PREVENTING THE DEVELOPMENT OF ANTIBIOTIC RESISTANCE IN WASTEWATER MATRICES BY HIGH ENERGY IRRADIATION

R. HOMLOK[1], T. KOCSIS[2], K. KOVÁCS[1], G. KISKÓ[2], T. TÓTH[1], E. TAKÁCS[1], CS. MOHÁCSI-FARKAS[2], L. WOJNÁROVITS[1], L. SZABÓ[3]

[1]Institute for Energy Security and Environmental Safety, Centre for Energy Research, Konkoly-Thege Miklós út 29-33, H-1121 Budapest, Hungary

[2]Department of Microbiology and Biotechnology, Faculty of Food Science, Szent István University, Somlói út 14-16, H-1118 Budapest, Hungary

[3]National Institute for Materials Science, International Center for Young Scientists, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan

Abstract

Our project aimed at investigating the applicability of high energy irradiation methods to eradicate any impact that antibiotics have on microbial population in wastewater matrices. Over the course of our study, the effect of solutions containing synthetic wastewater matrix along with antibiotics on a resistant - sensitive mixed bacterial population is taken under scrutiny after electron beam irradiation. As a result, we show that by appropriately optimizing the dose requirement, electron beam treatment appears to be a promising method to eliminate the biological activity of antibiotics and thereby achieve similar microbial population dynamics as it is found without the presence of antibiotics. Our results take us closer to our ultimate goal, developing technologies to tackle the evolution of antibiotic resistant bacteria in water reservoirs contaminated with antibiotics.

1. Introduction

Nowadays, one of the most important challenges of environmental protection is preservation and improvement of water quality. One of the greatest problems is the continuous decrease and pollution of our water reservoirs. A wide range of pollutants is released into our natural waters through industrial and urban wastewater pipelines all over the world. From the aspect of water pollution, several pharmaceuticals, especially antibiotics have a very harmful effect on the living environment among these environmental contaminants. The usage of broad-spectrum antibiotics has increased significantly in the past decades. Entering our environment through production processes, expired or discarded products, or by natural biological selection antibiotics can accumulate in both drinking and wastewater. Conventional water and wastewater technologies are not effective in the removal of these micropollutants [1]. This can result in many adverse consequences for the environment: antibiotic residues may concentrate in plants and animals; integrate into the living organisms and contribute to the spread of antibiotic resistance, which presents a global problem. As to demonstrate the escalated nature of this issue, it appears that more than 90% of the bacterial strains from seawater are resistant to more than one and a fifth of them to at least five antibiotics [2].

During development of antibiotic resistance several genetic processes occur in bacteria thereby microbes can acquire resistance against antibiotics. As a result, resistant or even multi-resistant microorganisms have emerged. Microbes are able to pass these resistant genes to related or even non-related species by horizontal gene-transfer that permits the rapid spread of resistance [3].

The spread of resistant and multi-resistant bacteria and genes poses a serious risk to humanity. One of the seedbeds for the development of resistant bacteria and genes is the reservoirs of municipal wastewater treatment plants. Antibiotics, resistant genes, and bacteria can enter the natural water through purified

wastewater, which means additional threats. The officially established quality requirements for purified wastewater do not cover the measurement and control of antibiotic concentrations. However, this issue should not be ignored and more regulations will be needed on antibiotic restriction in the near future. In our research, we focus on two goals: the abatement of antibacterial activity in wastewater by applying appropriate technology and the development of an adequate analytical method with high reliability.

Ionizing radiation can be a suitable technique for reducing the antibacterial activity in purified communal wastewater and inactivating bacteria and genes in effluent water. The advantage of the technique is that it does not require any additives and the presence of floating particles do not interfere the reactions taking place in water. Additionally, it is worth noting that this method can be easily fit into the existing technology process (flow system). With the practical application of research results the probability of the antibiotic resistance development can be reduced.

2. Description of the work

In a previous work [4] we have developed a microbiological assay to assess the effects of transformation products of certain antibiotics in course of electron beam treatment on the population dynamics of a resistant and sensitive *Staphylococcus aureus* mixed bacterial population.

The present work aims to answer the question whether the electron beam treatment is able to reduce effectively or eliminate completely the biological effects of erythromycin and piperacillin on bacteria in a complex wastewater matrix. While in our previous work, the antibiotics were irradiated in dilute aqueous solutions, in the present work the treatment is done in a model wastewater matrix, in order to have more insight about a real scenario. We get a more detailed picture whether this treatment can reduce the possibility of the development of antibiotic resistance through the removal of the biological activity of antibiotics from the wastewater. We are looking for the absorbed dose requirement that could eliminate the effect of antibiotics on microorganisms in a model wastewater, so that we could reduce the possibility for the development of resistance to the given antibiotic in the given matrix.

3. Experimental

3.1. Compounds investigated

Erythromycin (CAS Registry No. 114-07-8), piperacillin sodium salt (CAS Registry No. 59703-84-3), and humic acid (CAS Registry No. 1415-93-6) were obtained from Sigma-Aldrich (St. Louis, MO). Inorganic constituents of the assay medium such as $NaHCO_3$, K_2HPO_4, $MgSO_4$, and $(NH_4)_2SO_4$ were purchased from Reanal (Budapest, Hungary). Purified water was prepared with an Adrona B30 system (Adrona, Riga, Latvia).

In the microbiological experiments, sodium chloride (catalog no. 1.06404.1000), peptone (catalog no. 1.11931.1000), and bacteriological agar (catalog no. 1.01615.1000) were from Merck (Darmstadt, Germany). Trypto-casein soy broth (CASO, product BK046HA) was purchased from Biokar Diagnostics (Solabia Group, Pantin, France).

For the irradiation, we prepared solutions 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.

3.2. Experimental methods

For the irradiation, we used a vertically mounted Tesla Linac LPR-4 type linear electron accelerator, which delivers short pulses of electrons with 4 MeV energy, 800 ns duration and 50 Hz repetition frequency. We applied a wide range of treatment times to provide an increasing absorbed dose.

Microbiological assay

We chose *Staphylococcus aureus* as test microorganism. This Gram-positive bacterium can take part in several types of horizontal gene transfer events (HGT) [5, 6, 7, 8, 9, 10], thereby, it is a good candidate for screening the development of resistance in a mixed bacterial population. Therefore, this species may provide an appropriate approach reflecting a worst case scenario according to which an advanced oxidation process should be optimized.

The test is based on the population dynamics of a mixed bacterial culture in response to the presence of antibiotics in a concentration range well below the minimum inhibitory concentration (MIC) in a synthetic wastewater matrix. We added sensitive and resistant subtypes of *Staphylococcus aureus* in a 1:1 ratio to the test medium (antibiotics were irradiated in a model wastewater matrix) and determined the fraction of resistant mutants after incubation for 24 hours by simple colony counting.

Test medium

We determined the total colony count (sensitive + resistant) on agar plates including sterile water, 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 minimum inhibitory concentration (MIC).

The selection of the test medium has a key role in the experiments. In conventional antimicrobial testing methods the nutrient broth facilitates bacterial growth. However, limited sources are available for bacteria in the case of wastewater matrix that would presumably lead to appreciable distortion from real wastewater samples. Especially, enhanced bacterial growth may intensify the development of resistance by increasing the probability of *de novo* gene mutation events and exchange of genetic information, which cannot be the case in the environment. The chosen culture medium reflects a real wastewater sample [4].

Model wastewater matrix: We used humic acid to represent the dissolved organic carbon content (DOC) of a real wastewater matrix [5, 6]. We also applied the natural alkalinity condition by adding the corresponding amount of $NaHCO_3$ into the medium. Furthermore, the following inorganic constituents were also applied: 7.1 ppm (0.05 mM) $(NH_4)_2SO_4$, 7 ppm (0.04 mM) K_2HPO_4, and 0.71 ppm (2.88 µM) $MgSO_4 \cdot 7H_2O$ [7].

A schematic representation for preparing the medium is shown in Fig. 1.

Cell Counting

We performed colony counting by preparing dilution series of the assay medium and measuring equal amount from each member of the dilution series on trypto-casein soy broth (CASO) agar plates. After spreading (100 µL) the sample evenly on the surface, we incubated the plates at 37 °C for 24 h. To determine the colony count for the resistant bacteria, molten agar was spiked with erythromycin and piperacillin. To prevent thermal degradation of the antibiotic before spiking of the antibiotic solution, the molten agar was cooled to 40 °C and after homogenization it was poured on the plates immediately. Only resistant cells grow on the surface of the agar plates containing the antibiotic at a concentration above the MIC. The total colony count (sensitive + resistant) was determined on agar plates containing no antibiotics.

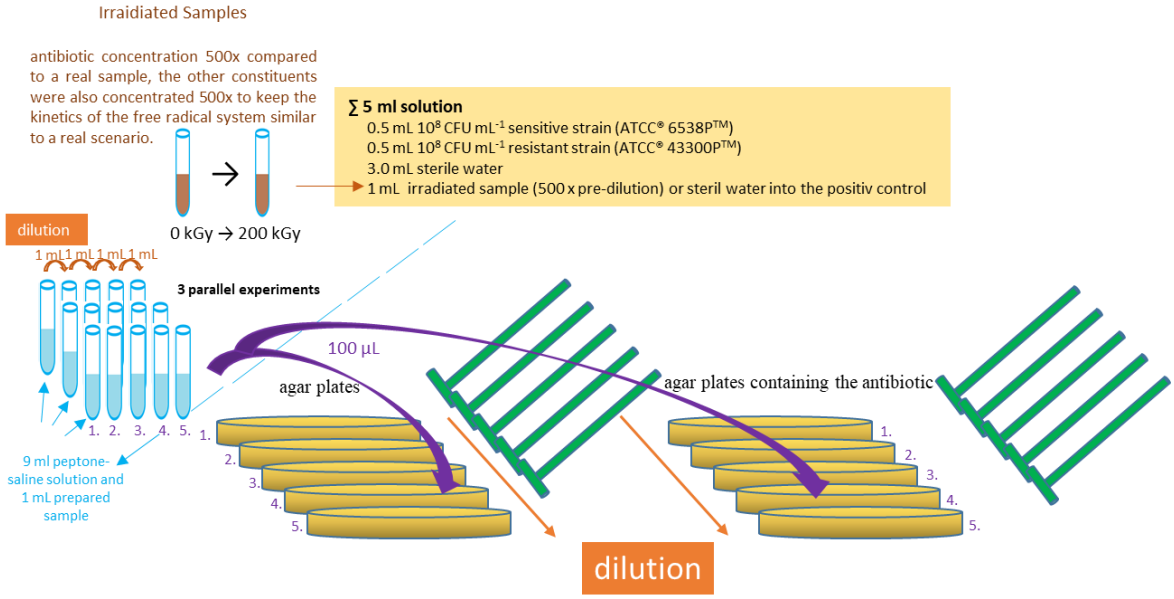

Fig. 1. Schematic representation for the preparation of the medium and colony counting.

In the first step, after an appropriate incubation period, I counted the number of colonies on Petri dishes. The yellow colonies were the resistant subtype, while the white colonies belonged to the sensitive subtype. This color difference allowed the two types of strains to be clearly distinguishable with the naked eyes when counting.

As part of the experiment I counted the number of resistant cells on the antibiotic-containing plate and the total number of cells on the control plate without antibiotic, then I calculated the average of them and determined the fraction of resistant strains compared to all the colonies with both antibiotics (erythromycin and piperacillin) and their associated controls.

4. Results of the microbiological assay

The effect of the subinhibitory level of antibiotics has been the subject of high scientific interest, since several studies pointed out that even very low level of antibiotic concentrations can select for antibiotic resistance and can have an impact on the population dynamics of the microbial community [11,12]. Fig. 2A shows the fraction of resistant bacteria after subinhibitory level of erythromycin (0.125 µg mL^{-1}, 4-times below the MIC for the sensitive *S. aureus* strain) is introduced into the system (after 24 h incubation time at 30 °C). It is clear that even at this low concentration, the resistant subtypes are significantly enriched in the population. In order to eliminate this biological effect, we investigated the applicability of electron beam irradiation. The irradiation was done on solutions containing 0.5 mM (0.37 g L^{-1}) erythromycin or piperacillin (0.26 g L^{-1}) in order to be able to work in a convenient dose range for the accelerator, and the model system with the bacteria was spiked with an appropriate amount of the treated solution similarly to the untreated sample. After irradiation the solutions were 500 times diluted.

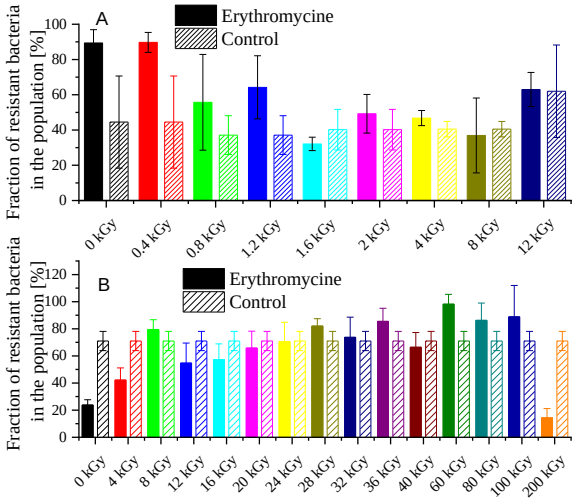

Fig. 2. (a) Fraction of resistant bacteria in a population that was spiked with erythromycin sample irradiated in pure aqueous solution. (b) fraction of resistant bacteria in a population that was spiked with a model wastewater matrix sample containing erythromycin and subjected to electron beam irradiation.

It appears from Fig. 2A that relatively low absorbed dose is not sufficient to eliminate the effect of erythromycin on the bacterial population, at 0.4 kGy there is not much change in the fraction of resistant bacteria compared to the control sample. Since at 1.6 kGy there can be still a significant difference observed between the control and antibiotic containing sample, we might conclude that at ~1.6 kGy we can reach a dose that might be considered appropriate for eliminating the biological effect of erythromycin. In respect to piperacillin (also 0.125 µg mL^{-1} concentration, 8-times below the MIC for the sensitive *S. aureus* strain), it appears that a higher dose is necessary to reach an appropriate treatment stage where there is definitely no significant difference between the antibiotic containing and control sample (Fig. 3A). The minimum dose requirement in this system was chosen to be 4 kGy. It was shown in our previous studies that in case of penicillin derivatives several products form that can retain the biological activity of the original molecule [15]. This phenomenon might lie behind the higher dose requirement in case of piperacillin compared to erythromycin.

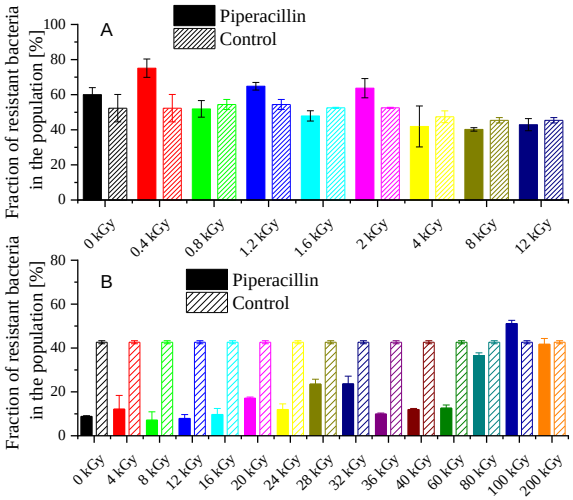

Fig. 3. (a) Fraction of resistant bacteria in a population that was spiked with piperacillin sample irradiated in pure aqueous solution. (b) fraction of resistant bacteria in a population that was spiked with a model wastewater matrix sample containing erythromycin and subjected to electron beam.

When irradiation is done in a complex wastewater matrix, markedly different system is generated, which can eventually give rise to considerably different outcomes. The fraction of resistant bacteria is shown in Fig. 2B and 3B for erythromycin and piperacillin, respectively, after incubating the system at 30 °C for 24 h. In this case the final concentration of the system with the bacteria was adjusted to be equal to a model synthetic wastewater sample applied in our previous studies [4,13,14]. The final antibiotic concentration was 2 μg L^{-1}, which was considered as a realistic level in wastewater samples based on a previous study [16]. Surprisingly, while in Fig. 2A the resistant bacteria dominates the population at 0 kGy when even lower antibiotic concentration is applied in pure water, the resistant bacteria seem to becomes the population dominated by the sensitive strain in the model wastewater (Fig. 2B). While in our first experiment (Fig. 2A) 125 μg L^{-1} concentration is applied (4 and 8-times below the MIC for the sensitive *S. aureus* strain for erythromycin and piperacillin, respectively), in the second experiment (Fig. 2B and Fig. 3B) the concentration is 2 μg L^{-1} (250 and 500 -times below the MIC for the sensitive *S. aureus* strain for erythromycin and piperacillin, respectively). This phenomenon is very interesting, and might be due to a substantial fitness cost of carrying the *mecA* gen and expression of the altered penicillin binding proteins in response to the antibiotic present (for the resistant *S. aureus* strain, see [14]), while the sensitive strain remains unaffected and has no such a fitness cost, eventually giving a competing advantage under such circumstances. As the samples are irradiated it appears that after ~8 kGy absorbed dose the bacterial population is unaffected in case of erythromycin. Nevertheless, too high absorbed dose seems to be again disadvantageous presumably due to some products forming from the humic acid. This matter will be further investigated through control experiments using only synthetic wastewater matrix. Furthermore, in case of piperacillin we can see that the population is dominated by the sensitive strain until 60 kGy, this phenomenon might be explained again in terms of substantial fitness costs of being resistant. As we have mentioned before, in case of piperacillin the forming products can also retain some antimicrobial activity, which was investigated in our previous study [15]. At higher doses again we assume that probably products from humic acid exhibits some effect on the population, as we can see in the case of piperacillin solutions (Fig. 3B), the population becomes dominated by the resistant mutant.

It must be noted that the absorbed doses are shown for the concentrated sample, and in fact for a real scenario these values should be divided by 500 (500x concentrated solutions were applied), and those dose requirements must be tested on a real scenario.

It should also be mentioned that although at first sight it appears to be delighting that the amount of resistant subtypes are suppressed, it must be recognized that they are under continuous effect of the antibiotic and this circumstance might lead to slow but further mutation events as it was shown in other cases [17].

5. Summary

While there are many studies focusing on the elimination of the antimicrobial activity of different antibiotics, there is a lack of knowledge about their subinhibitory effects, which is actually the case in wastewater samples and in the environment. Previously, we developed a microbiological assay to monitor the biological effect of subinhibitory level of antibiotics on resistant – sensitive bacterial cultures. As a continuation of our work, we applied electron beam irradiation to eradicate any effect the antibiotics have on these mixed microbial populations. Our study started with a simplified system using pure aqueous solutions of the antibiotic, followed by experiments on a more complex synthetic wastewater matrix. This step-by-step approach allowed us to have more understanding on the population dynamics of the chosen sensitive-resistant bacterial culture, adding to the continuing progress in this scientific field. Furthermore, we have also identified dose requirements important for the application of electron beam treatment to tackle the evolution of antibiotic resistance in wastewater matrices. As a next step, we are planning to further deepen our knowledge about the population dynamics in response to the presence of antibiotics, and apply even more complex systems with bacteria present during the irradiation. The findings in this study support

our ultimate aim to realize the implementation of electron beam treatment to tackle antibiotic resistance development in the environment.

6. Future plans

We have developed a microbiological assay to assess the effects of transformation products of electron beam treatment on the population dynamics of a resistant and sensitive mixed bacterial population. Using this method we plan two series of experiments, in one of them wastewater matrix with bacteria will be used, in the other wastewater-matrix with antibiotic and bacteria. In these cases, we will add bacteria to the samples prior to irradiation.

Our plan to measure the emergence and transmission of antibiotic resistance in the bacteria cell by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS). The instrument allows antimicrobial susceptibility testing which provides rapid differentiation between resistant and susceptible strains regardless of the microbial species, antimicrobial agent in question, or underlying resistance mechanism. The technique is a relevant tool for the detection of antibiotic resistance and opens new avenues for both clinical and experimental microbiology. In addition, understanding dispersal barriers is not only key to evaluate risks, but also to prevent resistant pathogens, as well as novel resistance genes, from reaching humans.
The future aim: methods will be elaborated for measuring the concentration of the bacteria in the wastewater effluent, and for measuring both the concentration and the antimicrobial activity of the antibiotics. The dose required to eliminate specific antibiotic contaminants will be established and appropriate methodologies will be developed for the treatment of bio-hazards in irradiated wastewater using electron beam accelerators.

7. References

[1] Kümmerer, K. Antibiotics in the aquatic environment – A review – Part I. Chemosphere. 75, 417-434 (2009)
[2] Baquero, F., Martínez, J.L., Cantón, R. Antibiotics and antibiotic resistance in water environments. Current Opinion in Biotechnology 19, 260-265 (2008)
[3] Pesti, M. (szerk) Általános mikrobiológia. Dialóg Campus Kiadó, Budapest
[4] Szabó, L., Steinhardt, M., Homlok, R., Kovács, K., Illés, E., Kiskó, G., Belák, Á., Mohácsi-Farkas, Cs., Takács, E., Wojnárovits, L. A Microbiological Assay for Assessing the Applicability of Advanced Oxidation Processes for Eliminating the Sublethal Effects of Antibiotics on Selection of Resistant Bacteria, Environ. Sci. Technol. Lett. 4, 251-255 (2017)
[5] Enright, M.C., Robinson, D.A., Randle, G., Feil, E.J., Grundmann, H., Spratt, B.G. (2002): The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). Proc. Natl. Acad. Sci. U. S. A. 99, 7687-7692 (2001)
[6] World Health Organization. Antimicrobial Resistance: Global Report on Surveillance. WHO Press, Geneva (2014)
[7] Kay, E., Vogel, T.M., Bertolla, F., Nalin, R., Simonet, P. In situ transfer of antibiotic resistance genes from transgenic (transplastomic) tobacco plants to bacteria. Appl. Environ. Microbiol. 68, 3345-3351 (2002)
[8] Spinks, J.W.T., Woods, R.J. An Introduction to Radiation Chemistry, third ed., John Wiley & Sons, New York, 1990.
[9] Buxton, G.V., Greenstock, C.L., Helman, W.P., Ross, A.B. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals ($^{\bullet}OH/^{\bullet}O^{-}$) in aqueous solution, J. Phys. Chem. Ref. Data 17, 513-886 (1988)
[10] Martin, M.V., Ruiz, G.T., Gonzalez, M.C., Borsarelli, C.D., Mártire, D.O. Photolytic and radiolytic oxidation of humic acid, Photochem. Photobiol. 88, 810-815 (2012)

[11] Gullberg, E., Cao, S., Berg, O.G., Ilback, C., Sanfegren, L., Hughes, D., Andersson, D.I. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 7, e1002158 (2011)
[12] Sandegren, L. Low sub-minimal inhibitory concentrations of antibiotics generate new types of resistance. Sustain. Chem. Pharm. 11, 46-48 (2019)
[13] Szabó, L., Szabó, J., Illés, E., Kovács, A., Belák, Á., Mohácsi-Farkas, Cs., Takács, E., Wojnárovits, L. Electron beam treatment for tackling the escalating problems of antibiotic resistance: eliminating the antimicrobial activity of wastewater matrices originating from erythromycin, Chem. Eng. J. 321, 314-324 (2017)
[14] Szabó, L., Gyenes, O., Szabó, J., Kovács, K., Kovács, A., Kiskó, G., Belák, Á., Mohácsi-Farkas, Cs., Takács, E., Wojnárovits, L. Electron beam treatment for eliminating the antimicrobial activity of piperacillin in wastewater matrix. J. Ind. Eng. Chem. 58, 27-32 (2018)
[15] Szabó, L., Tóth, T., Engelhardt, T., Rácz, G., Mohácsi-Farkas, Cs., Takács, E., Wojnárovits, L. Change in hydrophilicity of penicillins during advanced oxidation by radiolytically generated OH compromises the elimination of selective pressure on bacterial strains. Sci. Total Environ. 393-403, 551-552 (2016)
[16] Xu, W., Zhang, G., Li, X., Zou, S., Li, P., Hu, Z., Li, J. Occurrence and elimination of antibiotics at four sewage treatment plants in the Pearl River Delta (PRD), South China, Water Res. 41, 4526-4534 (2007)
[17] Wistrand-Yuen, E., Knopp, M., Hjort, K., Koskiniemi, S., Berg, O.G., Andersson, D.I. Evolution of high-level resistance during low-level antibiotic exposure. Nat. Commun. 9, 1-12 (2018)

Recent Achievements on the Removal of Biohazardous Pollutants by Radiation

REPORT OF THE 2ND RESEARCH COORDINATION MEETING

National Center for Nuclear Science and Technology, Tunis, Tunisia

2-6March 2020

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Vienna, Austria, 2020

OUTLINE

1. ***INTRODUCTION AND OBJECTIVES OF THE MEETING***
2. ***HIGHLIGHTS OF THE MEMBER STATES PRESENTATIONS***
3. ***DISCUSSION***
4. ***CONCLUSIONS***
5. ***RECOMMENDATIONS***
6. ***ANNEX I- LIST OF PARTICIPANTS***
7. ***ANNEX II- AGENDA***
8. ***ANNEX III –FULL COUNTRY REPORTS***