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Research article

Investigating the short- and long-term effects of antibacterial agents using a real-time assay based on bioluminescent *E. coli*-lux

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ABSTRACT

We have previously established that the *E. coli*-lux assessment is a convenient tool for rapid measurements of the kinetical features of short-term toxicity caused by various factors. In this study, kinetic measurements of seven specifically acting model antibacterials (i.e., polymyxin B, chloramphenicol, nalidixic acid, kanamycin, deoxynivalenol, erythromycin and tetracycline) and two metals ($AgNO_3$ and $CdCl_2$) against *E. coli*-lux through a bioluminescence- and optical density-based real-time assay that combined short- and long-term toxicity assessments were performed. Bacteria were exposed to antibacterials and the effects were reported as the half-maximum effective concentration (EC_{50}) after 30 min and 10 h. Regarding the 10-hour endpoints, all reference compounds, except deoxynivalenol, showed dose-response inhibition in the studied concentration range. The analysis of chloramphenicol, kanamycin, erythromycin, tetracycline and nalidixic acid clearly revealed the limitations of short-term inhibition tests. No significant differences were observed between the results obtained from luminescence inhibition and growth inhibition assays. The kinetical data from measurements provide differentiation between bacteriostatic and bactericidal mechanisms of various types of antibacterial agents. The combined assessment of short- and long-term effects reduces the risk of the underestimation of toxicity due to an inaccurate endpoint selection. The cost-efficient and fully automated *E. coli*-lux assessment technique may offer possibilities for high-throughput screening procedures.

1. Introduction

Bacterial growth and metabolism can be disrupted by a variety of physical treatments as well as by chemical and biological agents. The huge variety of existing methods for assessing the viability of microorganisms reflects its importance in the field of micro- and immunobiology. In addition, recently, for initial toxicity testing of organic and inorganic compounds, bacteria are an attractive and practical alternative to eukaryotic organisms. Viability in bacteria is conventionally estimated using plate counting and agar dilution methods (Amsterdam, 1996; Li et al., 1996). Although conceptionally simple, the approach by using colony counting is laborious, time-consuming and insensitive especially for slow-growing organisms. Moreover, the results do not provide real-time information about the effects of antimicrobial agents, because the efficacy is observed after overnight incubation. Optical density (OD) measurements provide real-time information, but the inability to distinguish between metabolically active and inactive bacteria restricts the application of this method.

One of the most well-known bacterial tests in toxicity screening is the Ames assay using *Salmonella typhimurium* (Claxton et al., 2010). Other methods for the viability assays include the usage of naturally luminescent bacteria, or bacteria with transformed reporter genes, such as bacterial luciferase. Although luciferases have structural and functional differences among bacteria, they all emit bioluminescence (BL) with a maximum at 490 nm (Poinar et al., 1980). This emission originates from the oxidation of a long chain fatty aldehyde and a reduced flavin mononucleotide:

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + h\upsilon_{490nm}$$

Luminescent bacteria can serve as good indicators for toxic substrates, because the degree of toxicity is directly proportional to the light loss. For this reason, they have been widely used in toxicology research (Hassan et al., 2016). The marine luminescent bacteria, *Vibrio fischeri* (also known as *Photobacterium phosphoreum* or *Aliivibrio fischeri*) and *Vibrio harveyi*, have already been implemented in diverse biological testing methods. One major disadvantage of the *V. fischeri*

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method, carried out according to EN ISO 11348, is the very short exposure time, which results in a low sensitivity in regard to substances with delayed effects (Froehner et al., 2002). Furthermore, the temperature suggested for the *V. fischeri* Microtox™ assay (+15 °C) is not compatible with conventional plate luminometers, and the needed high salinity (2% NaCl) is not appropriate for mimicking certain environments. A combined method for the assessment of acute and chronic effects using *V. fischeri* has been established (Menz et al., 2013; Westlund et al., 2018). The methods are, however, time-consuming with an analysis time of up to 24 h. Instead of using one test with a fixed analysis time, the usage of a variety of bioassays has been strongly encouraged (Kokkali and van Delft, 2014).

Methods using bioluminescent *Escherichia coli* have also been introduced (Lehtinen et al., 2006; Kurvet et al., 2011; Atosuo and Lilius, 2011). We have previously developed a bioluminescent *E. coli*-lux by transforming the bacterium *Escherichia coli* K-12 with a plasmid that included modified genes for the bacterial luciferase enzyme and its fatty aldehyde substrate (luxABCDE) from the soil bacterium *Photorhabdus luminescens* (Atosuo et al., 2013). Expression of the transformed operon produces the luciferase holoenzyme complex that results in bacterial cells emitting a bioluminescence signal, which can be measured in a real-time basis without any addition of external substrate. We have showed that the *E. coli*-lux assessment is a convenient tool in measurements of kinetical features of the serum complement system, neutrophil activity both *in vivo* and *in vitro* and the toxicity of various chemicals (Atosuo and Lilius, 2011; Atosuo et al., 2013; Atosuo, 2015; Atosuo and Suominen, 2019). However, in the previous studies about *E. coli*-lux, the effects of antimicrobial chemicals and the human defense system have been studied mainly in a minimal media that supported no bacterial reproduction and the limited analysis of long-term toxicological effects on bacterial reproduction. Moreover, in these studies, the analysis times have been relatively short (i.e., 30–120 min), and until now, no measurements have been carried out using the *E. coli*-lux assay to assess both the short- and long-term effects of antibacterial agents. A combined investigation of short-term and delayed toxicity can help to understand a toxicants mode of action and reduce the risk of underestimating its toxicity.

In this study, we investigated the possibility of measuring the short- and long-term antibacterial activities of different agents against *E. coli*-lux cells through a real-time basis by using bioluminescence and OD measurements to access the metabolic activity of bacterial cells and growth, respectively. The possibility to distinguish different antibacterials between the bacteriostatic and bactericidal mechanisms was also inspected. Seven specifically acting toxicants (i.e., polymyxin B, chloramphenicol, erythromycin, tetracyclin hydrochloride, deoxynivalenol, kanamycin and nalidixic acid) and two heavy metal compounds ($AgNO_3$ and $CdCl_2$) were included in our research.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical grade. Agar, tryptone and yeast extract were obtained from Difco Laboratories (Detroit, MI, USA) and glycerol from Fisher Bioreagents (Fair Lawn, NJ, USA). Ampicillin sodium salt (AMP, 90%, 69-52-3), polymyxin B sulfate (PmB, 1405-20-5), chloramphenicol (CAM, 98%, 56-75-7), nalidixic acid (NAL, 98%, 389-08-2), deoxynivalenol (DON, 99.5%, 51481-10-8), tetracycline hydrochloride (TCN, 95%, 64-75-5), erythromycin (ERY, 98%, 114-07-8) and kanamycin A (KAN, 99.5%, 25389-94-0) were purchased from Sigma-Aldrich (Chemie GmbH, Steinheim). Silver nitrate ($AgNO_3$, 7761-88-8) and cadmium chloride ($CdCl_2$, 10108-64-2) were acquired from J.T. Baker Chemical Co. and Scharlab (S.L., Barcelona), respectively. White polypropylene microplates with a clear bottom were purchased from Thermo Scientific (Waltham, MA, USA).

2.2. Bacterial strain

For toxicity assays, a constitutively bioluminescent *E. coli* K-12 strain M72 *(Sm^RlacZ(Am)ΔbiouvrBΔtrpE42[λn7(Am)N53(Am)ca857ΔH1])* constructed earlier in our laboratory (Atosuo et al., 2013) was used in our study. This construct designated as *E. coli*-lux carried luxABCDE genes from *Photorhabdus luminescens* in a *pEGFP* plasmid and was identified by resistance for ampicillin.

2.3. Preparation of E. coli-lux suspension

E. coli-lux cells were precultivated overnight in 5 mL Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 5 g NaCl, pH 7.4) in a shaker (250 rpm) at 37 °C. The overnight culture was diluted (1:3000) with fresh LB and further cultivated and incubated in a shaker at 37 °C until an OD_{600} = 0.45, as measured with the Ultrospec 7000 (GE Heathcare, United Kingdom), was reached. At this OD, the cells were in a logarithmic growth phase and the culture contained ~ 10^9 cells/ml. The cells were then harvested by centrifugation at 3000 rpm (Sorvall TC6, DuPont), washed twice in LB and re-suspended in 10 times less volume of LB containing 25% glycerol prior to aliquoting into 50 μL portions and storage at -80 °C. All *E. coli*-lux media contained 100 μg/mL of ampicillin in order to sustain the selection pressure.

2.4. Precultivation of E. coli-lux

Before luminometric and optical density measurements, *E. coli*-lux was precultivated by adding 50 μL of freezer stock to 5 mL LB and then incubated for 2 h in a shaker (250 rpm) at 37 °C until an OD_{600} = 0.25 was reached. After incubation, bacterial cells were harvested by centrifugation at 2500 *g*, washed twice with and resuspended in 5 mL of LB. All media used for *E. coli*-lux contained 100 μg/mL ampicillin to sustain the selection pressure.

2.5. Kinetical luminometric and optical density measurements

A precultivated bacterial culture dilution (25 μL corresponding to approximately 1×10^5 to 2×10^5 bacterial cells) was mixed with 75 μL of LB broth in the wells of a clear-bottom 96-well plate. Plates were incubated in a Hidex Sense Microplate Reader (Hidex Oy, Turku, Finland) at 37 °C for 30 min. For each antibacterial compound, eight sequential exponential dilutions were prepared in LB medium. The concentration range for all reference compounds was from 1×10^{-4} mg/mL to 1×10^3 mg/mL. After addition of 100 μL per well of the corresponding sample, a kinetic measurement loop for over 10 h, containing detection of optical density (absorbance; λ = 600 nm) and luminescence (counts of photons per second, CPS; integration time = 1 s), was performed at 30 °C. A temperature of 30 °C was used instead of 37 °C to prevent an excess of evaporation of samples. Each antibacterial concentration was measured in three parallel wells. Each plate, loaded with samples, also contained five wells of LB broth only, as the background measurement, and five wells of precultivated bacteria mixed with pure LB broth, as the negative controls. Measurements were carried out every 10–30 min. To prevent sedimentation of the cells and to ensure the cells received oxygen sufficiently, the plate was shaken at 150 rpm during the incubation period and in between the kinetic measurements.

2.6. Data analysis

The raw data acquired from Hidex Sense Microplate Reader was inputted in Microsoft Excel v. 2016 (Microsoft Corporation, Redmond, Washington, USA) and analyzed at two different end points. The background signal was measured from a well containing only LB medium and this reading with three standard deviations added was subtracted from the readings obtained from the experimental wells. The luminescence inhibition was calculated after 30 min and 10 h. Additionally, the growth

inhibition from the OD values was evaluated after 10 h. Inhibition of the luminescence ($LI_{\%}$) by a certain concentration of chemical was calculated as presented in formula (1).

$$LI_{\%} = 100\left(\frac{I_{NC} - I_T}{I_{NC}}\right) \quad (1)$$

I_{NC} = average luminescence intensity of bacteria in the control solution in relative luminescence units (RLU) after 30 min or 10 h of measurement; I_T = average luminescence intensity of bacteria exposed to certain concentration of chemical after 30 min or 10 h of measurement.

The measured optical density was used for calculation of the growth inhibition ($GI_{\%}$) according to formula (2).

$$GI_{\%} = 100\left(\frac{OD_{NC} - OD_T}{OD_{NC} - OD_0}\right) \quad (2)$$

OD_{NC} = average optical density of the negative controls after 10 h; OD_T = optical density of bacteria exposed to certain concentration of chemical after 10 h of measurement; OD_0 = average optical density of the negative controls after sample addition.

Half-maximal effective concentration (EC_{50}) values for short-term (30 min) and long-term (10 h) luminescence inhibition as well as growth inhibition (10 h) were determined from concentration versus $LI_{\%}$ and $GI_{\%}$ curves, respectively. The analysis of concentration-response relationships was performed using non-linear, logistic regression according to formula (3). The goodness-of-fit was evaluated with reduced chi-square (χ^2) values. The graphs were prepared using Origin v. 2016 (OriginLab Corporation, North Hampton, MA, USA).

$$y = \frac{X_1 - X_2}{1 + \left(\frac{x}{EC_{50}}\right)^p} + X_2 \quad (3)$$

X_1 = initial value; X_2 = final value; EC_{50} = x value in the curve that is midway between X_1 and X_2 parameters; p = hill slope.

3. Results

3.1. The kinetics of bioluminescence emission and growth of E. coli-lux

The kinetics of the luminescence signal and optical density were analyzed by measurements of microplate wells loaded entirely with negative controls (e.g., untreated cultures). The testing plate was analyzed for 16 h at 30 °C. Figure 1 shows the development of luminescence and optical density in negative controls under the testing conditions during 16 h of measurement. As depicted, the luminescence signal stayed roughly the same during the first 2.5 h of incubation, a period of time that *E. coli*-lux needs in these conditions to reach a critical cell density before exhibiting an increase in the luminescence signal (i.e., quorum sensing) (Miyashiro et al., 2014).

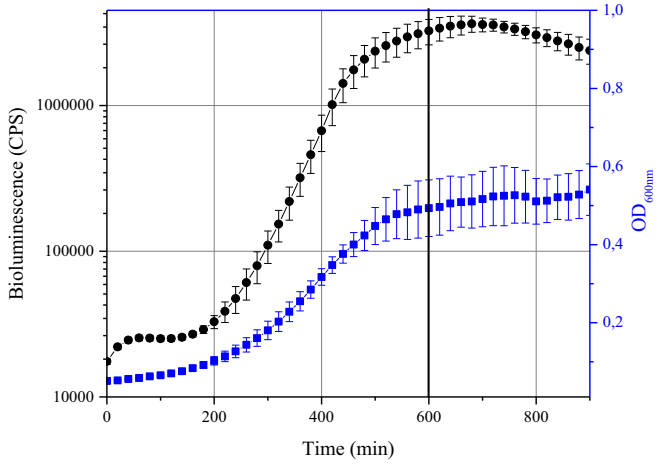

Figure 1. The kinetics of luminescence signal (counts per second, CPS) (●) and optical density (OD_{600nm}) (■) of the negative controls during the 16 h of measurement, n = 60.

The transition in growth from exponential to stationary phase was reached after approximately 9 h of incubation. The luminescence maximum was reached after approximately 11 h. We chose 10 h as an appropriate exposure time for the analysis of long-term luminescence and growth inhibition. Within this time, the OD doubled approximately 3.5 times, which was suitable for growth inhibition analysis, and the bacteria reached the state of full luminescence induction. All the measurements were performed in the inner 60 wells of the microplate to avoid the bias in luminescence measurements created by possible edge effects.

Seven specifically acting compounds and two heavy metals were used in the analysis of their antibacterial effects against constitutively luminescent *E. coli*-lux. Plotting the luminescence and OD over time provided information about the relationships between the rate of metabolic activity, growth and the concentration of the antibacterial agent. Figure 2 shows the effects of reference compounds acting in a short-term manner (i.e., PmB, $AgNO_3$ and $CdCl_2$) on luminescence signal and OD over time. The number of *E. coli*-lux cells at the beginning of the measurements was relatively low. At the highest concentrations of these agents, a significant decrease in the BL, but not in the OD signal was observed within 30 min of measurement, reflecting the much more limited dynamic range of the OD measurements (Figure 2B, D and F). The measurement dynamics, therefore, allowed us to observe the short-term effects only with the BL. Within 30 min, $AgNO_3$ and $CdCl_2$ established antibacterial effects at the concentration of 1000 μg/mL and 100 μg/mL (Figure 2A and C). Polymyxin B established antibacterial effects also at the concentration of 10 μg/mL (Figure 2E). During the long-term exposure, antibacterial effects emerged also at the lower concentrations as the increase in the BL signal was lowered and/or delayed in the logarithmic growth phase when compared to that of the negative control. The concentrations, which affected the development of the BL signal also affected the development of OD signal.

Figures 3 and 4 show the effects of the reference compounds that showed only long-term effects (i.e., chloramphenicol, nalidixic acid, kanamycin sulphate, erythromycin and tetracycline), on luminescence signal and OD over time. Erythromycin, nalidixic acid and chloramphenicol mainly inhibited the increase of BL at certain concentrations, whereas the highest concentrations of kanamycin sulphate (Figure 3E) and tetracycline (Figure 4C) also caused a marked drop in the BL signal during the pre-logarithmic phase. With all of the studied antibacterials, the inhibition of BL signal after 10 h of incubation appeared to reflect the inhibition of bacterial growth. With deoxynivalenol, no antibacterial effects were observed at any of the concentrations, and the BL and OD signals were similar to that of the negative control throughout the measurement time (Figure 4E and F).

3.2. Acute short- and chronic long-term toxicity effects of reference compounds

The short-term toxicity calculations were performed with three out of the nine reference compounds. Within 30 min of exposure, dose-response curves were obtained for polymyxin B, $AgNO_3$ and $CdCl_2$. The effect of polymyxin B on *E. coli*-lux was very rapid. The effects were observed within 20 min of exposure and the EC_{50} value for luminescence inhibition was 0.25 μg/mL after 30 min of exposure. The metal ions, Ag^+ and Cd^{2+}, also influenced the emitted luminescence signal, and EC_{50} values of 7.0 μg/mL and 55.4 μg/mL were obtained, respectively. Figure 5 shows the summarized concentration-response relationships of the short-term toxicity analysis. The EC_{50} values are represented in Table 1.

Regarding the 10-hour endpoints, all reference compounds, except deoxynivalenol, showed dose-response inhibition in the studied

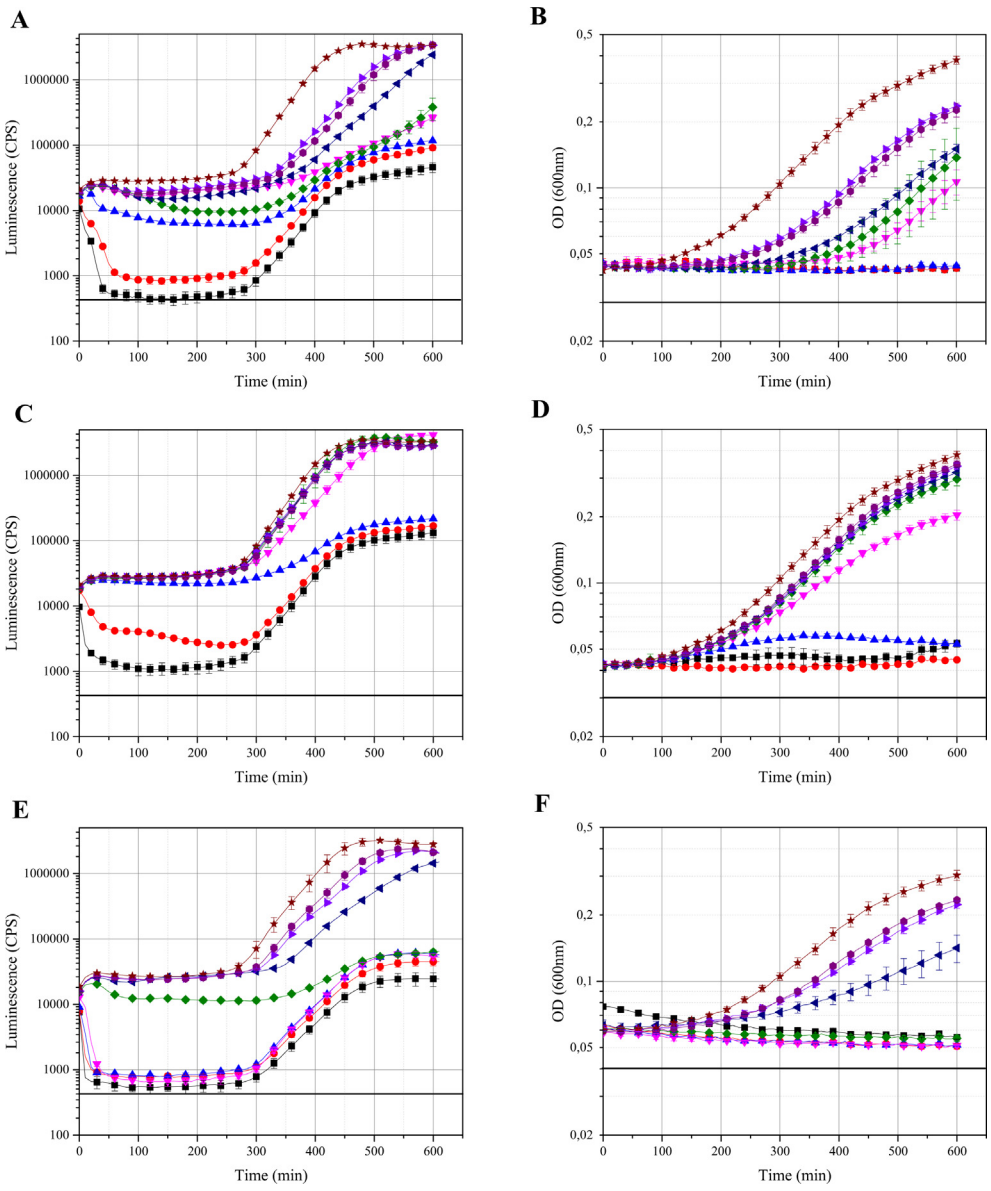

Figure 2. The kinetics of the activities of three antibacterial agents at a short-term rate measured as viability. Luminescence (CPS) and growth (OD_{600nm}) of target bacteria exposed to different concentrations of A & B) $AgNO_3$, C & D) $CdCl_2$ and E & F) polymyxin B. (■) 1000 μg/mL, (●) 100 μg/mL, (▲) 10 μg/mL, (▼) 1 μg/mL, (◆) 0.1 μg/mL, (◀) 0.01 μg/mL, (▶) 0.001 μg/mL, (●) 0.0001 μg/mL and (★) 0 μg/mL. The horizontal line represents the background signal +3 times the standard deviation.

concentration range. Because of the different mechanisms of action, the time interval from exposure to visible effects varied between the antibacterials. An increase in the incubation time resulted in an increase in the cell sensitivity to an antibiotic. Among the specifically acting antibacterials, polymyxin B showed the highest toxicity with the luminescence inhibition EC_{50} value furthermore decreasing from the 30-minute toxicity value of 0.25 ± 0.013 μg/mL to 0.010 ± 0.004 μg/mL after 10 h of exposure. The growth inhibition value of 0.009 ± 0.003 μg/mL correlated well with the luminescence inhibition. Metallic compounds also allowed the detection of lower EC_{50} values after 10 h. The analysis of chloramphenicol, kanamycin, erythromycin, tetracycline and nalidixic acid clearly, once again, revealed the limitations of short-term inhibition tests. All antibacterials affecting the protein and/or DNA synthesis of bacterial cells revealed relatively similar EC_{50} values after 10 h of exposure. Furthermore, EC_{50} values obtained from growth inhibition tests were similar to the luminescent inhibition values. The lowest sensitivity was obtained with erythromycin with EC_{50} values of 50.1 ± 5.8 μg/mL and 10.7 ± 1.3 μg/mL for luminescence and growth inhibition, respectively (see Figure 6).

4. Discussion

In the present study, the kinetics of antibacterials with different mechanisms of action were followed in real-time using luminometric and photometric methods. The antimicrobials were chosen to represent different target systems of *E. coli* cells (e.g., cell membrane integrity, protein synthesis, DNA replication and general enzyme inhibition). The bioluminescence reporter has several advantageous properties, which include a rapid response to metabolic changes, an excellent sensitivity, a large dynamic range and the possibility to collect data continuously. Similar to several other prokaryotic and eukaryotic bioassays, our *E. coli*-lux assessment represents a general toxicity test. It is noteworthy that the results obtained from the reference compounds show a response in which all cellular and molecular mechanisms are integrated under one reporter

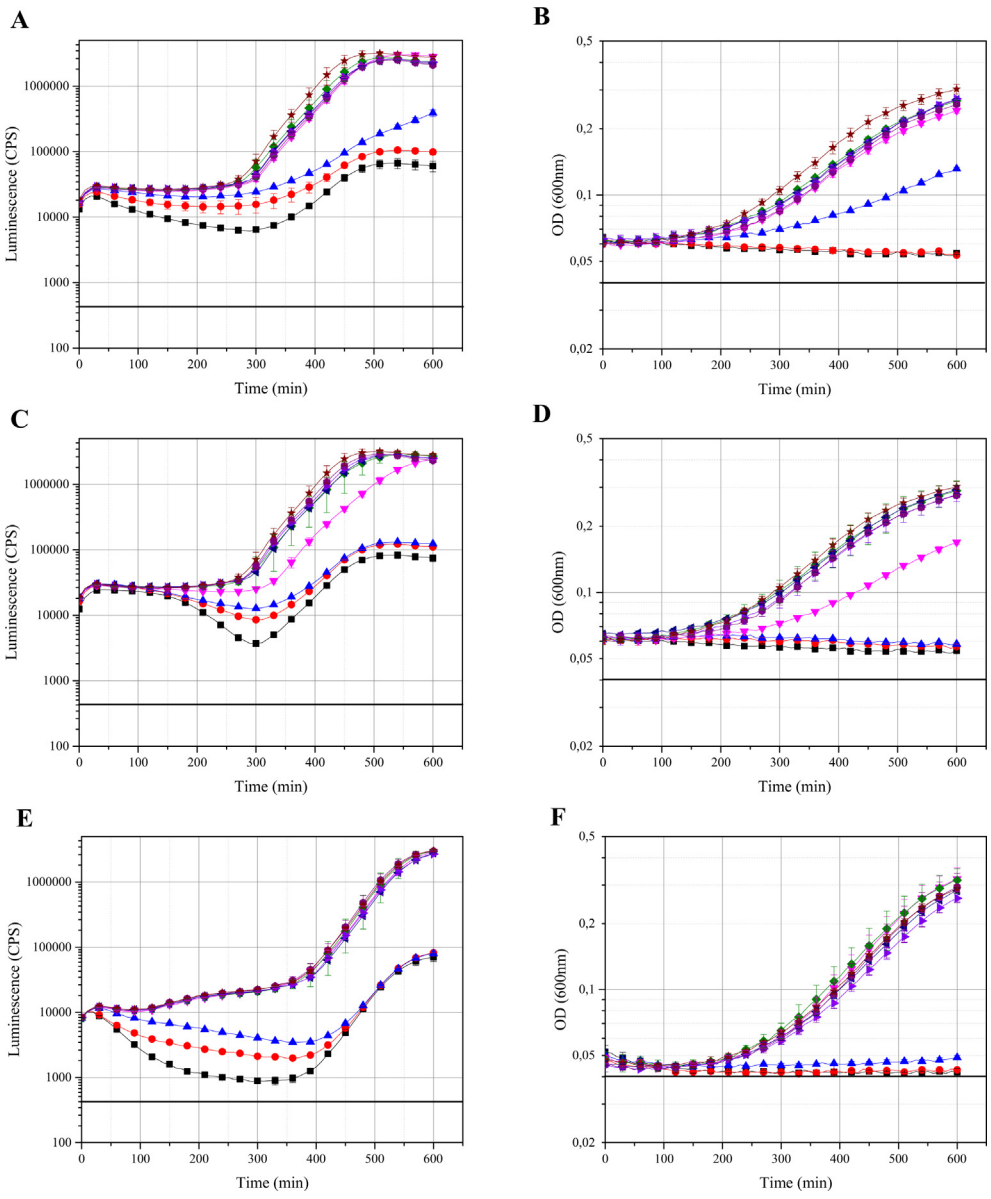

Figure 3. The kinetics of the activities of three antibacterial agents at a long-term rate measured as viability. Luminescence (CPS) and growth (OD_{600nm}) of target bacteria exposed to different concentrations of A & B) chloramphenicol, C & D) nalidixic acid and E & F) kanamycin sulphate. (■) 1000 μg/mL, (●) 100 μg/mL, (▲) 10 μg/mL, (▼) 1 μg/mL, (◆) 0.1 μg/mL, (◀) 0.01 μg/mL, (▶) 0.001 μg/mL, (⬢) 0.0001 μg/mL and (★) 0 μg/mL. The horizontal line represents the background signal +3 times the standard deviation.

system being either bioluminescence or growth. Therefore, definitive conclusions cannot be drawn about the mechanisms of the antimicrobial agents.

The measurements performed in this study mainly allowed us to assess the bacteriostatic and bactericidal effects of different agents. Bacteriostasis means the inhibition of bacterial growth without destruction of the cells and was observed when the bioluminescence and OD signal remained lower than that of the control cells but did not decrease. The effect of bacteriostatic agents can be reliably observed only if the composition of the test medium supports the renewal of bacterial population within the analysis time as in our study (Figure 1). The decrease in the bioluminescence signal, in turn, represented bactericidal effects, e.g., killing of the bacteria in our study. Destruction of bacterial cells, or bacteriolysis, can in general be observed by a decrease in the OD signal. In our study, the initial bacterial number was however relatively low and did not allow us to assess bacteriolytic effects reliably.

Polymyxin B is a water-soluble cationic antibiotic product by the bacterium *Paenibacillus polymyxa*. Acting like a detergent, it alters the structure and finally disrupts the bacterial cell membrane (Katz et al., 2003). This bactericidal effect was also observed in our study (Figure 2C). The toxic mechanism of metals is mostly due to the interference with cofactor metals in the active sites of enzymes (Nies, 1999). Silver is known to inhibit the respiratory chain of *E. coli* and prevent DNA replication by its condensation (Bragg and Rainnie, 1974; Matsumura et al., 2003). The highest concentrations of 1000 and 100 μg/mL of $AgNO_3$ and $CdCl_2$ had bactericidal effects against *E. coli*-lux. $CdCl_2$ at the concentration of 10 μg/mL inhibited the bacterial growth (Figure 2A and B). $AgNO_3$ seemed to have some level of effect against *E. coli*-lux at every concentration studied. Part of this might be because of the oxygen radicals created by $AgNO_3$ (Pandian et al., 2010). The effects of cadmium might also be partly because of reactive oxygen species (Liau et al., 1997). With metals, it is also possible that some of the short-term BL inhibition is because of the interference of luciferase action and that the bactericidal effects are obtained in a delayed manner.

Chloramphenicol (CAM) is a water-soluble and broad-spectrum antibiotic and was isolated first from *Streptomyces venezualae*. It inhibits bacterial protein synthesis by targeting the 50S subunit of the bacterial ribosome that prevents attachment of aminoacyl tRNA (Schifano et al., 2013). Its effect is reported to be bacteriostatic, which was also observed in this study (Figure 3A). Nalidixic acid (NAL) is a synthetic quinolone

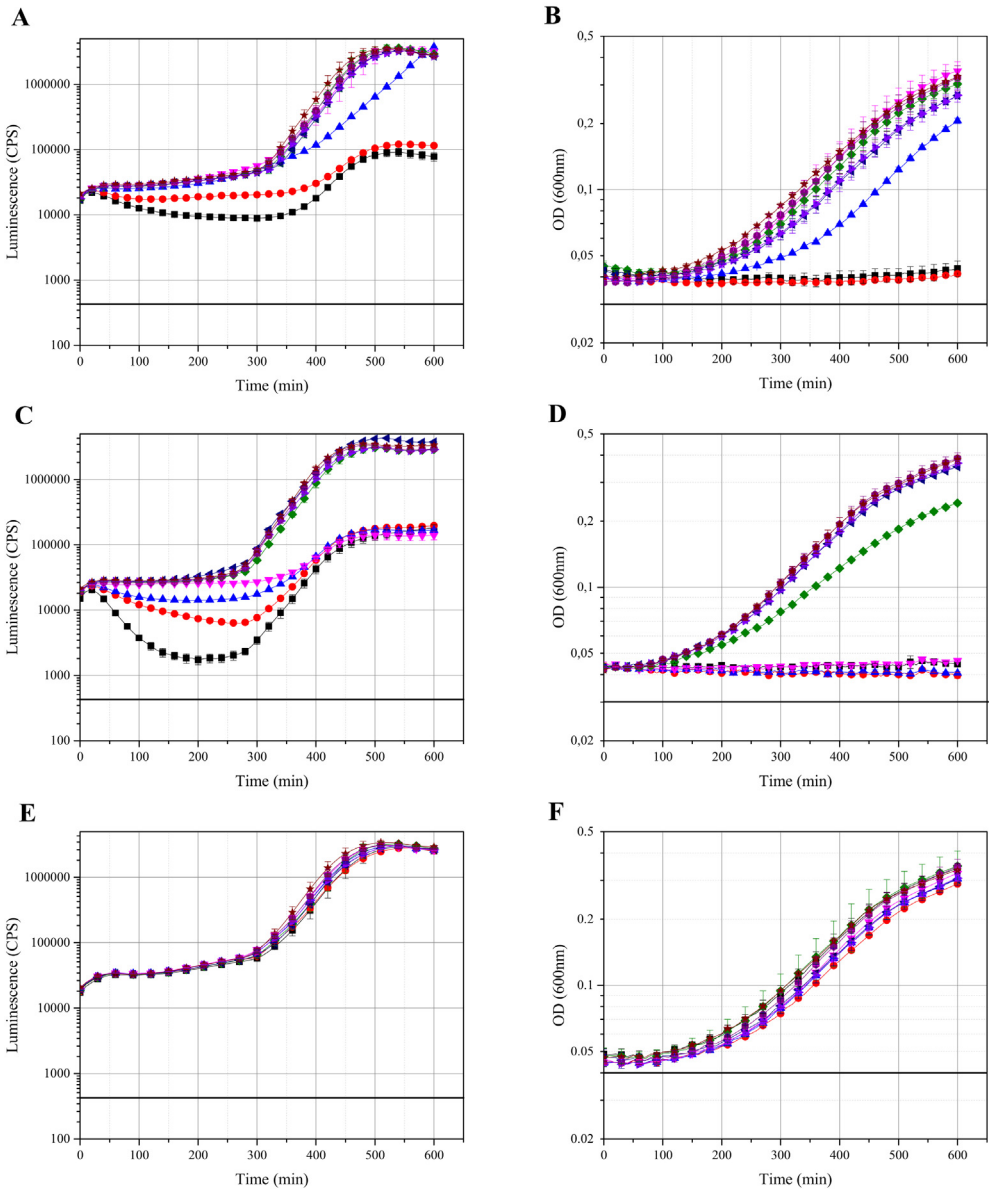

Figure 4. The kinetics of the activities of three antibacterial agents at a long-term rate measured as viability. Luminescence (CPS) and growth (OD_{600nm}) of target bacteria exposed to different concentrations of A & B) erythomycin, C & D) tetracyclin and E & F) deoxynivalenol. (■) 1000 μg/mL, (●) 100 μg/mL, (▲) 10 μg/mL, (▼) 1 μg/mL, (◆) 0.1 μg/mL, (◀) 0.01 μg/mL, (▶) 0.001 μg/mL, (⬢) 0.0001 μg/mL and (★) 0 μg/mL. The horizontal line represents the background signal +3 times the standard deviation.

antibiotic that arrests DNA synthesis in the bacterial cells by binding to the DNA gyrase enzyme, which is essential in DNA replication (Goss et al., 1965). In our study, nalidixic acid had mainly bacteriostatic effects (Figure 3B). It is, however, reported to also have bactericidal effects at higher concentrations (Stevens, 1980). Kanamycin (KAN) binds to the 30S subunit of bacterial ribosome, and therefore interferes with protein synthesis (Moazed and Noller, 1987). It is reported to be a bactericidal agent, which supports our findings (Figure 3C). Erythromycin (ERY) displays bacteriostatic effects by binding to the 50S subunit of the bacterial ribosome, which interferes with aminoacyl translocation (Liang and Han, 2013). Here, bacteriostatic effects were observed clearly in Figure 4A. Tetracyclines (TCN) are broad-spectrum antibiotics, which bind to the 30S and 50S subunits of the bacterial ribosome and also to the 40S and 60S subunits of eukaryotic ribosomes (Chopra and Roberts, 2001). The effect of tetracycline is essentially bacteriostatic, but it also displays bactericidal effects, which were also observed in our study, when high concentrations of tetracycline were used (Figure 4B). Deoxynivalenol (DON), also called vomitoxin, is a class B trichothecene mycotoxin, produced by certain *Fusarium* species. It is a strong protein synthesis inhibitor in eukaryotic cells with the mechanism of action being at the level of protein synthesis. We wanted to test the effects of DON on *E. coli* cells to access the possible cross-reactivity with bacterial protein synthesis. As can be seen from Figure 4C, no short-term or delayed effects were obtained in the used concentration range of DON. The goodness-of-fit for each concentration-response curve was evaluated with reduced χ^2 values (Table 1). The goodness-of-fit testing revealed a strong fit in the non-linear logistic regression model used in our study, as the χ^2 values were relatively close to one.

Luminescence-based *E. coli* methods for long-term toxicity assessments have been validated as potential probes for toxicity testing with similar kinds of antibacterials used here (Lehtinen et al., 2006; Kurvet et al., 2011). The major problem in the comparison of EC_{50} values lies in the fact that other studies have been conducted using not only different probe cells but also different test media, temperature, etc. Lehtinen et al. studied the antibacterial effects of polymyxin B, erythromycin, tetracycline and nalidixic acid in LB medium with *E. coli* transformed with firefly luciferase. With nalidixic acid, our method demonstrated approximately a 15-fold higher sensitivity regarding the 10-hour luminescence inhibition (EC_{50} values of 1.8 ± 0.3 μg/mL vs. 27 ± 5.1 μg/mL) and approximately a 17-fold higher sensitivity regarding 10-hour growth inhibition

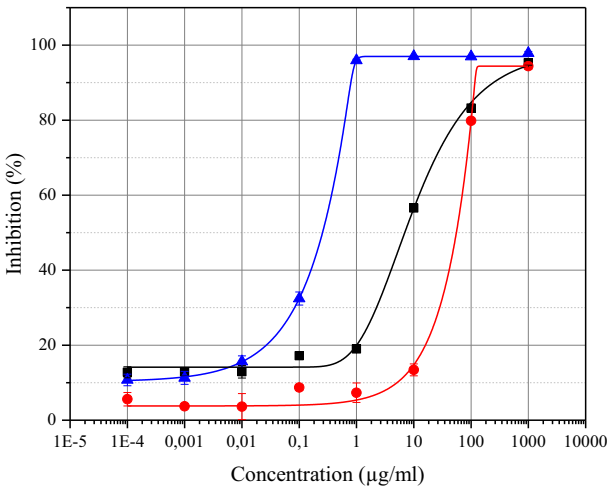

Figure 5. Short-term luminescence inhibition of *E. coli*-lux measured as a function of polymyxin B (▲), $AgNO_3$ (■) and $CdCl_2$ (●) after 30 min of exposure. The EC_{50}-values are presented in Table 1. The data was fitted using a non-linear logistic regression model. Error bars represent the standard deviation of mean from triplicate samples.

(EC_{50} values of 1.2 ± 1.0 µg/mL vs. 20 ± 4.9 µg/mL), when compared with results from our study (Table 1) and the study carried out by Lehtinen et al. (2006). Regarding polymyxin B, our method revealed approximately a 50-fold higher sensitivity in the 10-hour luminescence test (EC_{50} values of 0.01 ± 0.004 µg/mL vs. 0.5 ± 0.1 µg/mL) and a 45-fold higher sensitivity in the growth inhibition test (EC_{50} values of 0.009 ± 0.006 µg/mL vs. 0.4 ± 0.1 µg/mL), when compared with the study carried out by Lehtinen et al. (2006). For tetracycline, the LI value of 1.2 ± 0.2 µg/mL and the GI value of 0.2 ± 0.08 µg/mL were reported by Lehtinen et al. (2006) as being relatively similar with our results. As in our study, erythromycin showed the lowest sensitivity with a LI value of 17 ± 3.2 µg/mL and a GI value of 20 ± 6.4 µg/mL. The results were mainly at the same magnitude and could possibly be explained by the significantly higher cell number (i.e., 5×10^7 bacterial cells) used by Lehtinen et al. (2006), as well as a different luciferase enzyme (i.e., firefly luciferase) (Hartzen et al., 1997). Kurvet et al. reported an EC_{50} value of 1940 ± 1010 µg/mL for $CdCl_2$, which considerably varied from our value of 55.4 ± 6.7 µg/mL (Table 1) after 30 min of exposure. The reason for this might be the usage of saline solution with the formation of stable cadmium chloride complexes and the interference of cadmium dissolution.

The results obtained from the luminescence inhibition assay correlated well with the results from the growth inhibition assay. We have previously shown that the luminescence values emitted by *E. coli*-lux are strict measures of the number of viable cells (Atosuo et al., 2013). The

Table 1. Average EC_{50}-values and hill slopes of the dose-response curves of nine established antibacterial agents for *E. coli*–lux, as determined by luminescence inhibition (LI) after 30 min and 10 h and growth inhibition (GI) with photometry (OD) after 10 h of exposure. The mean of three independent experiments ±standard deviation is shown. For each fitted curve reduced χ^2 values are presented as a measure of goodness-of-fit.

Reference compound	Short-term LI			Long-term LI			Long-term GI		
	EC_{50}	Hill slope	χ^2	EC_{50}	Hill slope	χ^2	EC_{50}	Hill slope	χ^2
$AgNO_3$	7.0 ± 0.9	0.9 ± 0.2	15.7	0.015 ± 0.007	1.5 ± 0.3	15.2	0.04 ± 0.006	1.1 ± 0.3	6.0
$CdCl_2$	55.4 ± 6.7	1.6 ± 0.3	10.4	2.7 ± 0.7	8.4 ± 0.9	7.1	1.3 ± 0.2	1.7 ± 0.2	1.3
PmB	0.25 ± 0.013	2.4 ± 0.2	2.5	0.010 ± 0.004	2.6 ± 0.7	3.4	0.009 ± 0.003	8.0 ± 0.6	2.7
NAL	n.d.	-	-	1.8 ± 0.8	3.4 ± 1.7	3.3	1.2 ± 0.1	1.4 ± 0.2	1.1
CAM	n.d.	-	-	2.6 ± 0.7	2.8 ± 0.5	1.5	8.1 ± 0.9	2.2 ± 1.3	0.8
KAN	n.d.	-	-	0.6 ± 0.2	2.6 ± 0.9	0.9	0.7 ± 0.2	3.1 ± 1.8	0.9
TCN	n.d.	-	-	0.4 ± 0.2	7.7 ± 1.0	5.8	0.2 ± 0.08	1.8 ± 0.1	2.0
ERY	n.d.	-	-	50.1 ± 5.8	3.0 ± 1.3	4.2	10.7 ± 1.3	8.1 ± 2.5	3.4
DON	n.d.	-	-	n.d.	-	-	n.d.	-	-

In the experimental results, mean ± SD are ranges for three separate measurements. All EC_{50}-values are µg/mL, if no toxicity was observed within the concentration range, the sample was marked with "n.d."

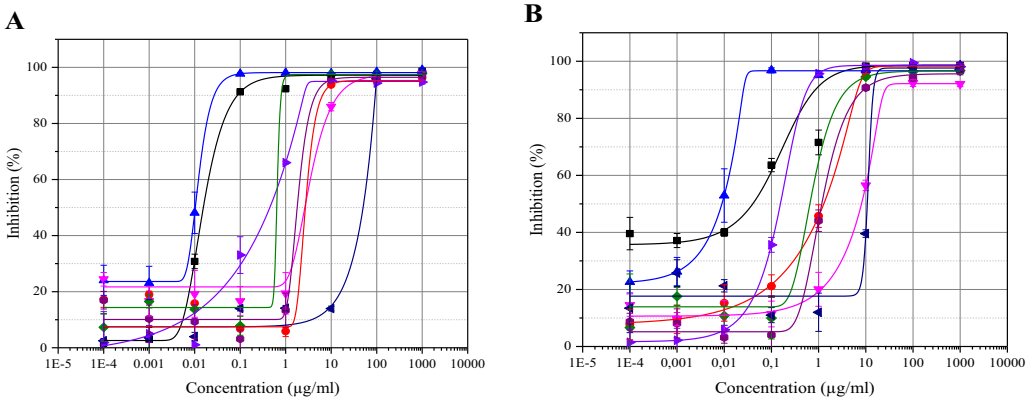

Figure 6. Long-term A) luminescence and B) growth inhibition of *E. coli*-lux measured as a function of polymyxin B (▲), $AgNO_3$ (■), $CdCl_2$ (●), nalidixic acid (●), chloramphenicol (▼), kanamycin sulphate (◆), tetracycline hydrochloride (▶) and erythromycin (◀) after 10 h of exposure. The EC_{50}-values are presented in Table 1. The data was fitted using non-linear logistic regression model. Error bars represent the standard deviation of mean from triplicate samples.

luminometric approach provides a reliable estimation of bacterial viability, killing and inhibition of growth, because the luminescence signal is connected to the metabolic activity of the bacterial population. The luminescence approach, however, can reveal the changes in the metabolic activity of the bacteria more reliably than the measurement of OD. In our study, this was revealed with several reference compounds, which during the first measurement caused a decline in the BL signal, but this was later reversed by a small viable bacterial population that had survived the acute toxicity effects. No changes in the cell densities were, however, detected photometrically, because the corresponding change in OD was too low to measure.

It is well known that the composition of the test medium has a strong impact on the toxicity test results. Minimal media such as 2% NaCl and M9 are good choices for analyzing most of the compounds in short-term inhibition assays. For long-term toxicity analysis, however, more nutrition-rich media are required. Furthermore, we were able to assess the short-term and delayed toxicity of metallic compounds. Previous reports have revealed a marked loss of sensitivity when analyzing metallic compounds and have outlined the need for growth medium with minimal complexing as possible when analyzing heavy metals (Menz et al., 2013; Hassan and Oh, 2010). This may be because of the usage of complex nutrition broths or minimal media containing orthophosphates. The toxicity is lost through the formation of insoluble phosphate salts with positively charged metal ions. In this study, our test system used LB broth, which does not contain orthophosphates, and therefore the bioluminescence and growth inhibition tests can be carried out also with metal ions. Nutritionally rich LB broth also supports intensive bacterial growth that keeps the duration of measurement within reasonable limits.

5. Conclusions

The *E. coli*-lux assessment allows for a rapid and reliable method for the analysis of short- and long-term luminescence inhibition as well as growth inhibition within one test. It also allows the detection of bactericidal and bacteriostatic effects of various antibacterials with different mechanisms of action. In addition, the long-term toxicity test allowed for a more in-depth investigation of toxicity risks toward *E. coli*-lux by taking into account the changes in luminescence emission and optical density concurrently over time. The results obtained using the long-term toxicity method supported the fact that a short-term toxicity endpoint for some antibacterials might provide an underestimation of the toxicity. The combined assessment of short- and long-term effects significantly reduces the risk of underestimation of toxicity due to an inappropriate endpoint selection. Furthermore, our reliable, cost-efficient and fully automated *E. coli*-lux assessment offers possibilities for high-throughput screening procedures.

Declarations

Author contribution statement

Eetu N. Suominen: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Tuula Putus: Contributed reagents, materials, analysis tools or data.

Janne Atosuo: Conceived and designed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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