

DEVELOPMENT OF BACTERIAL OXIDATIVE STRESS ASSAYS: TOWARDS
USING FLUORESCENCE METHODS

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LIST OF ABBREVIATIONS

CFU	Colony forming unit
DDC	Diethyldithio carbamate
DMSO	Dimethyl sulfoxide
PI	Propidium iodide
SOD	Superoxide dismutase

ABSTRACT

Several classes of bactericidal antibiotics increase oxidative stress in bacteria by upregulating the production of reactive oxygen species. Reactive oxygen species are also produced by host immune cells as protection against infectious bacteria. Superoxide dismutase (SOD) is a defensive enzyme that protects bacteria from the damage caused by reactive oxygen species. It is hypothesized that the inhibition of SOD would increase bacterial cell death caused by oxidative stress. The goal of this study was to establish a fluorescence-based assay for the determination of the viability of *Escherichia coli* that have been treated with antibiotics and SOD inhibitors. The fluorescence-based assay was compared to traditional methods of assessing bacterial viability including spread plating and measuring growth by optical density. The concentration of *E. coli* cells and the type of 96-well plate used in the fluorescence microplate reader affected the ability of the fluorescence assay to accurately assess the bacterial viability. Quercetin, a Cu,Zn-SOD inhibitor, interfered with measurements due to fluorescence it emits when bound to targets in the cell. Diethyldithio carbamate (DDC), also a Cu,Zn-SOD inhibitor, caused a decrease of bacterial viability at high concentrations of inhibitor (100 μ M and 1000 μ M DDC) which was noted when using both fluorescence measurement methods and traditional optical density growth measurements. The effects of ampicillin, kanamycin and norfloxacin, representatives of three classes of bactericidal antibiotics, on cell viability were measured using fluorescence methods and spread plate methods. The affects of the antibiotics on the bacterial cells could not be accurately measured because using the fluorescence methods. *E. coli* incubated with DDC and antibiotic showed little to no difference from cells grown with DDC alone or antibiotic alone.

INTRODUCTION

1. ANTIBIOTICS:

Bacterial antibiotics are divided into two general groups: bactericidal antibiotics that kill bacteria and bacteristatic antibiotics that inhibit bacterial growth (Walsh 2000). There are three classes of bactericidal antibiotics that are divided by their mode of action: inhibition of cell-wall biosynthesis, inhibition of protein synthesis and inhibition of DNA replication and repair (Walsh 2000). β -lactams (e.g. ampicillin) weaken cell walls by preventing cross linkages in the peptidoglycan layer of the bacterial cell walls. This disruption of cell wall structural integrity leaves the bacteria vulnerable to changes in osmotic pressure and in danger of cell lysis. Aminoglycosides (e.g. streptomycin and kanamycin) target ribosomes and cause the mistranslation of proteins. Lastly, quinolones (e.g. norfloxacin) target DNA gyrases which are responsible for the uncoiling of double-stranded bacterial DNA that is formed by DNA replication. A recent study has shown that although these three classes have different bacterial targets, they all contribute to an increase in the oxidation of NADH by the electron transport chain which, leads to an increase in reactive oxygen species (Kohanski et al 2007). These reactive oxygen species damage DNA, proteins and lipids and induce bacterial cell death (Kohanski et al 2007).

2. OXIDATIVE STRESS IN BACTERIA:

The tolerance and intolerance of microorganisms to oxygen in various forms is well studied. Several forms of reactive oxygen species can cause damage in the cell and are formed as byproducts of aerobic metabolism. In aerobic metabolism the tricarboxylic

acid cycle makes NADH from NAD⁺. NADH and O₂ initiate the electron transport chain. Flavoproteins are a component of the electron transport chain. The flavoproteins have a flavin portion (e.g, FADH₂) that is responsible for donating electrons down the electron transport chain. However, FADH₂ can non-specifically donate electrons to any acceptor that may be in the region, such as O₂, which results in the formation of superoxide (O₂⁻) (Imlay 2003). Superoxide can react with hydrogen ions to form H₂O₂ and can in turn react with H₂O₂ to form hydroxyl radicals (OH·). Ferrous iron is also a key component in production of OH· via the Fenton reaction. Superoxide releases iron by damaging iron sulfur clusters in the cells further driving the formation of OH· (Imlay 2003). Hydroxyl radicals are extremely reactive with proteins, membrane lipids and DNA and therefore are highly toxic to bacterial cells (Kohanski et al 2007).

A recent report showed that bactericidal antibiotics upregulate the expression of genes that are essential components to the production of NADH by the TCA cycle and depletion of NADH by the electron transport chain. An increase in these metabolic elements causes rapid depletion of NADH and therefore results in a spike in the amount of superoxide present in the cell. The superoxide then leaches ferrous iron from iron-sulfur clusters and fuels the formation of destructive hydroxyl radicals that lead to cell death (Kohanski et al 2007).

Another source of reactive oxygen species that poses a problem for bacterial viability is the neutrophil. A neutrophil is a phagocytic cell in the blood that is responsible for the identification and detection of microorganisms. Neutrophils internalize bacteria by phagocytosis. Cytoplasmic vesicles called lysosomes fuse with the internalized bacteria to form a phagolysosome. The components within these vesicles

result in the death of the microorganism. One of these vesicles contains the enzyme NADPH oxidase. The enzyme is activated when phagocytosis is initiated and the resultant products are superoxide and hydroxyl radicals. These reactive oxygen species are then delivered for destruction of the microorganism within the phagolysosome (DeFranco et al 2007).

3. SUPEROXIDE DISMUTASE:

SOD protects the cell from reactive oxygen species by catalyzing a reaction that converts superoxide to O_2 and H_2O_2 , thus eliminating the potential to contribute to the formation of hydroxyl radicals. SOD is found throughout a vast number of organisms from bacteria to humans. In *E. coli* it exists in three forms which are known by the catalytic metal that is located at the active site; Fe-SOD, Mn-SOD and Cu,Zn-SOD. They also differ in location and defensive function.

Mn-SOD and Fe-SOD are coded for by the *sodA* and *sodB* genes respectively. They are found in the cytoplasm of the bacteria cell and scavenge O_2^- that is present as a result of the reduction of molecular oxygen by respiratory enzymes. Mn-SOD is highly protective against respiratory damage that results from O_2^- . Its expression is relative to the oxidative environment. When O_2^- increases there is an increase in the amount of Mn-SOD produced by the cell. Fe-SOD provides only moderate protection since its expression is constant in all oxidative conditions (Lynch and Kuramitsu 2000).

Cu,Zn-SOD is coded for by the *sodC* gene. Cu,Zn-SOD are found in the periplasm of *E. coli*. The *sodC* gene is expressed during stationary phase growth. *E. coli* is sensitive to exogenously produced reactive oxygen species immediately after entering

stationary phase (Gort et al 1999). The production of Cu,Zn-SOD in the periplasm protects against the reactive oxygen species that can diffuse across the membrane from external sources.

As previously mentioned, the control of gene expression, the structures and functions of the SOD enzymes found in *E. coli* are extensively reported making it specifically an ideal selection for this research. *Escherichia coli* is a typical bacterial model that is widely used due to its ease to culture in the lab and 20 minute generation time. There is extensive information available about the organism.

4. SOD INHIBITION:

The Summers lab has found that quercetin inhibits Cu,Zn-SOD (personal communication). Quercetin is a flavonol that is found in many fruits and vegetables (Fig. 1). Much research has gone into quercetin's interaction with various biomolecules. Quercetin has been shown to chelate metal ions to form active complexes (Tan et al 2009). However, the inhibition of Cu,Zn-SOD is not due to the sequestering of metal ions from the active site of the enzyme but rather the structure of the flavonol B ring. The IC_{50} of quercetin for the inhibition of Cu,Zn-SOD has been measured at approximately 640 nM. Inhibition of the enzyme by quercetin is irreversible (Summers and Seischab unpublished).

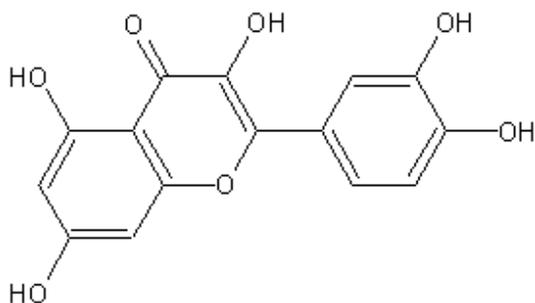


Figure 1. Structure of quercetin.

DDC, is a copper chelating agent (Fig. 2). It is a known Cu,Zn-SOD inhibitor that works by sequestering copper from the active site of the enzyme. DDC and other inhibitors that function by chelating metals are not appropriate for use as drugs since they can non-specifically bind metals which may be important cofactors in many enzymatic processes within the cell. However, DDC has been well-studied and its ability to inhibit Cu,Zn-SOD specifically isolated from *E. coli* (Hirata and Hayaishi 1975) makes it an optimal candidate for use in developing a biological assay.

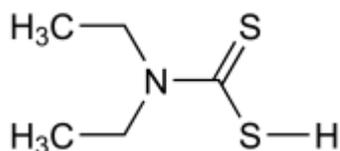


Figure 2. Structure of diethyldithio carbamate.

5. MICROBIOLOGICAL METHODS:

Traditional methods for enumerating bacteria include both plate counting and measuring of optical density by absorbance spectroscopy. Although these methods have been widely practiced they can be expensive and/or time consuming. New technology

and equipment have led to the development of methods that may prove to be more efficient in labor, equipment and time (Hoerr et al 2007).

Plate counting is performed by diluting a sample of bacteria and spreading it onto the media surface in a plate. It is assumed that the bacteria are diluted so that each single cell forms a colony on the surface of the plate after a period of incubation. Many factors can affect the ability of a cell to form a colony so the measurements are reported as colony forming units (CFUs) and not as numbers of viable cells. To estimate the cytotoxicity of an antibiotic, a dose-response curve can be generated by counting the number of CFUs remaining after incubation with a range of concentrations of the antibiotic in question.

The optical density of the cells is measured using a spectrophotometer. However, both cells that are dead and alive can affect the turbidity of the culture. This method can also be used to determine the effects of a compound on the growth of the bacteria. Again, this method does not directly measure whether cells are dead or alive but only their ability to grow in the presence of the specific compound.

Newer methods of detecting bacterial viability have been developed and are starting to become more widely used, replacing cumbersome and time consuming methods. The LIVE/DEAD *BacLight* bacterial viability kit (LIVE/DEAD® *Baclight*TM 2008) is a fluorescence-based kit that can be used with a fluorescence microscope, a fluorescence microplate reader and a flow cytometer. The kit contains two nucleic acid stains that differentially stain live and dead bacterial cells. Both excited at 485 nm. SYTO®9 used by itself stains all cells green by emitting fluorescence at 520 nm. It is able to penetrate the cell membrane of healthy bacteria to bind to nucleic acids in the

cytoplasm. Propidium iodide stains cells red by emitting fluorescence at 612 nm. However, propidium iodide is only able to penetrate the bacterial membrane when it has become compromised. Propidium iodide has a higher affinity for nucleic acids and can displace the SYTO®9 dye when present. Also the green fluorescence from the SYTO®9 dye is quenched in the presence of propidium iodide by fluorescence resonance energy transfer (FRET) (Hoerr et al 2007). FRET occurs when the excitation energy of one fluorophore (e.g. SYTO®9) is directly transferred to another fluorophore (e.g. propidium iodide) when they are in close proximity. Since the fluorescence intensity of SYTO®9 decreases in the presence of propidium iodide as the percentage of live cells decreases, comparing the fluorescence intensity of the SYTO®9 and propidium iodide dyes indicates the percentage of live cells (Fig. 3). Using this kit with a 96-well plate in a fluorescence microplate reader to determine the effects of SOD inhibition under oxidative stress conditions could be an ideal high throughput alternative to traditional methods.

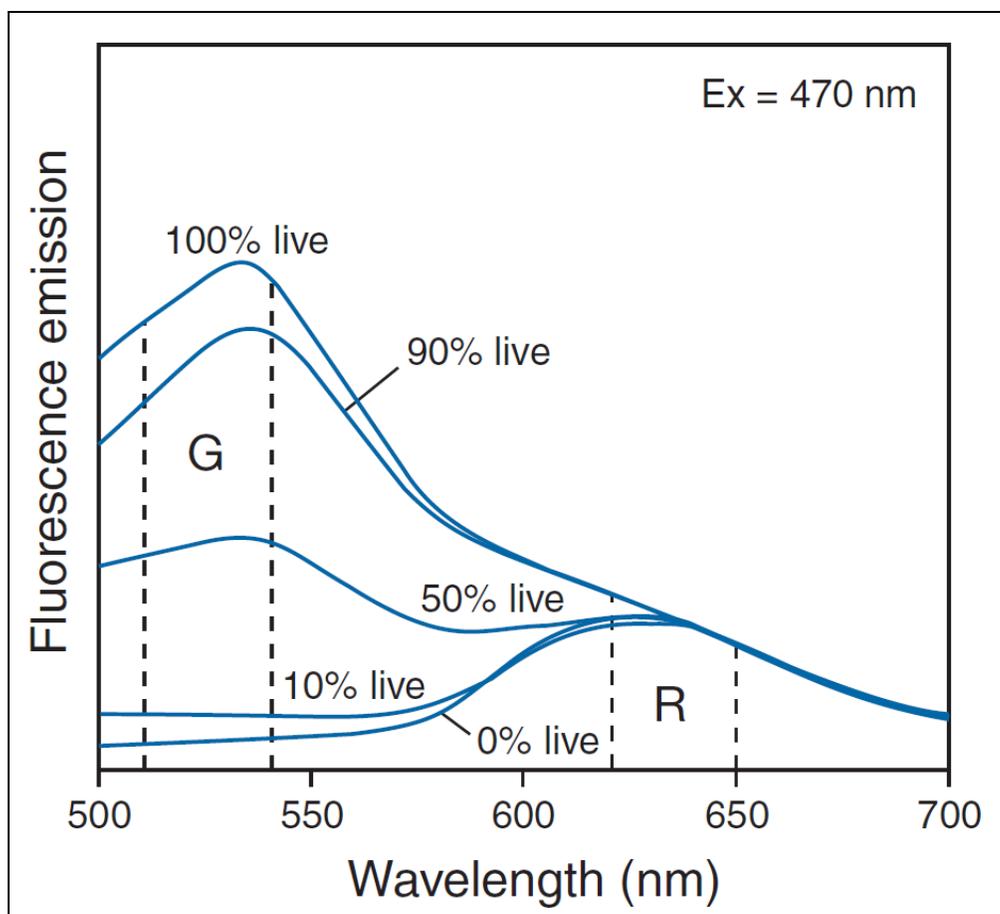


Figure 3. Viability analysis of bacterial suspensions. The viability of different proportions of live and isopropanol-killed *Escherichia coli* was assessed using the reagents in the LIVE/DEAD® BacLight™ Bacterial Viability Kits. Live bacteria are stained fluorescent green (G) by SYTO® 9 stain, and dead bacteria are stained fluorescent red (R) by propidium iodide. Bacterial suspensions simultaneously incubated in the two stains and then excited at 470 nm exhibit a fluorescence spectral shift from green to red as the percentage of live bacteria in the sample is decreased (LIVE/DEAD® BacLight™ 2008).

SIGNIFICANCE

SODs are important defensive enzymes that help to protect bacteria against superoxide produced both by the bacteria and external sources. Antibiotics are known to affect the oxidative environment within bacteria by increasing the amount of reactive oxygen species present (Kohanski et al 2007). Therefore, a “two-hit” mechanism in which antibiotics work in conjunction with a compound that inhibits SOD may enhance the effect of the drugs. The inhibitor compounds may enhance the immune response by inhibiting SOD that protects against an oxidative attack by host cells. This mechanism may be employed in the fight against clinically significant bacteria, such as *Mycobacterium tuberculosis* which depends on SOD activity for survival (Piddington et al 2001).

SPECIFIC AIMS

To test the two-hit mechanism hypothesis and identify from a library of compounds an inhibitor that may be useful in drug development, an accurate and efficient high throughput assay must be established. The purpose of this study was to develop a fluorescence-based assay which included selection of a bacterial model, determining an inhibitor compound to use, establishing a protocol for using the high throughput bacterial viability assay and comparing the results of the assay to traditional microbiological methods.

MATERIALS AND METHODS

1. MAINTENANCE OF CELL CULTURES:

An *Escherichia coli* strain (ER2566 NEB) was provided by Dr. Chris Coburn as a frozen stock that was stored in a glycerol solution at -70°C . The culture was revived by adding the frozen culture to 5 ml of LB media in a 15 ml conical tube and incubating at 37°C while shaking at 200 rpm. A stab culture was made by inoculating stab agar with bacteria from the liquid culture and incubating overnight at 37°C . The stab culture was stored in the dark at room temperature. For experiments, bacterial cultures were grown overnight at 37°C on LB agar plates. Isolated colonies were used to inoculate liquid LB media that was incubated at 37°C while shaking at 200 rpm.

2. BACTERIAL GROWTH CURVES:

Growth curves were determined by measuring the optical density at 600 nm using a spectrophotometer. LB media was pre-heated by incubating for 20 minutes at 37°C . The media was then inoculated with cells from an isolated colony from a streak plate that was grown overnight at 37°C . The culture was incubated at 37°C and shaken at 200 rpm shaking. The OD_{600} was measured every 20 minutes. Once the OD_{600} exceeded 1.0 the cultures were diluted prior to measuring. Measurements were taken until the bacteria reached late stationary phase.

3. SPREAD PLATE ASSAYS:

Effects of antibiotics on E. coli growth:

A 25 ml culture was grown until the culture reached early log phase ($OD_{600} \approx 0.2$). 1980 μ l aliquots of culture were put into six 15 ml tubes. From 100 mM stock solutions, a 10-fold dilution series of ampicillin, kanamycin and norfloxacin was prepared using the appropriate solvent. 20 μ l of each dilution was added to each tube resulting in a range of concentrations from 0.1 μ M to 1000 μ M. A positive control was prepared by adding 20 μ l of solvent. The treatments were then incubated until the control reached stationary phase. Each treatment was then diluted 1:100 in 0.85% NaCl. Then a 10-fold dilution series was performed to obtain dilutions of 1:1,000 to 1:1,000,000. 100 μ l of each dilution was spread evenly on an LB agar plate in triplicate to yield dilutions of 1:10,000 to 1:10,000,000 (Fig. 4). The plates were incubated 24 hours at 37° C and colonies were counted to determine the numbers of CFUs.

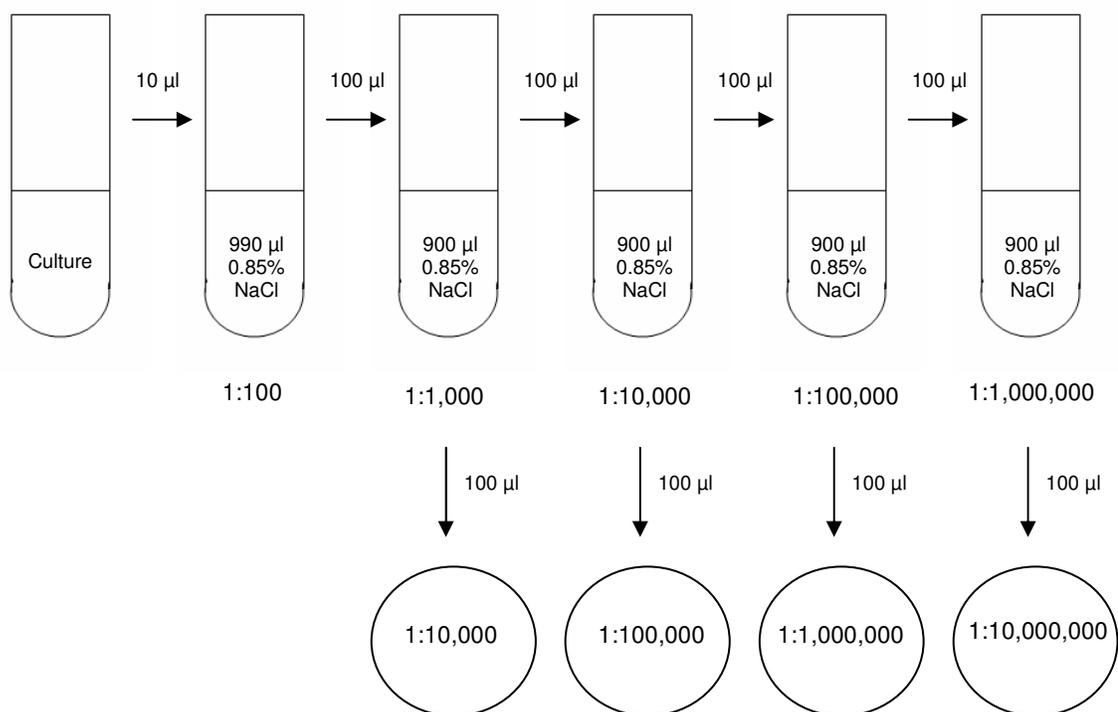


Figure 4. Dilution set up for spread plate enumeration of E. coli.

4. GROWTH CURVE ASSAYS:

Effects of Cu,Zn-SOD inhibitor on E.coli growth:

A 25 ml culture was grown until the culture reached early log phase ($OD_{600} \approx 0.2$). 3960 μ l aliquots of culture were placed into six glass culture tubes. From a 100 mM stock solution, a 10-fold dilution series of inhibitor was produced. 40 μ l of each dilution was added to the culture tubes to achieve an inhibitor treatment of 0.1 μ M to 1000 μ M. One tube received 40 μ l of solvent for a positive control. The treatments were incubated at 37° C with 200 rpm shaking. The OD_{600} was measured for all tubes every 20 minutes using a Spec20+ until the positive control reached stationary phase.

Effects of antibiotics and Cu,Zn-SOD inhibitor on E. coli growth:

A 25 ml culture was grown until the culture reached early log phase ($OD_{600} \approx 0.2$). 3920 μ l aliquots of culture were placed into seven glass culture tubes. From a 100 mM stock solution, a 10-fold dilution series of inhibitor was produced. 40 μ l of each dilution was added to the culture tubes to achieve an inhibitor treatment of 0.1 μ M to 1000 μ M. 40 μ l kanamycin, ampicillin or norfloxacin were added to the tubes with the inhibitor at a concentration that represents approximately a 50% inhibition of growth as determined by the spread plate bacterial enumeration. One tube received 40 μ l of antibiotic and 40 μ l of inhibitor solvent for a control. The final tube remained untreated and received no inhibitor or antibiotic but only solvent. The treatments were incubated at 37° C and shaken at 200 rpm. The OD_{600} was measured for all tubes every 20 minutes using a Spec20+ until the untreated control reached stationary phase.

5. FLUORESCENCE ASSAYS:

Dye saturation of SYTO®9 nucleic acid stain:

A 25 ml culture was grown at 37° C and at 200 rpm shaking. Cells were harvested at early stationary phase (8 hours). Cells were spun down for 10 minutes at 4300 rpm using an SH3000 rotor in a Sorvall RC 5C Plus centrifuge. The cells were then resuspended in 0.85% NaCl solution. The cell concentration was adjusted to 2.0×10^8 cells/ml by measuring the OD₆₇₀ with a Spec20+ and diluting with 0.85% NaCl buffer solution until it measured 0.06 (LIVE/DEAD® BacLight™ 2008). A dilution of a 3.34 mM SYTO®9 stock solution was performed to obtain a range of concentrations from 0.25 µM to 4 µM. 100 µl aliquots of cells at a concentration of 2.0×10^8 cells/ml were added to a 96-well plate. 100 µl of each dye concentration were added to cell suspensions resulting in a final dye concentration range of 0.125 µM to 2 µM. The plate was incubated at room temperature in the dark for 15 minutes. Using a POLARstar OPTIMA fluorescence microplate reader the suspensions were excited at 485 nm and the fluorescence intensity was measured at an emission wavelength of 520 nm.

Propidium iodide concentration determination:

A 25 ml culture was grown to late log phase by incubating at 37° C and shaken at 200 rpm. Cells were spun down for 10 minutes at 4300 rpm as above. The cells were resuspended in 2 ml of 0.85% NaCl solution. One ml of the cell suspension was added to 20 ml of 0.85% NaCl solution and 1 ml was added to 20 ml of 70% isopropyl alcohol. The two tubes were incubated at room temperature for one hour and suspensions were gently mixed every fifteen minutes. After the incubation period, cells in 0.85% NaCl

were assumed to be 100% live and cells in the 70% isopropyl alcohol were assumed to be 100% dead. Cells were spun down for 10 minutes at 4300 rpm using an SH3000 rotor in a Sorvall RC 5C Plus centrifuge. The cells were then rinsed with 0.85% NaCl, spun down, and resuspended in 20 ml of 0.85% NaCl. Using a Spec20+ the OD_{670} for both dead and live suspensions was adjusted to 0.06 to achieve a concentration of 2.0×10^8 cells/ml. The cells were then aliquoted into five 15 ml sterile conical tubes in live to dead ratios of 0:100, 10:90, 50:50, 90:10 and 100:0. A stock solution of 20 mM propidium iodide was diluted to a range of 4 μ M to 200 μ M. A solution of 5 μ M SYTO®9 was prepared by adding 2 μ l of 3.34 mM SYTO®9 to 2668 μ l of ultra pure water. 350 μ l of 5 μ M SYTO®9 solution was added to eight 1.5 ml centrifuge tubes. 350 μ l of each propidium iodide concentration was also added to achieve a SYTO®9 concentration of 1.5 μ M and a propidium iodide concentration ranging from 2 μ M to 100 μ M. In a 96-well plate, 100 μ l of cell suspensions ranging from 0% to 100% live cells were aliquoted (8X). For each range of cell viabilities, 100 μ l of dye solution with varying concentrations of propidium iodide and the optimal concentration of SYTO®9 was added. This brought the final concentration of SYTO®9 to 1.25 μ M for all wells and a range of 1 μ M to 50 μ M propidium iodide in contact with cells for the incubation period. The cells were incubated for 15 minutes in the dark at room temperature. The suspensions were then excited at 485 nm and emission was measured for both 520 nm (SYTO®9) and 612 nm (propidium iodide) using a fluorescence microplate reader.

Preparing cells for a standard curve:

A 25 ml culture was grown to late log phase by incubating at 37° C and at 200 rpm shaking. Cells were spun down for 10 minutes at 4300 rpm using an SH3000 rotor in a Sorvall RC 5C Plus centrifuge. The cells were resuspended in 2 ml of 0.85% NaCl solution. One ml of the cell suspension was added to 20 ml of 0.85% NaCl solution and 1ml was added to 20 ml of 70% isopropyl alcohol. The two tubes were incubated at room temperature for one hour and suspensions were gently mixed every fifteen minutes. After the incubation period, cells in 0.85% NaCl were assumed to be 100% live and cells in the 70% isopropyl alcohol were assumed to be 100% dead. Cells were spun down for 10 minutes at 4300 rpm using an SH3000 rotor in a Sorvall RC 5C Plus centrifuge. The cells were then rinsed with 0.85% NaCl, spun down, and resuspended in 20 ml of 0.85% NaCl. Using a Spec20+ the OD₆₇₀ for both dead and live suspensions was adjusted to 0.06 to achieve a concentration of 2.0×10^8 cells/ml. The cells were then aliquoted into five 15 ml sterile conical tubes in live to dead ratios of 0:100, 10:90, 50:50, 90:10 and 100:0. 100 µl of the varying ratios of live to dead cell suspensions are aliquoted into wells in a 96-well plate. A SYTO®9 dye solution and a propidium iodide dye solution were created. The solutions were mixed in a 1:1 ratio. 100 µl of the mixed dye solution was aliquoted into each well. The suspensions were then excited at 485 nm and emission was measured for both 520 nm (SYTO®9) and 612 nm (propidium iodide) using a fluorescence microplate reader.

Cytotoxicity of antibiotics:

A 25 ml culture was grown to early log phase. 1980 μ l aliquots of culture were placed into six 15 ml tubes. The remaining culture was returned to the incubator until late stationary phase and then was processed to determine a standard curve. From 100 mM stock solutions, a 10-fold dilution series of ampicillin, kanamycin and norfloxacin was prepared. 20 μ l of each dilution was added to the culture tubes to achieve an antibiotic treatment of 0.1 μ M to 1000 μ M. One tube received 20 μ l of solvent for a positive control. The treatments were incubated at 37° C with 200 rpm shaking until the positive control reached late stationary phase (3 hours). Cells were spun down for 10 minutes at 4300 rpm using an SH3000 rotor in a Sorvall RC 5C Plus centrifuge. The cells were then rinsed with 0.85% NaCl, spun down, and resuspended in 10 ml of 0.85% NaCl. Using a Spec20+ the OD₆₇₀ for all suspensions was adjusted to 0.06 to achieve a concentration of 2.0×10^8 cells/ml. 100 μ l of treated cells were aliquoted in triplicate with 100 μ l of dye solution. The plates were incubated in the dark for 15 minutes at room temperature. Cells were excited at 485 nm and the fluorescence intensity at 520 nm and 612 nm was measured and compared to a standard curve to assess viability.

Cytotoxicity of Cu,Zn-SOD inhibitor in a culture:

A 25 ml culture was grown to log phase by incubating at 37° C and at 200 rpm shaking for three hours. 1980 μ l aliquots of culture were placed into six 15 ml tubes. Remaining culture was returned to the incubator until late stationary phase and then was processed to determine a standard curve. From a 100 mM stock solution, a 10-fold dilution series of inhibitor were performed. 20 μ l of each dilution was added to the

culture tubes to achieve an inhibitor treatment of 0.1 μM to 1000 μM . One tube received 20 μl of solvent for a positive control. The treatments were incubated at 37° C and shaken at 200 rpm until the positive control reached late stationary phase (3 hours). The timing was determined by the expression of Cu,Zn-SOD. Cells were spun down for 10 minutes at 4300 rpm. The cells were then rinsed with 0.85% NaCl, spun down, and resuspended in 10 ml of 0.85% NaCl. Using a Spec20+ the OD₆₇₀ for all suspensions was adjusted to 0.06 to achieve a concentration of 2.0×10^8 cells/ml. 100 μl of treated cells were plated in triplicate with 100 μl of dye solution. The plates were incubated in the dark for 15 minutes at room temperature. Cells were excited at 485 nm and the fluorescence emission at 520 nm and 612 nm was measured and compared to a standard curve to assess viability.

RESULTS

1. OPTIMIZATION OF CELL ENUMERATION TECHNIQUES: Optical density, spread plate and fluorescence

Optical density method for determining E. coli growth curves:

Growth curves were established under varying conditions, namely different incubation volumes, mixing conditions and spectrophotometers. A SpectraMax 190, a Nanodrop and a Spec20+ were all used to determine growth curves by measuring the OD₆₀₀ at time intervals. When using the SpectraMax 190, a UV-Vis microplate reader, measurements were taken by pipetting 150 µl of culture into a 96-well plate. The culture was mixed in the plate for 5 seconds before the measurement was taken. The SpectraMax 190 readings indicated that the incubation time to reach stationary phase was approximately 5 hours and 20 minutes for 25 ml of media in a 50 ml conical tube. However, later attempts to repeat this curve were unsuccessful and consistent readings from the SpectraMax 190 were not possible with the current set up of the instrument. The Nanodrop, a small volume UV-Vis spectrometer, was also inconsistent in its measurements. Readings were taken by transferring 10 µl of culture and placing into a microcentrifuge tube. The tube was gently vortexed immediately prior to taking a measurement. 3 µl aliquots of sample were placed onto the instrument's optical pedestal which then drew the sample into a column to be read. At each time increment, multiple reads from the same sample were highly variable and measurements from cultures incubated in parallel were inconsistent. To use the Spec20+ approximately 4 ml of culture were poured into a glass culture tube and measured. Once the OD₆₀₀ exceeded

1.0 the measurements were taken by diluting 40 μl of culture with 3960 μl of LB media (1:100). These measurements produced a consistent and repeatable growth curve.

Growth curves were also determined for varying size culture containers which directly contributed to the amount of available oxygen. Specifically, growth curves were determined for cultures containing 10 ml of media in a 50 ml conical tube and 25 ml of media in a 250 ml flask. The 10 ml culture that was grown in a 50 ml conical tube, entered log phase after 120 minutes and entered stationary phase after 480 minutes. The 25 ml culture entered log phase after approximately 100 minutes and continued until stationary phase began at approximately 380 minutes (Fig. 5). These data was used in further experiments in which bacteria were harvested at specific stages of growth.

For cytotoxicity assays, bacteria from a pre-culture must be removed during log phase and treated with antibiotic or inhibitor. Therefore, a growth curve was determined for a 2 ml culture that was removed during log phase of a 25 ml pre-culture and grown to stationary phase. The 2 ml culture was prepared by adding 1980 μl of log phase pre-culture and diluting with 20 μl of LB media. The culture continued exponential growth and entered stationary phase after 160 minutes (Fig. 6).

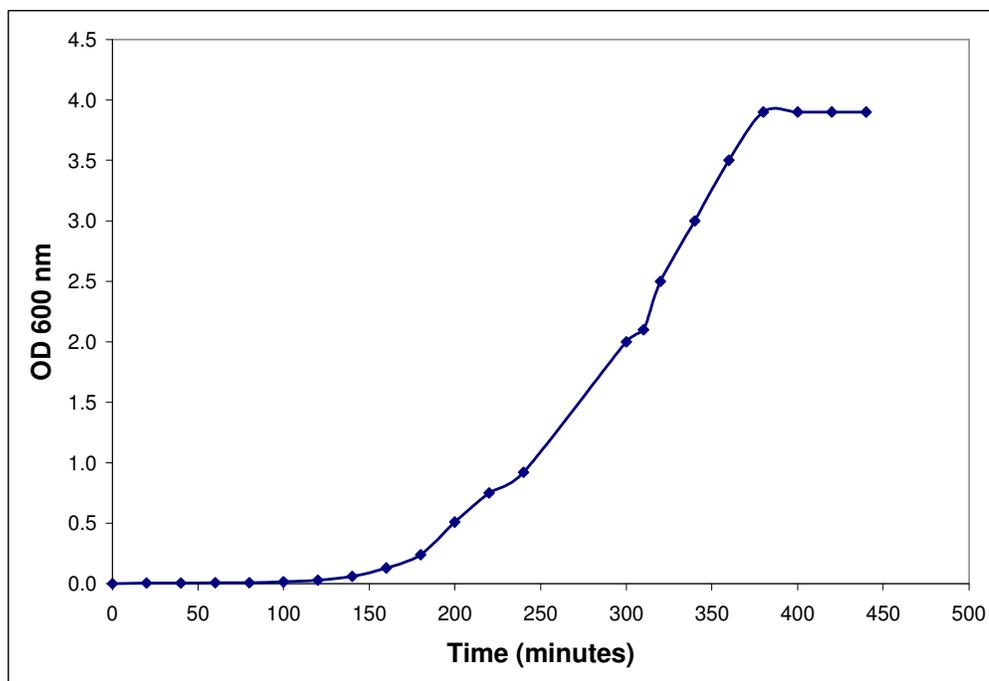


Figure 5. Growth curve for 25 ml culture of *E. coli* (ER2566). The culture was grown at 37° C with shaking at 200 rpm in a 250 ml flask containing 25 ml of LB media. The optical density at 600 nm was measured in increments using a Spec20+ until bacteria reached stationary phase.

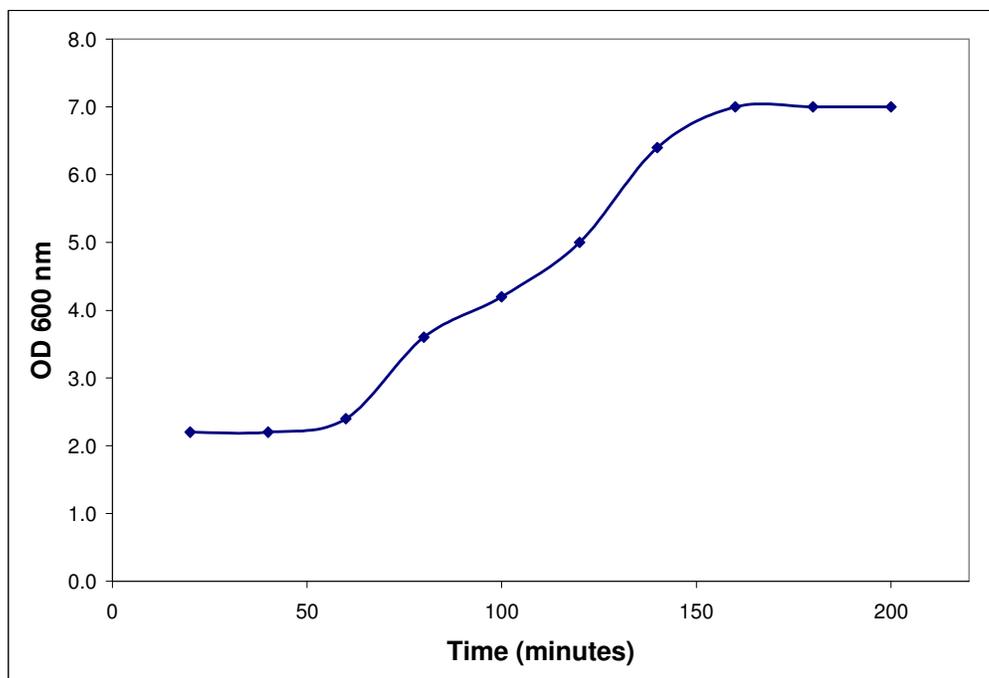


Figure 6. Growth curve for 2 ml culture of *E. coli* (ER2566). 1980 μ l were removed from a 25 ml pre-culture that had grown to log phase and 20 μ l of LB media was added. The optical density at 600 nm was measured in increments using a Spec20+ until bacteria reached stationary phase.

Spread plate method for determining antibiotic cytotoxicity:

Several experiments were performed to optimize the spread plate enumeration of bacteria. Initially, a dilution in which plates were treated with 100 μ l and 1 ml of cell suspension was used. Plates with 1 ml of suspension displayed growth across the entire plate surface and could not be counted. 100 μ l treatments had well isolated colonies. The experiment was repeated with the same protocol but after the plates were spread with a suspension they were left to air dry for 1 hour before being placed in the incubator. However, the bacterial growth again spread across the entire plate for treatments of 1 ml. A new dilution was then performed so each plate would only be treated with 100 μ l of cell suspension (Fig. 4). This dilution resulted in countable colony separation. It was also observed that plates that had been poured several days in advance and were allowed to dry more quickly and successfully absorbed the cell suspension. The final dilution range of 1:10,000 to 1:10,000,000 yielded a countable colony separation for all the antibiotic treatments.

Fluorescence microplate reader method for determining E. coli viability:

Two factors were found to affect the optimization of concentrations of SYTO®9 and propidium iodide for the fluorescence assay, namely the concentration of cells in the cell suspension and the type of 96-well plate (Table 1).

Table 1. *Effects of E. coli concentration and plate type on optimal fluorescence dye concentrations.* Concentration of cells was determined by measuring the optical density using a Spec20+. Fluorescence measurements were taken in a fluorescence microplate reader for determination of SYTO®9 and propidium iodide concentrations.

CELLS (10⁸ cells/ml)	TYPE OF 96- WELL PLATE	SYTO®9 (µM)	PROPIDIUM IODIDE (µM)
1.5	Costar 3695 White	1.0	12.5
2.0	Costar 3695 White	0.75	2.0
2.0	Costar 3915 Black	1.25	4.0

Effects of cell concentration on concentrations of SYTO®9 and propidium iodide:

The cells must be saturated with dye for the fluorescence intensity measurement to accurately depict the viability of all cells present in the suspension. To avoid dye quenching and to minimize dye waste, an excess of dye in the reaction must also be avoided. This concentration of SYTO®9 is then used to determine the concentration of propidium iodide that should be used to accurately determine the amount of viable cells.

The saturation of the SYTO®9 dye was determined for a cell concentration of 1.5×10^8 cells/ml and 2.0×10^8 cells/ml. A range of SYTO®9 concentrations from 0.5 nM to 5 µM were incubated with each cell suspension. For the lower concentration of bacteria, the fluorescence intensity plateaued at SYTO®9 concentrations above 1 µM (Fig. 7). For the higher concentration of bacteria, the fluorescence intensity reached its peak close to a SYTO®9 concentration of 1 µM. However, at this concentration of cells a decrease in fluorescence intensity occurred as the concentration of SYTO®9 increased from 1 µM to 5 µM (Fig. 8).

In determining the concentration of propidium iodide for each concentration of cells it was found that the optimal concentration of propidium iodide to dye cells at a

concentration of 1.5×10^8 cells/ml was $12.5 \mu\text{M}$. The appropriate concentration of propidium iodide to dye cells at 2.0×10^8 cells/ml was lower (data not shown).

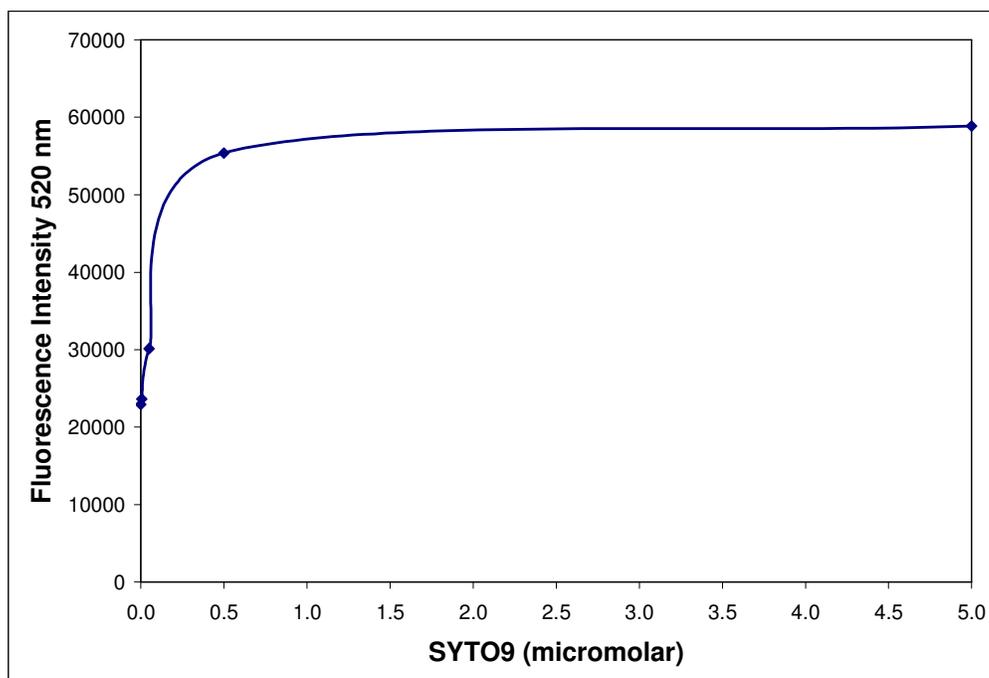


Figure 7. Saturation curve of a 1.5×10^8 cells/ml suspension of *E. coli* for SYTO@9. Cells were grown to late stationary phase and then suspended in sterile saline. The suspension was diluted to an OD_{600} of 0.05 using a Spec 20+ and diluting with 0.85% NaCl. Cells were incubated with a range of SYTO@9 from 0.5 nM to $5 \mu\text{M}$. Measurements were performed by means of a fluorescence microplate reader at an excitation wavelength of 485 nm and emission of 520 nm in a Costar 3695 96-well plate.

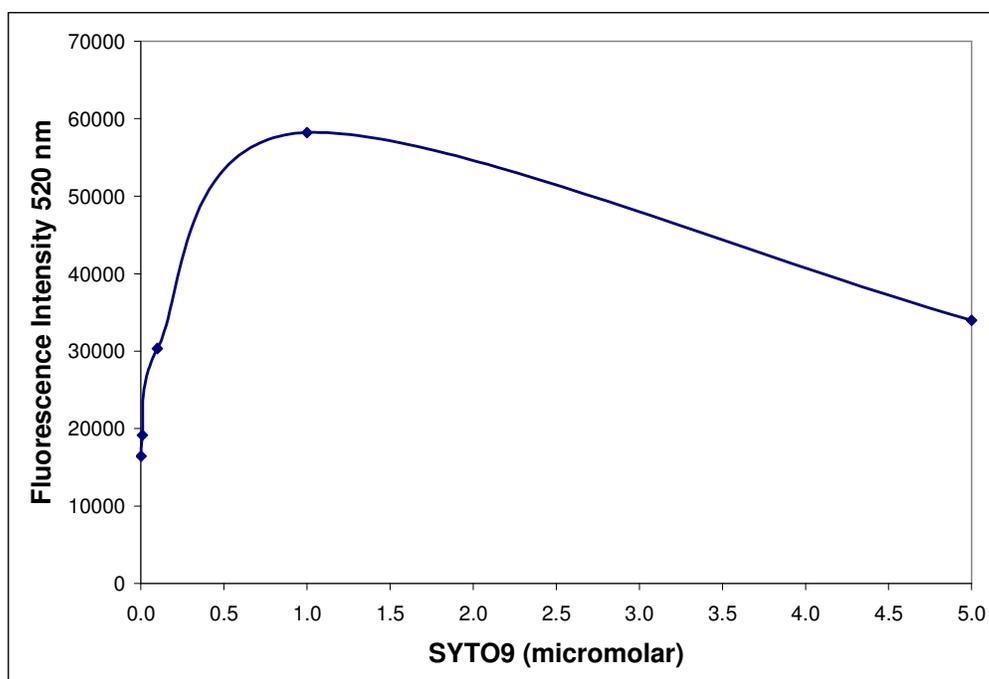


Figure 8. Saturation curve of a 2.0×10^8 cells/ml suspension of *E. coli* for SYTO@9. Cells were grown to late stationary phase and then suspended in sterile saline. The suspension was diluted to an OD₆₇₀ of 0.06 using a Spec20+ and diluting with 0.85% NaCl. Cells were incubated with a range of SYTO@9 from 0.5 nM to 5 μM. Measurements were performed by means of a fluorescence microplate reader at an excitation wavelength of 485 nm and emission of 520 nm in a Costar 3695 96-well plate.

Effects of plate type on the saturation concentration of SYTO@9:

Several different types of 96-well plates are available for fluorescence measurements. Two plates were compared to determine the effect of each plate on optimizing the concentrations of each dye. For both experiments the concentration of cells was adjusted to 2.0×10^8 cells/ml. With the Costar 3695 plate, which is white (opaque), the saturation concentration of SYTO@9 was 0.75 μM (Fig. 9). The second plate was Costar 3915, which is black and reduces/eliminates the possibility for crosstalk between wells on the plate. The SYTO@9 saturation in this plate was 1.25 μM (Fig. 10)

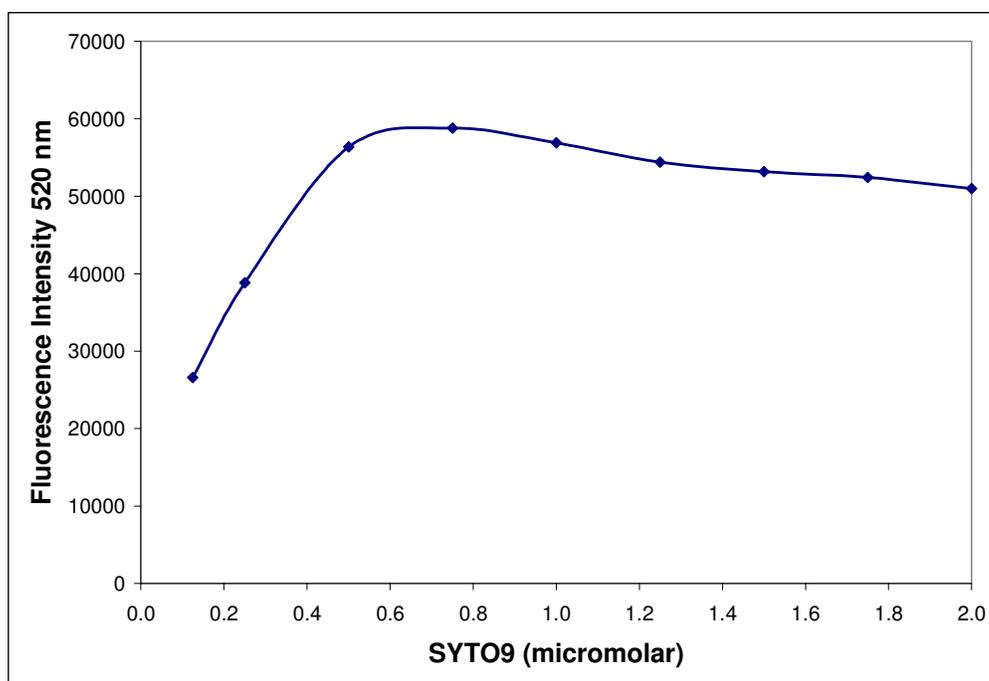


Figure 9. Saturation curve for SYTO®9 using a Costar 3695 96-well plate. Cells were grown to late stationary phase and then suspended in sterile saline. The suspension was diluted to concentration of 2.0×10^8 cell/ml. Cells were incubated with a range of SYTO®9 from 0.1 μ M to 2 μ M. Measurements were performed by means of a fluorescence microplate reader at an excitation wavelength of 485 nm and emission of 520 nm in a Costar 3695 96-well plate.

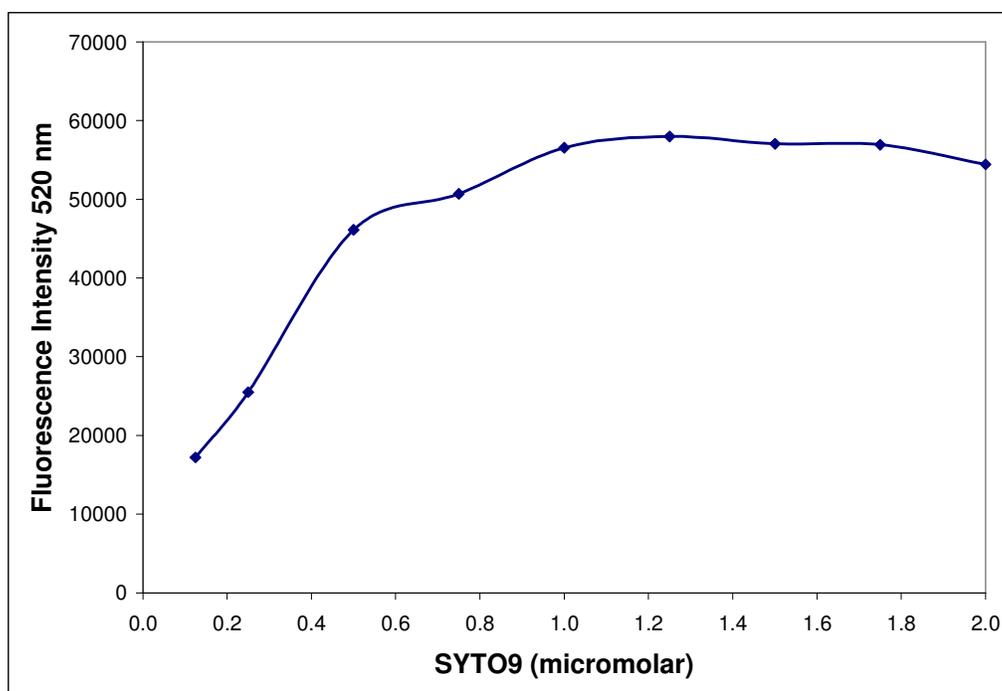


Figure 10. Saturation curve for SYTO@9 using a Costar 3915 96-well plate. Cells were grown to late stationary phase and then suspended in sterile saline. The suspension was diluted to concentration of 2.0×10^8 cell/ml. Cells were incubated with a range of SYTO@9 from 0.1 μM to 2 μM . Measurements were performed by means of a fluorescence microplate reader at an excitation wavelength of 485 nm and emission of 520 nm in a Costar 3915 96-well plate.

Effects of plate type on propidium iodide concentration:

A range of concentrations of propidium iodide along with SYTO@9 were incubated with cells at a concentration of 2.0×10^8 cells/ml. The concentration of SYTO@9 used with each type of plate was determined from the saturation curve, being 0.75 μM for the Costar 3695 white plate and 1.25 μM for the Costar 3915 black plate. The cells were aliquoted in ratios ranging from 0% to 100% live for each concentration of propidium iodide. The green/red (520 nm/612 nm) emission was measured at each concentration of propidium iodide and plotted against the percent of live. A linear regression line was determined and the R^2 value calculated.

A large range of propidium iodide concentrations were tested for the white plate (1 μM to 50 μM). Concentrations of 2.5 μM propidium iodide and 1.0 μM propidium iodide demonstrated the most linear relationships with R^2 values of 0.9983 and 0.9996 respectively in the white plate (Fig. 11). Using this and similar data not shown, an estimate that 2.0 μM propidium iodide would be suitable for staining cells in the white plates. This concentration was used to determine standard curves for future assays in the Costar 3695 96-well plates.

A smaller range of concentrations of propidium iodide were tested in the black plates based on the results from the white plate. Concentrations of 4 μM and 2 μM had the most linear relationship with R^2 values of 0.9982 and 0.9980 (Fig. 12). After repeated attempts with each concentration, 4 μM maintained a R^2 closest to one. This concentration was used to determine standard curves for future assays in the Costar 3915 black plate.

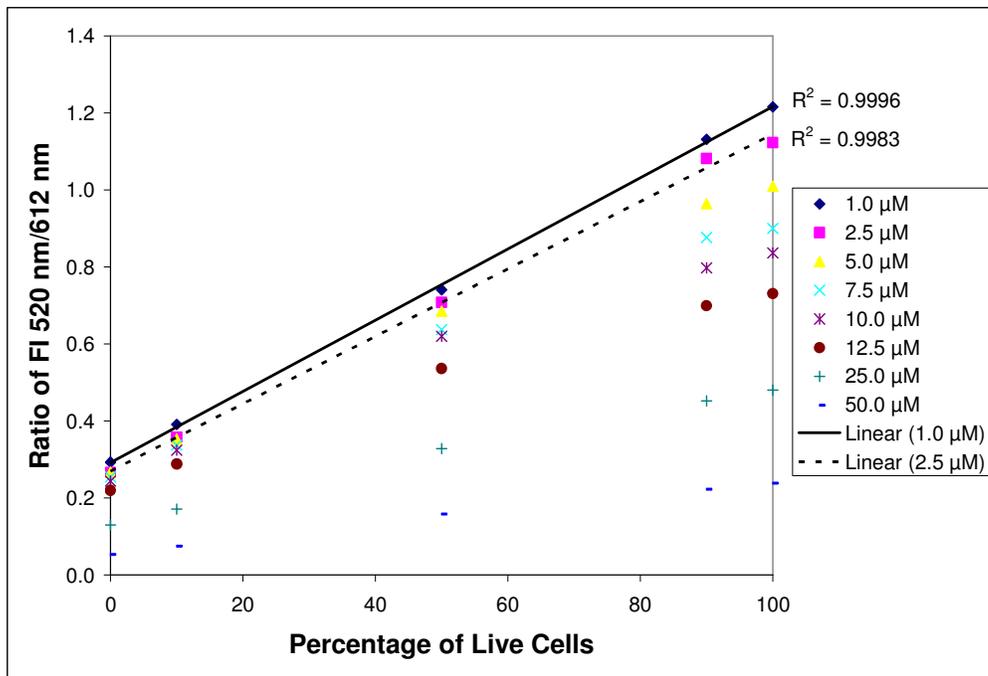


Figure 11. Propidium iodide concentration determination in Costar 3695 96-well plate. Cell suspensions of 0% to 100% live at a concentration of 2.0×10^8 cells/ml were incubated with $0.75 \mu\text{M}$ SYTO®9 and $50.0 \mu\text{M}$ to $1.0 \mu\text{M}$ propidium iodide. In a Costar 3695 96-well plate the fluorescence intensity at 520 nm and 612 nm was measured after exciting cells at 485 nm in a fluorescence microplate reader. The linearity between the ratio of fluorescence emitted at 520 nm/612 nm and percent of live bacterial cells was determined for cells.

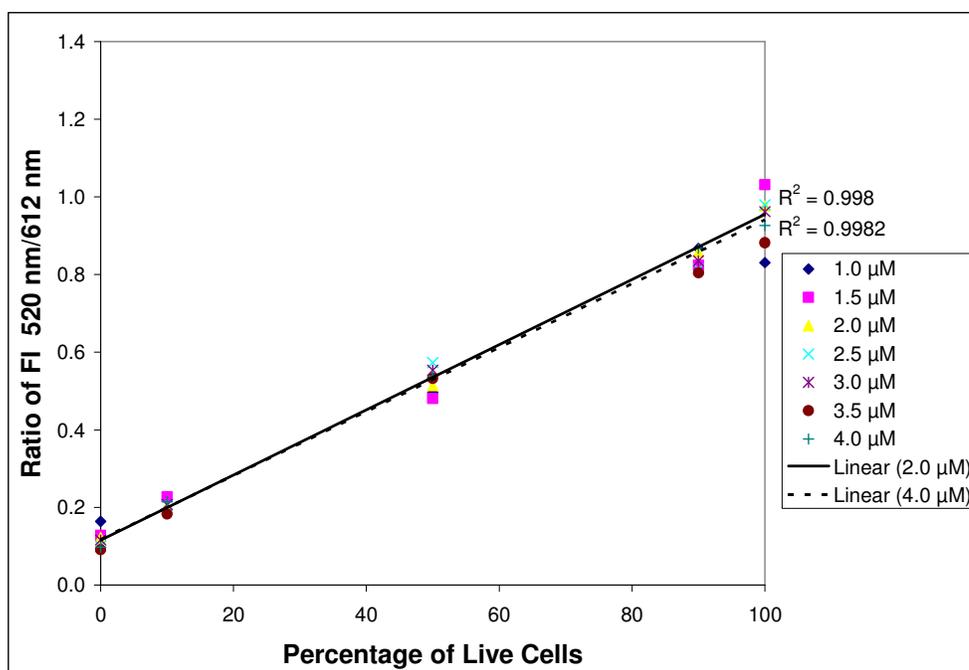


Figure 12. *Propidium iodide concentration determination in Costar 3915 96-well plate.* Cell suspensions of 0% to 100% live at a concentration of 2.0×10^8 cells/ml were incubated with $1.25 \mu\text{M}$ SYTO®9 and $4.0 \mu\text{M}$ to $1.0 \mu\text{M}$ propidium iodide. In a Costar 3915 96-well plate the fluorescence intensity at 520 nm and 612 nm was measured after exciting cells at 485 nm in a fluorescence microplate reader. The linearity between the ratio of fluorescence emitted at 520 nm/612 nm and percent of live bacterial cells was determined.

Other factors affecting the standard curve:

A standard curve was created for each experiment using dead cells that have been killed with isopropyl alcohol and live cells that were suspended in 0.85% NaCl. The fluorescence measurements for the standard curve and treated cells must be taken in the same plate, therefore appropriate timing of the experiment must be determined. Initially, cells for the standard curve were prepared for plating before the treated cells were prepared. Under these conditions the R^2 values for the standard curve were variable across experiments. To determine the effect of time on the standard curve two sets of cells were prepared. The first set of cells rested on the lab for more than an hour (the

time required to prepare treated cells for plating) while a fresh set of cells for a standard curve were prepared. Six standard curves were determined using the cells that rested on the bench and those that were prepared immediately before measurement. The standard curves produced by the fresh cells were more consistent and maintained an R^2 value closer to 1.

2. APPLICATION OF ASSAYS:

SOD Inhibitors:

Two Cu,Zn-SOD inhibitors were selected for study. Quercetin irreversibly inhibits the enzyme but does not sequester copper from the active site. It is unlikely to interfere with metal cofactors involved in other biological processes. It has also been studied for its therapeutic properties in other cell types (e.g tumor cells). DDC does inhibit Cu,Zn-SOD by sequestering copper and is not a good candidate for drug development due to its possible non-specific interactions with other metal cofactors. However, DDC is well studied and commercially available. It is not intrinsically fluorescent making it an optimal candidate for the development of the fluorescence assay.

Quercetin

Effect of DMSO on cell viability determined by fluorescence assay:

To determine the effect of inhibitor compounds on cell viability it must be demonstrated that the solvent for the compound alone does not affect cells. Quercetin went into a solution of pH 11 but not at concentrations high enough to produce the 0.1 μM to 1000 μM range desired for the assay. Quercetin was also found to be soluble in dimethyl sulfoxide (DMSO). However, DMSO is known to alter cell membranes. To

demonstrate that DMSO alone did not affect viability, the cells were treated with DMSO. DMSO was incubated with *E.coli* cells in a culture and a suspension at a concentration that would be present for quercetin cytotoxicity assays (1% DMSO). Both treatments demonstrated that 1% DMSO alone did not affect the viability of the bacterial cells (Fig. 13).

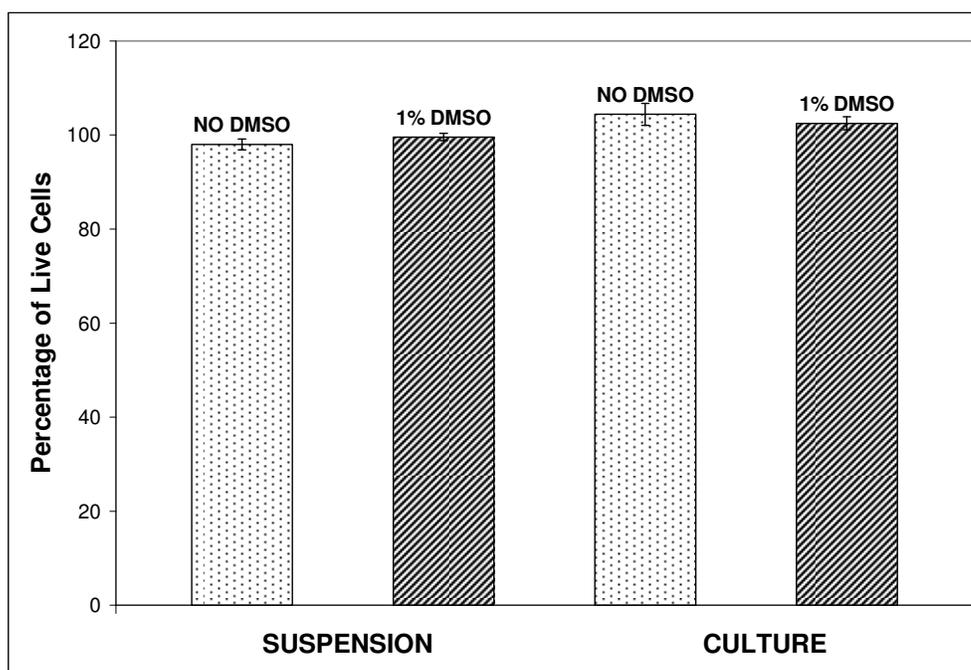


Figure 13. *Effects of DMSO on cell viability.* Cells were incubated with 1% DMSO in culture and in a suspension of 0.85% NaCl. The cells were adjusted to a concentration of 2.0×10^8 cells/ml and incubated with $0.75 \mu\text{M}$ SYTO®9 and $2 \mu\text{M}$ propidium iodide at room temperature in the dark for 15 minutes. Measurements were taken in a Costar 3695 96-well plate. Error bars represent the standard error of the mean.

Effects of quercetin on cell viability determined by fluorescence assay:

Quercetin was screened for its effect on cell viability in both a suspension of cells and in a log phase culture of cells. In the suspension of cells with quercetin the fluorescence measurements at concentrations of quercetin above $100 \mu\text{M}$ consistently showed a reduction in intensity in both the SYTO®9 emission channel (520 nm) and the

propidium iodide emission channel (612 nm). Fluorescence measurements of cells treated with quercetin in a culture when compared to a standard curve indicated the percentage of live cells greatly exceeding 100%.

DDC

Effects of DDC on cell viability determined by fluorescence assay:

DDC is a Cu,Zn-SOD inhibitor that is soluble in water making it unnecessary to determine the effect of its solvent on the cell viability. To determine whether fluorescence of DDC could interfere with the assay, the inhibitor alone was excited at 485 nm and the emission measured at 520 nm and 612 nm. The compound alone emits very little fluorescence in these channels, thus reducing the possibility of the compound's fluorescence interfering with the fluorescence emissions of the assay dyes.

DDC, at a concentration range of 0.1 μM to 1000 μM DDC, was screened for its effects on the viability of cells growing in a culture. It was observed that growth was greatly reduced for cells growing in the presence of 100 μM and 1000 μM DDC. The fluorescence assay showed that at low concentrations of DDC the cells were 100% viable. Cell viability decreased to 36% at 100 μM DDC but increased to 64% at 1000 μM DDC (Fig. 14).

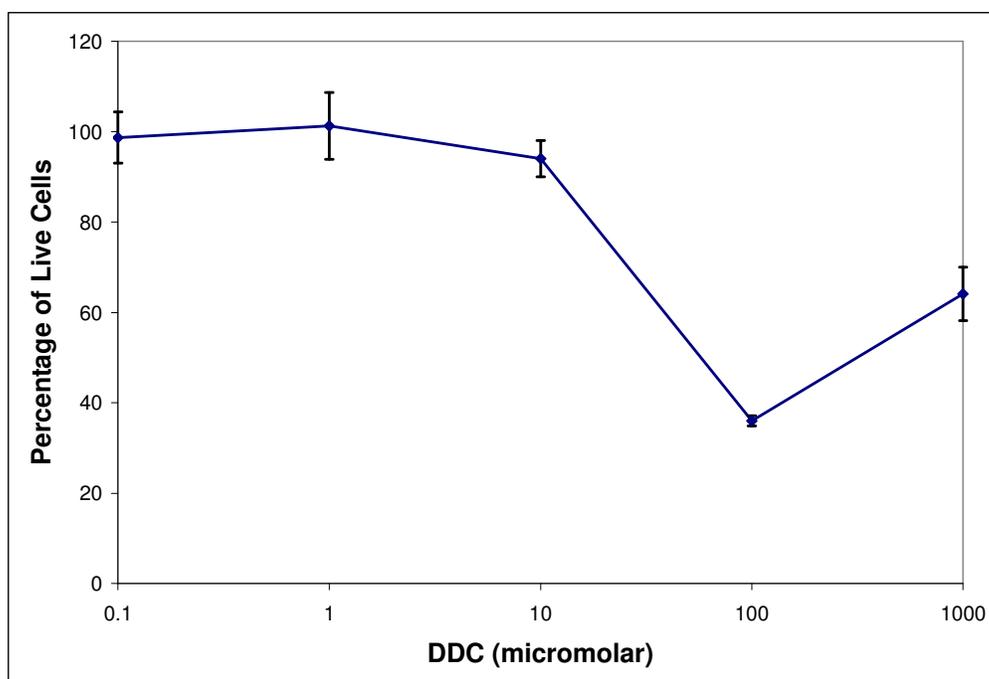


Figure 14. *Effects of DDC on cell viability using the fluorescence assay.* Cells were incubated with a range of concentrations of DDC from 0.1 μM to 1000 μM . Cells were suspended in 0.85% NaCl and the concentration was adjusted to 2.0×10^8 cells/ml using a Spec20+. Cells were incubated with 1.25 μM SYTO®9 and 4 μM propidium iodide. Fluorescence measurements were taken in a Costar 3915 96-well plate. Error bars represent the standard error of the mean.

Effects of DDC on E. coli determined by optical density:

Bacterial growth was determined by measuring the optical density. When the bacteria had reached an OD_{600} of 0.2, the cells were incubated with a range of DDC from 0.1 μM to 1000 μM until an untreated control reached stationary phase. At the lower concentrations (0.1 μM to 10 μM DDC) the growth curves did not deviate from the control. However, 100 μM and 1000 μM DDC incubations slowed the growth of the bacteria. Notably, the 1000 μM DDC treatment had more growth than the 100 μM DDC treatment (Fig. 15).

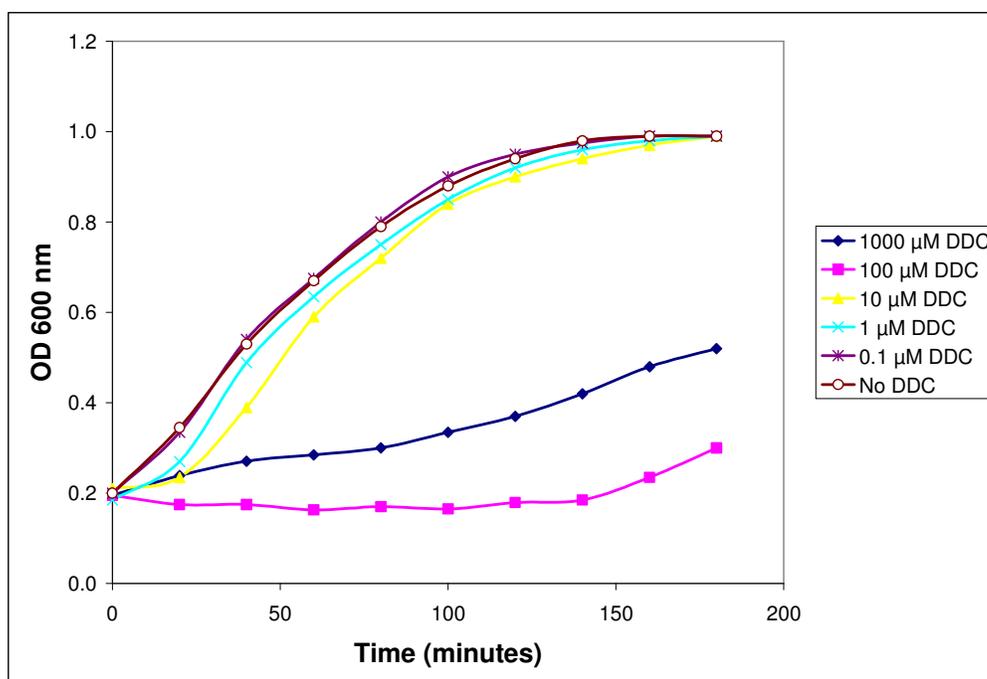


Figure 15. *Effects of DDC on cell growth.* A pre-culture was grown until it reached early log phase ($OD_{600}=0.02$). The cells were then incubated with concentrations of DDC ranging from 0.1 μM to 1000 μM . The OD at 600 nm was measured in time intervals using a Spec20+ until an untreated culture reached stationary phase.

Antibiotics:

Three classes of bactericidal antibiotics were chosen for this study because they have been shown to cause cell death by increasing bacterial oxidative stress (Kohanski 2007). Kohanski used ampicillin as a representative of the β -lactams, kanamycin as a representative of the aminoglycoside family and norfloxacin from the quinolone class. For this reason, the same antibiotics were used for the development of the fluorescence assay.

Determining the cytotoxicity of antibiotics using spread plate bacterial enumeration:

A range of concentrations of 0.1 μM to 1000 μM antibiotic were incubated with *E.coli* that had reached an OD_{600} of approximately 0.2. After several hours of incubation,

cells were diluted and spread on LB agar plates. After a 24 hour incubation period the CFUs were counted. The concentration of ampicillin which was estimated to inhibit growth of CFUs by half was 5 μM ampicillin (Fig. 16). For the kanamycin treatment, there were 1.56×10^6 CFUs at 1 μM and, 1.73×10^8 CFUs at 10 μM kanamycin (Fig. 17). For norfloxacin CFUs were reduced by approximately 50% between 0.1 μM and 1.0 μM norfloxacin (Fig. 18).

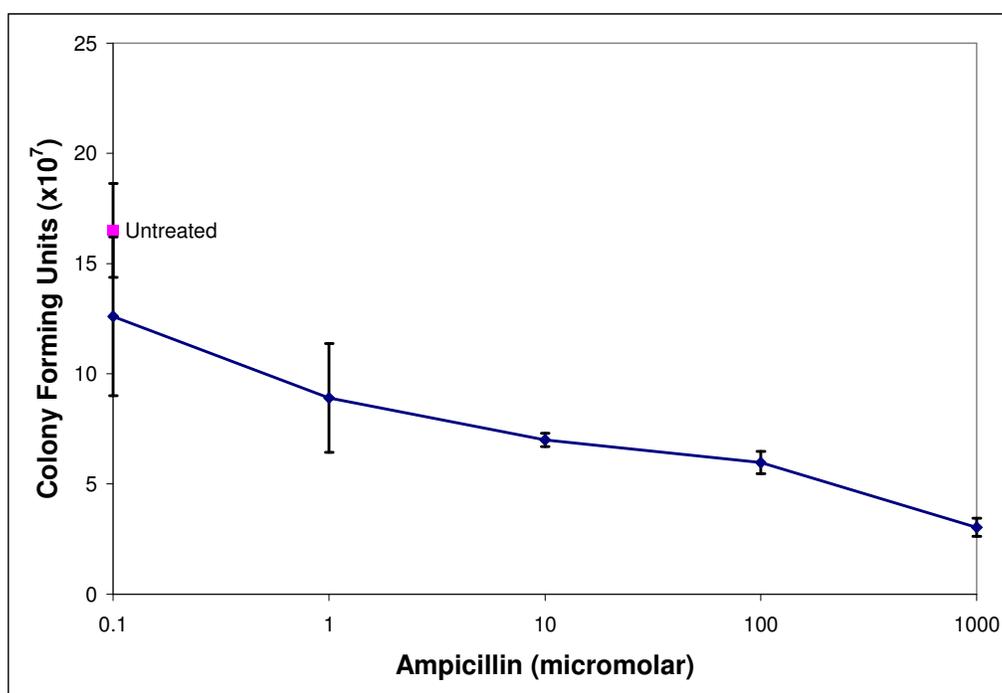


Figure 16. *Determining cytotoxicity of ampicillin by spread plate method.* A pre-culture was grown to early log phase ($\text{OD}_{600}=0.02$). Cells were incubated with a range of ampicillin from 0.1 μM to 1000 μM . Cells were diluted in 0.85% NaCl and spread onto LB agar plates. CFUs were counted after cells were incubated overnight at 37° C. The square represents the untreated control. Error bars represent the standard error of the mean.

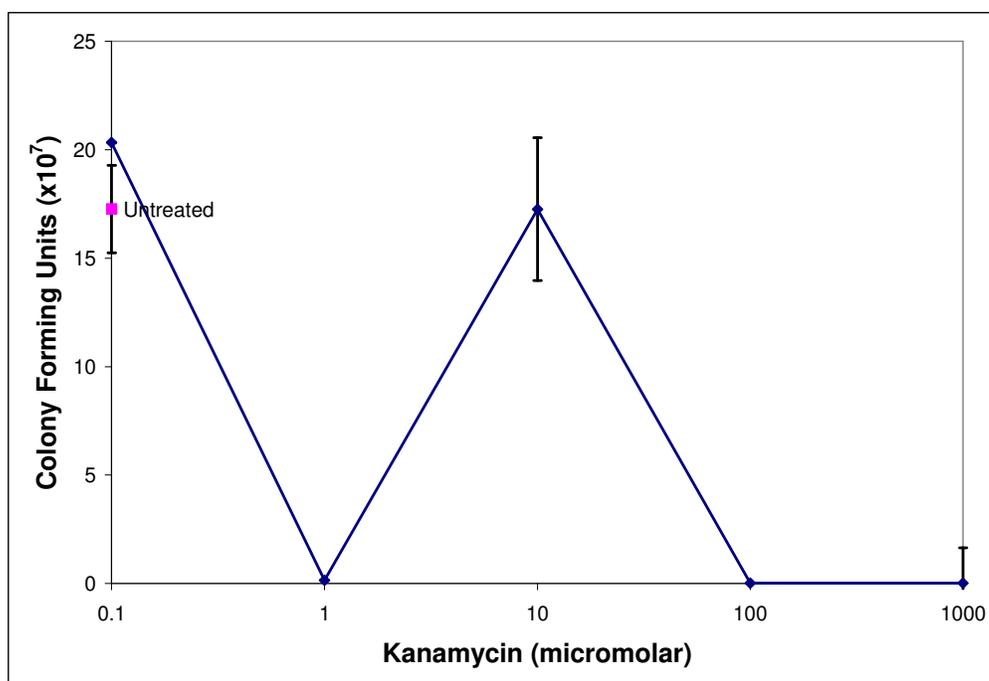


Figure 17. *Determining cytotoxicity of kanamycin by spread plate method.* A pre-culture was grown to early log phase ($OD_{600}=0.02$). Cells were incubated with a range of kanamycin from 0.1 μM to 1000 μM . Cells were diluted in 0.85% NaCl and spread onto LB agar plates. CFUs were counted after cells were incubated overnight at 37° C. The square represents the untreated control. Error bars represent the standard error of the mean.

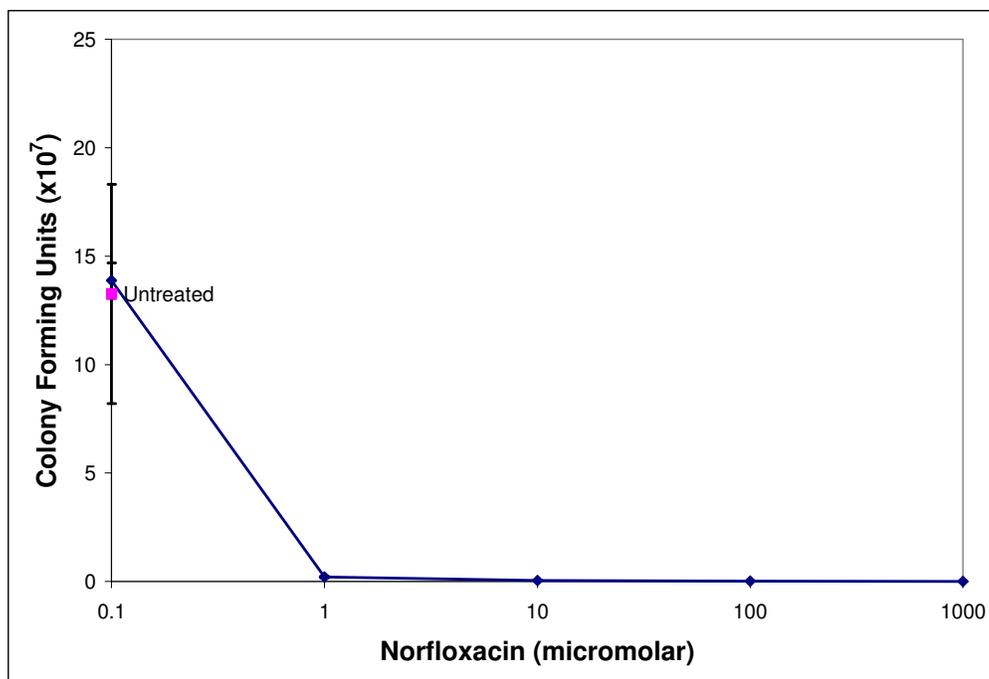


Figure 18. *Determining cytotoxicity of norfloxacin by spread plate method.* A pre-culture was grown to early log phase ($OD_{600}=0.02$). Cells were incubated with a range of norfloxacin from 0.1 μM to 1000 μM . Cells were diluted in 0.85% NaCl and spread onto LB agar plates. CFUs were counted after cells were incubated overnight at 37° C. The square represents the untreated control. Error bars represent the standard error of the mean.

Determining the cytotoxicity of antibiotics by fluorescence assay:

Cells were harvested from a pre-culture and incubated with antibiotic as in the spread plate protocol. After treatment, cells were washed and suspended in a 0.85% NaCl solution at a concentration of 2.0×10^8 cells/ml. The cells were incubated with nucleic acids stains and measurements were taken to assess viability in Costar 3915 96-well plate. The fluorescence assay indicated that the cells incubated with ampicillin at 0.1 μM to 10 μM were approximately 100% live. Cells incubated with 100 μM ampicillin had a mean of 87% live and cells incubated with 1000 μM ampicillin were 80% live on average (Fig. 19). Visual inspection of the culture tubes indicated an inhibition of growth for cells incubated with 100 μM and 1000 μM ampicillin. In

kanamycin experiments in a Costar 3695 plate percent live cells decreased with increasing antibiotic concentrations. The concentration that resulted in approximately 50% viability was 60 μM (Fig. 20). However, in a Costar 3915 plate these data were not repeatable. Although it was observed that 100 μM and 1000 μM concentrations of antibiotic greatly decreased growth all treatments reported viability much higher than 100% live. In norfloxacin experiments using a Costar 3915 plate, the percent viability decreased to approximately 20% at norfloxacin concentrations of 1.0 μM and 10 μM . However, at 100 μM and 1000 μM norfloxacin viability increased 38% and 59% respectively (Fig. 21).

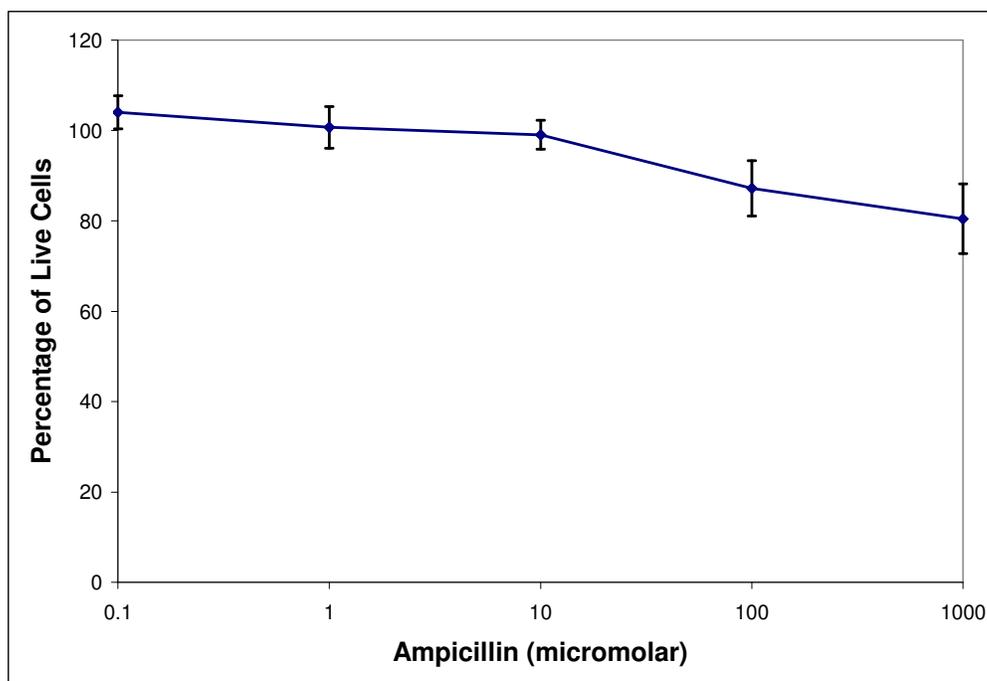


Figure 19. Cytotoxicity of ampicillin using fluorescence assay. A pre-culture was grown to early log phase ($\text{OD}_{600}=0.02$). Cells were incubated with a range of ampicillin from 0.1 μM to 1000 μM . Cells were suspended in 0.85% NaCl and diluted to 2.0×10^8 cells/ml using a Spec20+. Suspensions were incubated with 0.75 μM SYTO@9 and 2 μM propidium iodide at room temperature in the dark. Measurements were taken in a Costar 3695 plate. Error bars represent the standard error of the mean.

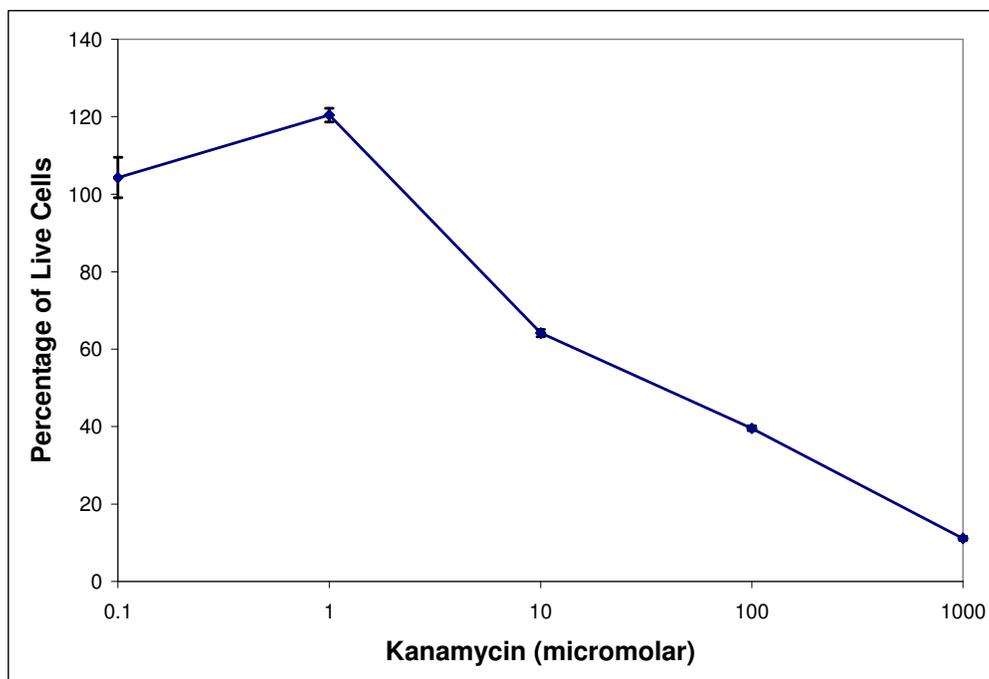


Figure 20. *Cytotoxicity of kanamycin using fluorescence assay.* A pre-culture was grown to early log phase ($OD_{600}=0.02$). Cells were incubated with a range of kanamycin from 0.1 μM to 1000 μM . Cells were suspended in 0.85% NaCl and diluted to 2.0×10^8 cells/ml using a Spec20+. Suspensions were incubated with 0.75 μM SYTO@9 and 2 μM propidium iodide at room temperature in the dark. Measurements were taken in a Costar 3695 plate. Error bars represent the standard error of the mean.

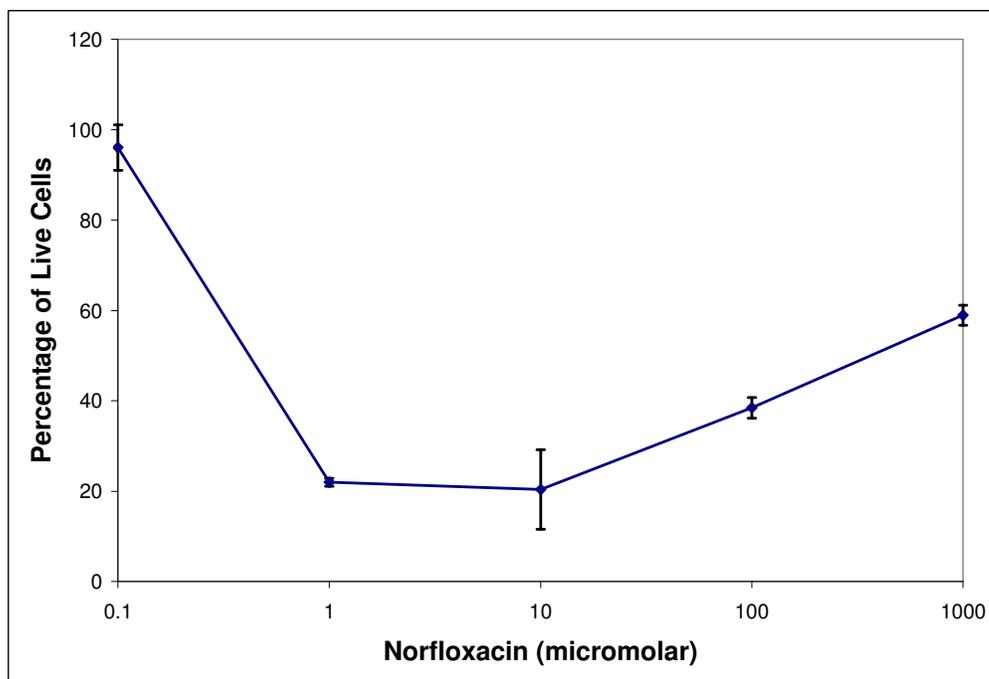


Figure 21. *Cytotoxicity of norfloxacin using fluorescence assay.* A pre-culture was grown to early log phase ($OD_{600}=0.02$). Cells were incubated with a range of norfloxacin from 0.1 μM to 1000 μM . Cells were suspended in 0.85% NaCl and diluted to 2.0×10^8 cells/ml using a Spec20+. Suspensions were incubated with 1.25 μM SYTO@9 and 4 μM propidium iodide at room temperature in the dark. Measurements were taken in a Costar 3915 96-well plate. Error bars represent the standard error of the mean.

Effects of antibiotics on the cytotoxicity of DDC determined by optical density:

Cells were incubated with a range of concentrations of DDC from 0.1 μM to 1000 μM . They were also incubated with a concentration of antibiotics that represented approximately a 50% reduction in CFUs as determined by the spread plate method or the fluorescence method. The concentration of ampicillin used was 5 μM . Treatments of 0.1 μM to 10 μM DDC with antibiotic did not deviate from the antibiotic only control. As in the experiment with DDC alone the 100 μM DDC had the slowest growth rate and 1000 μM growth was reduced (Fig. 22). The concentration of kanamycin incubated with the bacteria and DDC was 60 μM . The treatments of 0.1 μM DDC and 1.0 μM DDC with 60 μM kanamycin grew at a rate close to the antibiotic only treatment. The 10 μM DDC and

60 μM kanamycin treatment, however, demonstrated an inhibition in growth not observed with the DDC alone. The higher concentrations of DDC (100 μM and 1000 μM) followed the same pattern as seen in the DDC only treatment and the DDC and ampicillin treatments (Fig. 23). Cells were incubated with 1 μM norfloxacin initially but several measurements were taken with seemingly no effect of this concentration on growth. The experiment was abandoned and repeated with a concentration of 10 μM norfloxacin. A 0.1 μM DDC to 10 μM concentration of DDC with norfloxacin showed no notable difference from the growth rate of the antibiotic alone treatment. Again 100 μM and 1000 μM had a decreased growth rate but the 100 μM treatment after 60 minutes began to increase its growth rate beyond that of the 1000 μM DDC treated culture (Fig. 24).

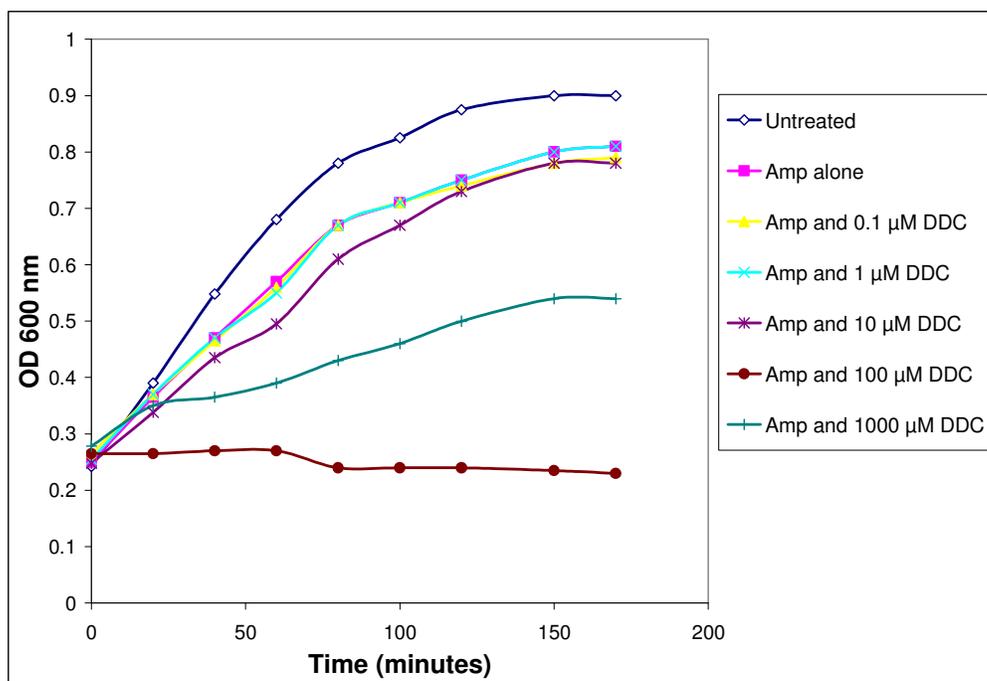


Figure 22. Effects of ampicillin and DDC on *E. coli* growth using growth curves. A pre-culture was grown to early log phase. Cells were then incubated with 5 μM ampicillin and a range of DDC from 0.1 μM to 1000 μM until an untreated culture reached stationary phase. OD_{600} was measured at time intervals using a Spec20+.

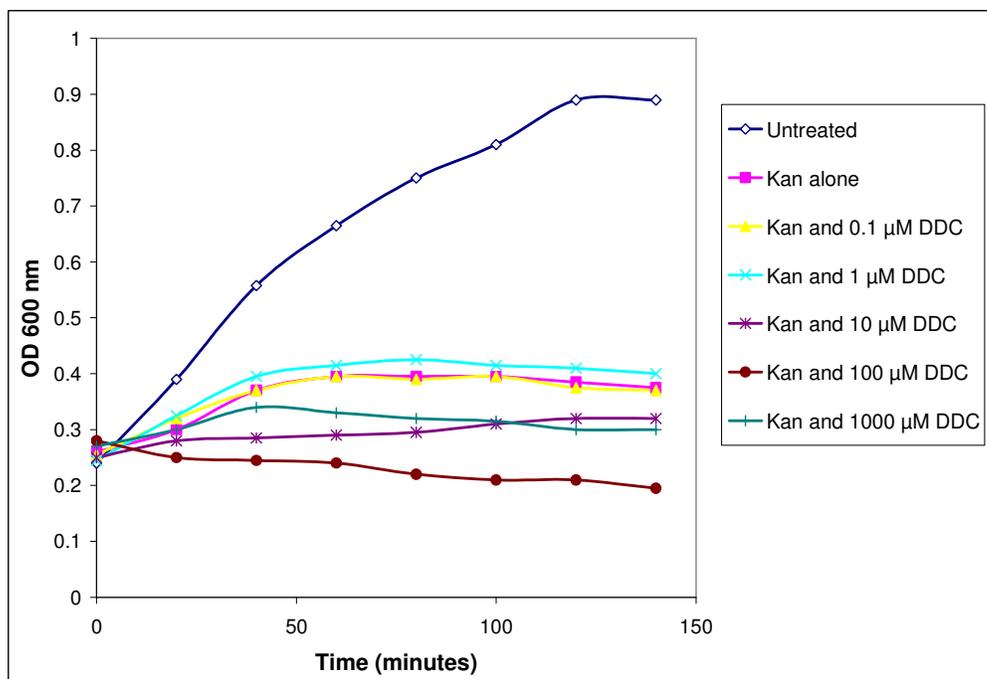


Figure 23. Effects of kanamycin and DDC on *E. coli* growth using growth curves. A pre-culture was grown to early log phase. Cells were then incubated with 60 µM kanamycin and a range of DDC from 0.1 µM to 1000 µM until an untreated culture reached stationary phase. OD₆₀₀ was measured at time intervals using a Spec20+.

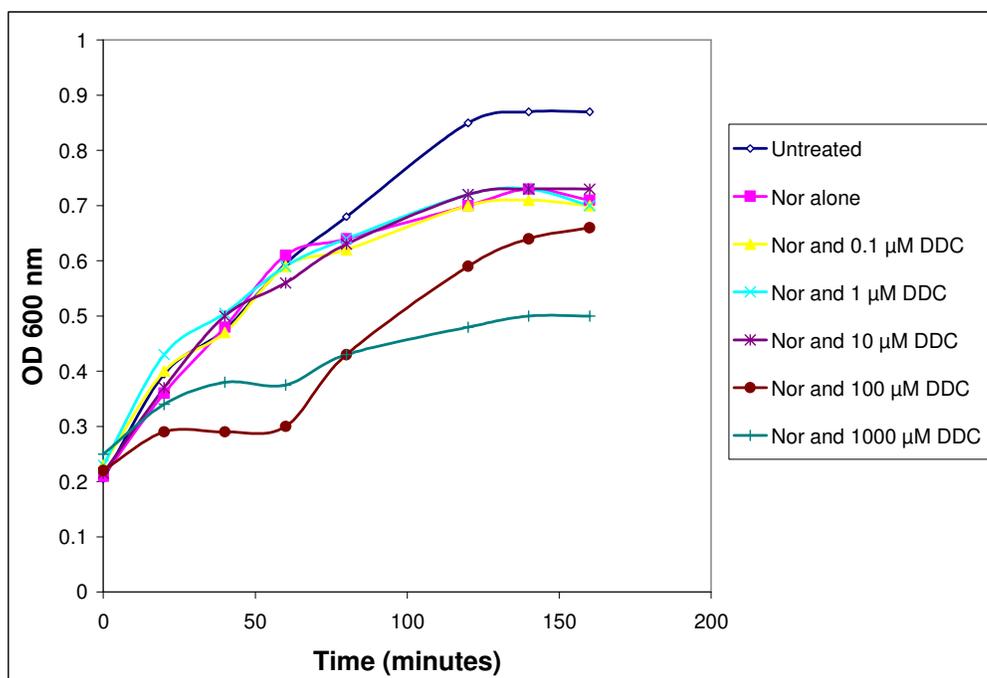


Figure 24. Effects of norfloxacin and DDC on *E. coli* growth using growth curves. A pre-culture was grown to early log phase. Cells were then incubated with 10 µM norfloxacin and a range of DDC from 0.1 µM to 1000 µM until an untreated culture reached stationary phase. OD₆₀₀ was measured at time intervals using a Spec20+.

DISCUSSION

Effects of cell concentration:

It was important to consistently and accurately measure the concentration of cells in a culture or suspension for many of the experiments performed. However, it was also a goal to streamline the assay for high throughput experiments. In order to determine the instrumentation that achieves consistency, accuracy and efficiency the optical density was measured using a SpectraMax 190, a Nanodrop and a Spec20+.

The SpectraMax190 is a UV-vis microplate reader that has the high throughput capabilities that is desirable for the assay. The microplate reader determines the optical density of 150 μ l aliquots of sample and is capable of reading large numbers of samples in a short period using 96-well plate. Measurements taken using this instrument to determine a growth curve were not repeatable. Multiple aliquots of the same sample were not consistent. The angle of the optics in the instrument needs to be adjusted to use the instrument to accurately measure the optical density for bacteria samples. Therefore this method was abandoned until the instrument can be optimized.

The Nanodrop is an instrument that measures the optical density of a small amount of sample. The sample is pipetted onto a pedestal and the sample is drawn into a small column where the measurement takes place. 3 μ l aliquots were used for the measurement of the bacterial cell concentration. As with the SpectraMax 190, the data were not repeatable and the multiple measurements from the same sample were highly variable. It is possible that the small amount of sample measured increases the probability that bunching or settling of cells could cause inconsistency of the measurement within a sample. Although the Nanodrop can quickly measure the optical

density of a sample the inconsistency seen in the measurements and the inability to read multiple samples makes it impractical for use with the fluorescence assay.

Finally, the Spec20+ was used to measure optical density for the determination of a growth curve (Fig. 5 and 6). The Spec20+ is not the ideal instrument for long-term use with the fluorescence assay. The Spec20+ can only measure the optical density of one sample at a time and the volume of that sample needs to be approximately 3 to 4 ml. If the volume of the sample is less then a dilution of the sample is measured and needs to be discarded. The instrument does not quickly measure optical density. It must warm up for approximately 20 minutes before use and every read can take several minutes. However, the measurements made in this study were consistent and repeatable. Therefore, although the instrument was not ideal it was used to determine cell concentrations for future experiments until a better alternative should arise.

Once the Spec20+ was determined to most accurately and consistently measure the optical density of the bacterial concentrations in suspension and in culture the effects of the cell concentration on the fluorescence assay were examined. SYTO®9 saturation curves were performed for bacterial suspensions with concentrations of 1.5×10^8 cells/ml ($OD_{600}=0.05$) and 2.0×10^8 cells/ml ($OD_{670}=0.06$) (Fig. 7 and 8). At the higher concentration of cells the amount of fluorescence intensity between 1 μ M and 5 μ M SYTO®9 decreased due to quenching. Although the saturation concentration of SYTO®9 did not greatly differ for the two concentrations of bacteria the optimal concentration of propidium iodide was affected. The optimal concentration of propidium iodide was approximately six times greater for the lower concentration of cells (1.5×10^8 cells/ml) showing that the concentration of cells can affect the fluorescence assay. If the

concentration of cells is too high then quenching of SYTO®9 could occur. If the concentration of cells is too low then the concentration of propidium iodide may be insufficient to label all dead cells. For all further experiments the concentration of bacteria was adjusted to 2.0×10^8 cells/ml, as suggested by the manufacturer of the fluorescence kit.

The effect of the cell concentration on the ability of the dyes to accurately label bacteria cells reinforces the importance of using instrumentation that precisely measures cell concentrations. It should be noted that microscopy can also be used to enumerate bacteria; attempts using a hemacytometer are not discussed here. Although the Spec20+ was determined to be the most effective instrument available, certain factors must be taken into consideration when evaluating the data from the fluorescence assay. The Spec20+ takes analog measurements. When measuring bacteria the culture or suspension is mixed to distribute the cells. The movement of the cells in the tube causes the needle to fluctuate. In order to get an accurate reading a wait time of 45 seconds to 1 minute until the needle settles must occur. In this time there is the possibility that cells may settle to the bottom of the tube. This also leads to variations in concentrations across treatments. All treated cells were diluted to the standard cell concentration of 2.0×10^8 cells/ml required for the accurate measurement by the fluorescence assay. The process of adjusting the cell concentrations for each treatment is an inaccurate one. It was impossible to ensure that all the tubes had the same number of cells and it must be recognized that this variation of cell concentrations may have had an effect on the fluorescence intensity measurements since the effects of cell concentration on the fluorescent dyes had been shown. It must also be noted that a tube containing all viable

cells with intact membranes affect the optical density differently than cells that have compromised membranes or have lysed.

Effects of plate type on fluorescence measurements:

Several different types of 96-well plates are manufactured for use in a fluorescence microplate reader. The Costar 3695 plate is white (opaque). It may allow some cross talk between the wells. The Costar 3915 plate is black and reduces any fluorescence cross talk between wells. Originally, the Costar 3695 white plate was used. In determining the effects of antibiotics or other compounds on the viability of the bacteria, a standard curve was prepared and plated in adjacent wells and the treated cells were aliquoted in wells that were not adjacent to one another. Some of the readings obtained suggested that the treated cells were more than 100% live when compared to the standard curve. This could be explained in several ways but it was hypothesized that cross talk between the adjacent wells altered the fluorescence measurements affecting the standard curve. The treated wells that were separated did not experience cross talk so the comparison to the standard curve resulted in artificially high percent live readings. Based on this hypothesis, further experiments used the Costar 3915 black plate. This plate should eliminate all cross talk and does not require the separation of treatments in the plates. In doing so the optimal concentration of both SYTO®9 and propidium iodide for the Costar 3915 black plate was determined showing that the type of plate used affects the standard curve and the concentrations of dyes that should be used to assess viability. The black plate required an increase in concentration of SYTO®9 from 1 μM to 1.25 μM

and an increase in the propidium iodide concentration from 2 μM to 4 μM from the white plate (Fig. 9, 10, 11 and 12).

Optimizing the protocol for the determination of the standard curve:

Once the concentration of SYTO®9 and propidium iodide were optimized the protocol for obtaining the standard curves could be optimized. The standard curves should fit a linear model (i.e., have an R^2 value close to 1). Initially, the cells for the standard curve were prepared before the treated cells were prepared. Due to the variability in the amount of time it took to prepare treated cells by using the Spec20+, an inconsistency in the standard curve R^2 value was observed. An experiment was performed comparing the standard curve of cells that had sat on the lab bench for over an hour and cells that were freshly prepared. This experiment reflected that the standard curve should be performed as close to the plating and fluorescence measurement as possible in order to keep the standard curve consistent. Assistance in the lab and experience increased the efficiency and decreased the time it took to prepare cells for fluorescence measurement. However, the effect of time on the results of the standard curve and presumably the treated cells further reiterates the need to expedite the process by finding an instrument that more quickly and accurately measures optical density.

Quercetin effects on cell viability using the fluorescence assays:

The Cu,Zn-SOD inhibitor, quercetin, was screened for its effect on the viability of bacterial cells. Several challenges were encountered when screening the compound. DMSO, the solvent of quercetin has known biological and cellular implications. Also the

intrinsic fluorescence of quercetin and fluorescence enhancement when bound to biomolecules in the cell have been shown to interfere with other fluorescence-based assays (Clarke et al 2001).

Quercetin is soluble in DMSO which is used in cell biology to increase membrane permeability. To ensure that the 1% DMSO that the bacteria would be exposed to in the screening assay did not affect the viability of the bacteria, a control experiment with DMSO alone was conducted. The bacterial cells were incubated with 1% DMSO in a log phase culture and in a sterile saline cell suspension. There was no notable difference between the controls that were not treated with DMSO and the treated cells (Fig. 13). Both the untreated and treated cells were approximately 100% live when compared to a standard curve (Fig. 13). Based on these results it was assumed that the concentration of DMSO present in the experiment to screen quercetin would not affect the viability of cells. Also any effects of membrane integrity by DMSO did not apparently cause changes in the ability for propidium iodide to enter the cells resulting in false negative (dead) assessment of cells.

Quercetin at varying concentrations was incubated with *E. coli* cells in a log phase culture as well as a cell suspension. The culture indicated percent viability much greater than 100%. The suspension of cells notably had a reduction of fluorescence intensities at concentrations of quercetin greater than 100 μM . Studies have shown that quercetin can cause a decrease in fluorescence emission in other fluorescence-based assays. For example, at a quercetin concentration of 100 μM the fluorescence intensity at 530 nm was decreased by 33% for a particular fluorescence based assay (Zou et al 2003). This is consistent with the results obtained with the fluorescence assay used in our experiments

in which a reduction of fluorescence intensity was noted at 520 nm emission at concentrations of 100 μM quercetin and greater. Quercetin also increases in fluorescence when bound to targets within the cell. It has been used to track bioavailability in the cell and binds to many targets. When bound, it can be visualized by confocal microscopy (Nifli et al 2007). This fluorescence may cause quenching or interference with measuring the fluorescence intensity of the nucleic acid stains, SYTO®9 and propidium iodide. Also the high affinity binding of quercetin to nucleic acids could inhibit the binding of the stains in the viability assay affecting the emissions and skewing the viability determination. Based both on experimental results and research into the compound's fluorescence interference potential, quercetin was abandoned as a test compound to be used in further development of the fluorescence assay and exploration into the results of SOD inhibition on cell viability under oxidative stress conditions.

DDC effects on cell viability:

DDC is soluble in water and therefore the effects of the solvent did not need to be tested for its effects on cell viability alone. In order to validate the fluorescence assay DDC was incubated with cells at varying concentrations of DDC and the growth of the cultures was measured using a Spec20+. Lower concentrations of DDC between 10 μM and 0.1 μM did not affect the growth of the bacteria. However, at concentrations of 100 μM and 1000 μM growth was inhibited. Notably, growth of the bacteria in the presence of 100 μM DDC was inhibited more than cells incubated with 1000 μM DDC (Fig.15). Possibly a pathway may be initiated at a higher level of stress that limits the effects of high concentrations of DDC on growth. The fluorescence assay using the same

concentration range of DDC showed that approximately 100% of the cells were alive at concentrations from 10 μM to 0.1 μM and at 100 μM cells were 36% live and 64% live at 1000 μM DDC (Fig. 14). The repetition of this phenomenon not only suggests that is caused by a biological process and not a lab error, but also that the fluorescence assay can reproduce results observed with more traditional methods and would be useful in the screening of more inhibitor compounds.

Effects of antibiotics on cell viability;

Cells in a log phase culture were incubated with various concentrations of antibiotic until an untreated culture reached stationary phase. Two methods were employed to assess the viability of the cells in each treatment. Cells were diluted in sterile saline and spread onto media. Plates were incubated overnight and the number of CFUs was counted. In a separate experiment cells were pelleted and suspended at a concentration of 2.0×10^8 cells/ml and incubated with SYTO®9 and propidium iodide to determine cell viability by fluorescent staining. These two methods were used to approximate a concentration of antibiotic that resulted in 50% viability to use as a base concentration in further assays. The two methods of determining cell viability both measure indicators of viability and assumptions are made that allow us to infer the actual percent live. The spread plate method assumes that the cells are diluted to the point that a single cell is spread on the plate and forms a colony, which may not always be the case. The indicator of viability is the ability for the cell to multiply. The fluorescence method measures membrane integrity as an indicator of cell viability. Although the results of the spread plate method could not be directly compared to the fluorescence data, comparison

of the results was used to examine the fluorescence assay for use with antibiotics. This also exposed potential problems with both methods.

The spread plate method generally results in a false low estimation of bacteria numbers (MacKenzie and Gould 1993). This can be due to several problems with the method. Bacteria adhere to each other more readily in the presence of antibiotics resulting in a bunching of cells that form a single colony instead of a single organism. Also the solvent in which the bacteria are diluted for plating, such as water or sterile saline, can lead to cell lysis and an inaccurate estimation of cell death caused by the antibiotic alone.

The fluorescence assay involves manipulation of the bacterial cells that could result in cell death and lead to inconsistency in readings. Cells are centrifuged and vortexed or hand-mixed for resuspension. This can cause cell lysis. Cells that are prepared for a standard curve that is used to determine the viability of treated cells are handled the same as the cells that have been treated with the antibiotics. However, the osmotic sensitivity and the sensitivity to mechanical mixing vary based on the type of antibiotic used. Cell lysis not only leads to inaccurate measurement of cell death caused by the antibiotic but also can attribute to problems with the spectrophotometric adjustment of cell concentrations. As has already been discussed, the concentration of bacteria cells in suspension affects the determination of cell viability by affecting the concentration of both the SYTO®9 and propidium iodide dyes that should be used. Therefore, the inaccuracy in the adjustment of cell concentration using the spectrophotometer leads to inaccuracy in the fluorescence measurements.

After using both methods to determine the concentration of each antibiotic that resulted in approximately 50% reduction CFUs or viability, the concentration determined was used for measuring the effects of DDC in conjunction with the individual antibiotics. This process also resulted in a third method of determining cell viability by comparing cell growth in an untreated control and in the presence of antibiotic that should result in approximately 50% growth compared to control. This provided another form of validating the fluorescence assay or revealing problems with both methods.

Effects of ampicillin on cell viability:

Using the spread plate method, 5 μM ampicillin resulted in approximately 50% of the CFUs than were observed with no ampicillin (Fig. 16). However, the fluorescence assay indicated that cells were 80% live at the highest concentration of ampicillin, 1000 μM (Fig. 19). This discrepancy could be attributed with certain problems with determining cell viability with β -lactams. Gram-negative bacteria in the presence of β -lactams, such as ampicillin, form filaments which can contain many cells. These filaments can lead to fewer numbers of CFUs since a group of bacteria are yielding a colony rather than individuals (MacKenzie and Gould 1993). The filamentation of the cells does not affect the fluorescence readings. When the antibiotic is removed as cells are prepared for the fluorescence readings, the filaments are divided. However, the concentration of cells to be used in the fluorescence assay is determined by adjusting the optical density of cells suspended in 0.85% NaCl using a Spec20+ which is inaccurate after bacteria are exposed to antibiotics (MacKenzie and Gould 1993). The light-scattering properties of bacteria are changed when the membranes of the cells are

compromised. Therefore, the problem of accurately estimating cell concentrations after the exposure to a β -lactam, such as ampicillin, is particularly an issue because this class of antibiotic works by disrupting formation of the cell wall.

After estimating the concentration 5 μ M ampicillin as yielding approximately 50% viability, cells were incubated with this concentration and varying concentrations of DDC. When examining the controls of untreated cells and those incubated with only 5 μ M ampicillin, growth of the bacteria with this amount of antibiotic is much higher than 50% (Fig. 22). This could be attributed to any of the aforementioned issues including inaccuracy in optical density readings due to membrane integrity or filamentation of bacterial cells and artificially low numbers on spread plates due to filamentation or lysis in saline buffers.

Effects of kanamycin on cell viability:

Using the plate method, kanamycin showed an extreme decrease in CFUs at 1 μ M kanamycin treatment but a much higher number of CFUs at the 10 μ M kanamycin treatment (Fig. 17). The low numbers of CFUs at 1 μ M could be attributed to cell lysis in the sterile saline solvent, bunching of cells that yielded lower colony numbers or cell lysis that resulted from vortexing. However, since all treatments were handled in a similar fashion it is more likely that the results are due to a pharmacodynamic or pharmacokinetic effect. Pharmacodynamics relates to the concentration of the drug and how that contributes to the effect on the target. Sub-minimum inhibitory concentrations (MIC) of antibiotics can affect the population numbers of exposed bacteria (Odenholt 2001). Pharmacokinetics examines time dependency on the effects of a drug and

interactions between a drug and its target. The amount of time the *E. coli* were exposed to kanamycin could also cause variable counts. Bacteria that are exposed to concentrations close to the MIC can display a regrowth when the concentration dips below the MIC for an extended period of time (Craig 1998). This could explain the large numbers of CFUs seen at the 10 μM kanamycin treatment.

The fluorescence-based assay showed the percent live remained near 100% and decreased at higher concentrations (Fig. 20). The concentration that yielded approximately 50% viability was 60 μM kanamycin. This concentration was used when incubating cells with varying concentrations of DDC and determining the affect on the growth of the bacteria. When comparing an untreated control to a control treated only with 60 μM kanamycin the growth was reduced by more than 50% (Fig. 23). This suggests that the fluorescence assay accurately determined the concentration that yielded approximately 50% viability.

Effects of norfloxacin on cell viability:

The spread plate method indicated that almost all cells were killed at concentrations of norfloxacin above 1 μM (Fig. 18). The fluorescence assay also indicated a significant decrease in cell viability between 0.1 μM and 1 μM norfloxacin but notably an increase in percentages of live cells was observed increasing from 10 μM to 100 μM norfloxacin (Fig. 21). When observing the effects of 1 μM norfloxacin on the growth of cells using the Spec20+, no difference was seen. When the concentration of norfloxacin was increased to 10 μM , which both the spread plate and fluorescence assay indicated viability measurements of 20% or less, the rate of growth was only minimally

decreased from an untreated control (Fig. 25). The CFUs were artificially low most likely due to a disruption in plating time that occurred causing the 1 μM treatments and higher to spend a longer time in the diluent possibly resulting in cell lysis.

The fluorescence assay indicated a decrease in viability between 0.1 μM and 1 μM norfloxacin but an increase from 10 μM to 1000 μM . This may also be explained by a pharmacokinetic or pharmacodynamic effect. Given time the regrowth of the *E. coli* may have occurred when the interaction between the drug and the bacteria cells effected the concentration of the drug present.

Effects of antibiotic on the cytotoxicity of DDC:

The antibiotics showed little affect on the cytotoxicity of DDC. Antibiotics have been shown to increase cytoplasmic reactive oxygen species (Kohanski et al 2007). DDC is a Cu,Zn-SOD inhibitor and is located in the periplasm. The inhibition of the Cu,Zn-SOD may not effect the oxidative stress within the cell caused by the antibiotics, therefore cell viability was not affected when the inhibitor and antibiotics were used in conjunction.

However, some differences in the growth of the *E. coli* in the presence of DDC and antibiotic were observed. A treatment of 10 μM DDC with kanamycin showed decreased growth from cells that were incubated with lower concentrations of DDC which was not seen with the other antibiotics (Fig. 23). Stress caused by the presence of the antibiotic and the DDC could have caused a negative effect on cell viability or growth. In the norfloxacin growth curves, the *E. coli* incubating with 100 μM DDC and norfloxacin increased growth after 50 minutes incubation and grew to an optical density

close to those treated with low concentrations of DDC and antibiotic (Fig. 24). However, the 1000 μM DDC treatment remained at low optical densities throughout the experiment as with the other antibiotic-inhibitor growth experiments. Under the conditions of 100 μM DDC and norfloxacin treatment, it is possible that pathways were initiated that may have allowed for this regrowth.

APPENDIX

STAB AGAR (1L)

10 g tryptone
5 g NaCl
6 g agar
5 g yeast extract
1 ml 1 N NaOH
1 mg cysteine
1 L sterile H₂O

LB LIQUID MEDIA (1L)

10 g tryptone
5 g yeast extract
5 g NaCl
1 ml 1 N NaOH
1 L sterile H₂O

LB PLATE MEDIA (1L)

10 g tryptone
5 g yeast extract
5 g NaCl
1 ml 1 N NaOH
15 g agar

AMPICILLIN SOLUTION (100 mM)

0.37 g ampicillin
10 ml sterile H₂O

KANAMYCIN SOLUTION (100 mM)

0.34 g kanamycin sulfate
5 ml sterile H₂O

NORFLOXACIN SOLUTION (100 mM)

0.32 g norfloxacin
10 ml 0.1 M NaOH

QUERCETIN SOLUTION (100 mM)

0.03 g quercetin
1 ml DMSO

DDC SOLUTION (100 mM)

0.22 g DDC
10 ml sterile H₂O

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