Development of a rapid method for assessing the efficacy of antibacterial photocatalytic coatings

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Abstract

Visible-light activated photocatalytic coatings may represent an attractive antimicrobial solution in domains such as food, beverage, pharmaceutical, biomedical and wastewater remediation. However, testing methods to determine the antibacterial effects of photocatalytic coatings are limited and require specialist expertise. This paper describes the development of a method that enables rapid screening of coatings for photocatalytic-antibacterial activity. Relying on the ability of viable microorganisms to reduce the dye resazurin from a blue to a pink colour, the method relates the time taken to detect this colour change with number of viable microorganisms.

The antibacterial activity of two photocatalytic materials (bismuth oxide and titanium dioxide) were screened against two pathogenic organisms (*Escherichia coli* and *Klebsiella pneumoniae*) that represent potential target microorganisms using

traditional testing and enumeration techniques (BS ISO 27447:2009) and the novel rapid method.

Bismuth oxide showed excellent antibacterial activity under ambient visible light against *E. coli*, but was less effective against *K. pneumoniae*. The rapid method showed excellent agreement with existing tests in terms of number of viable cells recovered. Due to advantages such as low cost, high throughput, and less reliance on microbiological expertise, this method is recommended for researchers seeking an inexpensive first-stage screen for putative photocatalytic-antibacterial coatings.

Keywords

Antibacterial activity, rapid method, Visible light photocatalysis, titanium dioxide, bismuth oxide, resazurin

1.0 Introduction

Microorganisms are ubiquitous. Many species can exist in a viable state for long periods of time, from several hours to several weeks on surfaces and up to several years depending on environmental conditions (Verran, 2002; Kusumaningrum *et al.*, 2003; Liao and Shollenberger, 2003; Wilks *et al.*, 2005; Fuster-Valls *et al.*, 2008; Møretrø *et al.*, 2010; Redfern and Verran, 2017). Pathogenic species with low infectious doses can be transferred from these surfaces many hours after initial contamination, contributing to the spread of disease including antibiotic resistant bacteria and viruses such as influenza and coronavirus (Wilks *et al.*, 2006; Casanova *et al.*, 2010; Greatorex *et al.*, 2011; Gerhardts *et al.*, 2012; Pérez-Rodríguez *et al.*, 2013). Furthermore, with access to nutrients, microorganisms can colonise the surface and form biofilms (Matz *et al.*, 2004; Codd *et al.*, 2005; Driscoll *et al.*, 2007; Banat *et al.*, 2010; Møretrø *et al.*, 2010). Surface contamination and

subsequent biofilm formation has major implications in certain domains where the hygienic status of the environment is paramount. Industries such as food, beverage, pharmaceutical, biomedical and wastewater remediation all require that microbial populations (bacteria, fungi and viruses) are controlled to prevent the spread of infectious disease, limit horizontal gene transfer, avoid product spoilage, and reduce operating costs (Choi *et al.*, 2007; Simões *et al.*, 2010; Bruscolini *et al.*, 2015; Priha *et al.*, 2015; Redfern and Enright, 2020). Amongst a host of strategies aimed at controlling, reducing or preventing such contamination and colonisation, the application of photocatalytic thin film coatings to surfaces is one approach (Foster *et al.*, 2011).

In recent years, the most commonly reported photocatalyst has been titanium dioxide (TiO_2). The material first received interest due to Fujishima's discovery of the photocatalytic splitting of water by titanium dioxide (Fujishima and Honda, 1972). Since then, there has been extensive study and development of TiO_2 photocatalysts (Fox and Dulay, 1993; Hoffmann *et al.*, 1995; Diebold, 2002; Verran, 2002; Allen *et al.*, 2008; Fujishima *et al.*, 2008; Caballero *et al.*, 2009; Foster *et al.*, 2011; Nakata and Fujishima, 2012; Lang *et al.*, 2014; Low *et al.*, 2017). Research has been focused on TiO_2 for three reasons; it has a high stability and activity compared to similar semiconductors, in addition to a low cost (Carp *et al.*, 2004). However, ultraviolet light (UV) is required for activation. UV comprises less than 5% of total photons of solar light, rendering the photocatalyst relatively inefficient (Dalrymple *et al.*, 2010). Additionally, it is likely that only outdoor coatings would be activated by sunlight at all, as indoor fluorescent lighting contains few UV photons. To be more useful, the photocatalyst must be activated by less energetic photons. Therefore, recent research has focussed on visible-light activated photocatalysts. A common

strategy to induce visible-light activity for TiO_2 has been by doping with other elements (Farahani *et al.*, 2011; Kelly *et al.*, 2014; Ratova *et al.*, 2015; Low *et al.*, 2017; Wu *et al.*, 2017; Yoon *et al.*, 2018).

Alternatively, photocatalysts other than TiO_2 with inherent visible light activity have explored. For example, bismuth oxide has high visible-light activity, and also significant photocatalytic-antibacterial properties (Ratova *et al.*, 2018).

Current methods to determine the antibacterial properties of photocatalytic materials can be time-consuming, complex, be affected by changing environmental conditions and require significant microbiological expertise (Redfern *et al.*, 2018a). Therefore, there currently is a need for a more accessible and rapid method to determine the photocatalytic-based antibacterial activity of thin film coatings.

The standardised test method BS ISO 27447:2009 (British Standards Institution, 2009) is one of the most commonly used methods to assess the antibacterial activity of a photocatalytic material. While originally designed for UV-activated materials, it has since been adapted for use with visible-light activated photocatalysts (Sadowski *et al.*, 2015). It encompasses the inoculation of coatings with specified numbers of microbial cells in a known volume, followed by irradiation. At pre-determined irradiation times, surviving cells are removed from the surface into a known volume of diluent for enumeration. Since the number of cells is unknown, the enumeration procedure requires the dilution of the cell suspension and the inoculation of agar plates with known volumes (typically 0.1 mL) of known dilutions of the suspension. Each cell inoculated onto the agar grows to form one colony, thus after incubation, the number of colonies growing on the agar plates is counted (the dilution which presents the highest countable number of colonies is used), and the number of cells

in the original suspension is calculated. This method requires competent microbiological expertise (culture maintenance, aseptic technique), and significant amounts of consumable materials (agar plates, diluents, pipettes). Each coating must be tested in the dark (control) as well as under irradiation. The method is also time-consuming, requiring replicate samples and repeat experiments to enable statistical testing of results. For example, testing one coating would require three replicate coupons per sampling time (at least three) for light and dark treatments (total of 18 coupons). At each sampling time, the cell suspension obtained by removal of surviving cells from the surface would be diluted to 10^{-8}, and each dilution would be inoculated onto two or three agar plates (288 plates required in total for duplicate plates) for incubation – and then the experiment is repeated. An experienced microbiologist would be able to identify any contaminants growing on the agar plates, and would also ensure that the dilution series is accurate (approximately ten-fold reduction in colony counts with each dilution) and that duplicate plates have colony counts that are in agreement.

This procedure can be divided into four main steps: inoculate the presumptive photocatalytic surface with a known number of bacteria in suspension, irradiate the surface for a given time, recover the cells, and then enumerate the colonies produced by the viable recovered bacteria. A pertinent issue with this procedure is the amount of time required; in addition to irradiation time (up to 24 hours) and culture time (18-48 hours), the number of worker hours required to prepare media, inoculate, recover, dilute, spread and count is significant. A faster test, potentially with fewer steps, might encourage more researchers to explore antibacterial properties using a prescribed test, promoting standardisation.

Colorimetric methods may provide a simpler alternative; such methods may involve the colour change of a dye, linked to the metabolic activity of viable cells. Methylene blue, used for assessing photocatalytic activity, can also be decolourised (reduced) by the metabolic activity of microorganisms. Both methylene blue and resazurin have long been used in the dairy industry as a rapid indicator of the quality of milk (the time taken to decolourise the dye is related to the extent of contamination in the milk and to its quality) (Nixon and Lamb, 1945; Twigg, 1945; Muhammad *et al.*, 2009; Silanikove and Shapiro, 2012).

Resazurin reduces to resorufin by bacterial metabolic co-factors, becoming highly fluorescent (Ex: 550 nm, Em: 583 nm) and resulting in a visible colour change from blue to pink (Candeias *et al.*, 1998; González-Pinzón *et al.*, 2012). Unlike some metabolic-based assays such as MTT/XTT, resazurin does not affect the viability of the cells (Barua *et al.*, 2017). The use of resazurin for determination of photocatalytic-antibacterial properties has been shown to be very effective (Lilja *et al.*, 2012; Robertson *et al.*, 2015).

This paper describes a method whereby a photocatalytic surface is inoculated, irradiated, and the remaining viable organisms subsequently recovered in a resazurin growth medium (RGM). The shorter the time taken to detect a colour change, the more microorganisms are present. Comparing this time-to-detection (TTD) to a standard curve can accurately estimate the number of viable cells in the solution. A comparison between the TTD of a photocatalytic surface and a control surface gives some indication of the antibacterial properties of the active surface, should the TTD take longer than the control (or is not attained). In this work, the suspension is incubated whilst concurrently being subject to video recording. Colour change in the suspension, indicative of surviving microorganisms from the surface, is

indicated on the video footage, and visually determined by rapidly scanning and identifying TDD manually (i.e. when the researcher can see the change of colour). The method can replace the enumeration step of the standard method, reducing the time, cost and expertise required to identify putative antibacterial surfaces and determine their antibacterial efficacy.

In order to demonstrate the effectiveness of the novel detection method, a range of putative photocatalytic antibacterial surface coatings were produced. Visible-light activated bismuth oxide coatings were compared with titanium dioxide. The antibacterial properties of the materials were determined using the current standard test method (BS ISO 27447:2009), and concurrently by the resazurin rapid method to assess how similar the test outcomes are.

2.0 Methods

2.1 Preparation of reagents and maintenance of cell culture

To prepare the resazurin solution, 0.251 g resazurin sodium salt (Merck, Gillingham) was mixed with 100 mL sterile distilled water to make a 10 mM solution. This stock solution was diluted to a final concentration of 0.5 mM (0.01%w/v) (Vazquez-Sanchez *et al.*, 2015) and filter sterilized using a 0.2 µm filter (PALL, Port Washington). Sterile saline 0.85%w/v (Saline tablets BR0053, Oxoid, Basingstoke) and sterile nutrient broth (Nutrient broth, CM0001, Oxoid, Basingstoke) were prepared according to manufacturer's instructions. A stock of RGM was then prepared by aseptically combining the three ingredients (Table 1).

Table 1: Concentrations of reagents used to produce RGM.

Reagent	Percentage by volume, %	Volume in one litre, mL
Sterile 0.85% w/v saline	89	890

Sterile nutrient broth	10	100
Filtered 0.5 mM resazurin solution	1	10

The RGM was designed so the 'final' concentration of resazurin would be 5 µM (0.0001%w/v), similar to other published studies (e.g. Csepregi *et al.*, 2018). This concentration ensured the use of minimal reagent while maintaining effective microbial detection. The RGM was dispensed aseptically into 100 mL volumes and maintained at 4 ºC for a maximum of six months.

2.2 Maintenance and standardisation of microbiological cultures

Two pathogenic bacterial species used in existing test methods (British Standards Institution, 2009; Mills *et al.*, 2012; Egamberdieva *et al.*, 2017), *Escherichia coli* 8739 (ATCC® 8739™, American Type Culture Collection, Manassas) and *Klebsiella pneumoniae* 40602 (Manchester Metropolitan University, Manchester) were selected for this study.

The organisms were stored long-term in a -80°C freezer (Hubálek, 2003; De Paoli, 2005) in 1.5 mL volumes of 30% glycerol suspension, prepared by combining 300g of glycerol (Fisher Scientific, Hampton) with one litre of sterile distilled water. When required, the microorganism was subcultured from the freezer stock by aseptically taking a loopful of frozen material and streaking onto a nutrient agar plate (Oxoid, Basingstoke). The plate was incubated at 37 °C for 18 hours, and checked for purity. This stock plate was stored in the fridge at 4 °C and used for assays for up to 4-6 weeks, after which, a fresh stock plate was produced from the freezer stock.

To carry out microbiological assays, a standardised washed cell suspension was prepared. This enables the inoculation of the test surface with a known number of

microorganisms. In addition, washing the cells removes residual nutrients (salts, sugars, amino acids, etc.) and by-products formed during the growth of the cells. These nutrients and by-products may have unintended effects on the assays, such as providing additional nutrients leading to additional proliferation of the cells, and acting as preferential or alternative reaction targets for the resazurin dye molecules (or any active antibacterial agents), leading to inaccurate results.

To prepare a standardised washed cell suspension, one colony from the stock plate was inoculated into 10 mL of fresh sterile nutrient broth and incubated at 37 °C for 18 hours with shaking at 180 rpm. Cells were harvested by centrifugation (2300G, 10 minutes), resuspended in 10 mL of sterile saline and mixed using a vortex mixer (Rotamixer Vortex-Mixer, HTZ Limited, Croydon) for 10 seconds. The suspension was centrifuged again (2300G, 10 minutes), then resuspended in sterile saline and adjusted to an optical density of 0.9-1.1 at 540 nm (Jenway 6305 UV/Visible Spectrophotometer, Chelmsford). This was then diluted 1:10 in sterile saline to make a standardised washed cell suspension of approximately 10^8 CFU mL^{-1} (confirmed via ten-fold serial dilution and colony counting).

2.3 Standard curve

To calibrate bacterial load against TTD, a curve was constructed. Simulating the method of inoculating surfaces and recovery used in the standard test (British Standards Institution, 2009), without in this case an active antibacterial surface, a washed cell suspension of each organism was prepared. This was diluted 10-fold eight times, thereby providing a range of bacterial concentrations ($1x10^7$ CFU mL^{-1} to $1x10^1$ CFU mL^{-1}, assuming the original concentration to be around $1x10^8$ CFU mL^{-1}). Sterile glass coupons (1 cm x 2 cm) were inoculated with either 50 µL of each

dilution (one coupon per dilution) or with sterile saline (as a bacteria-free, non-inoculated control, to assess the effect of nutrient broth on the resazurin) in duplicate, thus yielding two biological replicates. A 1 cm x 2 cm piece of polyethylene (SLS, Nottingham), washed in 70% ethanol and left to dry in a Class 2 biosafety cabinet (Bio 2+ Class 2 Microbiological Safety Cabinet, Envair Ltd, Haslingden), was gently placed on top of each inoculum (taking care not to spill any inoculum over the edge of the coupons). The covered glass coupons were then immediately and aseptically transferred to 5 mL sterile plastic containers ('bijou' bottles, SLS, Nottingham) containing 5 mL of RGM and vortex mixed for 60 s to remove cells from the surface. To determine the number of viable cells present in the resultant suspension, a 10-fold serial dilution to 10^{-8} was carried out, and the Miles-Misra viable count method was used (Miles *et al.*, 1938; Redfern *et al.*, 2018b; Hickey *et al.*, 2019). Briefly, a fresh sterile nutrient agar plate was divided equally into eight sectors, and 20 µL of each dilution aliquoted onto each section in triplicate (thereby reducing the number of agar plates required for the experiment). After the inoculum drops had dried, the plates were inverted and incubated for 12-18 hours, after which the number of colonies was counted and the number of viable cells recovered calculated (the Miles-Misra method enables colony counts to be made using far fewer plates than the (more accurate) 0.1 mL spread inoculum method described above).

Concurrently, the coupons and polyethylene films were removed from the bijou bottles using sterile forceps. These bottles were then incubated at 37 °C in an orbital incubator at 180 rpm for 18 hours, whilst being filmed by a video recorder (Victure AC800 Action Camera, Victure). The recorder was set to capture one still image every 5 seconds at a resolution of 1080p, creating date- and time-stamped time-

lapse footage. The video footage was loaded onto a computer and rapidly scanned (quickly fast-forwarded and rewound using the cursor, described by the term 'scrubbing') using a free commercially available video player (VLC media player, VideoLAN, https://www.videolan.org/). The time (from the video timestamp) of the colour change from blue to pink (detected by eye), occurring as the resazurin was being reduced to resorufin by bacterial metabolic co-factors, was recorded as the TTD (Figure 1).

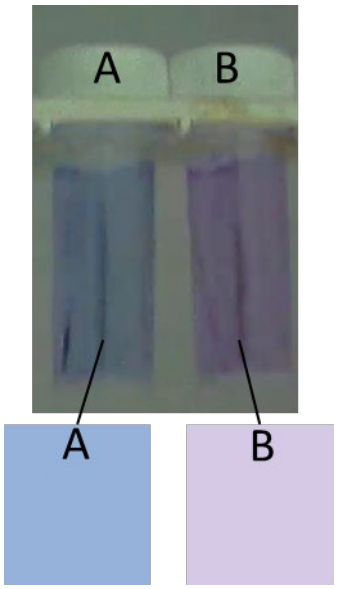

Figure 1: Still image excerpt from the digital camera recording, demonstrating time to detection, i.e., the point when the resazurin was first observed to change colour. The colour of the non-inoculated control sample (A) is compared to the test sample (B) while rapidly scanning through the video recording, to determine colour change by eye.

The TTD was plotted against the corresponding concentration of viable cells. This experiment was repeated three times.

2.4 Determination of antibacterial activity using both the TTD method and plate count method

To demonstrate the accuracy of the TTD as it related to viable cell counts, and to use the resazurin assay in its intended application, 1 cm x 2 cm glass coupons were coated with either photocatalytic anatase titanium dioxide (TiO_2), or photocatalytic

bismuth oxide (Bi_2O_3). The coatings were prepared via the magnetron sputter method. The deposition conditions were fully identical to the one described earlier for the deposition of bismuth oxide and titanium dioxide (Ratova *et al.*, 2018), with the exception of the films being deposited onto soda-lime glass slides in this case. For this purpose the glass slides, ultrasonically pre-cleaned in propanol prior to deposition, were mounted on a rotatable substrate holder positioned underneath two magnetrons. The deposition time was 30 min for each material; the films were post-deposition annealed in air for 30 min to enable crystal phase development at pre-identified temperatures: 400°C for bismuth oxide, 600°C for titanium dioxide. Following post-thermal treatment bismuth oxide was in the tetragonal β-phase, while titanium dioxide was in the anatase phase. The properties of the materials were analysed in detail in earlier work and the results can be found elsewhere (Ratova *et al.*, 2017; Ratova *et al.*, 2018).

The antibacterial capabilities of these materials were determined following an adapted BS ISO 27447:2009 (British Standards Institution, 2009) and the rapid TTD method described above. Each coupon was inoculated as previously described in the standard curve protocol with 20 µL of $1x10^6$ CFU mL^{-1} *E. coli* 8739, placed in a sterile petri dish, and exposed to ambient visible light irradiation (UV irradiance <0.01 mW/cm^2) for 0, 30, or 60 minutes. Non-irradiated controls for the photocatalytic surfaces were prepared by wrapping the petri dish in aluminium foil (Aluchef premier foil, Terinex. Bedford) to prevent light ingress. Each experimental condition (surface type and irradiation status) was performed in duplicate, yielding two biological replicates per experimental condition. After the prescribed time, each coupon was transferred to a bijou bottle containing 5 mL of RGM. This was vortex mixed for sixty seconds to remove cells from the surface. The coupons and polyethylene films were

removed from the bijou bottles using sterile forceps, and the resultant cell suspensions were enumerated via Miles-Misra viable count in triplicate and estimated via the resazurin method as described above.

Each bottle was monitored during rapid video scanning to identify colour change and determine the TTD. The TTD was then compared to the standard curve using the line of best fit equation to determine the 'estimated' initial concentration. This was compared with the 'counted' viable cell number. These experiments were repeated twice.

2.5 Statistical Analysis

Data were analysed for statistically significant differences, relationships and agreements, as appropriate using GraphPad Prism 7 for Windows and IBM SPSS Statistics version 25. Differences between groups were identified using 2-way ANOVA with multiple comparisons. Relationships between variables were investigated using linear regression models. Agreement between measurement methods was assessed using intraclass correlation coefficient analysis. The alpha level was set to 0.05; differences, relationships and agreements were considered significant if $p<0.05$.

3.0 Results

There was a very strong statistically significant correlation between the initial inoculum concentration and the time to detect a colour change (TTD) for *E. coli* ($R^2=0.985$, $p<0.001$, Figure 2).

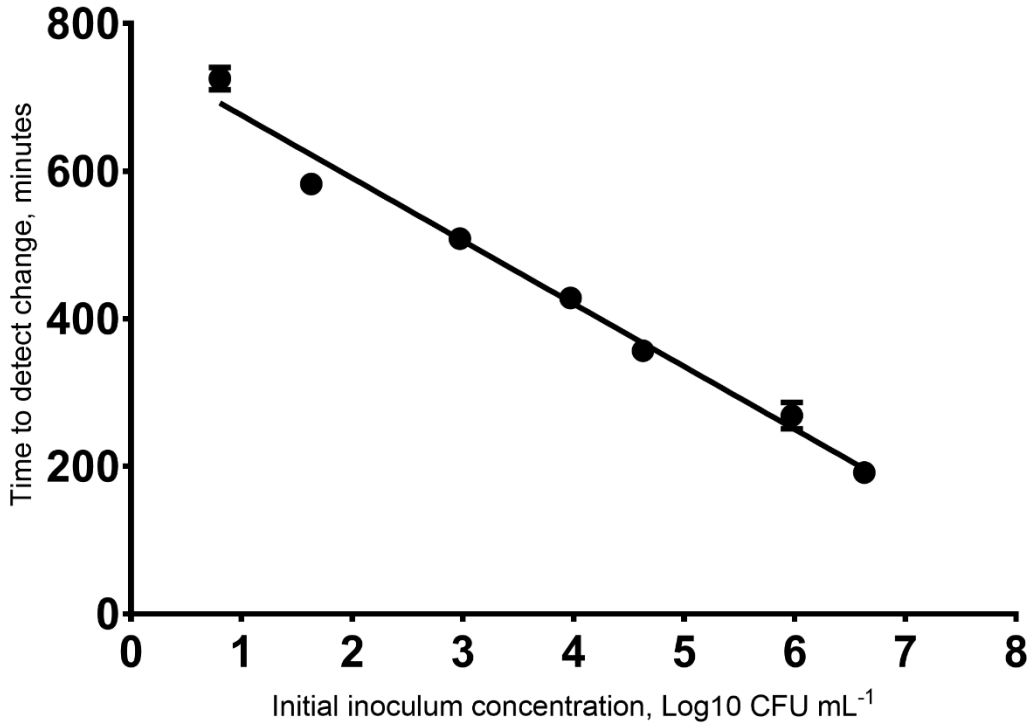

Figure 2: Standard curve of time to detection (TTD) and initial inoculum concentration for *E. coli*. A negative linear relationship between TTD and initial inoculum concentration was observed (R^2=0.985, p<0.001, n=6).

A similar result was observed for the *K. pneumoniae* (R^2=0.99, p<0.001, Figure 3).

No change in colour was observed in the bacteria-free controls within 18 hours.

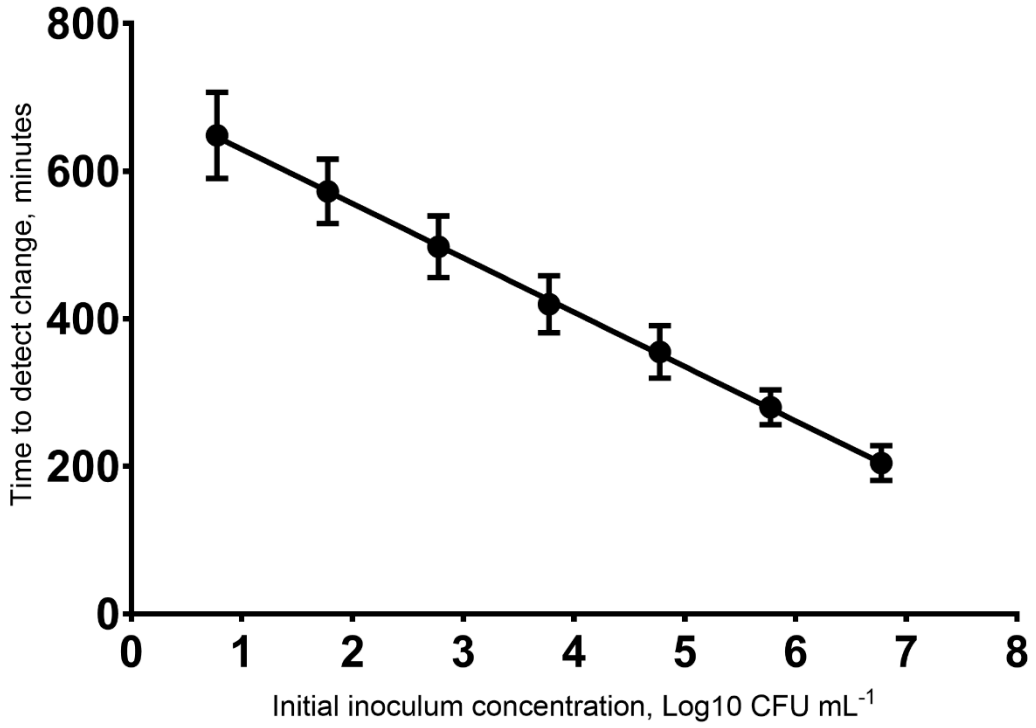

Figure 3: Standard curve of time to detection (TTD) and initial inoculum concentration for *K. pneumonia*. A negative linear relationship between TTD and initial inoculum concentration was observed (R^2=0.99, p<0.001, n=6).

These standard curves were subsequently used to estimate the number of viable cells recovered from the coated test surfaces. Irradiated bismuth oxide coatings reduced the number of viable *E. coli* by 1.2 Log10 CFU mL^{-1} after 30 minutes (p<0.001), and 2.6 Log10 CFU mL^{-1} after 60 minutes (p<0.001) (Figure 4A). A small but significant reduction of 0.85 Log10 CFU mL^{-1} in the number of viable *K. pneumoniae* on irradiated bismuth oxide was observed after 60 minutes (p<0.001) (Figure 4B). Similar small reductions in viability were observed after sixty minutes exposure to the irradiated titania for both *E. coli* (0.71 Log10 CFU mL^{-1}, p<0.001) and *K. pneumoniae* (0.49 Log10 CFU mL^{-1}, p=0.03). In addition, there was a reduction of 0.57 Log10 CFU mL^{-1} of *E. coli* exposed to non-irradiated bismuth oxide

for sixty minutes ($p<0.001$). No other antibacterial effects were observed for the ambient irradiated titanium dioxide coatings or dark controls.

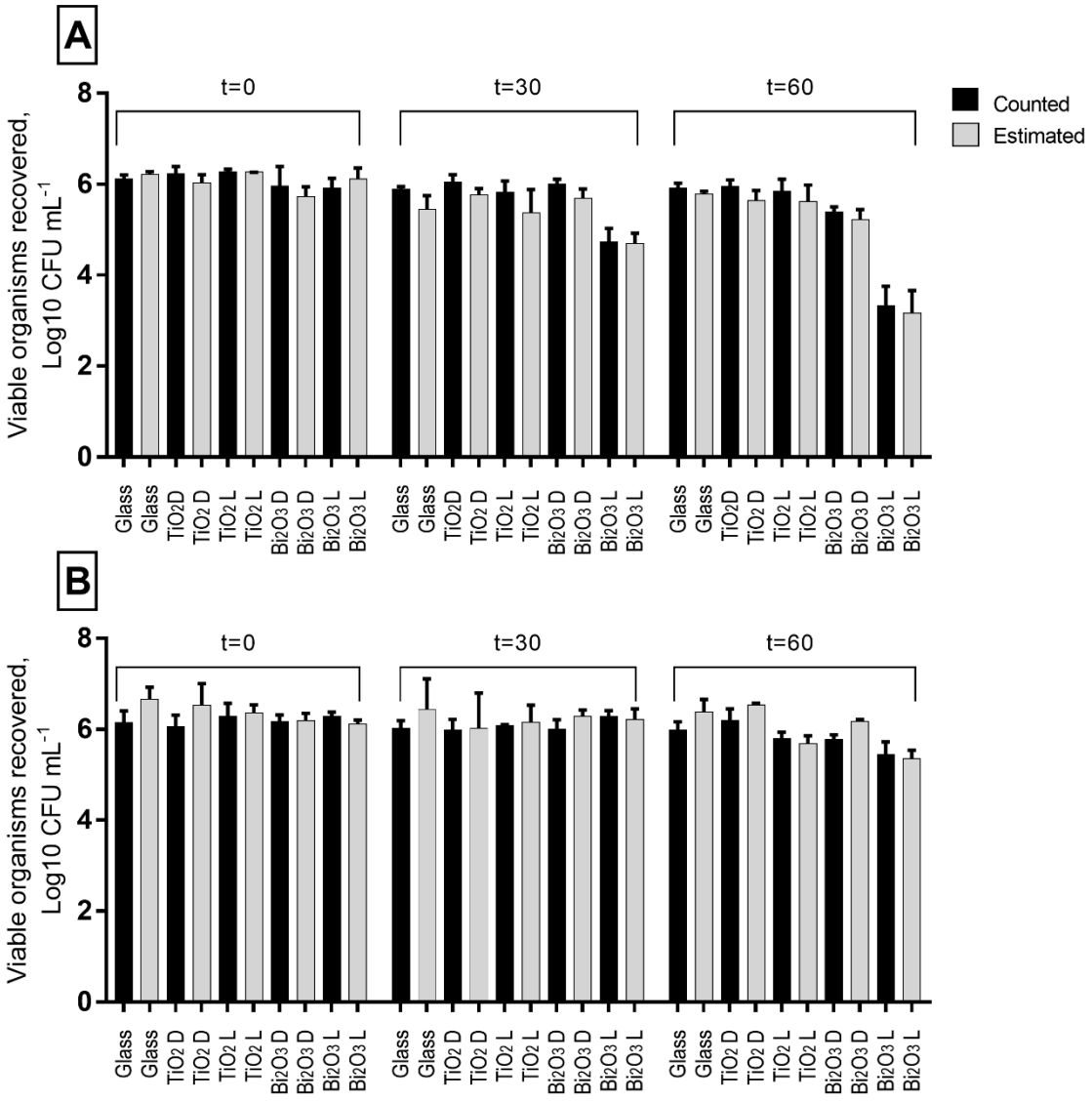

Figure 4: Counted (black) and estimated (by TTD - grey) viable cells for each experimental condition for A) *E. coli* and B) *K. pneumoniae*. TiO_2 = titanium dioxide. Bi_2O_3 = bismuth oxide. D = dark control. L = ambient light irradiation. N=4.

In terms of agreement between the methods, there were no significant differences between the mean number of viable cells recovered reported via the viable count method and estimated method for each experimental condition. To assess further the agreement and relationship between methods, the results were combined, and a

correlation analysis was performed. A significant strong correlation was noted between bacterial load reported via viable count and estimated method (Figure 5) ($r=0.64$, $p<0.001$).

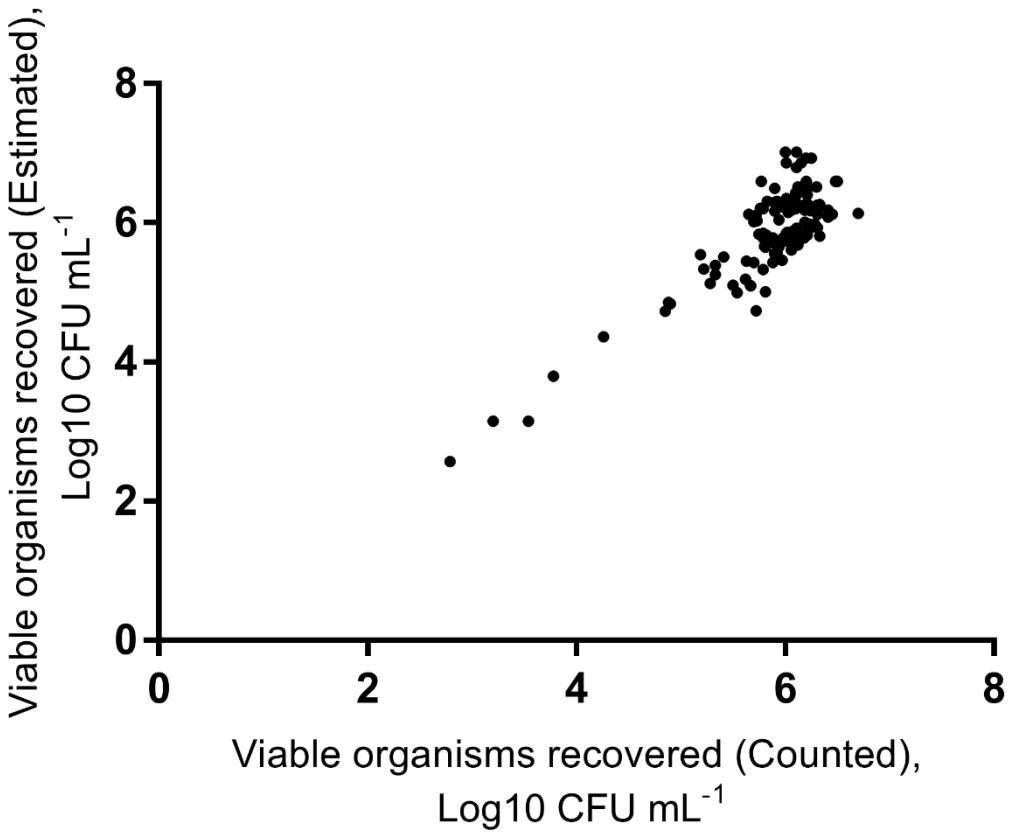

Figure 5: Correlation between counting and estimating the bacterial load ($r=0.64$, $p<0.001$, $n=120$). There was a strong correlation indicating that both methods were able to detect an increase in bacterial load.

As correlation analysis identifies the relationship between the methods, a Bland-Altman plot was used to assess the differences between the methods, identify bias, and construct limits of agreement (Bland and Altman, 1986; Giavarina, 2015).

The Bland-Altman analysis (Figure 6) showed that the rapid estimated method had a mean difference from the counted method (bias) of 0.019 Log10 CFU mL^{-1}, with 95% limits of agreement from -0.72 to 0.72 Log10 CFU mL^{-1}. This indicates that for 95% of cases, the number of recovered viable cells reported by the resazurin rapid

estimated method may be between 0.72 Log10 CFU mL^{-1} below and 0.72 Log10 CFU mL^{-1} above the result reported by a Miles-Misra viable count.

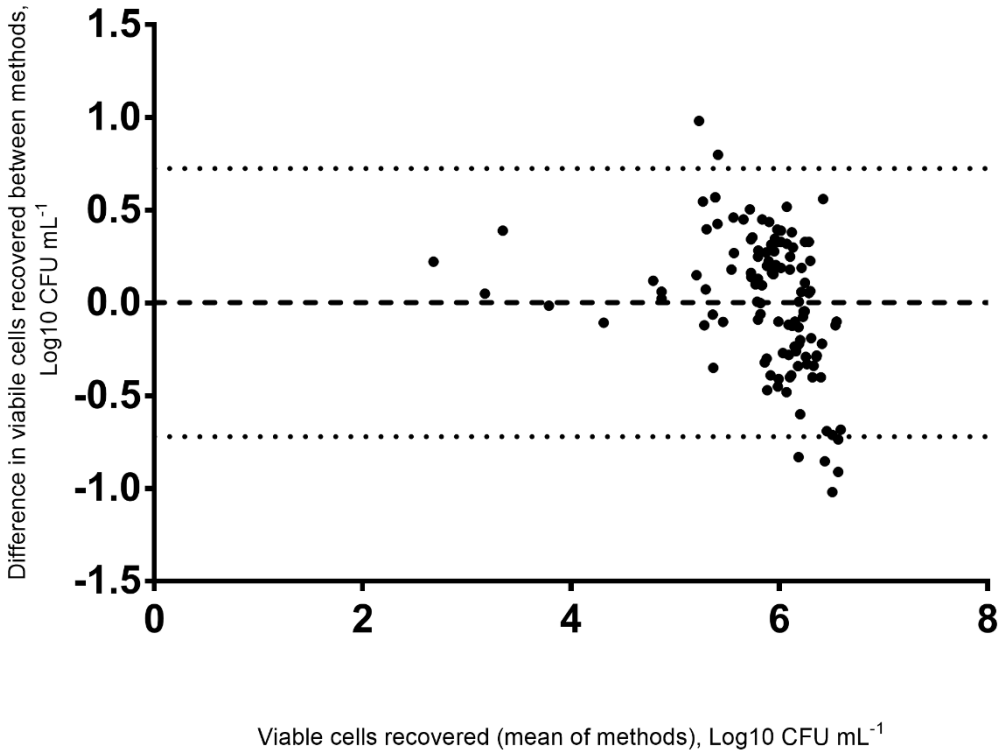

Figure 6: Bland-Altman analysis of the difference between counted and estimated measurements. Dashed line indicates mean difference (bias) between measurements (0.019 Log10 CFU mL^{-1}). Dotted lines represent 95% limits of agreement (-0.72 to 0.72 Log10 CFU mL^{-1}). The negligible bias and narrow limits of agreement indicate excellent agreement between methods. N=120.

Absolute agreement of the measurements obtained by each method was determined by intraclass correlation coefficient analysis. An intraclass coefficient of 0.846 (95% CI [0.786, 0.890], $p<0.001$) indicated excellent agreement between methods.

4.0 Discussion

Photocatalytic processes have been frequently reported as having the potential for inexpensive and sustainable inactivation of microbial pathogenic species. Applications where they may be useful include hospital environments (Reid *et al.*, 2018; Priyadarshini *et al.*, 2020), decontamination and remediation of water

(Ioannou-Ttofa *et al.*, 2019; Oliveira *et al.*, 2019; Redfern and Enright, 2020), high-frequency contact surfaces (Kim *et al.*, 2018), and in air purification systems (Mohamed and Awad, 2020). However, the translation of these reports to real-world systems may be limited by several factors. Although the antibacterial properties of titanium dioxide, the most commonly used photocatalyst (Chong *et al.*, 2010; Ratova *et al.*, 2018), have been extensively utilised in recent years (Das *et al.*, 2017), this photocatalyst may have a limited application in the intended environments due to the requirement for a UV source to activate the material. To realise the potential for photocatalytic inactivation of nosocomial, water, and skin pathogens, the material must be able to be activated by visible light, preferably solar or ambient fluorescent light (Caballero *et al.*, 2009; Chong *et al.*, 2010; Pelaez *et al.*, 2012). In comparison to papers focusing on titanium dioxide, there are few reports of the antibacterial properties of visible light-activated photocatalysts.

In this study, the titanium dioxide was not activated by the ambient light due to its large bandgap of 3.20 eV (Ratova *et al.*, 2020), and therefore was unable to reduce the microbial load of either *E. coli* or *K. pneumoniae* by a clinically meaningful amount. The bismuth oxide on the other hand, with a much narrower reported bandgap of 2.40 eV (Ratova *et al.*, 2018), was able to reduce the microbial load of *E. coli* by 93.7% after thirty minutes, and by 99.7% after sixty minutes, after exposure to ambient light. It is likely that different organisms may have different responses to the photocatalyst. Therefore, the intended application must be considered for photocatalytic antibacterial coatings, and test conditions should always closely emulate the intended application wherever possible (Redfern *et al.*, 2018a). Although photocatalytic antibacterial coatings provide a promising avenue for passive disinfection of surfaces, and broad spectrum activity has been demonstrated against

several medically relevant bacterial such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis*, penicillin-resistant *Streptococcus pneumoniae*, and multidrug-resistant *Pseudomonas aeruginosa* (Nakano *et al.*, 2013), the universality of the technology as an antibacterial cannot yet be claimed.

Results from the rapid estimation method gave excellent agreement with traditional counting methods. The construction of the standard curve demonstrated that cell concentration has a linear relationship with TTD of metabolic change of resazurin, with a higher concentration of initial inoculum resulting in a shorter TTD. This is due to each cell having a specific metabolic potential, with two identical cells having a combined metabolic capacity of twice the single cell and so on. While cells from the same strain and culture may have variation in terms of metabolic capacity (Nikolic *et al.*, 2017), the relationship between Log10 inoculum concentration (cell number) and TTD (metabolic capacity) was linear. In other words, as TTD is related to the metabolic potential of the bacterial community present, it follows that the linear relationship observed is actually a representation of the metabolic needs of the bacterial community. Therefore, given similar cells, the number of bacteria present is represented; higher concentrations of cells lead to a higher concentration of co-factors capable of reducing resazurin to resorufin. This standard curve experiment should be repeated for different strains of interest depending on the intended application. Therefore, future work could include the development of several standard curves for typical model and applied organisms of interest. These could include relevant Gram-positive nosocomial pathogens (which were not included in this study) such as multi-drug resistant *Staphylococcus aureus* (MRSA) and

vancomycin-resistant enterococcus (*Enterococcus faecium* and *Enterococcus faecalis*) (Kim *et al.*, 2018).

In addition to the excellent agreement reported between methods, the rapid method represents a significantly more accessible test, requiring less microbiological expertise (including reduced requirement for dedicated microbiology laboratory space) and laboratory plasticware and reagents (diluent, agar, petri dishes, etc.). Importantly, the time taken to performed the test is reduced compared to standard culture-and-count methods – in this study, bacterial concentrations as low as $1x10^1$ CFU mL^{-1} were able to be detected within 10-12 hours. Furthermore, this time reduction includes less time 'at the bench' for the user (facilitated by the reduction in diluting and plating from individual samples), highlighting further improvements in efficiency.

The use of resazurin for enumeration of microbial cells has been noted by other authors (González-Pinzón *et al.*, 2012; Lilja *et al.*, 2012; An *et al.*, 2020). In other studies, however, usually the fluorescence is monitored. While more sensitive than assessment of colour or absorbance monitoring (Rampersad, 2012), fluorescence monitoring systems require the use of more specialised equipment and so are less suitable for adoption by surface engineering or non-specialist groups, and therefore as rapid testing technique for assessment of antibacterial activity of photocatalysts. Furthermore, it has been shown that components of growth media can themselves cause increases in fluorescence, even in the absence of cells (Munshi *et al.*, 2014), contributing to potential measurement errors. By instead using an easily determined colour change with a dilute growth medium, the process is simplified, reducing potential measurement error. It has shown good correlation with other metabolic assay tests such as XTT/MTT (Hamid *et al.*, 2004; Pettit *et al.*, 2005; Peeters *et al.*,

2008), whilst having considerable improvements such as reduced toxicity (to both the operator and sample cells), reduced costs, reduced time to perform the test and fewer steps, improving usability and making it an attractive alternative for microbial enumeration. This resazurin TTD method is useful for first stage screening of antibacterial surfaces as a replacement for the costly and time-consuming enumeration step, which also requires some element of technical expertise and precision. The chief advantage of this test is its accessibility for non-specialists (requiring less microbiological expertise and experience) and low cost. It can identify reductions in cell number caused by an intervention using a simple redox dye and assessing a colour change by eye, a method that agrees well with established culture-and-count techniques. The inclusion of a digital automated camera with recording functions make this test semi-automated and enables increased throughput, as well as providing a data record. This initial screen enables selection of candidate antibacterial surfaces for more detailed investigation and quantitative testing via established standardised methods.

An important aspect to consider with reporting photocatalytic inactivation of microorganisms is accurate comparison between authors. Key variables to consider such as temperature, humidity and irradiation parameters, the inoculation size and vehicle, and recovery medium should be considered, along with the choice of control samples and number of test sample. As with many microbiological testing methods, transparency in reporting the methods is paramount (Parker *et al.*, 2018). Small differences in experimental variables can affect the overall number of viable cells recovered, both by affecting the viability of the cells (Redfern and Verran, 2017) and by affecting recovery parameters (Verran *et al.*, 2010), thus changing the photocatalytic-antibacterial efficacy reported (Foster *et al.*, 2010).

A key advantage of this method is that the time and cost of screening candidate materials is reduced. Therefore, if desired the number of experimental conditions can be increased with less investment than the culture-and-count method. For example, the number of exposure times investigated can be increased, providing a more detailed picture of the antibacterial properties of the material. It is vital that, if a photocatalyst is to be successfully employed, it must inactivate the microbial community quickly. This is because the reactive oxygen species generated during photocatalysis cause the bacterial cells to exhibit a stress or SOS response. This coordinated response may upregulate certain genes that mediate efflux pumps, mutation rate, biofilm formation, and horizontal gene transfer – all of which are elements that may contribute to antibacterial resistance (Redfern and Enright, 2020; Yin *et al.*, 2020). By including more exposure times, it can be ascertained that a candidate material exhibits rapid inactivation of microorganisms, in addition to long-term activity that may help prevent colonisation of the surface.

Recent publications have posited that antibacterial surface materials may assist the reduction in spread of multidrug-resistant bacteria (Ansari *et al.*, 2020). The ability for surface engineers to select candidate antibacterial surfaces more quickly has never been more pressing. The method presented here is rapid, requires no specialised equipment and can provide high-throughput quantitative results that can aid surface engineers assess the photocatalytic-antibacterial activity of candidate surfaces. It is hoped that this method may represent a stepping stone for authors to screen and select promising candidate surfaces for further analysis (such as more objective determination of the photocatalytic-antibacterial properties using BS ISO 27447:2009, and investigations into bacterial adhesion and retention), helping to

address ongoing global issues regarding antimicrobial resistance, water remediation, and spread of infectious disease.

5.0 Conclusion

In this paper, it was demonstrated that the number of viable cells estimated by the resazurin time-to-detection method was equivalent to the number of viable cells counted via a total count method, in the context of determining the antibacterial properties of a photocatalytic surface coating. The advantages of this method include low cost, high throughput, and no reliance on specialised equipment or extensive microbiological expertise. Therefore, the use of this method is recommended for researchers seeking an inexpensive first-stage screen for putative photocatalytic-antibacterial coatings.

Conflicts of interest: There are no conflicts to declare.

6.0 References

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