increasing worldwide. This may lead to health risks and even jeopardize human survival (e.g., contaminants in water, air, soil, etc.). Control and remediation of these pollutants are necessary to prevent us harming ourselves and future generations. Moreover, the increasing demand for drinking water in many countries often faces two main problems: the scarcity of drinking water and the proper processing and disposal of increasing amounts of wastewater. One solution to these problems is to process wastewater for reuse. The purification of wastewater has been and continues to be a prime environmental issue. Although conventional wastewater treatment systems such as shallow maturation ponds have the capacity to improve effluent quality, they are not sufficient to remove all contaminants (Von Sperling and Mascarenhas 2005). In contrast, disinfectant agents act by inducing biochemical changes in both pathogenic agents and the effluent (Blatchley et al. 1997). Wastewater disinfection methods include chlorination, ozonation and ultraviolet (UV) radiation.

The International Atomic Energy Agency (IAEA) has published many documents, which draw attention to the considerable potential of radiation technology to clean up waste discharges from various industrial and municipal activities (IAEA 2001). Irradiation treatment has proven to be a powerful tool in inactivating human pathogenic microorganisms in water, wastewater and sludge, as well as in food and medical products (Farooq et al. 1993; Lagunas-Solar 1995; Rawat et al. 1998; Basfar and Abdel Rehim 2002; IAEA 2004; Wang and Wang 2007).

One of the main advantages of such treatment is the fact that because irradiation is a physical process, no chemicals have to be used. The use of gamma radiation is justified by its capability of degrading organic matter and pathogenic organisms while retaining nutrients such as nitrogen and phosphorus, which are important if the wastewater is to be used for agricultural purposes (Rawat et al. 1998; Tahri et al. 2010). Furthermore, gamma irradiation as an alternative disinfection process may be superior to conventional chlorination/dechlorination method, in terms of the effluent toxicity response attributable to the disinfection process (Thompson and Blatchley 1999).

The microbicidal mechanism of ionizing radiation could act in two ways: directly by damaging the nucleic acids (single- and double-strand breaks) or indirectly where radicals originated from water radiolysis, mainly OH free radicals, H atoms and solvated electrons e^-_{aqu}, interact with intra- or extracellular molecules. The effect of the indirect action by radicals may be reduced by the presence of scavengers such as bicarbonate, but can also be enhanced by the presence of oxygen (von Sonntag 1994). The response of the microorganisms to ionizing radiation could be influenced by several factors such as composition of the irradiation medium (presence of protectors, sensitizers), irradiation atmosphere (e.g., air, N_2, vacuum), temperature, water content of the cell, age of the microorganisms, energy source (e.g., gamma radiation, neutrons) and dose rate (Silverman 1983).

Previous reports (Ridenour and Armbruster 1956; Lowe et al. 1980; Hill 2003; Taghipour 2004) showed that enteric bacteria in sewage and liquid wastewater are quite easily inactivated by ionizing radiation, although there are no studies with respect to the influence of dose rate on the response of enteric bacteria to gamma radiation.

The aim of this study was to analyze the influence of dose rate and substrate composition on the inactivation response of natural microbiota present in wastewater samples in order to identify the parameters that could influence the efficiency of wastewater treatment by gamma radiation. Furthermore, the significance and applicability of radio-resistant microbial survivors of irradiated wastewater (ISW) on wastewater biodegradation should be investigated.

Materials and methods

Sampling

The wastewater samples were collected from two different treatment plants.

1. A municipal wastewater treatment plant (MWTP) in Lisbon, Portugal, with a flow rate of 54,000 m^3 effluent per day from a population of 215,000 habitants; the main steps of this MWTP are (1) gravity and pumping, (2) screening, (3) grit, oil, fat and grease removal, (4) primary sedimentation, (5) activated sludge reactor, (6) secondary sedimentation, (7) sand filters and (8) UV irradiation as tertiary treatment. Samples of 1 l of volume were collected in sterilized flasks from effluent before the UV treatment and transported at 4 °C to the laboratory.
2. A Portuguese slaughterhouse treatment plant (SWTP) producing approximately 143 ton of hog meat daily and generating solid residues and wastewater. Wastewater treatment which is a continuous process including anaerobic and aerobic digestion and sedimentation lagoons takes 7 days until discharge to the river. Samples of 5 l were taken in sterilized flasks from primary-treated wastewater (after mechanic screening that included the removal of solids) and transported at 4 °C to the laboratory.

Irradiation process

Glass flasks with 0.5 l of wastewater samples were exposed to gamma radiation from a semi-industrial Co-60 facility located at the Campus Tecnológico e Nuclear (Bobadela, Portugal). The irradiations of the MWTP samples were carried out at room temperature and simultaneously at the dose rates of 0.08, 0.8 and 9 kGy/h. The target doses were 0.1 up to 1.2 kGy for the inactivation studies of coliforms and 5.1 up to 40.3 kGy for the inactivation studies of mesophilic population. The SWTP samples were irradiated at room temperature and simultaneously at the dose rates of 0.9 and 3 kGy/h. Doses were established from 0.1 up to 1.2 kGy for coliforms and 5.1 up to 40.3 kGy for total microbiota. Triplicate samples of each type of wastewater (3 × 0.5 l of wastewater in glass flasks) were used per absorbed dose (three absorbed doses per dose rate; e.g., 0.1, 0.5 and 1.2 kGy) for the tested dose rates. Non-irradiated samples were also treated with all the assays. The irradiation conditions for the performed assays are summarized in Table 1.

Dose rates were experimentally determined using a Fricke dosimeter as described in ASTM (1992). Absorbed doses were monitored using calibrated routine dosimeters (measurement uncertainty of ±2.5 %; Perspex, Harwell, UK).

Inactivation studies

To determine total coliforms before and after irradiation, the multiple-tube fermentation method was used and results are reported in terms of the most probable number (MPN) of coliforms present per 100 ml. Three sets

Table 1 Gamma radiation dose rates and dose ranges applied to study the inactivation of microbial populations in wastewater samples from municipal wastewater treatment plant (MWTP) and slaughterhouse wastewater treatment plant (SWTP)

Target microorganism(s)	Substrate	Dose rate (kGy/h)	Applied dose range (kGy)
Total coliforms	MWTP	0.8	0.1–1.2
		0.08	
	SWTP	3.0	
		0.9	
Total mesophilic microbiota	MWTP	0.8	5.1–40.3
		0.08	
	SWTP	3.0	
		0.9	
Methylobacterium spp.	TSB	9	5.3–40.2
		0.8	
		0.08	
	WW	9	
		0.8	
		0.08	

WW, sterilized MWTP sample, *TSB* Tryptone Soy Broth

(dilutions of 10^{-1}, 10^{0} and 10^{1}) of five Durham tubes containing Lauryl Sulphate Broth (LSB, Merck, Germany) were prepared for each sample. The tubes were incubated at 37 °C for 7 days. Confirmation of the results for the tubes with gas production was carried out using Brilliant Green Bile Lactose Broth (BRILA, Merck, Germany). Results were observed during incubation at 24, 48 and 72 h and 7 days after irradiation, and the number of coliforms was calculated using standard statistical tables (APHA 1998).

To determine the total counts for the mesophilic microbiota before and after irradiation, the spread-plating technique was used. For each sample, two decimal serial dilutions (10^{-1}; 10^{-2}) in physiological saline solution (0.9 % NaCl) were performed and three replicas of an aliquot (0.1 ml) of each dilution were plated in Tryptic Soy Agar (TSA, Oxoid, UK). The growth conditions were aerobic incubation at 30 °C for 14 days. Colony-forming units were counted after 24, 48 and 72 h and 7 and 14 days after irradiation. Results were expressed in colony-forming units per milliliter (CFU/ml).

These protocols are based on standard methods for the examination of water and wastewater (APHA 1998).

Characterization of natural contaminants in wastewater samples

The obtained colonies in the inactivation studies were macroscopically (e.g., looking at pigmentation, texture, shape), microscopically and biochemically typed by gram staining, catalase activity and oxidase production. The isolates were organized into typing groups according to Bergey's Manual of Determinative Bacteriology (Holt 1994). The frequency of each phenotype was calculated based on the number of isolates and their characterization. The most frequent radio-resistant strains in each wastewater sample were identified by means of miniaturized biochemical kits (API system, bioMérieux, France).

Biodegradation studies

The radio-resistant strain isolated from the MWTP samples was inoculated (approximately 10^{5} CFU/ml) in 0.5 l of two different suspending media, Tryptone Soy Broth (TSB, Oxoid, England) and municipal wastewater (WW) that had been sterilized by autoclaving (30 min at 121 °C). The cultures were irradiated at the Co-60 facility in a similar way as the MWTP samples. The range of applied doses was 5.3 up to 40.2 kGy. The number of surviving cells before and after irradiation was determined by the spread-plating technique, as described in section "Inactivation Studies."

The radio-resistant strain isolated from the SWTP sample was inoculated (approximately 10^{2} CFU/ml) in 250 ml of wastewater samples from the slaughterhouse that had been sterilized by irradiation at 30 kGy (dose rate 3 kGy/h) and by autoclaving (30 min at 121 °C). All experiments were carried out aerobically in 500-ml flasks at 30 °C with constant shaking (125 revolutions per minute—r.p.m.). The growth of the microbial strain in the two substrates was monitored spectrophotometrically (1800-UV, Shimazdu) by measuring culture turbidity at 600 nm.

Data analysis

Origin software version 7.5 (OriginLab Corporation, Northampton, USA) was used for data analysis. D10-values in kGy, which is the dose required to reduce a microbial population by 90 % (one-decimal logarithm reduction), were obtained from a linear regression model (reciprocal of the slope) of the logarithm of the surviving fractions as a function of absorbed radiation dose (kGy).

Results

Inactivation studies

To quantify the inactivation response of the total coliforms and microbial mesophilic populations of the studied wastewater samples, the D10-value of each population was calculated for the different dose rates.

Coliforms were used as indicators of pollution by fecal origin. Because these microorganisms could contribute to potential health risk hazards, it was considered important to assess their inactivation response to irradiation. The total coliform load in the analyzed wastewaters was 2×10^3 MPN/ml for MWTP samples and 3×10^6 MPN/ml SWTP samples, respectively. For both types of wastewater samples, there was an exponential decrease in the coliform population with an increase in dose from 0.1 to 1.2 kGy. The corresponding D10-values ranged between 0.15 and 0.24 kGy (Table 2).

The bioburden values obtained for the microbial mesophilic population were 9×10^4 CFU/ml and 3×10^7 CFU/ml for MWTP and SWTP wastewater samples, respectively. The inactivation response of the mesophilic microbiota was found to depend on both irradiation condition and type of wastewater sample (Table 2). The D10-values of the mesophilic microbiota increased about four times when the dose rate was ten times lower, for the same sample of MWTP wastewater, and in the same way, for the slaughterhouse effluent (SWTP) samples, the D10-values doubled when the applied dose rate was three times less.

Characterization of wastewater microbiota

Before and after irradiation with different doses, the isolates of wastewater samples were phenotyped to analyze the dynamics of the microbial population with absorbed doses. The results indicated that for the natural microbiota of both wastewater types, the most frequent morphotype was gram-negative, catalase-positive, oxidase-negative rods (>45 %), which are included in the coliform group. At the higher dose rates tested for both types of wastewater samples (0.8 kGy/h for MWTP and 3 kGy/h for SWTP) and with increasing absorbed dose, the observed major morphological type decreased in relative frequency, and other groups of bacteria were isolated, such as microorganisms of the genus *Bacillus* (>60 %), which are more radio-resistant due to their spore-forming capacity. In the MWTP samples irradiated at lower dose rate (0.08 kGy/h) and at the target doses of 10, 15 and 20 kGy, the surviving microorganisms appeared as a homogenous population of pink-pigmented bacteria, with methylotrophic characteristics putatively identified as *Methylobacterium* spp.. Equally, for the SWTP samples irradiated with 15 kGy at the lower dose rate (0.8 kGy/h), the microorganisms isolated revealed to be a homogenous population of yellow-pigmented bacteria, identified as *Stenotrophomonas maltophilia*.

Biodegradation studies

Two experiments were performed with the isolated radio-resistant bacteria to evaluate a possible biodegradative function of these microorganisms as part of the wastewater microbiota. In the first experiment, the pink-pigmented isolate was inoculated in two different substrates, TSB (usually used as a nutrient-complete culture medium) and WW (sterilized municipal wastewater sample), and irradiated (5.3 up to 40.2 kGy) at three different dose rates (0.08, 0.8 and 9 kGy/h). The response of the radio-resistant microorganism on the six different irradiation conditions suggested, based on the D10-values, a higher radio-resistance on WW than on TSB substrate (except for the dose rate of 9 kGy/h) with an increasing trend of D10-value (i.e., a higher radio-resistance) with decreasing dose rate (Table 3). These observed differences in the D10-values of the pink-pigmented isolate might be due to: (1) a protective

Table 2 Mean D10-values (± standard error) for total coliform population and microbial mesophilic population for wastewater samples before UV treatment from municipal wastewater treatment plant (MWTP) and from slaughterhouse wastewater treatment plant (SWTP) mechanic screening pretreatment

	Sample	Dose rate (kGy/h)	D10-value (kGy)
Total coliforms	MWTP	0.8	0.15 ± 0.01
		0.08	0.16 ± 0.01
	SWTP	3.0	0.24 ± 0.01
		0.9	0.22 ± 0.02
Total mesophilic microbiota	MWTP	0.8	3.6 ± 0.2
		0.08	16.8 ± 0.5
	SWTP	3.0	2.2 ± 0.1
		0.9	4.0 ± 0.4

Table 3 Mean (± standard error) D10-values of the pink-pigmented radio-resistant isolate (*Methylobacterium* spp.) suspended into two substrates (wastewater, WW and Tryptone Soy Broth, TSB) irradiated at three different dose rates

Dose rate (kGy/h)	Irradiation substrate	D10-value (kGy)
9	TSB	2.9 ± 0.2
	WW	2.0 ± 0.1
0.8	TSB	14.1 ± 0.3
	WW	19.4 ± 0.1
0.08	TSB	21.0 ± 0.4
	WW	43.9 ± 0.2

effect of the wastewater chemical components that react with water radiolysis products, such as hydroxyl radicals, decreasing their availability to reach the microbial cells and hence lowering the apparent inactivation rate and (2) a dose rate effect—the microorganisms have more time to repair from radiation injuries at a lower dose rate that is reflected in higher surviving fractions after irradiation. The results also demonstrate a rising difference between the D10-values of the two substrates with the diminishing of the dose rate. This observation could lead to the formulation of the hypothesis that the investigated highly radio-resistant microorganisms are able to metabolize some components of the wastewater that would allow for a more efficient repair of any breaks of molecular bonds that might have occurred during irradiation.

The second experiment was performed to test this hypothesis. The strain of *S. maltophilia* was inoculated in two types of samples of the same slaughterhouse effluent (one sterilized by gamma radiation and the other autoclaved). *Stenotrophomonas* sp., a gram-negative genus widely distributed in the environment, is a potential genus for bioremediation because its members are able to metabolize a wide variety of recalcitrant compounds such as aromatic and phenolic compounds. Therefore, this genus has attracted increasing attention in both environmental and biotechnological applications (Guzik et al. 2009; Greń et al. 2010; Chen et al. 2011). The obtained results on the growth of this strain revealed different growth rates for distinct growth phases (Fig. 1). The lag phase (initial/adaptation phase of the growth curve) was shorter in ISW than in autoclaved wastewater (ASW), indicating that the physiological adaptation of the strain to the irradiated substrate is simpler. Furthermore, the exponential or log phase is more pronounced in ISW than in ASW, demonstrating a higher rate of biomass growth in the irradiated substrate. These preliminary results corroborate with the hypothesis that ionizing radiation could render the nutrients more available for bacterial growth. However, besides the assessment of bacterial growth it will be also important to evaluate the degradation kinetics of wastewater organic content.

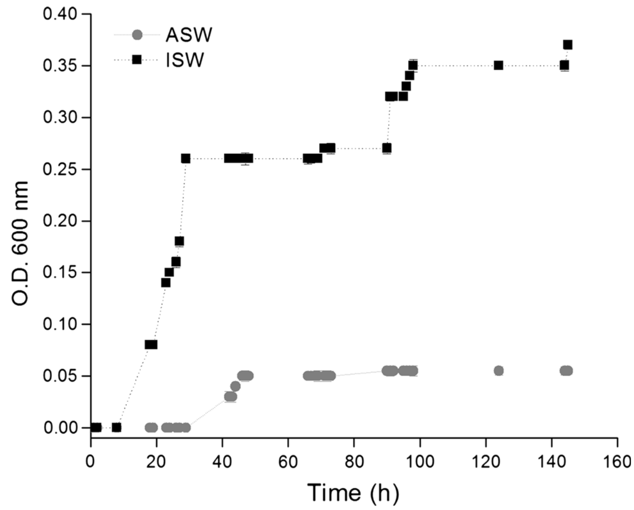

Fig. 1 Growth curve (optical density—OD, absorbance at 600 nm) of *Stenotrophomonas maltophilia* radio-resistant strain in slaughterhouse wastewater samples sterilized by ionizing radiation (ISW) and autoclaved (ASW). *Error bars* correspond to 95 % confidence intervals about mean values ($n = 3$; $\alpha = 0.05$). *Dotted lines* serve as a guide for the eye

Discussion

The results obtained highlight the influence of dose rate and substrate type on the gamma inactivation response of microorganisms. To the best of our knowledge, there is no reported study on the inactivation of coliforms by gamma radiation of SWTP samples. However, the obtained data are consistent with those of Rawat et al. (1998) indicating that the D10-values for total coliforms in domestic wastewaters range from 0.2 to 0.3 kGy. More specifically, for a dose rate of 0.8 kGy/h, Rawat et al. (1998) reported D10-values for total coliforms of 0.20 and 0.22 kGy, for primary and secondary effluents from a municipal sewage treatment plant (MSTP), respectively. In another study, Basfar and Abdel Rehim (2002) reported a D10-value of 0.3 kGy, at a dose rate of 12.7 kGy/h, for total coliforms on unchlorinated secondary effluents from an activated sludge treatment plant. Also, Tahri et al. (2010) found D10-values of 0.32 and 0.30 kGy at a dose rate of 0.18 kGy/h, for the inactivation of total coliforms present in primary and secondary wastewaters, respectively, from an urban wastewater treatment plant. These results also point out that inactivation response of total coliforms by gamma radiation is not significantly influenced by the dose rate and substrate composition, probably due to the high sensibility of this type of microorganisms to gamma radiation. The effect of gamma radiation dose rate on *Escherichia coli* inactivation in secondary effluent samples from a wastewater treatment plant was investigated by Taghipour (2004), also indicating no significant impact of dose rate on *E. coli* inactivation.

In the present study, a higher microbial radio-resistance to gamma radiation was found at lower dose rates, which could be related to the fact that microbial enzymes may have more time to repair radiation-induced cell damage, contributing to microbial growth, to a reduction in the rate of inactivation and, consequently, to higher D-10 values. It is noted that the dose rate effect on microbial inactivation response to low-LET ionizing radiation was also reported by other authors (Lopez-Gonzalez et al. 1999).

The use of gamma radiation for wastewater treatment could lead to the selection of radio-resistant microorganisms if the irradiation geometry (e.g., source(s) geometry) and doses applied are not suitable or adequate. Radio-resistant bacteria have already been isolated in various experiments as the survivors of high doses of ionizing radiation (Anderson et al. 1956; Welch and Maxcy 1975; Shukla et al. 2007). Previous studies have also reported of isolated highly pigmented radio-resistant bacteria in a very low relative percentage with respect to total bacterial number (Anderson et al. 1956). This fact is in agreement with the results obtained in the present study, in which the isolation of the radio-resistant pigmented bacteria was just achieved at high radiation doses when the other major frequent bacteria were already inactivated. The characteristics of these bacteria, considering the mutagenic action of ionizing radiation, have to be studied in order to assess their taxonomic position and significance as part of the residual flora of the irradiated products. Nevertheless, the potential use of radio-resistant bacteria in biotechnological applications in areas such as bioremediation, waste reduction, biosensors and biodecontamination has already been reported elsewhere (Binks 1996).

The ionizing radiation technology could be a useful instrument for bioremediation, since microorganisms and chemical species depend on each other in a dynamical way (Jo et al. 2006; Melo et al. 2008). The results obtained in the present study suggest that lower dose rates could lead to an improved bioremediation process. This is more effective for wastewater substrates, due to the effect of ionizing radiation on their degradation, producing molecular forms easier to be metabolized by bacteria, consequently, diminishing the chemical wastewater pollution. Furthermore, the large irradiation period required when low dose rates are used allows the repair mechanisms of bacteria to respond more efficiently. Higher dose rates showed to be more efficient for the disinfection process and could be applied in the tertiary treatment, as mentioned before by other authors (Sabbagh et al. 2014).

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