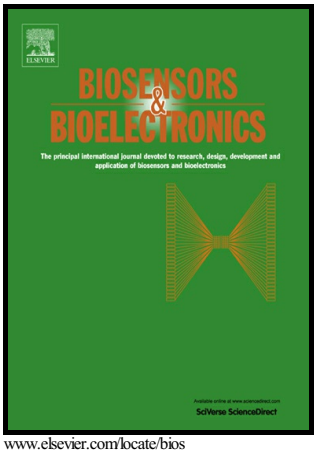

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www.elsevier.com/locate/bios

PII: S0956-5663(17)30278-6
DOI: http://dx.doi.org/10.1016/j.bios.2017.04.029
Reference: BIOS9690

To appear in: *Biosensors and Bioelectronic*

Received date: 20 January 2017
Revised date: 9 April 2017
Accepted date: 21 April 2017

Cite this article as: N. Shah and D.C. Naseby, Efficacy of benzalkonium chlorid against bioluminescent *P. aeruginosa* ATCC9027 constructs, *Biosensors an Bioelectronic,* http://dx.doi.org/10.1016/j.bios.2017.04.029

Efficacy of benzalkonium chloride against bioluminescent *P. aeruginosa* ATCC9027 constructs

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1.0 Abstract

Whole cell biosensors have been seldom used in the pharmaceutical and cosmetics industries for preservative efficacy testing (PET). According to several pharmacopoeias, preservatives should be tested for microbial activity using traditional viable count techniques; the use of whole cell microbial biosensors potentially provides an alternative, fast, and efficient method. The aim of the study was to assess the applicability of *Pseudomonas aeruginosa* ATCC9027 and its validated bioluminescent strains for preservative efficacy tests using benzalkonium chloride (BKC). Applicability of five constitutively-expressed bioluminescent strains was evaluated for preservative efficacy tests (PET) using bacterial replication, bioluminescence and fluorescence in a three-way study. PET using BKC showed no significant difference between bioluminescence and enumeration. Good correlations between bioluminescence, colony-forming units (CFU) count and fluorescence were obtained for BKC concentrations (R>0.9) between 0.0003%-0.0025% against strains containing the constructs lys-pME*lux*, lpp-pME*lux* and tat-pME*lux*. Furthermore, two-

way ANOVA analysis showed that the bioluminescent method and traditional plate counting method were equivalent for concentrations of BKC (0.0003 - 0.01%) during preservative efficacy tests. PET testing with BKC showed that tat-pME*lux* (R>0.9) had consistently high correlation coefficients between CFU and relative bioluminescence; *P. aeruginosa* ATCC9027 tatH5-pME*lux* is the best construct for testing various antimicrobial agents.

Keywords:

Pseudomonas aeruginosa, Preservative efficacy testing, benzalkonium chloride, bioluminescence, whole-cell biosensor, rapid screening.

2.0 Introduction

Antimicrobial preservatives are usually added to medicinal products that do not have antimicrobial activity to increase the shelf life of products (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008; United States Pharmacopoeia, 2008). Water-based medicinal products, such as solutions, creams, emulsions, suspensions, injections and eye-drops are most prone to microbial contamination. The effectiveness of the preservative must be tested over a period of time. Such tests are normally referred to as Preservative Efficacy Tests (PET) or Antimicrobial Efficacy Tests (AET) (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008); in such tests individual ingredients including excipients/additives composing the medicinal products as well as the medicinal product containing the preservative in its final container is inoculated with certain microorganisms and incubated at a specified temperature. Samples are then tested to assess the number of viable organisms present at various intervals of time up to 28 days after inoculation (Lundov, Moesby, Zachariae, & Johansen, 2009). A significant reduction in the number of viable microorganisms in tests signifies that the preservative is effective. The criteria may vary slightly between different pharmacopeias.

Pseudomonas aeruginosa ATCC 9027, one of the test micro-organism to be tested by various pharmacopoeias (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008; United States Pharmacopoeia, 2008) is one of the most widespread opportunistic pathogenic microbes to have been detected in various medicinal products and cosmetics. Microbial contamination in pharmaceuticals, food and cosmetics has been a problem for several decades, partly due to the difficulty in detecting it. It is therefore imperative that testing protocols for preservative efficacy be improved, such that all microorganisms are detected in due time. In 2013, a study found *P. aeruginosa* contamination in unopened toothpaste (Tan, Tuysuz, & Otuk, 2013). This pathogen, known to cause microbial keratitis, was also detected in a study investigating various contact lens disinfection solutions (Amiri, Mohammadinia, Tabatabaee, Askarizadeh, & Behgozin, 2011), which therefore failed to comply with the microbiological requirements set by The International Organisation for Standardisation (ISO). A fatal episode involving a *P. aeruginosa* outbreak was observed in immunocompromised patients in a haematology unit in Italy, when the source of infection was heavily contaminated triclosan soap dispensers (Lanini et al., 2011). *P. aeruginosa* was also detected in cough syrups in 2009, when one of the preservative systems employed for a cough syrup failed to be effective (Khanfar, Khalil, & AbuJafal, 2009). Many cosmetic products across Europe were recalled between 2005 and 2008 due to contamination with *P. aeruginosa* or other bacterial species (Lundov & Zachariae, 2008). A study investigating an outbreak of disease caused by contaminated mouth swabs across hospitals in Norway found that it was linked to *P. aeruginosa* and identified the source of the outbreak as occurring at various points in the manufacturing process (Iversen et al., 2007). These are just a few examples that emphasize how detrimental and lethal to human life this pathogen can be. Numerous investigations have been done for several decades that highlight the urgent need for novel methods for testing the efficacy of preservative systems.

There is increasing concern over benzalkonium chloride (BKC) resistance, especially in *Pseudomonas aeruginosa*. Resistance of *Pseudomonas* to BKC was reported as early as 1969 (Adair, Geftic & Gelzer, 1969). One reason for this is because *P. aeruginosa* forms biofilms that provide a protective barrier, facilitate the formation of bacterial clusters and therefore provide bacteria with intrinsic resistance towards

some antimicrobial agents. *Pseudomonas* is able to adapt to BKC, a feature known as antimicrobial tolerance. Increased synthesis of proteins involved in oxidative stress response, alginate synthesis and synthesis of cell membrane components have also been reported (Sheldon, 2005; Szomolay, Klapper, Dockery & Stewart, 2005). Although BKC is able to penetrate through the biofilm layers, *Pseudomonas* cells take advantage of the delay in penetration, thereby showing intrinsic resistance (Bridier, Dubois-Brissonnet, Greub, Thomas & Briandet, 2011). Another reason for intrinsic resistance is the expression of efflux pumps that are able to actively remove BKC back into the extracellular environment. Such resistance mechanisms provide one of the major reasons for persistent and repeated infections caused by *Pseudomonas.*

BKC, a quaternary ammonium compound (QAC), is a synthetic nitrogenous cationic antimicrobial agent with amphoteric surface-acting properties (Baudouin, Labbe, Liang, Pauly & Brignole-Baudouin, 2010; Fazlara & Ekhtelat, 2012). BKC is widely used in the food industry, hospitals and households as a disinfectant and antiseptic sanitizer (Fazlara & Ekhtelat, 2012). In pharmaceutical preparations, BKC is primarily used as a biocide or antimicrobial preservative in eyewashes, nasal sprays (Graf, 2001; Hodges, Denyer, Hanlon & Reynolds, 1996; Lenoir, Adriaens & Remon, 2011; Marple, Roland & Benninger, 2004), ophthalmic solutions (Mohanty, Acharya & Mishra, 2012; Rase, Bodkhe, Kshirsagar & Bedi, 2011; Ryan, Fain, Lovelace & Gelotte, 2011), amongst others. Interestingly, benzalkonium chloride is also used as an antimicrobial agent in acrylic carpets (Khajavi, Sattari & Ashjaran, 2007).

BKC is a broad-spectrum antimicrobial agent that possesses activity against both gram-positive and gram-negative bacteria as well as fungi and viruses. BKC cannot be classified as an antibiotic due to its lack of selectivity (McDonnell & Russell, 1999). BKC possesses both bacteriostatic and bactericidal properties. BKC is thought to change the hydrophobicity of bacterial cell membranes, thereby altering the cell membrane functions. Bacterial cell membranes carry a net negative charge on their surface, with cations stabilising the negative charge (Fazlara & Ekhtelat, 2012).

The aim of this work was to assess the applicability of *Pseudomonas aeruginosa* ATCC 9027 and bioluminescent constructs for preservative efficacy tests with benzalkonium chloride. Bioluminescent constructs were constructed (Shah & Naseby, 2014) and validated (Shah & Naseby, 2015) according to the PDA technical report (2000) and International Organisation for Standardisation (1994a, 1994b, 1994c, 2000) previously. Five promoter-*lux* variants were evaluated in a biosensor system for preservative efficacy testing using the bioluminescence method, traditional plate counting method and fluorescence method.

3.0 Materials and methods

3.1 Whole-cell bioluminescent strains, media and solutions

The *Pseudomonas* bacterial strains used for determining MIC and efficacy of BKC in this study are summarised in Table 1. Transformed *P. aeruginosa* strains were maintained on tryptone soy agar (TSA) (Oxoid). Gentamicin (10 µg/mL) was employed where required. Each strain was grown in tryptone soy broth (TSB) for 24 hours aerobically at 32^{o}C and 100rpm on an orbital shaker (Thermo Scientific). Cultures were diluted tenfold in buffered peptone water (Oxoid) where required in the following sections.

3.2 Bioluminescence, colony forming units and fluorescence determination

Aliquots (1.0 mL) of each ten-fold dilution for each strain were transferred in to culture tubes (Fischerbrand disposable borosilicate glass culture tubes 12 x 75 mm) and bioluminescence was measured at a wavelength of 490nm using the Celsis®

Advance Luminometer (London, UK). The luminometer was maintained at 32^{o}C during all measurements. Buffered peptone water was used as a blank.

For enumeration, all of the *P. aeruginosa* strains were serially 10-fold diluted in buffered peptone water. Cell counts were determined by spread-plating 100 µL of the serially diluted cultures, onto TSA agar containing 10 µg/mL gentamicin and incubating at 32 ^{o}C for 48 hours.

Quantification of fluorescence was performed by preparing samples according to BacLight Live/Dead kit (Molecular Probes). Fluorescence of the samples was measured using Promega GloMax detection system with blue filters (excitation at 490 nm, emission at 510– 570 nm) and green filters (excitation at 525 nm, emission at 510–570 nm). A control with sterile 0.85% sodium chloride was used to determine background fluorescence.

3.3 Minimum inhibitory concentration determination for BKC

Stock BKC (Sigma-Aldrich®) was prepared in sterile distilled water at a concentration of 5.12% ($^{w}/_{v}$) and pH of 7.0. The stock was filter-sterilised and stored at 20^{o}C in the dark. Working stocks of BKC were prepared at a concentration of 0.16% ($^{w}/_{v}$) in sterile distilled water. Various concentrations of BKC ranging from 0.0003% to 0.02% were then prepared in tryptic soya broth for MIC determination. The volume of inoculum was set at 1% of total reaction volume in accordance with pharmacopoeial guidelines. Therefore, the inoculum volume used was 50 µL of cell culture for 5 mL total reaction volume.

Each of the suspensions of BKC were prepared, in triplicate, in sterile cell culture plates and incubated for 24 hours at 32^{o}C and 100 rpm. The absorbance for each cell culture was measured at 600nm and recorded after incubation.

3.4 Preservative efficacy test for BKC

BKC working solutions were prepared at various concentrations, ranging from 0.0003125% to 0.01%, in a total reaction volume of 100 mL.

All strains were grown in LB- broth at 32^{o}C until a concentration of 10^8 CFU/mL was obtained as described above. Following incubation, aliquots (1 mL) of all cultures were centrifuged at 13000 rpm for 1 minute. The supernatant was discarded and the pellets were each re-suspended in 1 mL of sterile distilled water. This wash procedure (in accordance with pharmacopoeial requirements) was repeated once more before final re-suspension in sterile distilled water. Ten-fold dilutions were performed for WT, pME4510 and pME-*lux* strains in sterile distilled water as these strains generally grew up to a concentration of 10^9 CFU/mL.

For preservative efficacy testing, 1 mL of each of the washed (and in some cases diluted) cultures were inoculated aseptically into various concentrations of the preservative and incubated at 22^{o}C and 150 rpm for 28 days. The final concentration of each of the strains in the PET testing was 10^6 CFU/mL.

Samples were retrieved in triplicate aseptically from each flask at various time points as per the preservative efficacy testing requirements: 0 hours, 6 hours, 24 hours, 7 days, 14 days, 21 days and 28 days. All samples were serially diluted 10-fold seven times in buffered peptone water (containing a mixture of peptides from partially hydrolysed proteins). Aliquots (1 mL) of each undiluted culture and diluted culture were transferred into culture tubes aseptically for bio-luminescence measurements. Aliquots (100 μL) of each undiluted and diluted culture were transferred onto TSB Agar plates for quantification of CFU counts. Fluorescence was also measured as described above.

3.5 Data analysis

Pearson's correlations between bioluminescence reading and plate count were performed at a significance level of $P<0.05$ for data obtained from each concentration of preservative.

Log unit reductions in plate count and relative bioluminescence were calculated as:

$$\frac{(\text{Log}_{10}\text{ CFU at time }_{t}\text{ and concentration }_{c}) - (\text{Log}_{10}\text{ CFU at time }_{t0}\text{ and concentration }_{c0})}{}$$

Equation 1: Log unit reduction

Two-way analysis of variance (ANOVA) at a significance level of $P<0.05$ was performed between bioluminescence and plate count data in order to determine the equivalence between these two methods.

4.0 Results

4.1 Minimum inhibitory concentration of BKC

The minimum inhibitory concentration of benzalkonium chloride was evaluated for all the strains by measuring absorbance at 600nm after challenge with a range of concentrations of BKC (0.0003% and 0.02%). Growth of wild-type, pME4510 and pME*lux* bacterial strains was observed in the negative controls (0% BKC), as well as dilute concentrations between 0.0003% and 0.005% (Table 2). The MIC of BKC for these three strains was therefore found to be 0.01%. The absorbance values obtained for wild-type, pME4510 and pME*lux* strains with 0% BKC were greater (>2.25) than those of the bioluminescent constructs (1.75). This was because these three strains grow up to 10^9 CFU/mL compared to the bioluminescent constructs that grow up to 10^8 CFU/mL. However, for all bioluminescent constructs the MIC was 0.00125%.

4.2 Preservative efficacy test for BKC

The effect of various concentrations of BKC was evaluated with all the strains exposed to preservative challenge. Bioluminescence, CFU count and fluorescence measurement were recorded at time intervals as defined by several pharmacopoeias. Wild-type *P. aeruginosa* and *P. aeruginosa* with either plasmid pME4510 or pME*lux* were used as negative controls for bioluminescence detection. Correlation coefficients and their significance are shown in Supplementary material Table 3.0. Log unit reductions in CFU and RLU are shown in Supplementary material Table 4.0. Equivalence between the bioluminescence method and traditional plate count method for BKC is shown in Supplementary material Table 5.0.

At high BKC concentration of 0.01%, negative control strains were 10^5 CFU/mL, bioluminescence was undetectable upon inoculation for bioluminescent constructs; however, CFU count in the range of 10^3 CFU/mL were observed for all bioluminescent constructs. Hence, there was a 3 log reduction in CFU immediately upon inoculation. A further 3 log reduction in CFU was observed by 6 hours. It was therefore shown that bioluminescence was undetectable at such high concentrations of BKC upon inoculation of the strains with considerable reduction in CFU count. No CFU count was detected beyond time 0.

Weak bioluminescence signals were detected (10^2 RLU/mL) at 0 hour only for the strong promoter lys challenged with 0.005% and 0.0025% BKC. No bioluminescence was detected at subsequent time points or for all other strains. For all strains, CFU count was 10^5 and 10^6 CFU/mL upon challenge with the same concentrations of BKC, respectively. No CFU count was detected beyond time 0. Correlations between bioluminescence and plate counts for lys strain challenged with 0.005% BKC were found to be statistically significant ($P<0.05$) with correlation coefficients of 0.8. This was because lys was the strongest promoter out of the five promoters tested and therefore it produced higher levels of bioluminescence. At BKC concentrations of 0.0025%, correlations between bioluminescence and plate count were significant ($P<0.01$), with correlation coefficients greater than 0.9. Relative fluorescence (RFU) measurements were taken to determine viability of cells with respect to CFU counts.

RFU readings were measured for strains challenged with 0.0025% BKC. RFU measurements for control strains and bioluminescent constructs were 10^5 and 10^6 RFU/mL, respectively, at only 0 hour time point. No further RFU readings were detectable for the subsequent time points.

Bioluminescence was detected at 0 hour for all constructs of lysS, lpp and tat (except ldcC and spc) challenged with 0.0125% BKC (Figure 1.0A). No bioluminescence was detected at subsequent time points or for all other strains. CFU counts were 10^5 and 10^6 CFU/mL for all control strains and bioluminescent strains, respectively (Figure 12.0B). No CFU count was detected after 0 hour. RFU measurements (Figure 1.0C) for control strains and bioluminescent strains were 10^5 and 10^6 RFU/mL, respectively, at time 0. No further RFU readings were detectable for the subsequent time points. Correlations between bioluminescence and CFU count, for all the strong promoter strains were significant ($P<0.01$) with correlation coefficients greater than 0.96. Viability testing of all the strains also resulted in significant correlations ($P<0.01$) between culturable cells and fluorescence, with correlation coefficients greater than 0.98. Correlations between bioluminescence and fluorescence were also significant ($P<0.01$), with coefficients greater than 0.96 for the bioluminescent constructs.

Bioluminescence was 10^3 and 10^4 RLU/mL for bioluminescent constructs with weak and strong promoters, respectively, when challenged with 0.0006% BKC (Figure 2.0A). Bioluminescence then reduced by 1 log unit at 6 hours for lys and lpp. No bioluminescence was detected up to 28 days for all other strains. CFU counts were greater than 10^5 and 10^6 CFU/mL, respectively, for all control strains and bioluminescent strains upon challenge with the same concentration of BKC (Figure 2.0B). CFU counts for bioluminescent constructs subsequently decreased to 10^4 CFU/mL at 6 hours and decreased further at 24 hours. No CFU counts were detected after 24 hours. RFU was measured for strains challenged with 0.0006% BKC (Figure 2.0C) and these were 10^5 and 10^6 RFU/mL for control strains and bioluminescent strains, respectively, at 0 hour. RFU for control strains subsequently decreased to 10^4 RFU/mL at 6 hours; however, for bioluminescent constructs RFU

reduced to 10^4 RFU/mL at 24 hours. No further RFU readings were detectable for later time points. Correlations between bioluminescence and CFU count were significant ($P<0.01$) for all bioluminescent constructs with coefficients greater than 0.99. Correlations between bioluminescence and fluorescence were significant ($P<0.01$). However, the coefficients were slightly lower and ranged between 0.86 – 0.97 for all bioluminescent constructs. Furthermore, significant correlations ($P<0.01$) between CFU and RFU were obtained for all bioluminescent constructs except lysR25. However, the coefficients ranged between 0.85-0.97. Correlations for lys were significant at $P<0.05$ with a coefficient of 0.85 indicating that not all cells were culturable. This was further confirmed when fluorescence was 10^4 RFU/mL at 6 and 24 hours but no CFU counts were detected for negative control strains at the same time points. A 2 log and 3 log reduction in CFU at 6 hours and 24 hours, respectively, was therefore observed for all bioluminescent strains. This related to a >2 log and 4 log reduction in bioluminescence at 6 hours and 24 hours, respectively.

Bioluminescence was 10^3 and 10^4 RLU/mL for bioluminescent constructs with weak and strong promoters, respectively, when challenged with 0.0003% BKC (Figure 3.0A). Bioluminescence decreased by a log unit for all promoters within 6 hours and by a further log unit for all promoters except ldcC and spc by 24 hours. No bioluminescence was detected from 24 hours up to 28 days for all strains. CFU counts were 10^6 CFU/mL (Figure 3.0B) for all strains at time 0. CFU counts for bioluminescent constructs decreased by a log unit by 24 hours and by 2 log units by 7 days. CFU counts for all bioluminescent strains except ldcC and spc were detected at 10^3 CFU/mL at 14 days followed by undetectable levels up to 28 days. No CFU counts were detected beyond 6 hours for the negative control strains. A similar pattern of fluorescence to CFU was found for all strains up to 24 hours. Relative fluorescence for negative control strains and bioluminescent strains (Figure 3.0C) declined even further to 10^3 RFU/mL and 10^4 RFU/mL, respectively, at 7 days. From 14 days onwards, negative control strains and ldcC and spc were undetectable. Strains lys, lpp and tat fluoresced consistently throughout the rest of the experiment. Correlations between bioluminescence, CFU count and fluorescence were significant ($P<0.01$). However, the correlations between bioluminescence and fluorescence

were slightly reduced for all bioluminescent strains except tat. Correlations between culturable cells and fluorescence were also reduced slightly for lys.

Correlations between CFU and RFU were significant ($P<0.01$) for negative control strains wild-type and pME4510 throughout 28 days when they were challenged with different concentrations of BKC. However, the correlations were slightly reduced to 0.89 and 0.93, respectively, when these strains were challenged with 0.0006% BKC. A similar pattern was found for the promoterless strain, where correlations were significant ($P<0.05$) and there was slight reduction in correlation coefficient to 0.85 with 0.0006% BKC challenge.

Bioluminescence was detected throughout 28 days for all promoter-*lux* constructs when challenged with 0% BKC (Figure 4.0A). CFU counts for all strains were approximately 10^6 CFU/mL and remained consistent throughout 28 days (Figure 4.0B). RFU measurements for all strains were 10^6 RFU/mL throughout 28 days and remained consistent (Figure 4.0C). The correlations between bioluminescence and CFU count were found to be weak and non-significant. However, correlations between culturable cells and fluorescence for all strains except ldcC and lys were significant ($P<0.05$) with correlation coefficients above 0.8 when conditions were starved for 28 days.

5.0 Discussion

5.1 Minimum inhibitory concentrations of BKC

All strains of *P. aeruginosa* were challenged with different concentrations of BKC, ranging from 0.0003% to 0.02%. The MIC for wild-type, pME4510 and pME*lux* was found to be 0.01%. This result has been confirmed by another study that found the MIC for wild type *P. aeruginosa* to be 0.01% (Osman, El-Hendawy, Hassan, Abdel-All, & Dina Ezzat, 2012). The MIC for bioluminescent constructs was found to be 0.00125%. Several studies have also shown that the MIC of BKC against *P.*

aeruginosa is non-uniform. Studies found the MIC to be 0.006% (Fazlara & Ekhtelat, 2012) or 0.0032% (Kamysz & Turecka, 2005). Yet another study indicated the MIC to be 0.025% after 120 hours of contact time (Khajavi et al., 2007).

The change in MIC from 0.01% for wild-type to 0.00125% for bioluminescent constructs suggests that the bioluminescent constructs are more vulnerable to BKC challenge than the wild-type. The studies suggest that the incorporation of the *lux* cassette under constitutive promoters may be detrimental to the cells by providing a metabolic load on the cells. Furthermore, incorporation of just the *lux* cassette into the plasmid had no effect on the MIC. However, further incorporation of promoters changed the MIC significantly. The strength of the promoters, however, did not have any effect on the MIC shift. Physical observations of growth of bioluminescent constructs compared to that of wild-type also showed that bioluminescent constructs produced significantly less biofilms at 24 hours. This may explain the metabolic load/detrimental effects of active expression of the *lux* cassette that result in cells being more susceptible to benzalkonium choride. Gilbert and Moore (2005) have shown a positive correlation between biofilm formation and increase in MIC. Changes in MIC in gram-negative bacteria may be due to certain factors that alter the susceptibility of bacterial cells against preservatives. These factors include alterations in phenotype, efflux pumps, expression of enzymes, chromosomal and plasmid-based alterations (Gilbert & McBain, 2003). They also suggested that plasmids provide low levels of resistance against preservatives.

5.2 Preservative efficacy tests for BKC

Preservative efficacy testing (PET) was performed according to the requirements of the pharmacopoeias. This study aimed at utilising the validated bioluminescent strains (Shah & Naseby, 2014; Shah & Naseby, 2015) to determine antimicrobial efficacy of BKC using the bioluminescence method of detection in parallel to the traditional plate counting method. Different concentrations of BKC, ranging from 0.01% to 0.0003% ($^{w}/_{v}$) were used for the PET against all *P. aeruginosa* strains used in the study.

The MIC of BKC against the wild-type was 0.01%. However, under starved conditions in preservative efficacy testing, the wild-type strain was susceptible at 0.00125% BKC. MIC determination is performed using growth medium, thereby providing cells with a chance to survive in the presence of antimicrobials by utilising the available energy sources. The change in biocide susceptibility relates to the fact that in nutrient-depleted environments (PET), the bacterial cells are slow-growing as opposed to nutrient-rich environments (MIC determination in presence of media) when the bacterial cells grow and form biofilms (Fazlara & Ekhtelat, 2012). In the presence of growth media, the cells were strong enough (unstarved) to provide intrinsic resistance against antimicrobials as compared to their susceptibility when the cells were starved during preservative efficacy testing. Biocide activity may be significantly compromised in nutrient-rich environments due to the biofilm formation in such environments. Nutrient depleted environments trigger a stress response by bacterial cells (Gilbert & McBain, 2003). Initiation of stationary phase leads to dormant phenotypes where cells adopt a 'resting' nature. A study (Foley, Marsh, Wellington, Smith & Brown, 1999) also showed increased rpoS expression in nutrient-rich environments. In nutrient limiting environments, the detrimental effect of metabolic burden has also been highlighted in a study where lack of glucose has shown increased susceptibility of *P. aeruginosa* to benzalkonium chloride (Mc Cay, Ocampo-Sosa & Fleming, 2010). Lack of magnesium, which is vital for *Pseudomonas* cell membrane development, has also been shown to increase susceptibility of the cells to antimicrobial agents (Manzoor, Lambert, Griffiths, Gill & Fraise, 1999)

Negative control strains were observed to be more vulnerable to BKC challenge than to bioluminescent constructs. This was indicated by the susceptibility of negative control strains at 0.0006% BKC, whereas the bioluminescent constructs were resistant to BKC at the same concentration. The promoterless strain also behaved in the same manner as the wild-type and hence it was more susceptible than the bioluminescent constructs. This indicated that the incorporation of the plasmid and therefore expression of the genes on the plasmid provided a protection against BKC for all bioluminescent cells. Growth of the promoterless strain in a manner similar to the wild-type suggested that it was not plasmid incorporation but rather the

expression of the *lux* cassette that was conferring resistance of bioluminescent constructs to BKC.

Most interestingly, the correlation between bioluminescence and CFU count in the absence of preservative was weak and non-significant. However, the correlation between culturable cell count and fluorescence was significant ($P<0.05$), with a correlation coefficient greater than 0.8. This indicated that the cells were culturable. However, their bioluminescence pathway was affected. This was further confirmed with a significant difference between the bioluminescent method and plate counting method when ANOVA was performed. Under starved conditions, bacterial cells lack the fundamental resources of energy production and therefore cannot perform certain energy-demanding reactions because of a lack of an energy source. One such example is the bioluminescence pathway, which is energy-demanding in the form of NADPH, $FMNH_2$ and ATP (Meighen, 1991). Other metabolic pathways include biofilm production and virulence determination.

High concentrations of BKC (0.01%-0.0025%) were found to be too toxic for the cells, as observed by immediate arrest in bioluminescence and significant reduction in CFU count, hence indicating an immediate bactericidal effect of the biocide. Typical in-use concentrations of BKC as antimicrobial agents are 0.01-0.02% (Osman et al., 2012; Rase et al., 2011; Ryan et al., 2011). The immediate bactericidal effect has been observed because cell membranes are virtually dissolved at such high concentrations of BKC (Lambert & Hammond, 1973). At high BKC concentrations, micelles are formed leading to solubilisation of the hydrophobic membrane (Gilbert & Moore, 2005). BKC also forms dimers, which interact strongly with the cell membrane, thereby exerting a stronger antimicrobial effect (Gilbert & Altaae, 1985).

Fluorescence detection with the aid of Molecular Probes® Live/Dead® BacLight™ staining comprising of two dyes Syto-9 and propidium iodide (Berney et al., 2007; Molecular Probes) discriminated between viable and non-viable live cells. This method of estimating viable and non-viable cells is a rapid method that has been used in numerous studies (Ferreira et al., 2011; Harrison et al., 2008). At BKC concentrations of 0.0006% and 0.0003%, correlations between bioluminescence,

CFU count and fluorescence were significant ($P<0.05$) for all bioluminescent constructs. However, a decrease in the correlation coefficients between fluorescence and bioluminescence and between fluorescence and culturability suggested that a few cells had become non-culturable. At low BKC concentrations, the membrane perturbations lead to loss of membrane fluidity and stable hexagonal arrangements of phospholipids are formed (Gilbert & Moore, 2005). Therefore cells may not be able to grow and replicate on media.

Although concentrations of 0.0006% BKC fulfilled the criteria of the pharmacopoeias, the latter does not take into account cells that are viable but undetected using the plate count method. This may further explain why microbial contamination has been a problem in numerous pharmaceutical products for several decades; a recent study by Tan *et al* (2013) found *P. aeruginosa* contamination in unopened toothpaste. Another recent study also showed the importance of detection of viable, non-culturable cells in the food industry (Elizaquível, Aznar & Sánchez, 2014). The capability of viable but non-culturable bacterial cells to cause product spoilage and potentially pose as a threat for infections has been shown in various studies (Colwell et al., 1996; Rahman, Shahamat, Chowdhury & Colwell, 1996). It is therefore imperative that testing protocols for preservative efficacy be improved such that all microorganisms bypassing the protocols are detected in due time. *P.aeruginosa* has been one of the many widespread opportunistic microbes to have been detected in various pharmaceutical preparations and cosmetics.

At a lower BKC concentration of 0.0006%, correlations between bioluminescence and CFU count were significant ($P<0.05$) for all bioluminescent constructs Correlations between bioluminescence and fluorescence were reduced. Decrease in bioluminescence prior to cell count was observed when cells were challenged with 0.0006% BKC; bioluminescence decreased by two log units within 6 hours, whereas fluorescence reduced by a half log unit. CFU count also reduced by two log units, suggesting that bioluminescence decreased before cell viability decreased. Initial reduction in bioluminescence followed by reduction in CFU count indicates that the binding of the biocide to membrane proteins and creating osmotic gradients such that the constituents of the cells leak out (Lambert & Hammond, 1973) leading to subsequent disturbances to optimum environments for luciferase reactions. This is

rapidly followed by reduction in CFU count where the cell constituents have leaked out to detrimental levels such that cell death is observed. Furthermore, denaturation of proteins and formation of protein aggregates (Gupta & Kaisheva, 2003) including the luciferase enzymes imposes an immediate detrimental effect on the bioluminescence pathway. Hence this phenomenon of reduction in gene expression prior to reduction in cell number may serve as an early indicator of the effects of preservative on microbial death and therefore the bioluminescent method may be used to detect widespread death of microbial cells during antimicrobial efficacy testing. Bioluminescence has been shown to give an earlier indicator of the health state of bacterial cells compared to traditional plate counting.

Correlations between bioluminescence, cell count and fluorescence measured at the minimum inhibitory concentrations of BKC were also significant for all the bioluminescent constructs. Analysis of variance tests give an indication whether the bioluminescent and plate count method were equivalent. These tests were performed at $P<0.05$. For various concentrations of BKC during preservative efficacy tests, it was found that there was no significant difference between the bioluminescent method and traditional plate counting method for concentration ranges of 0.0003% - 0.01% BKC as observed by P-values being greater than 0.05. This therefore indicated that the bioluminescent method may be used to detect microbes during antimicrobial efficacy testing. Previous studies have also utilised the bioluminescent method to examine the efficacy of biocides such as phenol, chlorhexidine digluconate and ethanol (Choi & Gu, 1999; Dhir & Dodd, 1995; Robinson et al., 2011). Testing model preservative systems used in pharmaceutical preparations against *P. aeruginosa* using the ATP bioluminescent method showed incompliance of the preservative systems to the pharmacopoeial requirements (Kramer et al., 2008). This was because initial ATP levels in *P. aeruginosa* were low (Kramer et al., 2008). ATP among other energy compounds are regarded as markers that inform about the global health state of bacterial cells. Therefore reduction in initial levels of such markers indicate immediate effect of antimicrobial agents on bacterial cells. Such immediate effects would not be detected by the ATP bioluminescent method since this method requires ATP extraction from cells, thereby presenting a physical disadvantage for detection of immediate effects. Coupling of

bioluminescence to cell viability with respect to constitutive gene expression provides a powerful, non-destructive tool for studies of bacterial growth under preservative challenge in real time.

Bioluminescent signals as a result of gene expression from P_{ldcC} and P_{spc} were weak. As a result the strains with these promoters showed weaker and non-significant correlations between bioluminescence and CFU counts when challenged with BKC concentrations between 0.00125% and 0.01%. There strains therefore are not suitable candidates for preservative efficacy testing.

6.0 Conclusion

Strong correlations between bioluminescence, CFU count and fluorescence were obtained for BKC concentrations between 0.0003% and 0.0025% against strains lys, lpp and tat. The bioluminescent method and traditional plate counting method were found to be equivalent for concentrations of 0.0003% - 0.01% BKC during preservative efficacy tests. To conclude, these bioluminescent constructs are therefore strong candidates for selection for incorporation into a biosensor system for preservative efficacy testing.

Preservative efficacy testing using traditional methods takes at least 28 days plus set up time and incubation time; additionally the analysis is very laborious and time-consuming. Furthermore, a range of concentrations of various preservatives need to be tested for antimicrobial efficacy, making the challenge test expensive, prolonged and laborious. Use of whole cell microbial bioluminescent constructs therefore provides a rapid, accurate, non-destructive and less laborious method that gives results in real time.

Acknowledgements:

This work was supported and funded by the School of Life and Medical sciences at the University of Hertfordshire, UK.

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Figures and Tables

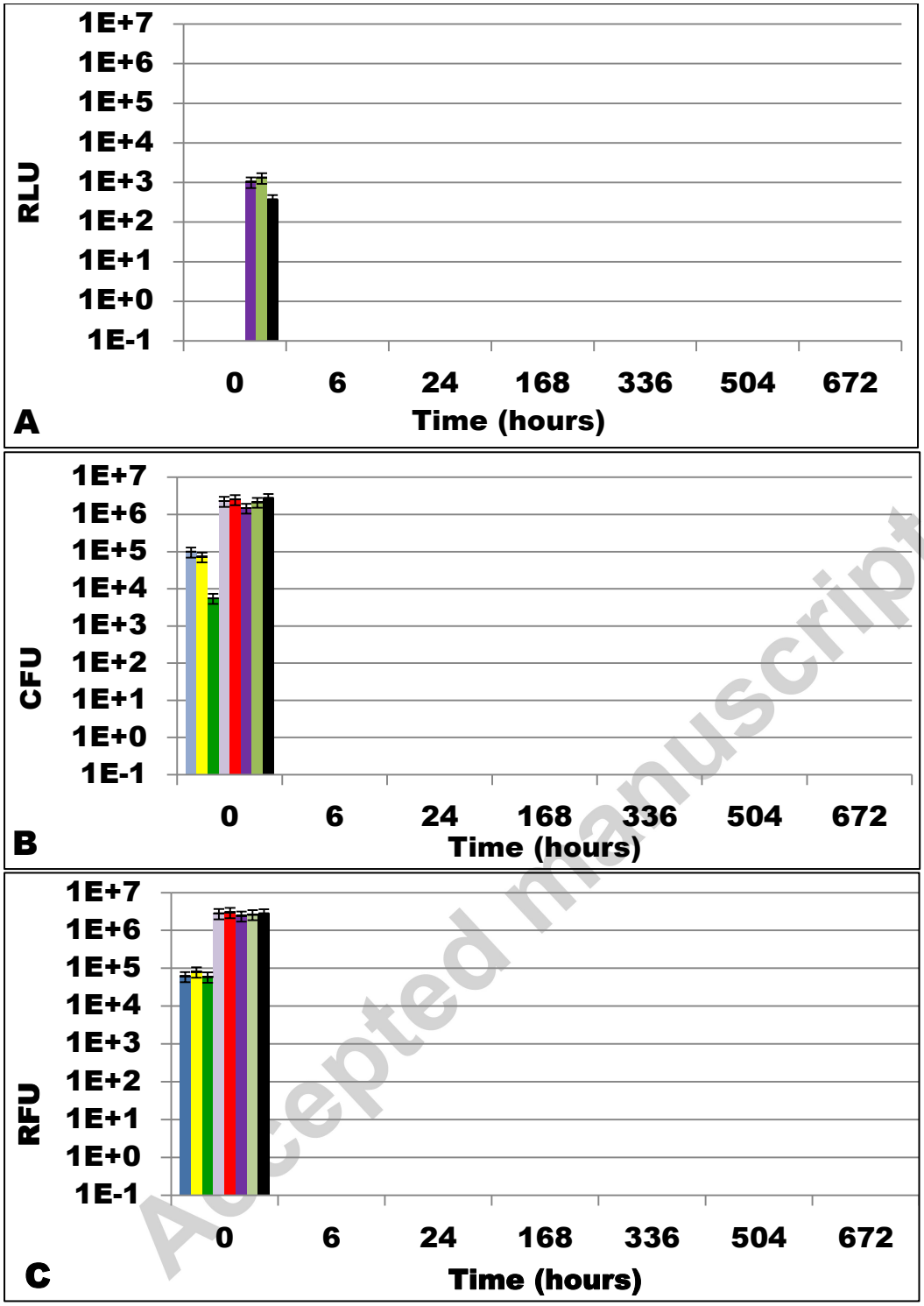


Figure 1.0: BKC efficacy test for all strains challenged with 0.00125% BKC. Measured for 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence. Error bars show standard error for n=3 replicates.

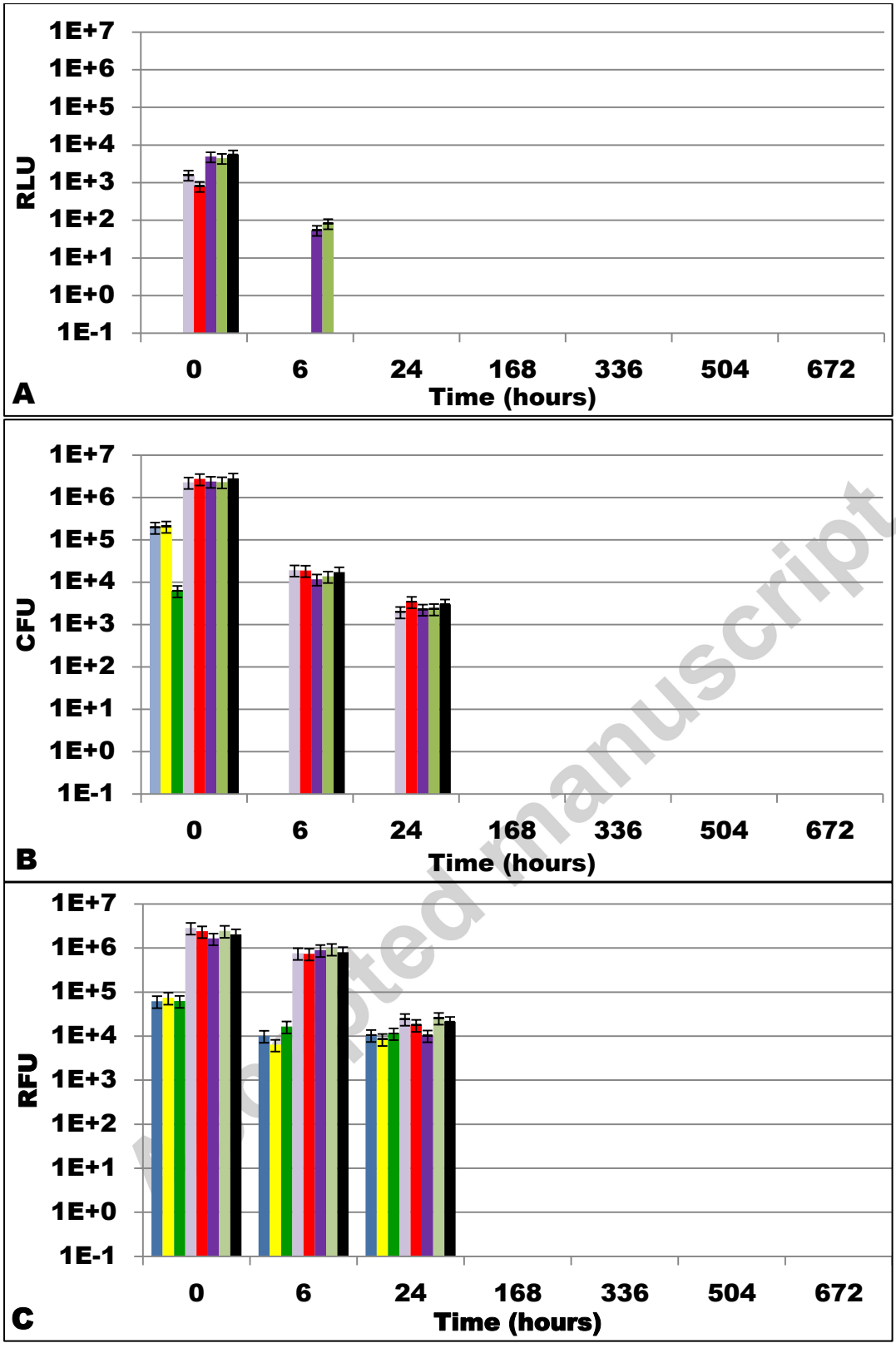
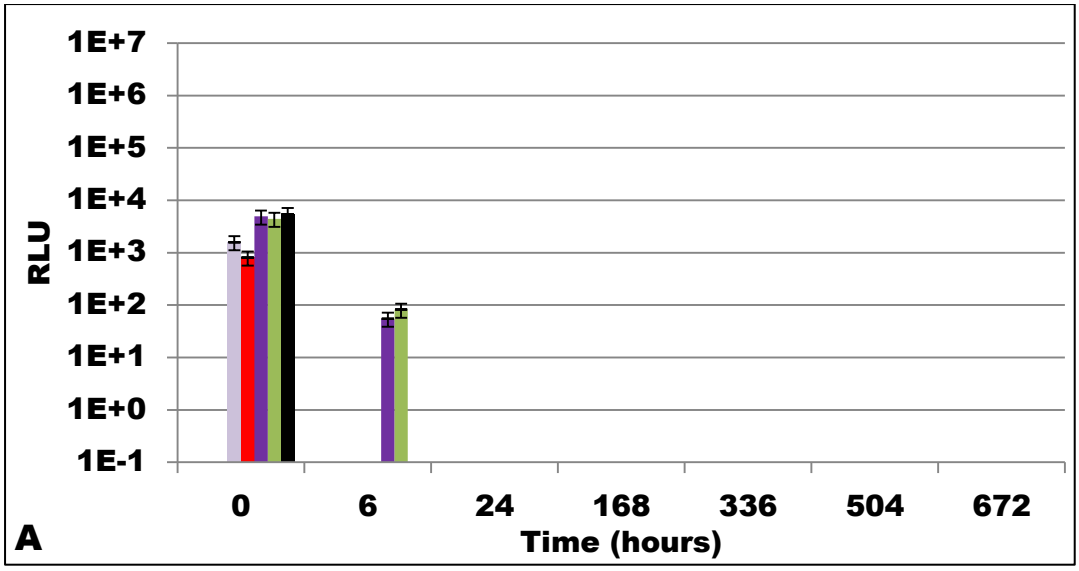
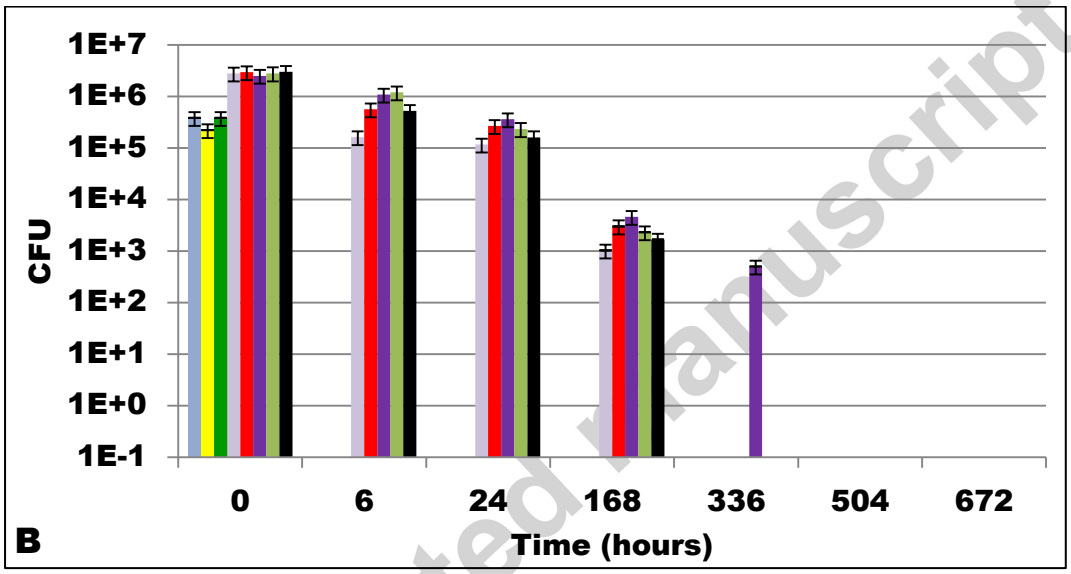


Figure 2.0: BKC efficacy test for all strains challenged with 0.000625% BKC. Measured for 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence. Error bars show standard error for n=3 replicates.

WT pME4510 pME*lux* ldcC spc lys lpp tat





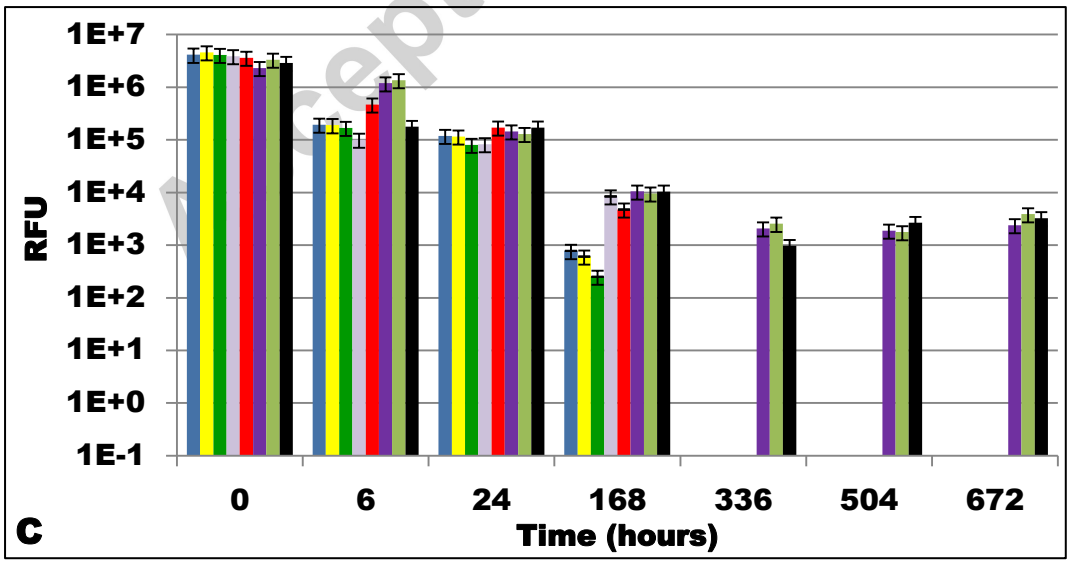
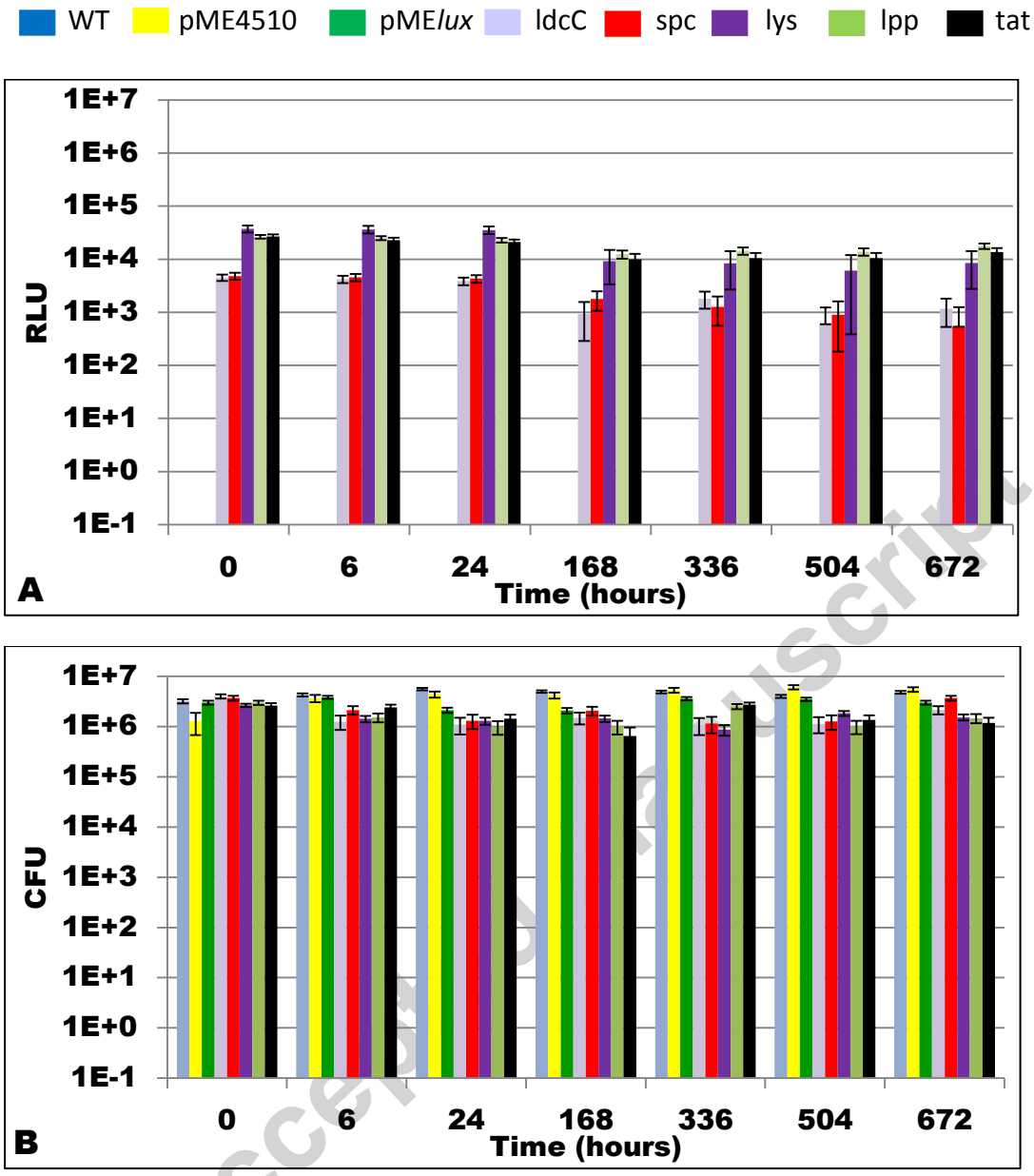


Figure 3.0: BKC efficacy test for all strains challenged with 0.0003125% BKC. Measured for 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence. Error bars show standard error for n=3 replicates.



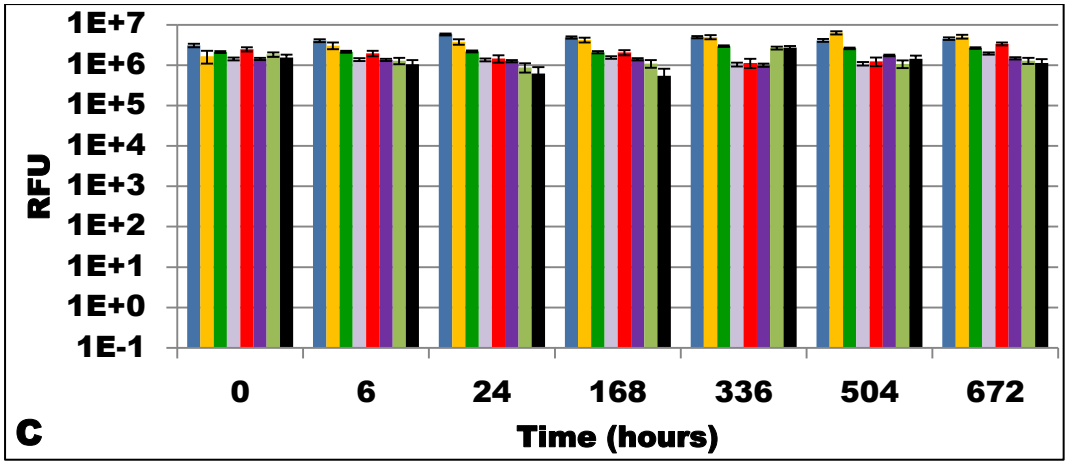


Figure 4.0: BKC efficacy test for all strains challenged with 0% BKC. Measured for 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence. Error bars show standard error for n=3 replicates.

WT pME4510 pME*lux* ldcC spc lys lpp tat

Table 1: Bacterial strains used in this study

Bacterial strain	Plasmid	Reference
***P. aeruginosa* ATCC 9027 (Wild-type)**	No plasmid	ATCC®
***P. aeruginosa* ATCC 9027**	pME4510	Rist & Kertesz (1998)
***P. aeruginosa* ATCC 9027**	pME4510-*lux*	Shah & Naseby (2014)
***P. aeruginosa* ATCC 9027**	pME4510-ldcC-*lux*	Shah & Naseby (2014)
***P. aeruginosa* ATCC 9027**	pME4510-spc-*lux*	Shah & Naseby (2014)
***P. aeruginosa* ATCC 9027**	pME4510-lys-*lux*	Shah & Naseby (2014)
***P. aeruginosa* ATCC 9027**	pME4510-lpp-*lux*	Shah & Naseby (2014)
***P. aeruginosa* ATCC 9027**	pME4510-tat-*lux*	Shah & Naseby (2014)

Table 2: Minimum inhibitory concentrations for BKC against all strains.

	Absorbance [a] at 600nm at various BKC concentrations (%)							
Strain ID	**0**	**0.0003**	**0.0006**	**0.0013**	**0.0025**	**0.005**	**0.01**	**0.02**
WT	2.40	2.42	2.39	2.30	2.36	0.80	0.10	0.13
pME4510	2.31	2.43	2.42	2.32	2.30	0.34	0.07	0.08
pMElux	2.35	2.28	2.20	2.15	2.17	0.10	0.06	0.09
ldcc	1.86	1.76	1.74	0.01	0.08	0.12	0.08	0.04
spc	1.85	1.77	1.72	0.01	0.09	0.11	0.06	0.03
lys	1.84	1.87	1.68	0.04	0.02	0.03	0.03	0.03
lpp	1.86	1.77	1.85	0.01	0.02	0.03	0.04	0.04
tat	1.86	1.78	1.77	0.01	0.01	0.10	0.11	0.01

[a] Absorbance was measured at 600nm.
Green indicates growth of *P. aeruginosa.* Red indicates no growth of *P. aeruginosa.*

Highlights

- The main point of the paper is to highlight the use of rapid alternative methods (as opposed to the traditional enumeration method that is laborious and requires long incubation times) to determine the efficacy of benzalkonium chloride on a pathologically notorious and pharmacopoeial-defined pathogen, P. aeruginosa. This paper highlights the use of bioluminescent bacteria as a whole-cell microbial biosensor when coupled to a luminometer, allowing results to be generated in real-time mode. Such a method is highly valuable to pharmaceutical industries, where time to generate results can be very limited.