HUNGARY

EFFECT OF ELECTRON BEAM IRRADIATION AND THE PRESENCE OF ANTIBIOTICS ON THE POPULATION RATIO OF RESISTANT/SENSITIVE BACTERIAL CULTURES IN MODEL WASTEWATER MATRIX

Renáta Homlok[1], Gabriella Kiskó[2], András Kovács[3], Tünde Tóth[1,4], Viktória Dobó[2], Erzsébet Takács[1], Csilla Mohácsi-Farkas[2], László Wojnárovits[1], László Szabó[1,5]

[1]Institute for Energy Security and Environmental Safety, Centre for Energy Research, H-1121 Budapest, Konkoly-Thege Miklós út 29-33, Hungary
[2]Department of Food Microbiology, Hygiene and Safety, MATE Institute of Food Science and Technology, H-1118 Budapest, Somlói út 14-16, Hungary
[3]Atomic Energy Engineering Company Ltd., H-1121 Budapest, Konkoly-Thege Miklós út 29-33, Hungary
[4]Department of Organic Chemistry and Technology, Budapest University of Technology and Economics, H-1111 Budapest, Szent Gellért tér 4, Hungary
[5]International Center for Young Scientists, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan
e-mail: homlok.renata@ek-cer.hu

Abstract

Control strategies against the spread of antibiotic resistance should be considered in wastewater treatment plants. It is important to understand how resistant bacteria behave in the presence of trace amounts of antibiotics, in order to implement appropriate measures. In our work, we examined the population dynamics of resistant/sensitive *Staphylococcus aureus* co-cultures. On the one hand, we gained insight into the effect of trace amounts of antibiotics (piperacillin and erythromycin) on bacteria in different wastewater matrices, and on the other hand, we studied the applicability of electron radiation to eliminate the antibacterial effect. Based on our results, trace amounts of antibiotics act on the resistant strain. Presumably, it triggers biological processes in resistant bacteria that do not provide a competitive benefit but disadvantage over the sensitive subtype, and the trace level of the antibiotic present does not appear to affect the sensitive strains. The effect of these conditions on population dynamics is reduced with the use of treatment with accelerated electrons, presumably due to the fact that the decomposition products of the components of the effluent matrix (such as humic acid) also contribute to the chemical transformations. Furthermore, it has become apparent that the presence of trace amounts of antibiotics on the one hand initiates biochemical processes in the resistant subtype and on the other hand sensitizes bacteria to the attack of free radicals generated during electron beam treatment. It is clear that better understanding is needed on the effects of trace level of antibiotics in environmental waters on the cellular response and population behavior of resistant bacterial cultures.

Introduction

Radiation sterilization has been developed as a safe and reliable technology and is widely used in the healthcare sector. By using it, the microbiological safety of food is improved and their shelf life is extended [1-2]. In addition, ionizing radiation has been admitted to be effective for reducing the bacterial load of wastewater and sewage sludge [3-6]. However, it should be contemplated that the species present in these diverse microbial populations show different sensitivities to irradiation [5]. Extensive DNA damage occurs in bacteria as a result of exposure to lethal radiation. These bacteria are unable to multiply. Some recent studies indicate that radiation-inactivated bacteria are still metabolically active. The mechanisms are different when heat sterilization is used [7-9]. Based on these, we assume that sensitive and resistant subtypes of the same bacteria may show different sensitivity to ionizing radiation. Therefore, we further investigated this issue and performed experiments with bacteria present during irradiation.

Experimental

Four types of model systems were used in the studies. This provided step-by-step insight into, how different external stressors affect the composition of a mixed resistant / sensitive bacterial culture. In the first series of experiments, the model effluent matrix was irradiated with antibiotics (erythromycin and piperacillin (separately)). In the second series of experiments, irradiation was performed without the addition of antibiotics. Bacteria were added only after irradiation in the first two series. In this way, we obtained an idea of the effect of the components and degradation products of the wastewater matrix (especially humic acid, since it appeared to affect bacterial cultures when subjected to oxidative treatment [10, 11]) on the microbial community.

In the first two series of experiments, for the irradiation, we prepared a solution that contained an antibiotic concentration 500x compared to a real sample, the other constituents were also concentrated 500x to keep the kinetics of the free radical system similar to a real scenario. This was necessary to work in a convenient dose range. After the irradiation, the samples were pre-diluted to the appropriate level for the microbiological assay. The doses indicated in the measurement results refer to the concentrated solutions. Based on these, it is possible to estimate the actual dose required in the different systems, based on a 500-fold dilution factor.

Finally, the last two sets of experiments (Fig. 1) were done in the presence of bacteria added to the wastewater matrix prior to EB treatment. In this case the original synthetic wastewater matrix was prepared with or without the antibiotic (non-concentrated samples).

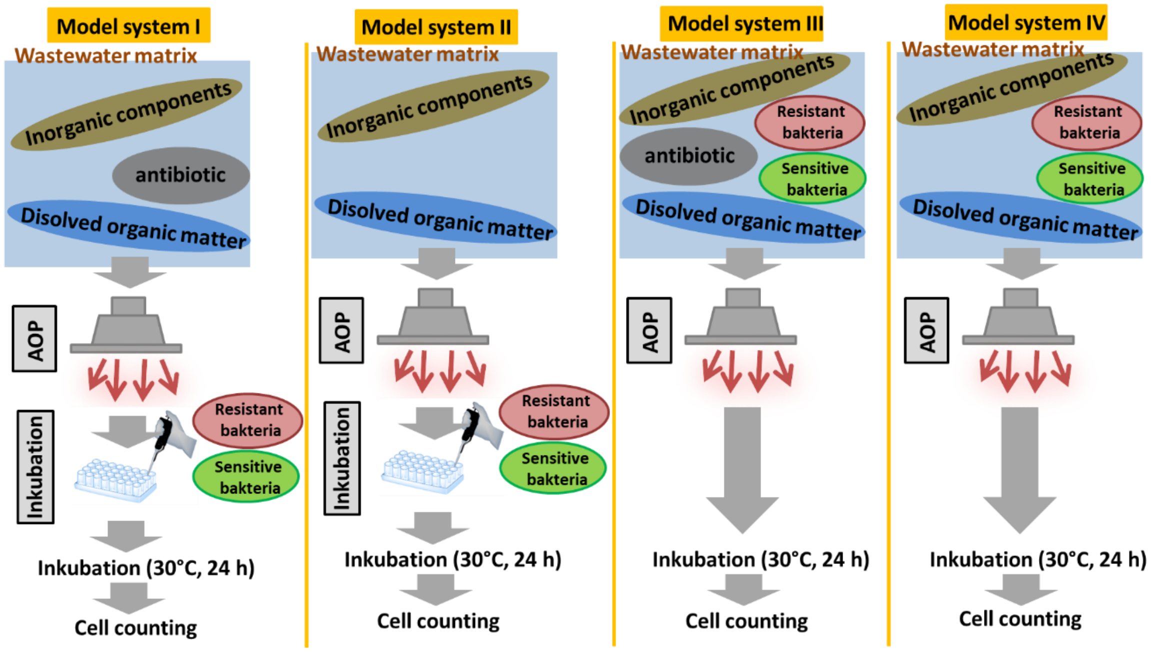

Fig. 1. Schematic representation of the model systems and the main processing steps applied in our study.

The electron beam (EB) treatment was performed using a Tesla Linac LPR-4 type linear electron accelerator. We developed this microbiological assay previously [10-11].

We selected sensitive and resistant *Staphylococcus aureus* (*S. aureus*) isolates (National Collection of Agricultural and Industrial Microorganisms, NCAIM, Szent István University) to monitor the change in antibacterial activity. In this test the dynamics of a mixed (sensitive/resistant) bacterial population gives information on the effects of antibiotics in a concentration well below the minimum inhibitory concentration (MIC). Inocula were prepared from an overnight culture (incubated at 37 °C) in the case of the sensitive strain, and in the case of the resistant one, the freshly innoculated cells were incubated for 72 h (at 37 °C) for the bacterial suspension preparation. This incubation time was sufficient to yield a culture containing dead cells with released genetic information, which is then available for the sensitive cells that may acquire resistance. The sensitive and resistant subtypes in a 1:1 ratio were added to the wastewater matrix prior to the advanced oxidation treatment. After the irradiation, the samples were incubated for 24 hours (at 30 °C). Colony counting was performed on trypto-casein soy broth

(CASO) agar plates. After spreading 100 µL samples evenly on the surface, the inoculated plates were incubated at 37 °C for 24 h and then the number of surviving colonies were counted.

The total colony count (sensitive + resistant) was determined on unspiked agar plates, while resistant cells were counted on agar plates spiked with the corresponding antibiotic reaching a concentration well above the minimum inhibitory level.

Only resistant cells grow on the surface of the agar plates containing the antibiotic above the MIC. Then the ratio of resistant colonies to the sensitive + resistant colonies was calculated.

Results and discussion

Using advanced oxidation treatment (here EB irradiation), the selective pressure on the bacterial population favouring the predominance of antibiotic resistant mutants can be eliminated. This is achieved when the fraction of resistant bacteria, within a statistically insignificant deviation, is the same as in the control sample (with no antibiotic added). In other words, the difference between the control sample and the sample containing the antibiotic (piperacillin) is no longer significant (based on statistical significance analysis using GraphPad Prism biostatistics software; multiple *t*-test analysis was applied assuming equal variances). A synthetic effluent wastewater was designed as a kinetically appropriate reflection of a real wastewater sample, spiked with antibiotics at environmentally relevant concentrations (2 µg L^{-1}).

Model wastewater matrix with antibiotics subjected to EB treatment

In the applied microbiological test, the change of the fraction of resistant bacteria in the population is monitored in response to various environmental stressors (antibiotics, wastewater components and their degradation products following EB treatment). The effect of these stressors was examined on the basis of significance analysis, and the deviation from the control samples was taken into account when evaluating the results.

In our present study, we examined low, environmentally relevant concentrations (and conditions, see Model System I in Figure 1), that are certainly below the minimum selective concentration. The results are shown in Figures 2 and 3 for erythromycin and piperacillin, respectively.

Only limited information are available about the effect of this environmentally relevant concentration on the cellular response in bacterial cultures [12, 13, 14, 15].

Fig. 2 shows an interesting phenomenon for untreated samples (0 kGy). Compared to the control sample (no antibiotic added), the fraction of resistant bacteria is significantly suppressed in populations containing trace amount of erythromycin and piperacillin (Fig. 2 and 3, respectively). Furthermore, the fraction of resistant bacteria in case of erythromycin increases following the EB treatment that is expected to degrade the antibiotic according to our previous studies [10, 11, 16].

For erythromycin, a dose of 8 kGy is sufficient to eliminate the initial existing biological effects on the population (Fig. 2). In case of piperacillin (Fig. 3), a higher dose appears to be required to eliminate the biological effects on the bacterial culture (Fig. 3).

We hypothesize that in this extremely low concentration range, the antibiotic has practically no effect on the growth rate of sensitive bacteria, while it might still trigger biological protecting responses in the resistant one. This extra cellular activity which does not lead to any advantage compared to the sensitive subtype might slow down the growth of the resistant one. This would give explanation for the population shift in this low concentration range (0 kGy samples).

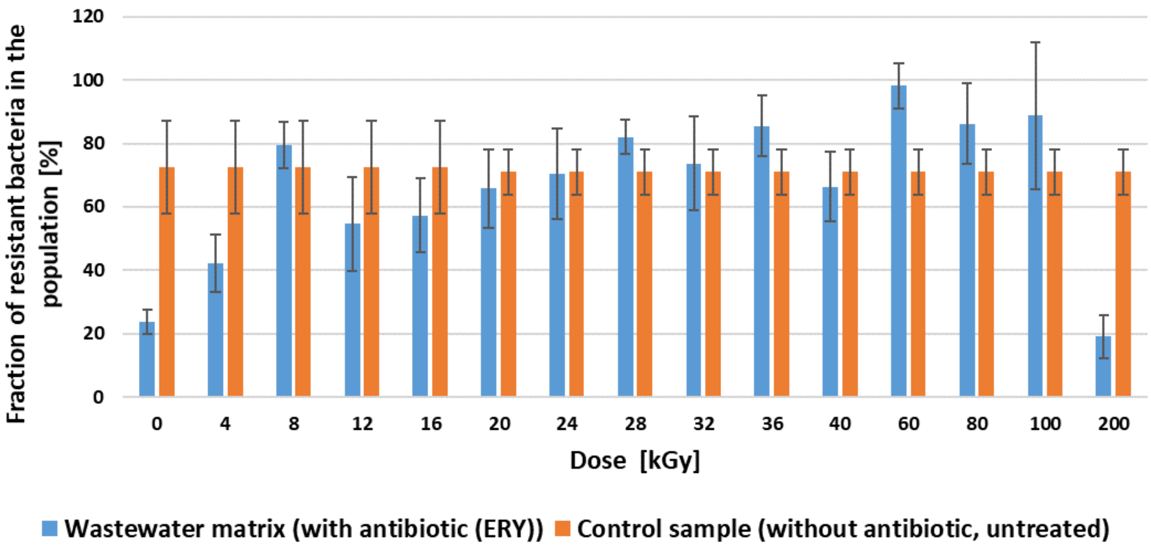

Fig. 2. Fraction of resistant bacteria in a population that was spiked with a model wastewater matrix sample containing erythromycin, and subjected to electron beam irradiation.

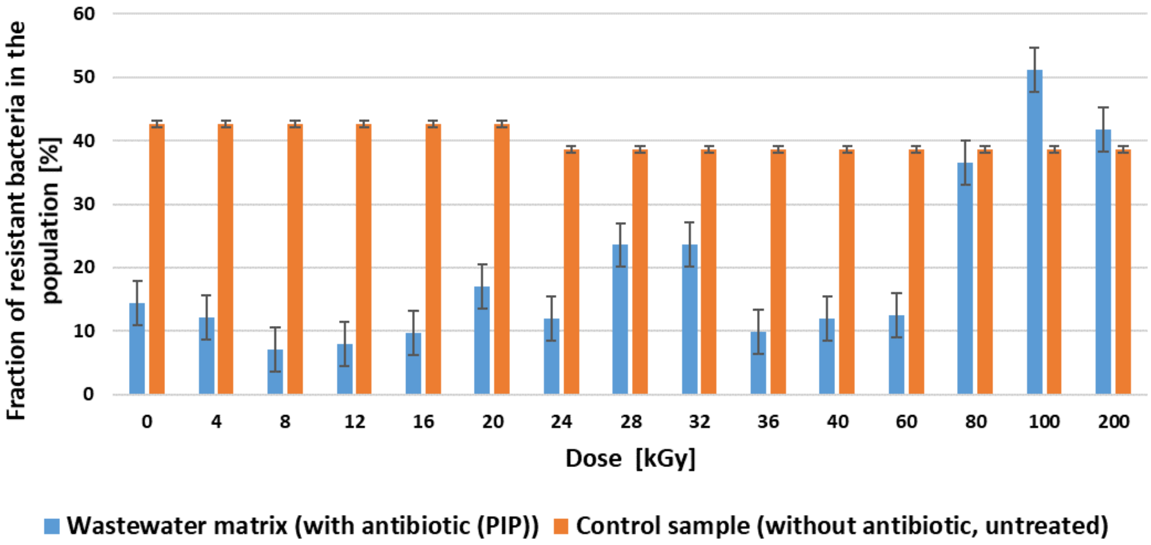

Fig. 3. Fraction of resistant bacteria in a population that was spiked with a model wastewater matrix sample containing piperacillin, and subjected to electron beam irradiation.

Another interesting phenomenon can be observed in Fig. 2 and 3. Higher deviations were observed between 60 and 200 kGy for erythromycin (Fig 2) and at 100 kGy for piperacillin (Fig. 3). In our previous studies some growth inhibition was already observed for both the sensitive and resistant subtypes when only synthetic wastewater matrix was irradiated with high absorbed doses: this phenomenon was attributed to the presumably forming polyphenolic degradation products of humic acid potentiating antimicrobial activity [10, 11].

If only the degradation products of humic acid affected the bacterial population, similar results could be expected in Fig. 2. and Fig. 3 for high doses. However, the results did not confirm this.

In addition, we can see an increase and decrease in the resistant fraction in Fig. 2 (see 60 and 200 kGy, respectively), while for piperacillin only an increase can be observed at 100 kGy (Fig. 3). These observations indicate that degradation products formed from antibiotics also have some effect on the population, in addition to the expected effects of other degradation products. These results prompted us to perform experiments using only the synthetic wastewater matrix, as outlined in Fig. 1, Model System 2.

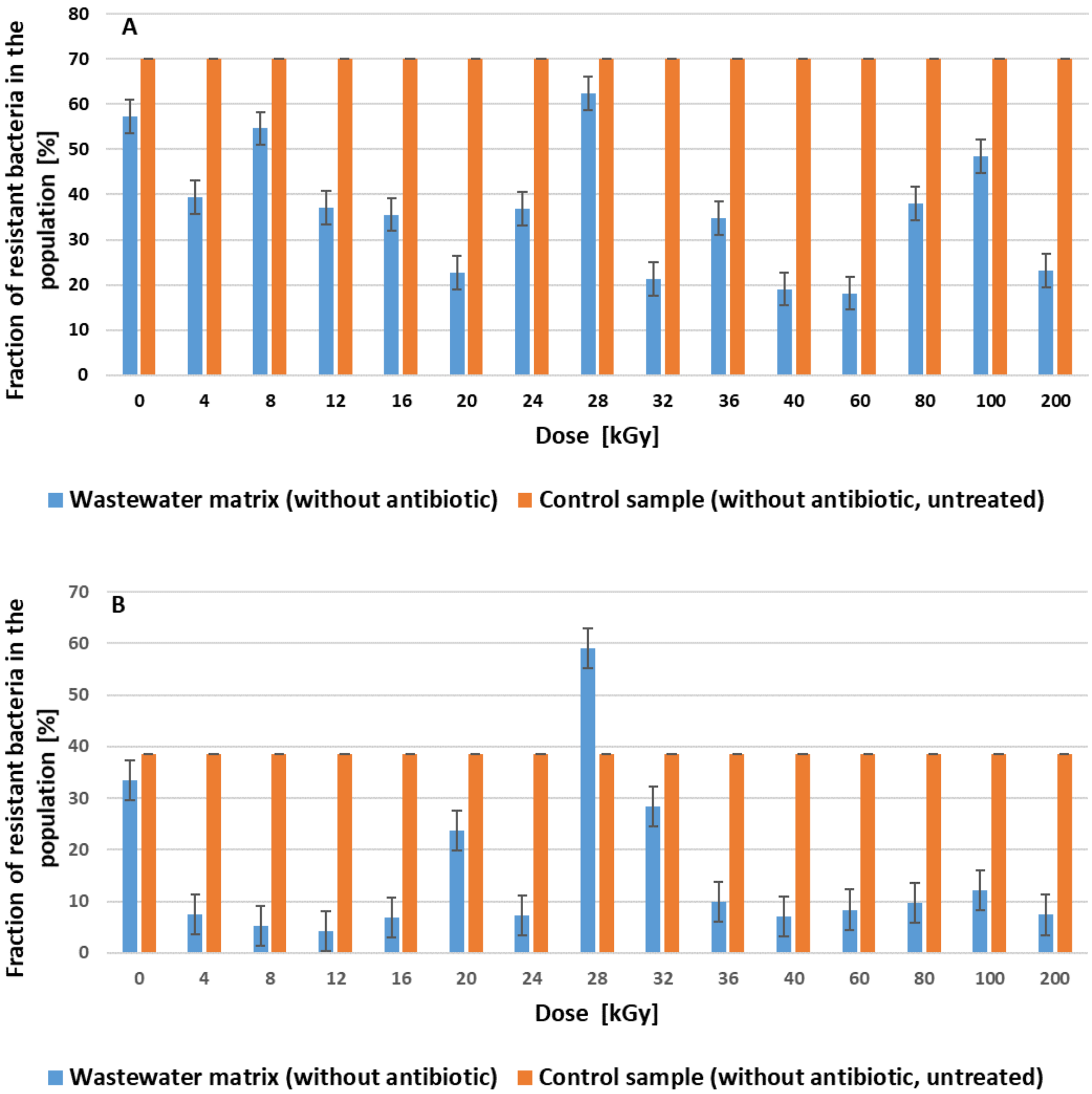

Fig. 4. Fraction of resistant bacteria in the population placed in a model wastewater matrix (no antibiotic added) and subjected to electron beam irradiation. Cell counting performed on plates containing (A) erythromycin or (B) piperacillin.

Fig. 4 shows the fraction of resistant bacteria when synthetic wastewater matrix is used without the presence of the antibiotic. Fig. 4A and 4B show the results of cell counts performed on plates containing erythromycin and piperacillin.

Comparing Fig. 4A and 4B, it is clear that higher resistant fractions can be obtained on plates containing erythromycin. This is due to the fact that the resistant strain shows some sensitivity to piperacillin. For piperacillin containing plates, it is important to keep this fact in mind when determining the cell number of the resistant subtype (I would mention that the data are still reliable because appropriate control samples were used). Furthermore, the tendency that can be seen on these figures (Fig. 4A and B) is very similar and obviously indicates that some degradation products following advanced oxidation treatment have an impact on the population dynamics of the bacterial culture.

Nevertheless, while in case of erythromycin in Fig. 2 quite different dose dependence can be seen (compared to Fig. 4), in case of the system with piperacillin (Fig. 3) the trend is very similar with increasing absorbed dose.

Fig. 4 and 3 shows that the fraction of resistant bacteria reaches the local maximum in the range of 24–32 kGy. We can also conclude that the increasing fraction of resistant bacteria at high absorbed doses (80–200 kGy) in Fig. 3 must be due to the products arising from piperacillin, this appears to hold also for erythromycin (Fig. 2, i.e. erythromycin degradation products might have an effect with increasing absorbed doses, except at 200 kGy). Therefore, in case of the piperacillin containing wastewater matrix (Fig. 3) one might anticipate that following EB treatment the products from the wastewater constituents other than the antibiotic determine mostly the shifting microbial population (besides prolonged treatment stages).

However, in the case of erythromycin (Fig. 2), it is difficult to explain rationally why the effect of degradation products from the matrix components cannot be clearly seen. In this case, more complex factors may affect the cellular response of resistant bacteria that cannot be explained by our current knowledge based on the available data. We will make further efforts to further study the cellular response in different settings.

Model wastewater matrix with antibiotics and bacteria added prior to EB treatment

The fraction of resistant bacteria in the population after irradiation is depicted in Fig. 5 ((Fig. 1, Model System 3–4). As the dose increases, the amount of bacteria continuously decreases. Bacteria are not capable of growing on agar plates after 5 kGy or higher absorbed dose.

Fig. 5 shows that resistant bacteria are significantly more sensitive to radiation treatment over the entire dose range (0.2–2 kGy) than sensitive bacteria when the corresponding antibiotic is present in the matrix. Fig. 6 represents the results in the samples without antibiotics.

In this case, the population of bacteria is not affected by low doses. Higher absorbed dose leads to significant distortions. This is due to the effect of the products formed from the components of the wastewater matrix on bacteria. Comparing the two figures (Fig 5A-B), the resistant bacteria show clearly higher sensitivity towards irradiation. This higher sensitivity does not appear in samples without antibiotics: the presence of the antibiotic makes the resistant subtype more sensitive to irradiation. This may be explained by some ongoing biological processes. The sensitive subtype is left intact at very low concentrations of the antibiotic. In contrast, the resistant subtype becomes more depleted, less prepared to the attack of free radicals. In addition, it can be recognized that a higher resistant bacterial fraction was obtained on plates containing piperacillin (Fig. 6B) compared to plates containing erythromycin (Fig. 6A). This observation may suggest that the resistant subtype that survived treatment in the effluent matrix eventually became more suitable for growing on piperacillin-containing plates.

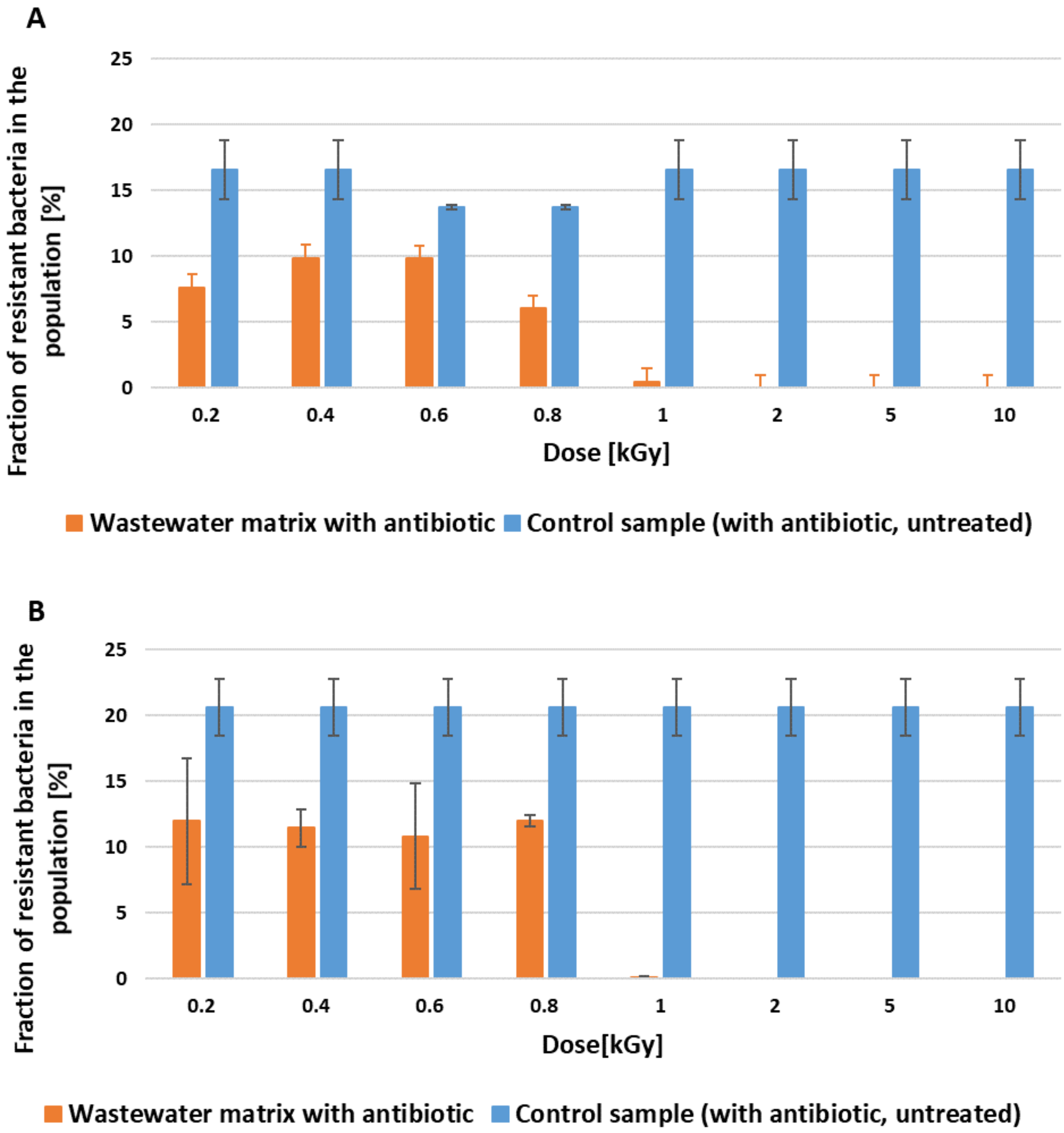

Fig. 5. Fraction of resistant bacteria in the population after EB treatment performed directly on a culture containing bacteria, wastewater matrix and either (A) erythromycin or (B) piperacillin at environmentally relevant concentrations (2 µg L^{-1}).

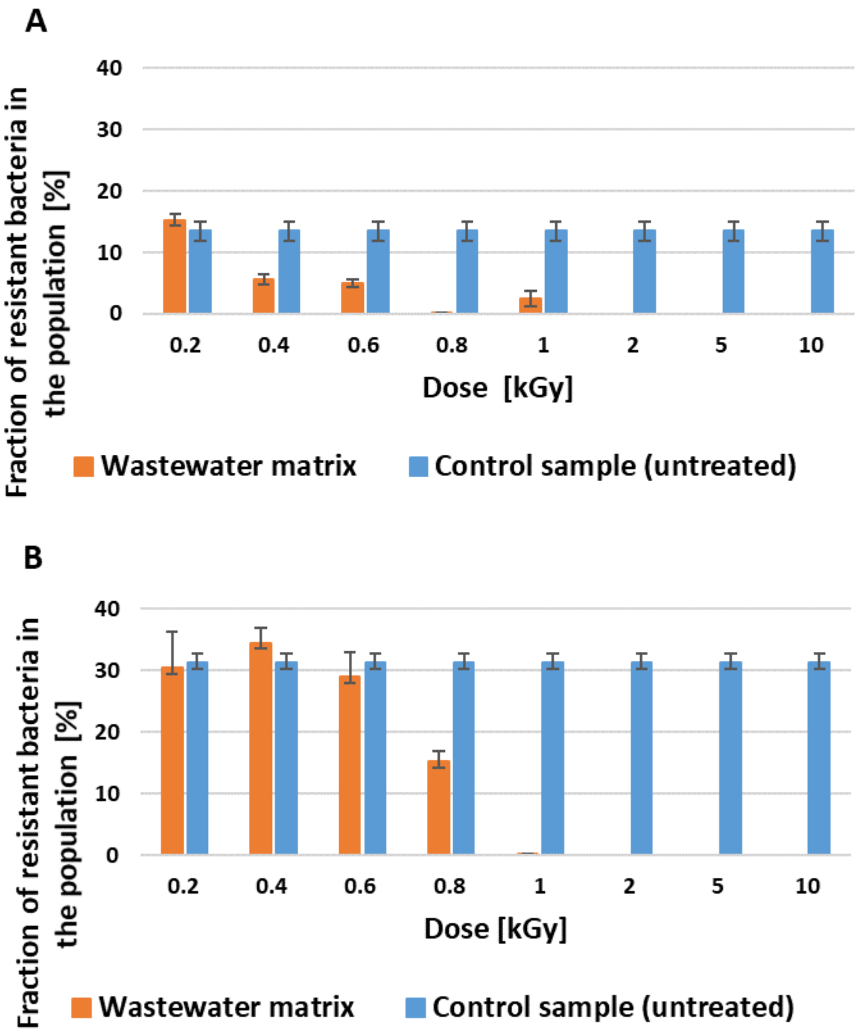

Fig. 6. Fraction of resistant bacteria in the population after EB treatment performed directly on a culture containing bacteria and wastewater matrix without antibiotics. Cell counting was performed on either (A) erythromycin or (B) piperacillin containing plates.

Conclusion

Based on our results, trace level of antibiotics does not bring about an advantage for the resistant bacteria in a mixed resistant / sensitive *Staphylococcus aureus* population. We assume, that trace levels of antibiotics initiate biological processes in the resistant bacteria from which they derive no benefit. Low concentrations of antibiotic have no effect on the sensitive subtype. Advanced oxidation treatment after optimisation can be a capable technique to eliminate these effects. Nevertheless, we also need to consider that the products from the effluent matrix also have an effect on the bacterial culture. Moreover, antibiotics present at trace amount in the wastewater matrix make resistant bacteria more sensitive towards the advanced oxidation treatment, which might be attributed again to the stimulation of biological processes disadvantageous for the resistant subtype under these conditions prior to the treatment.

Acknowledgements

The authors gratefully acknowledge the financial support of the International Atomic Energy Agency through the Coordinate Research Project F23033. This paper was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences. We also thank the National Collection of Agricultural and Industrial Microorganisms (NCAIM, Hungary) for kindly providing the bacterial strains.

References

[1] J. Farkas, Cs. Mohácsi-Farkas, Trends Food Sci. Tech., 22 (2011) 121.
[2] M. Spotheim-Maurizot, M. Mostafavi, T. Douki, J. Belloni, Rad. Chem. EDP Sciences, Paris (2008).
[3] International Atomic Energy Agency (IAEA), Radiation Processing: Environmental Applications, IAEA, Vienna (2007).
[4] P.H. Jones, D. Prasad, J. Water Pollut. Con. F, 40 (1968) R477.
[5] C. Praveen, P.R. Jesudhasan, R.S. Reimers, S.D. Pillai, Bioresour. Technol., 144 (2013) 652.
[6] R.L. Ward, J.G. Yeager, C.S. Ashley, Appl. Environ. Microb., 41 (1981) 1123.
[7] S.S. Bhatia, S.D. Pillai, Front. Microbiol., 10 (2019) 1.
[8] A.S.C. Hieke, PhD Thesis, Texas A&M University (2015).
[9] A.S.C. Hieke, S.D. Pillai, Front. Microbiol., 9 (2018) 1.
[10] L. Szabó, J. Szabó, E. Illés, A. Kovács, Á. Belák, Cs. Mohácsi-Farkas, E. Takács, L. Wojnárovits, Chem. Eng. J., 321 (2017) 314.
[11] L. Szabó, O. Gyenes, J. Szabó, K. Kovács, A. Kovács, G. Kiskó, Á. Belák, Cs. Mohácsi-Farkas, E. Takács, L. Wojnárovits, J. Ind. Eng. Chem., 58 (2018) 24.
[12] M.-C. Danner, A. Robertson, V. Behrends, J. Reiss, Sci. Total Environ., 664 (2019), 793
[13] N. Kraupner, S. Ebmeyer, J. Bengtsson-Palme, J. Fick, E. Kristiansson, C.-F. Flach, D.G.J. Larsson, Environ. Int., 116 (2018), 255
[14] S.V. Lundström, M. Östman, J. Bengtsson-Palme, C. Ritgersson, M. Thoudal, T. Sircar, H. Blanck, K.M. Eriksson, M. Tysklind, C.-F. Flach, D.G.J. Larsson, Sci. Total Environ., 553 (2016), 587
[15] X. Yi, C. Lin, E.J.L. Ong, M. Wang, B. Li, Z. Zhou, Environ. Pollut., 250 (2019), 437
[16] I. Reinholds, I. Pugajeva, I. Perkons, E. Lundanes, J. Rusko, G. Kizane, V. Nikolajeva, O. Mutere, Z. Petrina, L. Baumane, V. Bartkevics, Int. J. Environ. Sci. Technol., 14 (2017), 1969

IAEA
International Atomic Energy Agency

Recent Achievement on the Removal of Biohazardous Pollutants by Radiation

Report of the 3rd RCM for CRP F23033

Virtual

31 Jan – 4 Feb 2022

Working Material Produced by the International Atomic Energy Agency
Vienna, Austria, 2022