EFFECT OF ELECTRON BEAM IRRADIATION AND THE PRESENCE OF ANTIBIOTICS ON THE POPULATION RATIO OF RESISTANT/SENSITIVE BACTERIAL CULTURES IN MODEL WASTEWATER MATRIX WITH ANTIBIOTICS AND BACTERIA ADDED PRIOR TO ADVANCED OXIDATION TREATMENT

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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 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 more 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

Experiments (Fig. 1) were done in the presence of bacteria added to the wastewater matrix prior to the advanced oxidation treatment. In this case the original synthetic wastewater matrix was prepared with or without the antibiotic (non-concentrated samples).

The electron beam (EB) treatment was performed using a Tesla Linac LPR-4 type linear electron accelerator. Our method is based on a microbiological assay we developed 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 (in the form of 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}).

The fraction of resistant bacteria in the population after irradiation is depicted in Fig. 1. The concentration of the antibiotic in the effluent matrix is 2 µg L^{-1}. 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. 1 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. Figure 2 represents the results in the samples without antibiotics.

In this case, the population of bacteria is not affected by low doses. Longer treatment time 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 1A-B), the resistant bacteria show clearly higher sensitivity towards irradiation. This higher sensitivity does not appear in samples without antibiotics. This leads to the conclusion that that 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. 2B) compared to plates containing erythromycin (Fig. 2A). 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.

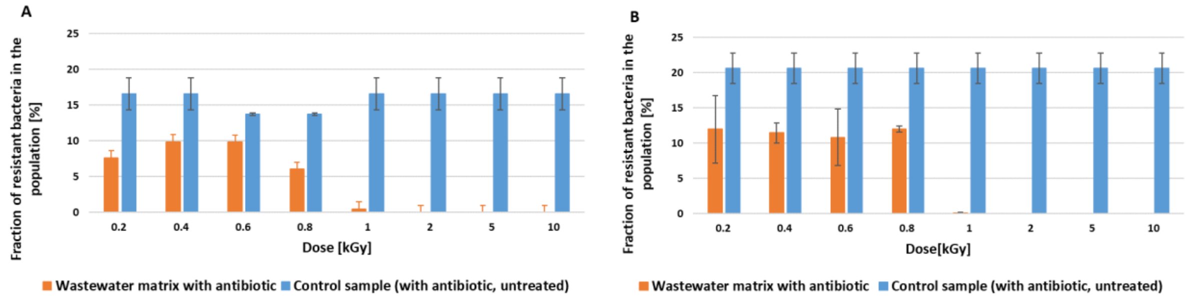

Fig. 1. Fraction of resistant bacteria in the population after advanced oxidation treatment performed directly on a culture containing bacteria, wastewater matrix and either (A) erythromycin or (B) piperacillin at environmentally relevant concentrations (2 μg L^{-1}).

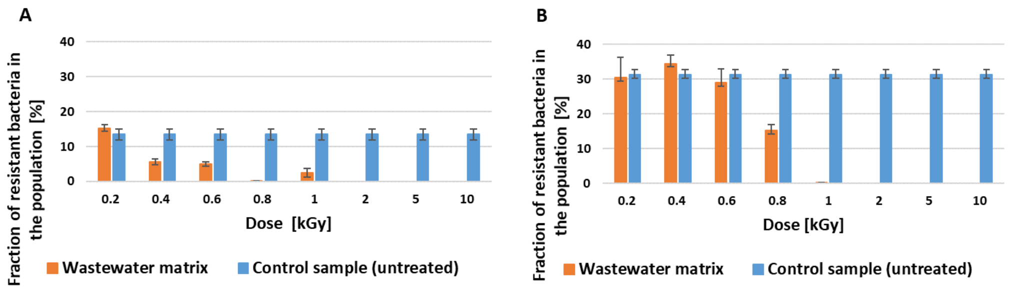

Fig. 2. Fraction of resistant bacteria in the population after advanced oxidation treatment performed directly on a culture containing bacteria and wastewater matrix without antibiotics. Cell counting was performed on either (A) erythromycin or (B) piperacillin containing plates.

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. According to our assumptions, it initiates biological processes in the resistant bacteria from which they derive no benefit. This amount of antibiotic has 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 disadvantageous biological processes in the resistant subtype under these conditions prior to the treatment.

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