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THE INTERNATIONAL ARABIC JOURNAL OF ANTIMICROBIAL AGENTS

2013
Vol. 3 No. 1:3
doi: 10.3823/725

# A novel binary biofilm model for the study of the development of antimicrobial tolerance in *Pseudomonas aeruginosa* PAO1 and *Escherichia coli*

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## Abstract

The primary aim of this study was to investigate the effect of species interactions on biofilm formation and the investigation of the susceptibility of component species towards the antimicrobial agent Benzisothiazolone (BIT) in binary biofilms (*Ps. aeruginosa* PAO1and *E. coli* ATCC 10000). The Sorbarod biofilm model [1] was used to establish mono-species and binary species biofilms under conditions of growth rate control by the utilisation of a Modified Chemically Defined Medium (MCDM). This MCDM was designed to allow the growth of both bacterial species (*Ps. aeruginosa* PAO1and *E. coli* ATCC 10000) in the same biofilm, whilst preventing either species from having a growth rate advantage over the other. Our results suggest that it is possible to use this model to investigate the consequences of environmental exposure of bacteria to sub-MICs of biocides and develop a comprehensive insight of their subsequent tolerance and resistance characteristics. These results indicate that it is possible to establish a binary biofilm in a modified chemically defined media, subjected to growth rate control and to induce tolerance in dual species (binary) biofilms in response to BIT. The mechanism of tolerance in binary biofilms towards this biocide was a gradual adaptive process, dependent upon the presence of the biocide itself. This study elucidates a novel technique for the establishment, control and operation of binary biofilms. It has yielded information regarding the use of passage approaches to develop antimicrobials tolerance in both mono- and binary species biofilms of medically important bacteria.

## Introduction

Many studies have focused on adhesion and biofilm formation by planktonic monocultures [2]. However, under natural conditions, true mono-species biofilms are comparatively rare and in most natural and industrial environments, biofilms are complex multi-species communities [3]. Although prominent in nature, much less is known about mixed population biofilms. Bacteria do not have uniform colonization and physiological properties [4], a feature that enables them to utilize different ecological niches. Therefore, one would predict that increasing species diversity of planktonic bacterial communities would lead to increased species diversity and overall cell density within biofilms [5]. A range of interactions has been observed among microorganisms in biofilms, such as antagonistic, mutualistic, competitive and commensal relationships [6, 7, 8, 9, 10, 11, 12]. For instance, competition among microorganisms for space and nutrients is a powerful selective force which has led to the evolution of a variety of effective strategies for colonization [6-13]. The mechanisms that control microbial interactions in multispecies biofilms are not fully understood [13-14]. The ecology of a biofilm is a complex equation of physicochemical and biological parameters. As with all levels of evolution, a complex web of interactions is central to the structure, composition, and function of any community [15]. Binary biofilms may be thicker and more stable than mono-species biofilms and this fact might influence their susceptibly to antimicrobials [16]. A two species system is simple enough to allow quantitative analysis of interactions and *in situ* speciation [17, 18, 19].

*Escherichia coli* is a Gram-negative bacterium that exhibits great tolerance towards many antimicrobial agents. [20] Reported that >90% of antibiotics having a natural origin have no activity against *E. coli*. When *E. coli* cells are exposed to antibiotics, (such as chloramphenicol or tetracycline) at concentrations slightly greater than their Minimum Inhibitory Concentration (MIC), resistant isolates are observed. This phenomenon occurs at high frequencies, results in cross resistance to other antibiotics and is called the multiple antibiotic resistance (mar) phenotype. [21] Found that *E. coli* cells deficient in glutathione were more sensitive to electrophilic biocides, such as formaldehyde and 2-bromo-2-nitropropane-1,3-diol. However, there was little difference in susceptibility towards surface active biocides, such as benzalkonium chloride. Thus, the up-regulation of the mar operon results in a multidrug-resistant phenotype in *E. coli* planktonic cultures. However, the up-regulation of the mar operon in biofilms was not supported by the work of [22] based on the fact that the level of mar was lower in biofilms as compared to the level reported in equivalent stationary-phase cultures grown in batch culture. On the other hand, [23] revealed that physicochemical reactions between biocide and non-living components of the biofilm can also result in an apparent cellular resistance.

Our previous findings [24] evaluated the efficacy of selected biocides on *Pseudomonas aeruginosa* biofilms grown within Sorbarod filter plugs and fed with Chemically Defined Medium (CDM). The antimicrobial effects of the selected biocides were assessed in this simplified biofilm system [1] by comparing sub-Minimum Inhibitory Concentrations (sub-MIC) of eluate and biofilm (attached) cells. The results of the study revealed that the induction of tolerance in mono-species (*Pseudomonas aeruginosa* PAO1) biofilms towards three biocides (Zinc Pyrithione (ZnPT), Cetrimide and Benzisothiazolone (BIT)) occurred in a step-wise fashion. This indicates that the process of adaptation in response to the sub-MIC presence of these biocides is probably a phenotypic process. In addition, these results support the possibility of a third mechanism of reduced biofilm susceptibility, in that *"some of the cells in a biofilm adopt a distinct, and relatively protected, biofilm phenotype"* [25]. However, upon subsequent growth of the tolerant cultures in the absence of the biocides the observed MICs declined. This indicates the phenotypic nature for the development of the biofilm tolerance process. In general, a phenotypic adaptation is a physiological modification demonstrating a change in response to environmental stimuli, but does not necessarily require a genomic modification. Although the MIC value decreased upon growth in the absence of the biocides, it did not revert to the initial MIC value. This suggests that the induced biocide tolerance is largely irreversible. In addition, there was no difference in the MIC value between adherent and eluate cells for ZnPT, but this was not the case with Cetrimide and BIT. For BIT, there was an immediate and sharp difference in MIC value between both types of cells and this continued for the whole series of passages. One possible explanation for this phenomenon is the neutralization of the activity of BIT in the presence of accessible thiol groups (-SH) in the EPS that surrounds the biofilm. The mode of action of BIT is an oxidative reaction with accessible thiol groups [26-27]. Hence, the presence of accessible thiol groups in the EPS will result in a greater MIC value for biofilm cells as compared to that of eluate cells.

This study is a continuation of our previous study [24] and builds upon the results achieved within that work. The main goal of this study was to evaluate the responses of binary biofilms when exposed to antimicrobial stress and compare these responses to those obtained with single-species biofilms. For that purpose, the induction of tolerance in *E. coli* ATCC 10000 biofilms against BIT was undertaken. This was followed by the determination of the effect of species interactions on biofilm formation and the investigation of the susceptibility of component species towards BIT in binary biofilms (*Ps. aeruginosa* PAO1and *E. coli*). The choice of BIT to induce tolerance in *E. coli* and in binary biofilms, is based on its unique feature of exhibiting differences in MICs between sessile and eluate cells [24]. Moreover, Scanning Electron Microscope (SEM) and Environmental Scanning Electron Microscope (ESEM) techniques were utilized in this study to analyze the biofilm architecture of mono-species and binary bacterial biofilms and to illustrate any differences in the morphology, composition and physiology between control biofilms and the ones treated with BIT (tolerant biofilms).

# Materials and methods

## Organisms and chemicals

Stock cultures of *Ps. aeruginosa* ATCC 15692 PAO1 (NCIMB 10548) and *E. coli* ATCC 10000 were obtained from the National Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen. Cultures were sub-cultured in either R2A medium supplemented with 1% glycerol [28] or a Chemically Defined Medium (CDM, replacing 0.5% succinic acid with glycerol [29-30]. Culture identification was determined using Gram-stain reaction and the API 20 E and API 20 NE test (Biomerieux, France). BIT was the kind gift of Nipa Ltd. All other reagents were purchased from Sigma (Poole, UK). The biocide was stored at room temperature until use. Concentrated and diluted solutions of the biocide were prepared on a daily basis and discarded after use, although concentrated solutions may be stored frozen (-20°C) if required.

## Preparation of Media

### *Chemically Defined Medium (CDM)*

CDM was prepared according to Dinning [31] and was modified by replacing 0.5% succinic acid with glycerol. This medium is made up from 4 solutions; solution A, solution B, solution C and solution D. Solution A was prepared by dissolving $K_2HPO_4$ (2.56g), $KH_2PO_4$ (2.08g) and $NH_4Cl$ (1.00g) in 900mL of distilled water. The volume was adjusted to 1L by adding distilled water. The pH was adjusted to 6.8 by either 1M HCl or 1M NaOH. The solution was sterilized by autoclaving at 121°C, 15 psi for 15 minutes. Solution B was prepared by dissolving ferric ammonium citrate (1.00g) and $CaCl_2$ (0.1g) in 100mL of distilled water. Solution B was sterilized by filtration through a 0.22 µm pore size cellulose acetate filter (Merck Eurolab Ltd, Lutterworth, UK) under vacuum. Solution C is a 1M glycerol solution and was prepared by dissolving 46.45g of glycerol in 400mL of distilled water. The volume was adjusted to 500mL by adding de-ionized water. The pH was adjusted to 6.0 by the addition of either 0.1M HCl or 0.1M NaOH. Solution C was sterilized by autoclaving at 121°C, 15 psi for 15 minutes. Finally, solution D was prepared by dissolving $MgSO_4.7H_2O$ (0.5g) in 900mL distilled water. The volume was adjusted to 1L by adding distilled water. The solution was sterilized by autoclaving at 121°C, 15 psi for 15 minutes. The CDM was then prepared by the aseptic addition of 5mL, 15ml and 10ml of solutions B, C and D respectively to a final volume of 1L of solution A. As a gelling agent, 15.45g technical agar (Oxoid number 3) was added when required. However, in order to obtain a specific CDM suitable for the optimal growth of both *Ps. aeruginosa* and *E. coli*, whilst preventing either species from exhibiting a growth rate advantage over the other, CDM was modified by increasing the concentration of nitrogen ($NH_4Cl$) in solution A from 0.0187 M to 0.0280 M in MCDM and decreasing the concentration of carbon in solution C from 1 M in CDM to 0.66 M in MCDM. These modifications will render the doubling time ($t_D$) for both bacteria approximately equal in the modified CDM.

## Chromogenic *E. coli*/Coliform Medium

Chromogenic *E. coli*/Coliform Medium CM956 (Oxoid LTD., Basingstoke, UK) is a differential agar, which provides presumptive identification of *E. coli* and coliforms in food and environmental samples. The agar contains two enzyme substrates to improve differentiation between *E. coli* and other coliforms. The first substrate allows specific detection of *E. coli* through the formation of purple colonies. This substrate is cleared by the enzyme glucuronidase, which is produced by approximately 97% of *E. coli* strains. The other substrate is cleaved by the enzyme galactosidase, which is produced by the majority of coliforms, resulting in rose/pink colonies [32]. This medium has been used in this study to differentiate between two types of bacteria; *Ps. aeruginosa* forming straw colonies and *E. coli* forming purple colonies. Aliquots (55.8g) of this medium were suspended in 1L of distilled water. This medium was sterilized by autoclaving at 121°C, 15 psi for 15 minutes.

### *Establishing a mono-species and binary biofilm for later analysis*

The processes required for the formation of an *E. coli* biofilm on a Sorbarod filter is similar to the establishment of a *Ps. aeruginosa* PAO1 biofilm [1, 24]. The Sorbarod filter was pre-wetted with 5 mL of 0.9% (w/v) sterile normal saline, and then inoculated with a mid-logarithmic phase culture (10mL). The plunger was withdrawn from a sterile, disposable 2mL syringe, in such a way as to leave the rubber plunger seal within the barrel of the syringe. The plunger was discarded. The syringe was attached, via the Luer lock, to a piece of sterile PVC tubing (10cm length, internal diameter 1cm) previously prepared to contain the Sorbarod filter. A sterile disposable needle (0.8 x 40 mm) was then inserted through the rubber seal within the syringe. Media inlet tubing was attached via the needle Luer lock and sterile modified CDM (MCDM) was delivered into the unit, which was placed within a 37°C incubator. After 48 hours, Sorbarod filters were removed and stored at -18°C for later analysis. For binary biofilms, the Sorbarod filter was inoculated with mid-logarithmic phase of *E. coli* culture (10 mL), which was, in turn, inoculated with MCDM at 37°C. The *E. coli* biofilm was incubated for 48 hours (until steady-state) before adding an aliquot (10 mL) of mid-logarithmic phase of a culture of *Ps. aeruginosa* to the Sorbarod filter. This step was performed in order to give *E. coli* a *'head start'* in establishing itself on the Sorbarod filter and forming the first monolayer of colonies before the addition of *Ps. aeruginosa*. The system was operated for 126 hours, in order to achieve the steady-state for both microorganisms.

### *Gradient Plate Technique*

The gradient plate technique was used to maintain the raised tolerant cells in the process stated above 24 hours before undergoing subsequent studies. Square Petri dishes (BDH; 20 cm x 20 cm), were used for this process. The plates were labeled and CDM agar (25mL), having a known concentration of antimicrobial agent calculated according to the following formula: 1.5 x MIC of biocide was added. The agar was allowed to set with the plates propped at a slight angle. An additional 25mL of the CDM agar devoid of biocide were poured onto the surface of the previous layer within the plates and allowed to set with the plates on the level. Plates were then stored, allowing the diffusion of the biocide to occur and thus, the formation of a concentration gradi-

ent across the plates. The surfaces of the plates were then inoculated with biocide-tolerant *Pseudomonas aeruginosa*. Following incubation for 24 hours, the length of the zone of inhibition (x) was measured and the MIC was calculated according to the following equation:

$$MIC\ (\mu g\ mL\text{-}1) = ((y\text{-}x) / y).[B] \quad \text{(Equation 1)}$$

Where, y = the length of the plate, x = the length of the zone of inhibition, [B] = concentration of biocide (μg $mL^{-1}$). The point on the surface of the plate at which growth ceased was approximately equivalent to the MIC.

### *Induction of tolerance*

The induction of BIT-tolerance in *E. coli*/*Ps. aeruginosa* binary biofilms was performed according to the method previously described by [33]. This approach is similar to the one described for *Ps. aeruginosa* biofilms in our previous work [24]. The Sorbarod model was established as described earlier and supplied with modified CDM, which was prepared to contain a quarter of the concentration of the biocide of the previously established MIC (MIC = 5 μg $mL^{-1}$, hence MIC/4 = 1.25 μg $mL^{-1}$). The eluate culture was collected during the last hour (the 48 hour for *E. coli* biofilm and the 126th hour for binary biofilm) and the biofilm filter was sacrificed to remove the adherent cells (in 10 mL, 0.9% (w/v) sterile normal saline). Aliquots (100μl) from the eluate and the biofilm cultures were spread plated onto sterile Chromogenic *E. coli*/Coliform agar. This step was performed in order to separate *Ps. aeruginosa* (straw) colonies from *E. coli* (purple) colonies. For MIC determination, a single colony from each bacterium was inoculated onto the surface of the sterile Chromogenic *E. coli*/Coliform gradient plate (this was performed in triplicates). The plates were incubated at 37°C for 48 hrs. The point at which growth ceased on the surface of the agar was an indicator of the approximate MIC value (Passage 1). The colonies growing in the region of the plate where the concentration of the biocide was the highest were considered to be the most tolerant and therefore, were used to inoculate the next passage. This process was repeated until 4 successive passages were inoculated in the presence of increasing MIC/4 of BIT. This process utilizes successively higher MIC/4 values I order to 'train' the cultures towards tolerance. This was the point at which the cells were deemed to be sufficiently tolerant (Passage 5) for our experimental purposes. Although the MICs may continue to increase with further exposure to biocides, an aliquot (10 mL) of the final cultures in the presence of biocide was inoculated onto a new Sorbarod filter, which was perfused with a fresh MCDM devoid of biocide. The MIC was re-determined via the gradient plate method (Passage 6). This was repeated until two successive passages were performed devoid of BIT.

### *Scanning Electron Microscopy*

Biofilm cell samples were taken from steady state biofilms after 48 h of establishment and perfusion with CDM, either with or without BIT. The biofilm samples were fixed with 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) and left overnight at room temperature. Samples were then rinsed twice with cacodylate buffer and placed in cacodylate buffer supplemented with 0.2% osmium tetroxide overnight. Following incubation, samples were rinsed twice with distilled water and were placed in 2% uranyl acetate (aq) overnight. The samples were then rinsed twice with distilled water before cleaving in liquid nitrogen. After dehydration in ethanol, samples were transferred to acetone and then critical point dried using a Baltec CPD 030 critical point drier. Samples were mounted on aluminium stubs using double-sided carbon tabs and then coated with 30 nm of Au / Pd in a Cressington 208 HR sputter coater. Samples were then viewed at 5 kV using a Hitachi S-4700 field emission electron scanning electron microscope.

## Results

### Induction of tolerance

### *Passages of E. coli biofilms*

The initial pre-exposure MIC value against BIT when grown in MCDM was 5μg $mL^{-1}$ and 4μg $mL^{-1}$ for biofilm and eluate cells, respectively during Passage 0. There was a marked difference between biofilm and eluate MICs during the whole passage process. Within the 48 hours of exposure to the biocide, the MIC had decreased to a value of 1.88 μg $mL^{-1}$ for biofilm cells and 0.98 μg $mL^{-1}$for eluate cells, Passage 1 (**Figure 1**). In subsequent passages the MIC started to increase in a step-wise fashion until Passage 4, where the MIC values attained 8.85μg $mL^{-1}$ and 3.5μg $mL^{-1}$ for biofilm and eluate cells respectively. Passage 5 demonstrated a marked increase in the MIC value for both biofilm (47.79 μg $mL^{-1}$) and eluate cells (40.85μg $mL^{-1}$). This represents an increase of 9.6-fold for the adherent cells and 10.2-fold for the eluate cells, when compared to the initial, pre-exposure value. At Passage 6, which is the first passage without the addition of biocide the MIC decreased sharply to 26.97 μg $mL^{-1}$ for the adherent cells and 16.20 μg $mL^{-1}$ for the eluate cells. Within the two successive passages in biocide-free medium, the MIC showed a marked decrease and at Passage 8, the MIC was 4.96 μg mL-1 (biofilm cells) and 3.95μg ml-1 (eluate cells).

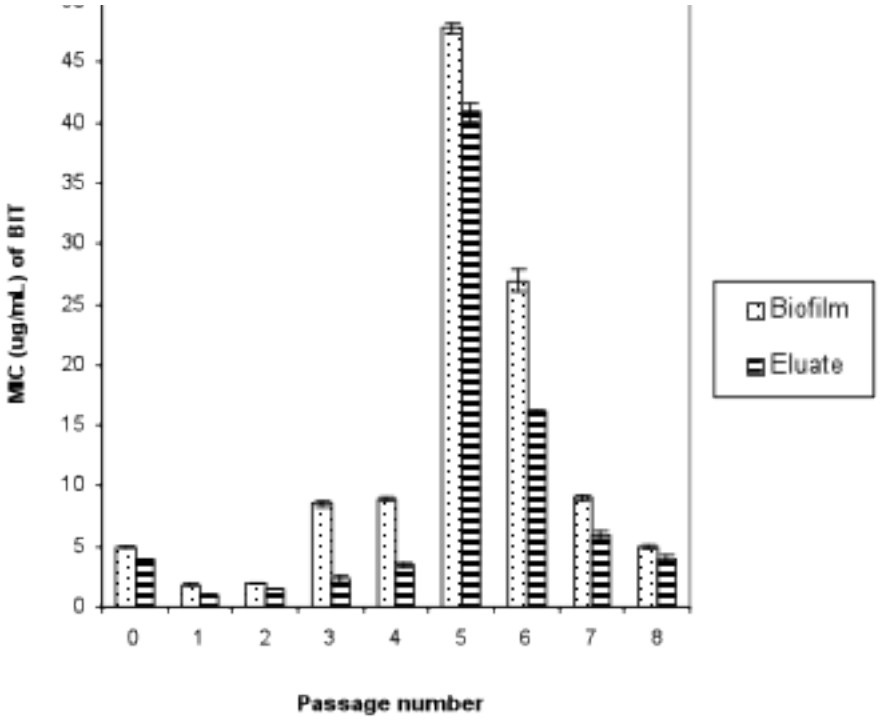


**Figure 1.** Increase in MIC for BIT against *Escherichia coli* ATCC 10000 in MCDM as a function of passage number. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. Passages 6, 7 and 8 were performed without the addition of biocide.

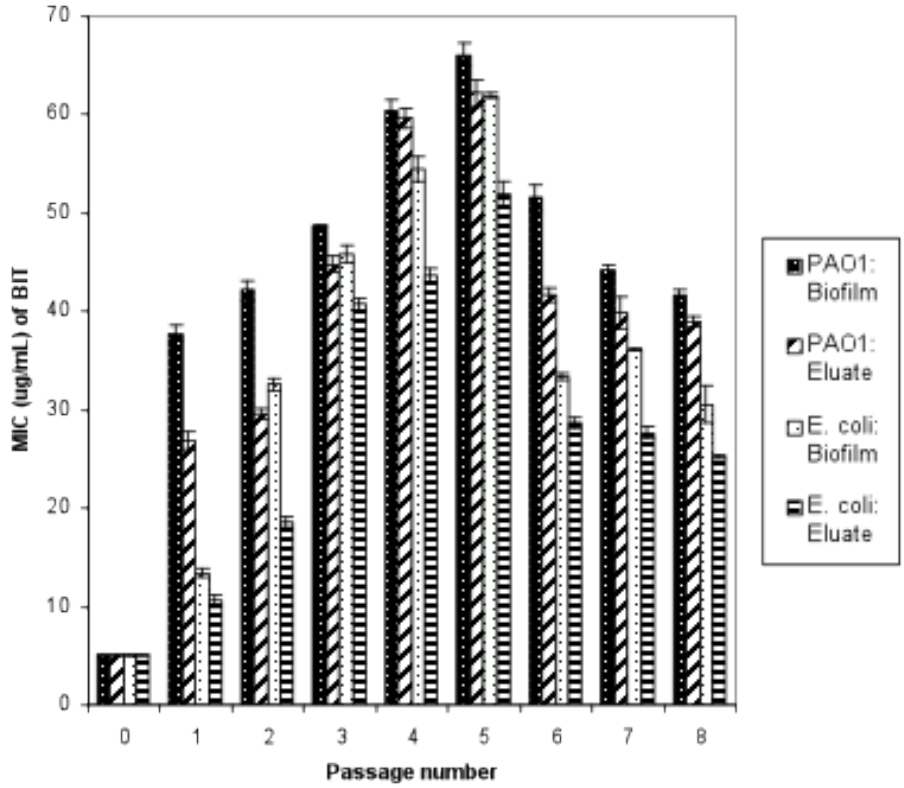


**Figure 2.** Increase in MIC for BIT against binary biofilms (*Pseudomonas aeruginosa* PAO1 and *Escherichia coli* ATCC 10000) in MCDM as a function of passage number. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. Passages 6, 7 and 8 were performed without the addition of biocide.

## Passaging of Binary Biofilms

By using the gradient plate method to calculate the MIC value, it was possible to differentiate between the MIC for *Ps. aeruginosa* and that for *E. coli*. Starting with *Ps. aeruginosa*, there was a considerable difference between the biofilm and the eluate MIC values. The original MIC was 5μg $mL^{-1}$ for both adherent and eluate cells. For Passage 1, there was a marked increase in the MIC value (37.76μg $mL^{-1}$) for biofilm cells and 26.88μg $mL^{-1}$ for eluate cells. This is an increase of 7.6-fold for the biofilm cells and 5.4-fold for the eluate cells (**Figure 2**). After Passage 1, the MICs continued to increase

gradually with every subsequent passage in the presence of biocide. At Passage 5, the MIC increased to 66.11µg $mL^{-1}$ for the adherent cells and 62.16µg $mL^{-1}$ for the eluate cells representing an increase of 13.2-fold for the sessile cells and 12.4-fold for the eluate cells when compared to the initial, pre-exposure value. At Passage 8, the MIC had decreased to 41.60µg $mL^{-1}$ (biofilm cells) and 38.93µg $mL^{-1}$ (eluate cells).

For *E. coli*, the initial, pre-exposure, value was 5µ g $mL^{-1}$ (biofilm and eluate cells). There was a marked difference in MIC values between adherent and eluate cells in each passage. At Passage 1, the MIC increased to 13.44µ g $mL^{-1}$ (biofilm cells) and 10.56µ g $mL^{-1}$ (eluate cells). In the following passage, there was a considerable increase in MIC values: 32.64µ g $mL^{-1}$ for the sessile cells and 18.56µ g $mL^{-1}$ for the eluate cells. For each subsequent passage, in the presence of an increasing concentration of biocide, the MIC continued to increase in a step-wise fashion. At Passage 5, the MIC increased to 61.91µ g $mL^{-1}$ (biofilm cells) and 52.29µ g $mL^{-1}$ (eluate cells). This is an increase of 12.4-fold and 10.5-fold adherent and eluate cells, respectively. The cells were then passaged in biocide-free medium and within the first 126 hours a significant decrease in MIC was observed and by Passage 8, the MIC had fallen to 30.54µ g $mL^{-1}$ and 25.20µ g $mL^{-1}$ (biofilm and eluate cells, respectively).

## Scanning Electron Microscopy

Environmental scanning electron micrograph (**Figure 3a**) shows two different types of fibres; (1) is an un-encapsulated fibre, whereas fibre (2) is an encapsulated one, colonized by *E. coli* mono-species biofilm. Fibre 2 is shown in a higher magnification in **Figure 3b**. The fiber is fully covered by the biofilm, but no individual bacterial cells can be distinguished. Micrograph (**Figure 3c**) illustrates a SEM surface image of an *E. coli* biofilm treated with BIT for 48 hours. The micrograph shows individual bacterial cells fixed within the exopolymer matrix. There are gaps in the EPS due to SEM treatment (considered as artifacts). The topography of *E. coli* biofilm surface treated with BIT is completely different than that for binary biofilm (**Figure 3d**).

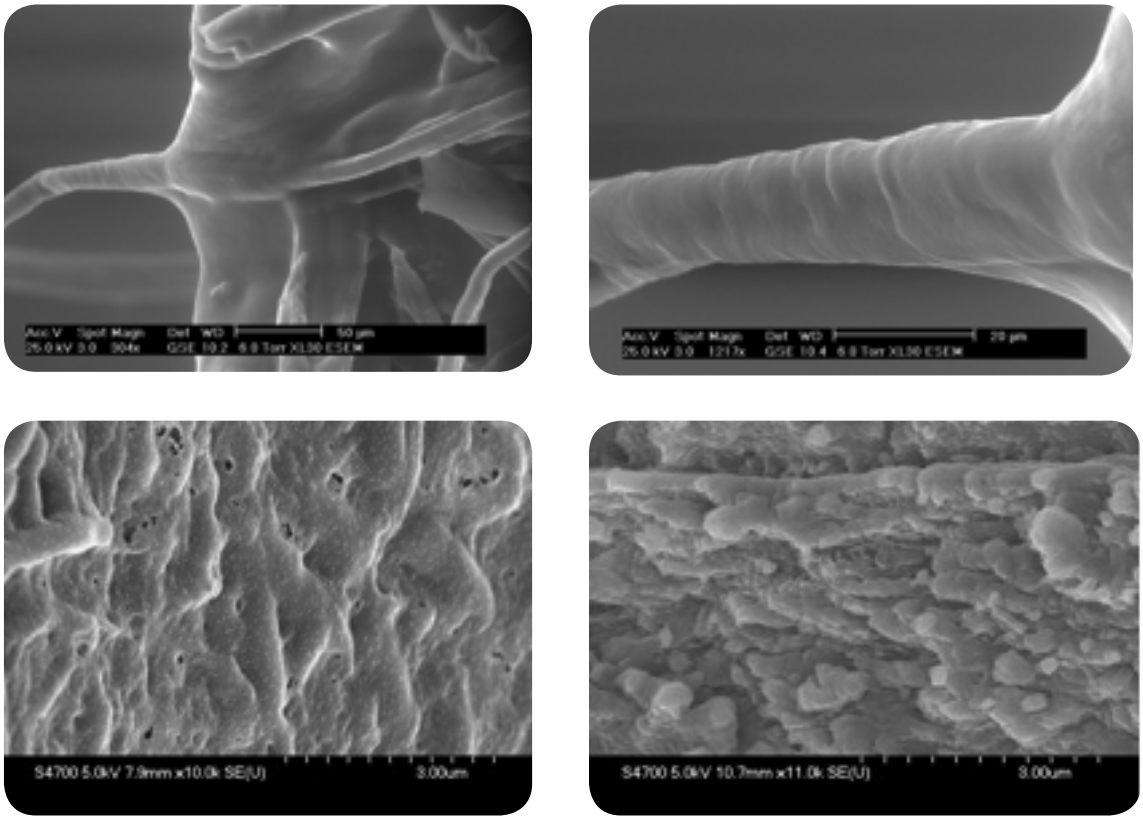

**Figure 3.** (**a**) ESEM image of *E. coli* control biofilm cultured in modified CDM for 48h (304x). (**b**) A Sorbarod fibre encapsulated by *E. coli* control biofilm (1217x). (**c**) The SEM image shows the surface of *E. coli* biofilm treated with BIT for 48h (10000x). Note the individual encapsulated cells and the gaps in EPS caused by sample preparation, considered to be artefactual. (**d**) SEM image of 77h binary biofilm, treated with BIT (11000x). The image illustrates that the bacteria altered their morphology upon their exposure to the biocide. Note the coagulated surface of EPS.

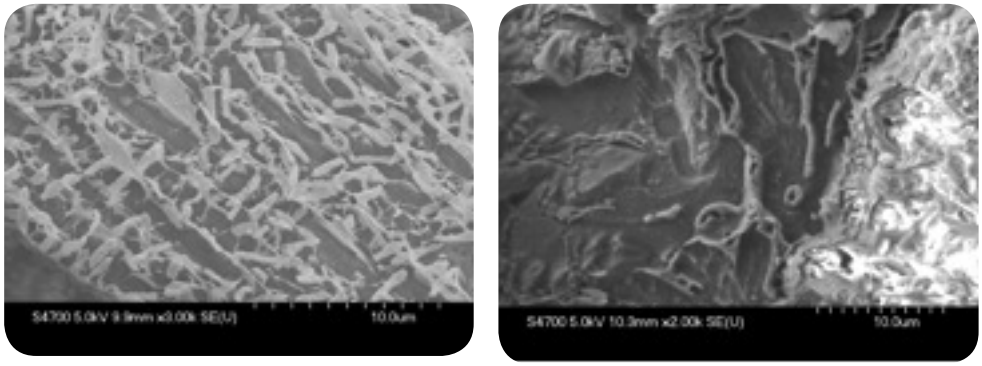

**Figure 4.** (**a**) Scanning electron micrograph of a 77h binary control biofilm grown on a Sorbarod filter (3000x). The image shows a monolayer of cells with conspicuous EPS associated with these cells. (**b**) SEM image of binary biofilm treated with BIT for 77h (2000x). Note the complete loss of spatial arrangement of cells and coagulation of EPS.

## Discussion

Genetic studies of single species biofilms have shown that they are formed in consecutive steps [34]. In addition, they require intercellular signaling [35-36] and reveal a profile of transcribed genes, which is different from that of planktonic cells [37]. However, in natural environments the biofilm is almost invariably a multispecies microbial community. Increased species diversity within the biofilm may provide spatial and temporal niches not available within the monoculture or may create microenvironments within the community [38, 39, 40]. Furthermore, species composition of binary or mixed biofilms is important in determining the response of component species under the effect of exposure to antimicrobial agents. In other words, the complexity of mixed biofilms may increase the antimicrobial resistance phenomena, increasing the difficulty in eradication of biofilms. Thus, the investigation and characterization of mixed cultures in planktonic and sessile state, as well as their responses to antimicrobial products, is of increasing importance for basic research as well as for ecological, medical and biotechnological applications.

Several studies have shown the impact of antimicrobial agents on biofilms [2]. However, the induction of tolerance in binary biofilm cultures (binary biofilm composed of equal numbers of *E. coli* and *Ps. aeruginosa*; hereinafter, referred to as binary biofilms) towards BIT has not been performed before. This could be considered a novel and beneficial idea to enable our understanding of the development of tolerance in natural biofilms. Initial experiments investigating the development of tolerance in binary biofilms towards BIT were performed by repeated passages in MIC/4 of the biocide. The gradient plate method using Chromogenic *E. coli*/Coliform agar was used for MIC determination in order to differentiate between *Ps. aeruginosa* (straw coloured) and *E. coli* (purple coloured) colonies in binary biofilms.

Mono-species (*E. coli*) and binary (*Ps. aeruginosa* PAO1 and *E. coli*) biofilms were subjected to the passage process in the presence of sub-MICs of BIT. The results obtained from the passage process for *E. coli* biofilm and eluate cells with BIT revealed a marked difference between biofilm MICs as compared to eluate cells (as was the case in *Ps. aeruginosa* mono-species biofilm and eluate cells; [24]. In addition, a complete reversion to the original, pre-exposure MIC value (Passage 0) for both biofilm and eluate cells was observed. This suggests that the induced tolerance is unstable and that the developed tolerance was probably the result of a phenotypic adaptation rather than a mutation.

Comparing the obtained results from the passage process for *E. coli* as a mono-species and in binary biofilms, one can conclude that the survival of *E. coli* upon exposure to BIT is enhanced by being part of a binary biofilm. *Ps. aeruginosa* provided a significant protection for *E. coli* against BIT, [5] illustrated that *Vogesella indigofera*, a betadine-resistant microorganism, enhanced the survival of *Pseudomonas putida*, a betadine-susceptible bacterium in mixed cultures. Therefore, resistant bacteria components in binary biofilms may protect more susceptible bacteria, again emphasizing that the induction of tolerance in binary biofilms towards a selected biocide is a complex phenomenon, influenced by community composition, biocide concentration and growth rate.

In comparing these results to the ones obtained from the passage process for *Ps. aeruginosa* mono-species biofilms against BIT [24], the MIC value for the first passage was 2µ g $mL^{-1}$ (for both types of cells), whereas the initial MIC in binary biofilms was 37.76µ g $mL^{-1}$ and 26.88µ g $mL^{-1}$ for the eluate cells. This represents a 17-fold and 10-fold increase for the biofilm and the eluate cells respectively when compared to the initial MIC. However, in binary biofilms, there was a 13-fold (biofilm cells) and 12-fold (eluate cells) increases in the MIC value, when compared to the initial, pre-exposure value after only 5 successive passages in the presence of biocide. Therefore, the induction of tolerance in *Ps. aeruginosa* binary biofilms was faster than that in mono-species *Ps. aeruginosa* biofilms. Thus, it can be suggested that the growth of species in a mixed biofilm clearly favours the sessile growth of each species, which may be a disadvantage in terms of sanitation.

The reduced growth activity exhibited by binary biofilms can be explained by the fact that the bacteria growing alone do not face cell-to-cell signaling stresses. In fact, the resulting binary biofilms seem to have cells in a latent state embedded in a matrix with more exopolymers (**Figure 3 c & d**), which may form a shield protecting the bacteria from stress factors and render the access of antimicrobials more difficult.

On the other hand, the MIC for both bacteria (in binary biofilms, Passage 8) did not revert to the original, pre-exposure, value. It is possible that further passages in biocide-free media could result in a complete reversion to the original, pre-exposure value (Passage 0). However, this was not done in these experiments due to both time constraints and that the mechanism and nature of reversion of tolerance was not a primary aim in these experiments. However, the observation of a gradual loss in tolerance suggests that the induced tolerance is unstable. Moreover, the gradual adaptation in MIC values and the gradual loss in tolerance (for mono-species and binary biofilms) indicate that the mechanism of tolerance is not a mutational event, but a specific intracellular phenotypic mechanism.

## Conclusions

The previous results indicate that it is possible to establish a binary biofilm under controlled growth rate and to induce tolerance in dual species (binary) biofilms towards BIT. The mechanism of tolerance in binary biofilms towards this biocide was a gradual adaptive process dependent upon the presence of the biocide itself. By comparing single and mixed biofilms, binary biofilms seemed to be less susceptible to the action of BIT, since biomass and activity reduction were only observed upon using high concentrations of the biocide. These results seem to indicate that, when growing two bacteria together within a biofilm, bacteria establish favorable microbial interactions that give them additional tolerance to the aggressive action of antimicrobials.

Electron microscopy has revealed the loss of the spatial arrangement of bacterial cells upon the exposure of binary cells to BIT. The topography of the surface of *E. coli* and binary biofilms is entirely distinct for each. Therefore, the SEM offered excellent resolution with the capacity to reveal complex shapes. Hence, each of the two techniques (SEM & ESEM) added a different dimension to our understanding of the spatial composition of biofilms. It is suggested that a combination of both techniques is required to overcome the problems of recognizing artifacts and to give the most accurate image of the true biofilm structure and organization. This study has yielded novel information and techniques regarding the use of passage approaches to develop antimicrobials tolerance in both mono-species and binary species biofilms of medically important bacteria. The results from these experiments suggest that it is possible to use these models to investigate the effects of environmental exposure of bacteria to sub-MICs of biocides and develop a complete understanding of their subsequent tolerance and resistance characteristics. Thus, this study highlights the need to develop suitable biofilm control strategies based on multispecies biofilms approaches and it gives emphasis to the question of the microbial complexity of biofilm nidus.

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