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A Microbiological Assay for Assessing the Applicability of Advanced Oxidation Processes for Eliminating the Sublethal Effects of Antibiotics on Selection of Resistant Bacteria

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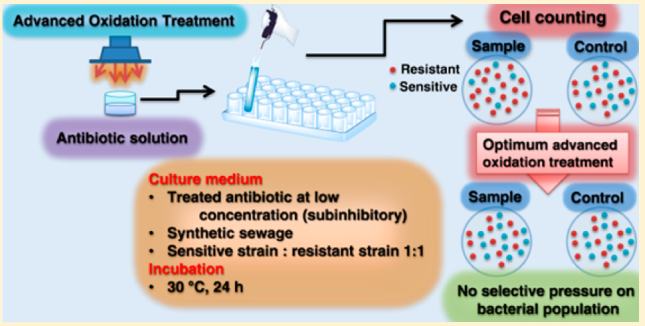
Supporting Information

ABSTRACT: Subinhibitory levels of antibiotics in reservoirs highly affected by anthropogenic activity, e.g., wastewater treatment plants, have a profound impact on the development and spread of resistant bacteria in the biosphere. As an aid, advanced oxidation processes (AOPs) have been suggested to eliminate the antibacterial activity of several antibiotics, and this activity has been followed using conventional antibiotic susceptibility tests. While the antibacterial activity can hardly be monitored below the minimum inhibitory concentration (MIC) with these assays, the selective pressure on a bacterial population might remain in this concentration range. To assess the applicability of an AOP for eliminating the subinhibitory effects of antibiotics on selection of resistant bacteria, a microbiological assay is introduced. The test is based on the dynamics of a mixed bacterial population in response to the presence of antibiotics in a concentration range well below the MIC in a synthetic wastewater matrix. Sensitive and resistant subtypes of *Staphylococcus aureus* in a 1:1 ratio are added to the test medium, and the fraction of resistant mutants is determined after incubation for 24 h by simple colony counting. By using electron beam irradiation as an AOP, we show that the assay provides a simple tool for determining the optimal treatment stage.



INTRODUCTION

The emergence of resistant pathogens has long overshadowed the success of medicine in the antibiotic era.[1] While the occurrence of resistance is ancient, it is in fact the excessive use of antibiotics that has precipitated this phenomenon.[2] On an evolutionary time scale, there is no doubt that resistant bacteria challenge human domination; however, the odds might be changed by taking appropriate actions in several fields, including the environmental framework.[3]

The widespread use and misuse of antibiotics has established a situation in which environmental bacteria are continuously exposed to subinhibitory concentrations of antimicrobial agents. It is now recognized that this low level of exposure has several impacts on cell physiology, eventually promoting the evolution and spread of antibiotic resistance within a wide range of microorganisms.[4] The phenomenon is even more pronounced in reservoirs highly affected by anthropogenic activity, including wastewater treatment plants.[5,6] It is of utmost importance to avoid the release of antimicrobial agents, resistant bacteria, and the corresponding genetic information from these hot spots to receiving water bodies.[7]

For this purpose, implementation of advanced oxidation technologies to treat the effluent wastewater has received considerable attention. In particular, elimination of the antibacterial activity of several antibiotics has been extensively studied by applying advanced oxidation treatment.[8−16] In these papers, the antibacterial activity was monitored using growth inhibition assays that assess the effect of rather high antibiotic concentrations. The effects of sublethal antibiotic levels also need to be tested as they are relevant to real environmental conditions. From the point of view of the development of resistance, one should further probe the efficiency of the treatment in eliminating any biological activity of the antibiotic

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that gives rise to the dominance of resistant species in a bacterial population. We propose this type of microbiological assay as an addition to the existing methods.

The dynamics of a bacterial population containing the resistant and sensitive subtypes of the same microorganism exposed to very low antibiotic concentrations has been studied.[17] Inspired by this work, we created a simple microbiological assay that can be used to assess the applicability of a certain treatment to eliminate the selective pressure of model antibiotics on a mixed (resistant-sensitive) bacterial population.

MATERIALS AND METHODS

Materials. 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).

Advanced Oxidation Treatment. Erythromycin and piperacillin stock solutions (0.5 mM each) were prepared and irradiated using a vertically mounted Tesla Linac LPR-4 type (Tesla V. T. Mikroel, Praha, Czech Republic) linear electron accelerator (Centre for Energy Research, Budapest, Hungary). The experimental setup has been described in detail in our previous work.[16] The absorbed dose is given in kilograys; 1 kGy is equal to 1 kJ kg^{-1}. As seen for other AOPs, in radiolysis mainly $^{\bullet}OH$ induces chemical degradation.[18] The total concentration of $^{\bullet}OH$ that was introduced into the solution within a certain time period can easily be calculated as described in our previous work.[16] Because other AOPs are also based on the reaction of $^{\bullet}OH$, this allows us to quantitatively link these processes.

Sample Analysis. The residual erythromycin concentration after the treatment was followed by a high-performance liquid chromatography−diode array detection method (limit of quantification of 0.6 μM); the details of the analysis have been published elsewhere, and they are also included in the Supporting Information.[16] The remaining piperacillin concentration was determined by high-performance liquid chromatography−mass spectrometry analysis (limit of quantification of 0.05 μM), and the details are included in the Supporting Information.

Bacterial Strains. *Staphylococcus aureus* was chosen as the test microorganism. This bacterium has acquired resistance at a tremendous rate, leading to the so-called "superbug" classification that denotes strains with multiple types of resistance.[19] Because it is one of the most important human pathogens with a wide resistance profile, it has long attracted the attention of many scientists.[20] It is also favorable that this Gram-positive bacterium can take part in several types of horizontal gene transfer events,[21] thereby being a good candidate for screening the development of resistance in a mixed bacterial population. Therefore, it is thought that this species provides an appropriate approach to reflecting a worst case scenario according to which an advanced oxidation process should be optimized.

To monitor the change in antibacterial activity in our previous work,[16] we selected sensitive and resistant *S. aureus* isolates (National Collection of Agricultural and Industrial Microorganisms, NCAIM, Szent István University) and also determined their resistance profile. The sensitive strain (ATCC 6538P) is approved by the Food and Drug Administration (FDA) for susceptibility testing, while the resistant one (ATCC 43300) is a methicillin-resistant *S. aureus* (MRSA) with known resistance against erythromycin and oxacillin and moderate susceptibility to piperacillin.[16,22] (It is important to mention that these strains need a Biosafety Level 2 laboratory according to the U.S. Public Health Service Guidelines, and it is the responsibility of the users to adhere to the biosafety regulations being enforced in their own country.) The minimum inhibitory concentrations (MICs) as a measure of the susceptibility of the sensitive strain have been determined to be 0.5 μg mL^{-1} (0.68 μM) and 1 μg mL^{-1} (1.93 μM) for erythromycin and piperacillin, respectively. Furthermore, the resistant strain has a MIC of 64 μg mL^{-1} (123.66 μM) for piperacillin, while it is fully resistant against erythromycin. The MIC values have been determined with the broth microdilution method as described previously.[16]

Inoculum. Inocula were prepared from an overnight culture (incubated at 37 °C) in the case of the sensitive *S. aureus* strain, and in the case of the resistant one, the freshly passaged cells were incubated for 72 h (at 37 °C) prior to preparation of the bacterial suspension. This incubation time is sufficient to yield a culture containing dead cells and released genetic information, which is then available for the sensitive cells in some cases to acquire resistance via horizontal gene transfer. This action was taken to reflect environmental conditions.

Bacterial suspensions were prepared from each strain at a concentration of 10^8 colony-forming units (CFU) per milliliter, and appropriate amounts were added to the medium to reach 10^7 CFU mL^{-1} with respect to the sensitive and resistant strains (1:1 ratio of resistant to sensitive cells).

Test Medium. When the medium for the assay was considered, we decided not to follow conventional antimicrobial testing methods in which nutrient broth is used to facilitate bacterial growth, because this would presumably lead to appreciable distortion from real wastewater samples where limited sources are available for bacteria. In particular, enhanced bacterial growth is expected to intensify the development of resistance by increasing the probability of *de novo* gene mutation events and exchange of genetic information at a rate not expected to occur in the environment. The culture medium was, therefore, rather a reflection of a real wastewater sample.

Humic acid from Sigma-Aldrich was taken to represent the dissolved organic carbon content (DOC) of a real wastewater matrix. Alternatively, one can also use a carbon source from the International Humic Substances Society (St. Paul, MN); however, this would inevitably increase the cost of the measurements. The amount of humic acid (~44% carbon content)[23] was equal to the average 7 ppm DOC of 28 effluent samples from the study of Keen et al.[24] The natural alkalinity was reported to be 105 ppm as $CaCO_3$ in this work, and we also applied this condition by measuring the corresponding amount of $NaHCO_3$ ($[HCO_3^-]$ = 0.97 mM) into the medium. Furthermore, the following inorganic constituents were also applied according to Seo et al.:[25] 7.1 ppm (0.05 mM)

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$(NH_4)_2SO_4$, 7 ppm (0.04 mM) K_2HPO_4, and 0.71 ppm (2.88 μM) $MgSO_4 \cdot 7H_2O$. The stock solutions were sterile filtered using hydrophobic PTFE syringe filters with a 0.22 μm pore size (Labex, Budapest, Hungary).

The untreated antibiotic sample was added to the medium to produce a concentration of 0.125 μg mL^{-1} (0.17 μM erythromycin and 0.24 μM piperacillin), being 4 and 8 times below the MIC of erythromycin and piperacillin, respectively, for the sensitive strain. The same dilution factor was then used for the treated samples. Positive controls were prepared by adding sterile water instead of the antibiotic sample.

Solutions (5 mL) were prepared in sterile test tubes, and three parallel experiments were performed. The test tubes were incubated at 30 °C for 24 h (incubation time also extended to 48 and 72 h) but not shaken. This temperature falls within the optimal range for biological activity in wastewater treatment plants.[26] All the microbiological work was performed under aseptic conditions. A schematic representation for the preparation of the medium is shown in Figure S1.

Cell Counting. Colony counting was performed by preparing dilution series from 1 mL of the assay medium and measuring 100 μL from each member of the dilution series on trypto-casein soy broth (CASO) agar plates (20 mL of agar in each plate). After the sample had been evenly spread on the surface, the plates were incubated at 37 °C for 24 h. To determine the colony count for the resistant bacteria, molten agar was spiked (using the sterile filters mentioned above) with erythromycin and piperacillin, achieving concentrations of 1 μg mL^{-1} (1.36 μM) and 2 μg mL^{-1} (3.86 μM), respectively. To prevent thermal degradation of the antibiotic before spiking of the antibiotic solution, the molten agar was cooled to 40 °C and after homogenization 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.

Statistical analysis was performed using the built-in multiple *t*-test function of GraphPad Prism to determine whether there is a significant difference between the control sample and the sample containing the antibiotic. Statistical significance was calculated without correction for multiple comparisons using α = 0.05%. The results are listed in Table S4. Computations assumed that all rows (control and corresponding antibiotic-containing samples) are samples from populations with the same standard deviation.

RESULTS AND DISCUSSION

Removal of Erythromycin and Piperacillin. Samples of erythromycin and piperacillin (0.5 mM each) were exposed to electron beam irradiation by applying a wide range of treatment times to provide an increasing absorbed dose (in a dilute aqueous solution, a higher antibiotic concentration needs to be applied compared to that in a real wastewater sample, where matrix constituents attenuate the effects, to stoichiometrically fit to the amount of hydroxyl radicals generated during the treatment and dilution needs to be performed to obtain the subinhibitory level for the assay).[16] The total amount of $^{\bullet}$OH introduced into the sample within a certain time period is shown in Figure 1. The concentration of erythromycin and piperacillin continuously decreases as the amount of $^{\bullet}$OH increases. It can be seen that while removal of erythromycin is more efficient when the stoichiometric ratio is low, it remains more persistent against an excess of $^{\bullet}$OH than piperacillin does.

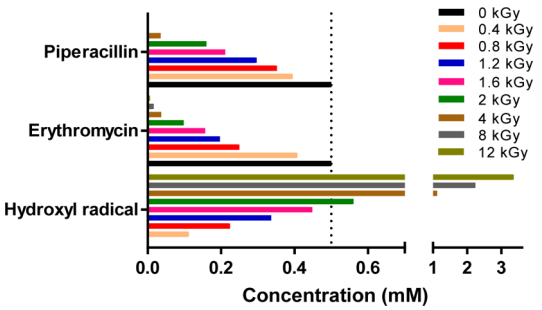

Figure 1. Total amount of $^{\bullet}$OH introduced into the system and the changing antibiotic concentration as a function of absorbed dose.

This phenomenon can be attributed to the scavenging capacity of the forming transformation products (TPs) eventually "protecting" the residual intact antibiotic molecules.

Effects of Treatment on the Selection of Resistant Bacteria. Bearing in mind that natural selection is occurring in any bacterial population, we evaluated the experiments in light of control samples in which the antibiotic (present at a sub-MIC concentration) is replaced with sterile water. The fraction of resistant bacteria in the samples after incubation for 24 h is shown in Figure 2. The aim of the advanced oxidation

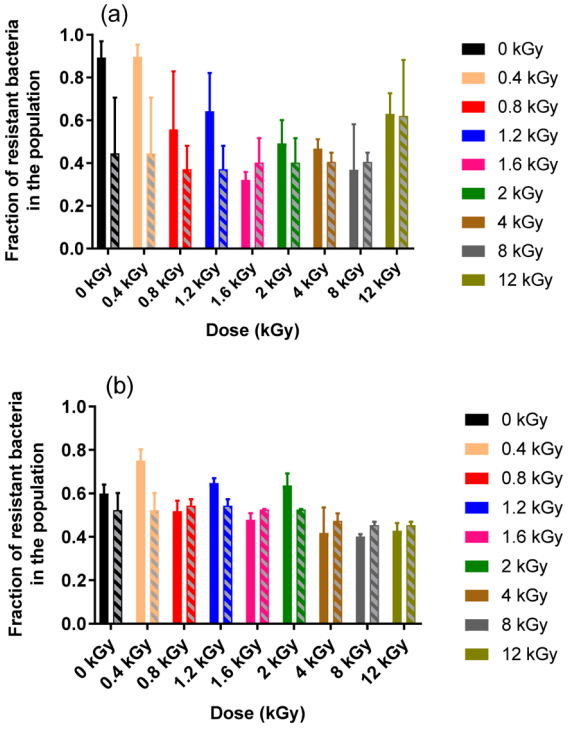

Figure 2. Fraction of resistant *S. aureus* in the bacterial population after incubation for 24 h in samples spiked with treated samples of (a) erythromycin and (b) piperacillin. Corresponding patterned columns show the control sample (no antibiotic added). Error bars show the standard deviation of three independent experiments.

treatment should be the elimination of selective pressure on the bacterial population favoring the predominance of resistant mutants. This is achieved when the fraction of resistant bacteria within a statistically insignificant deviation is the same as in the control sample. In other words, the difference between the control sample and the sample containing the antibiotic is no longer significant (statistical analysis shown in Table S4). In the

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case of erythromycin, a 0.4 kGy dose practically does not have any impact on the elimination of the selective pressure (Figure 2a). At 1.2 kGy, the difference between the control sample and the sample containing the antibiotic is still significant (Table S4). With 1.6 kGy as a starting point, there is no significant change from the control sample, and it can be stated that the selective pressure has eventually been eliminated.

In the case of piperacillin samples (Figure 2b) at 0.4 kGy, the fraction of resistant bacteria is significantly higher than in the untreated sample. Formation of biologically active molecules upon mild advanced oxidation treatment has frequently been found for penicillin derivatives,[15] and we have observed the same phenomenon for erythromycin, as well.[16] This peculiarity resides in the remaining pharmacophore (responsible for biological activity) during •OH-induced oxidation that favors other sites of the molecule and not the pharmacophore. A significant departure from the control sample was obtained at 1.2 and 2 kGy, and the selective pressure appeared to be eliminated thereafter. It follows that appropriate treatment was reached only at a stage at which all the intact piperacillin was transformed to additional products (Figure 1).

We were also interested in the effects of incubation time on the population dynamics of the mixed bacterial culture. Therefore, the incubation time was prolonged to 48 and 72 h for erythromycin samples. The results are shown in Figure S2. It can be seen that the products forming at 0.4 kGy do not have an effect on the fraction of resistant bacteria compared to results obtained using a 24 h incubation time. Probably because of the low oxidizing radical yield, the bacterial population could eventually overcome the effects of the products in the long term. However, at 0.8 kGy, the difference between the control and the sample containing the antibiotic remained appreciable (Figure S2). While after incubation for 48 h a significant difference was observed even at 2 kGy, no effect was noticed after 72 h. Because the prolonged incubation time did not yield additional information and led to some observations fading, we decided to apply the 24 h incubation time thereafter. Furthermore, we have also investigated the effects of passaging 1 mL of test medium after incubation for 24 h to freshly prepared, new test medium. However, we found that the culture in some cases started to decline, and no promising results were obtained. It should also be noted that while after incubation for 24 h the colony count remained the same as the initial value, it was cut in half after every 24 h thereafter (48 and 72 h). This is due to the limited nutrient source in the medium and further confirms the application of the 24 h incubation time rather than the prolonged incubation time.

A microbiological assay has been developed to assess the effects of transformation products of electron beam treatment on the population dynamics of a resistant and sensitive mixed bacterial population. The authors anticipate that this simple test can also be applied to other advanced oxidation techniques to determine the optimal treatment stage for eliminating the selective pressure on a bacterial population that is expected to drive the development of resistance in the environment. The test can be applied to different antibiotics or bacterial strains by constructing the resistant mutants via transformation of the corresponding resistance gene into the sensitive one, as shown by Gullberg et al.[17]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.7b00127.

Detailed description of sample analysis, schematic representation of the microbiological assay (Figure S1), statistical analysis (Table S4), and additional information about the effects of incubation time on the assay (Figure S2) (PDF)

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Notes

The authors declare no competing financial interest.

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