



# Development of High-Throughput Methods for Nano- and Bulk Silver Toxicity Assays Using Bioluminescent Recombinant *Pseudomonas* Wastewater Isolates

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**Abstract:** Silver get washed into sewerage systems and eventually to wastewater treatment plant (WWTP) due to its utilization in industries. This poses concerns about the toxicity of these particles to microorganisms which are involved in biodegradation of organic wastes in biological WWTP. *Pseudomonas species* (Biosensor cell A, B, C, D and E) originally isolated from WWTP and modified by incorporating a stable chromosomal copy of the lux operon (lux CDABE) derived from *Escherichia coli* S17 $\lambda$  pir were sensitive immediately upon addition of silver nanoparticles (AgNPs) and bulk silver in short terms of incubation ranging from 0 to 300minutes. Microtitre plate luminometre was used to generate detailed luminescence reduction data for the silver particles tested against the bacterial cells in various concentrations ranging from 9 $\mu$ g/ml to 2500 $\mu$ g/ml. The EC<sub>50</sub> values generated at various time points showed that the highest toxicity was observed at time point, 0 of incubation for both AgNPs and bulk silver (158 $\mu$ g/ml and 618 $\mu$ g/ml EC<sub>50</sub> values respectively); these EC<sub>50</sub> values also indicate that AgNPs are much more toxic than bulk silver. Two putative biosensors, E and D showed proportional responses of bioluminescence reduction with increasing toxicant concentrations up to 2500 $\mu$ g/ml, hence displaying dose-dependent responses, superior operational range and sensing capabilities; good features for toxicity assay. Therefore, the recombinant isolate can be used to assay the toxicity of silver particles.

**Keywords:** *Pseudomonas* Species, Recombinant, Bioluminescence, Biosensor Cells

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## 1. Introduction

Presently, silver nanoparticles make up the largest portion of nano-materials used in consumer products and has been found in over 300 products globally which make up approximately 30% of known nanoparticles-containing products [1]. The increase in usage of nano-silver has led to an increase number of these particles being released to wastewater treatment plant through wastes disposal from domestic and manufacturing processes [2]. There have been different studies on the synthesis, use and release of

nanoparticles (such as silver materials) into the environment. It has been reported that the released nanoparticles finally end up in the wastewater systems and subsequently gets into biological WWTP [3]. [4] reports that nano-silver releases ions when in contact with water and in aerobic conditions therefore its anti-microbial effect could be greater in aquatic than in terrestrial environments. Hence, environment condition is a determinant in the level of toxicity of nano-silver. [5] reports that autochthonous (indigenous) microorganisms are the most suitable for toxicity testing with prospective for in-situ relevance.

Initially, eco-toxicity studies were assayed using

vertebrates; however, these methods are time consuming, expensive and have some ethical issues related to animal 'rights'. Other methods such as growth OD assays, Gas-MS or HPLC can be time-demanding, prone to errors and are ecologically irrelevant (cannot assess the ecological influence) [6]. Hence, research is now focused on new technologies for toxicity measurement such as the use of embryo tests, algae, micro invertebrates, microorganisms, tissues, cell lines, nucleic acid and biochemical reactions [7]. The use of recombinant bioluminescent whole-cell biosensors is a quick, highly sensitive, inexpensive and relatively easy technique for toxicity assay and mechanism of toxicity [8]; In this method, a luminescent gene is incorporated into the genome of a sensor microorganism.

The source of luminescence found in *Vibrio fischeri* or *Photobacterium luminescens* which are naturally light-emitting organisms is the luxCDABE operon; catalysis of an enzyme (bacterial luciferase) is responsible for the luminescence in these organisms linked to the metabolic activity of the cells meaning that any inhibition in the enzymatic and cellular activity is shown by a reduction bioluminescence [7]; these organisms are not directly used for the eco-toxicity assay because they are rarely found in WWTP (ecologically irrelevant) and because they are obligatory symbionts that require unique nutritional supplements which are exclusively available from the host; they are not separable from their host, and therefore are not able to be cultured in the laboratory for studying [9]. *Pseudomonas spp.* are able to metabolize a wide variety of nutrients and are ecologically relevant and well adapted to WWTPs, which potentially makes them good indicator organisms for wastewater systems. Also, they are not growth-medium dependent [10].

[5, 10, 11] successfully developed bespoke biosensors from WWTPs which consist biosensor based on growth inhibition of *Pseudomonas spp.* that has lux-operon transferred from *E. coli* S17 $\lambda$  pir by transconjugation for assessing toxicity of wastewater pollutants. The reports are contradictory regarding the modes of toxicity and whether elevated toxicity of nanoparticles compared to bulk particles is truly a nano-effect or simply due to higher rate of ion release. [10] reported that nano-silver is more toxic than bulk silver due to the higher rate of ion release of nano-silver which conflicts the report from [5, 11] who reported that the higher toxic effect is due to the nano-scale size of silver nanoparticle. Also, some of these reports have shown that nano- and bulk silver reduced bioluminescence at certain concentrations; this has created basis for further research in this study.

This project aims to assay the toxicity effect of silver nanoparticles compared to the bulk silver by using recombinant bioluminescent whole-cell *Pseudomonas* species isolated from wastewater in order to broaden the knowledge of impacts of nano-silver toxicity on microorganisms in wastewater treatment.

## 2. Materials and Methods

### 2.1. Biosensor Cell Strains

Fifteen transconjugant *Pseudomonas* strains were obtained from the culture collection of Biotechnology laboratory at Edinburgh Napier University, United Kingdom. The creation of the recombinant strains has been previously reported to have been originally isolated from wastewater samples collected from four biological wastewater treatment plant (WWTP) (Cupar, Greengairs, East Calder and Newbridge WWTP) in central Scotland, prior to recombination. These strains have been stored in glycerol stock solution (50% v/v glycerol in dH<sub>2</sub>O) at -20°C for eight months.

### 2.2. Growth of Transconjugant *Pseudomonas Spp*

The fifteen biosensor strains used in this study were revived by inoculating them into fresh Luria-Bertani (LB) broth supplemented with Kanamycin monosulfate (Sigma-Aldrich) in duplicate. The LB liquid cultures were incubated overnight at 27°C at 200rpm and were observed for growth. The overnight cultures were streaked onto LB Agar using a sterile loop and incubated overnight at 27°C to create pure cultures. Discrete colonies were observed for characteristics and micro-morphology and staining reactions in comparison with bergey's manual of systematic bacteriology for identification [12].

### 2.3. Screening of the Transconjugant *Pseudomonas Spp* for Stable Bioluminescence Output

The transconjugants were screened for stable light emission when freshly subcultured from glycerol stock and when stored at 4°C for 72 hours. The transconjugants cultures grown overnight in LB at 27°C at 200 rpm were subcultured in fresh LB broth overnight under same condition. Subcultures of twelve transconjugants selected at random were taken forward to be screened for stable bioluminescence. Liquid LB subcultures of twelve transconjugants and control samples (including glow wells) in triplicates were transferred into a 96-well black microtitre plate (NUNC Thermo Scientific, Roskid, Denmark). Luminescence (RLU) was measured using a luminometre set to incubation temperature of 27°C for 2.25 hours at 15 minutes interval giving a total of nine readings. Tests were set up for luminescence output readings using the software 'OPTIMA' in the Computer systems connected to the luminometre and data generated were transferred to a Microsoft®Excel® 2010 sheet for analysis. Luminescence values were expressed as a percentage of a positive control with the highest level of luminescence and plotted against time. Transconjugants that showed stable luminescence upon subculture compared to the controls were stored at 4°C for 72 hours and then subcultured in fresh LB medium to measure for bioluminescence output repeating the previous procedure. A set of transconjugants that produced stable luminescence upon storage at 4°C was generated and grouped according to level of light emission (weak – 0 to 39%, medium – 40 to

69% and strong – 70 to 100% luminescence, RLU).

#### 2.4. Toxicity Assay of Silver Nanoparticles and Bulk Silver Using the Putative Biosensors

Transconjugants E and D were selected for AgNPs and bulk silver toxicity testing. These silver particles were tested in various concentrations against E and D grown to either mid-exponential phase or end-exponential phase.

##### 2.4.1. Preparation of Silver Particles Suspensions

Silver nanoparticles (AgNPs) powder with diameter of approximately 35nm (Nanostructured and Amorphous Materials, Houston, U.S.A.) and bulk silver powder with diameter 0.6 to 1.5µm (Nanostructured and Amorphous Materials, Houston, U.S.A) were used. The solutions were prepared by adding either AgNPs or bulk silver to 1% w/v Bovine Serum Albumin (BSA) solution in bijoux bottles to a final concentration of 5000µgml<sup>-1</sup>.

##### 2.4.2. Toxicity Tests of the Silver Particles

Two biosensor organisms, E and D were exposed to either silver nanoparticles or bulk silver at nine various concentrations. Thereafter, bioluminescence output from the biosensors were read using the luminometer in 96-well black microtitre plates at 30 minutes' intervals for 5 hours, recorded as the instrument's arbitrary relative light units (RLU). The microtitre plates were set up to contain bacterial inoculum and the silver particles suspension at a required %v/v concentration to make a final of volume of 100µl of Liquid culture plus Ag suspension per well prior to luminescence output readings. Incubation in the luminometer was at 27°C. Control wells were also included. Luminescence values of the test organisms were expressed as percentage of the control (untreated biosensor) in the instrument's arbitrary relative light units (RLU). All experiments were run two times on different days.

#### 2.5. Data Analysis

The toxicity data generated, consist of a series of bioluminescence output readings for two biosensor transconjugant organisms (experiments for each biosensor was performed separately) exposed to various concentrations of toxicants (silver particles). Bioluminescence output at each time point was measured as relative light units (RLU) which was normalized and converted to the percentage of the bioluminescence output of untreated biosensor samples, E or D. Toxicity response data of the biosensors (E and D) were analysed and compared to each other. Mean RLU of four transconjugant replicates was plotted against toxicant concentrations at each time point of 1 hour intervals for 5 hours. For the normalized data, luminescence values as % bioluminescence for each concentration and control were plotted against time.

The RLU versus toxicants concentrations curves were used to determine the approximate EC<sub>50</sub> at each time point by observing using a pencil and ruler. The normalized data was used to determine EC<sub>50</sub> values statistically using PriProbit

software.

## 3. Results

On the LB agar medium, colonies of twelve transconjugants were observed visually as circular in shape, cream (no pigmentation) in colour, entire margin, raised in elevation, rough in texture, glistening in appearance, large and medium sized and translucent. The twelve transconjugants were of the described colony characteristics on LB agar.

Luminescence curves for each transconjugant were obtained (curves not shown). All the transconjugants (A, B, C, D, E, F, G, H, I, J, K and L including the control, P- *P. putida* BS566: lux CDABE) tested, showed stable luminescence output over time before and after storage at 4°C for 72 hours and were grouped according to level of light emission (Table 1).

Light output measurements of each transconjugants before and after storage at 4°C were constant with no significant difference at each time point within 120 minutes except transconjugant B and K's luminescence output at 120 minutes which increases after storage at 4°C (Figure 1) and transconjugant E and J which dropped in luminescence output from approximately 50% to approximately 30%. Also, transconjugant A reduced in bioluminescence from 40% to below 20% upon keeping at 4°C for 72 hours (Figure 1) compared to when freshly subcultured from glycerol stock. Luria broth medium had 0.02% luminescence output (data not shown), hence, background light emission from the medium was negligible. Glow wells 1, 2 and 3 emitted 1%, 2% and 5% luminescence respectively (with difference of one-logarithm increment in light output as designed) showing that results are valid and the machine as functioned efficiently.

Upon the addition of Bovine Serum Albumin (BSA) solution to the silver material powder, AgNPs completely and rapidly (within 10 secs) settled to the bottom of the bottles before and after shaking forming black aggregates-like substance. The bulk silver powder which did not seem to form aggregates settled completely to the bottom of the bottle not as rapidly as AgNPs. They were able to stay in suspension for a bit longer time (approximately 3 minutes) before completely settling.

Luminescence curves for transconjugants tested against silver nanoparticles and bulk silver are similar and are shown in Figure 2 and Figure 3. Experiments were repeated two times for statistical standardization and there was no significant difference between repeat experiments. Background luminescence output (0.1%) by LB broth medium and the parent strain of the transconjugants was negligible. The addition of AgNPs or bulk silver in different concentrations resulted in reduction in luminescence output of the transconjugants (Figure 2). The EC 50 values calculated following challenge of transconjugant D with AgNPs at various time points (0, 60, 120, 180, 240 and 300 minutes) are shown in Table 2. EC 50 values of both AgNPs

and bulk silver were not calculated for transconjugant E because there were no 100% light reduction observed when challenged with bulk silver (Figure 2b and Figure 3b).

Figure 2 shows reduction in the luminescence output by transconjugant E when challenged with different concentrations of silver nanoparticles solution from 9 to 2500 $\mu\text{g/ml}$ . The highest toxicity with 100% light reduction of transconjugant E (reduction from 100% luminescence output to 0% luminescence output) was observed at time 0 minutes, just at the start of incubation with 1250 and 2500 $\mu\text{g/ml}$  of silver nanoparticles tested (Figure 2a). The same was observed when this transconjugant E was challenged with bulk silver, the highest toxicity (luminescence reduction from 100% to 20%) was at time 0 minutes with 1250 and 2500 $\mu\text{g/ml}$  of bulk silver solution tested (Figure 2b) and indeed this was the case for all toxicity tests of the silver materials performed; highest toxicity at 0 minutes (Figure 2 and 5); Responses of the transconjugants to the toxicants were maximal at the highest concentration (2500 $\mu\text{g/ml}$  of AgNPs and bulk silver solution). At the various concentration (0, 9, 19, 39, 78, 156, 312, 625, 1250, 2500  $\mu\text{g/ml}$ ) of both the AgNPs and bulk silver, luminescence was significantly reduced to levels below that of the untreated organism within 60 minutes (Figure 2). Decrease in light output was concentration-dependent; this was observed for AgNPs (Figure 2 a and c) and bulk silver (Figure 2 c and d). Although, only at concentrations of 2500 $\mu\text{g/ml}$  did bulk silver significantly inhibit bioluminescence compared to

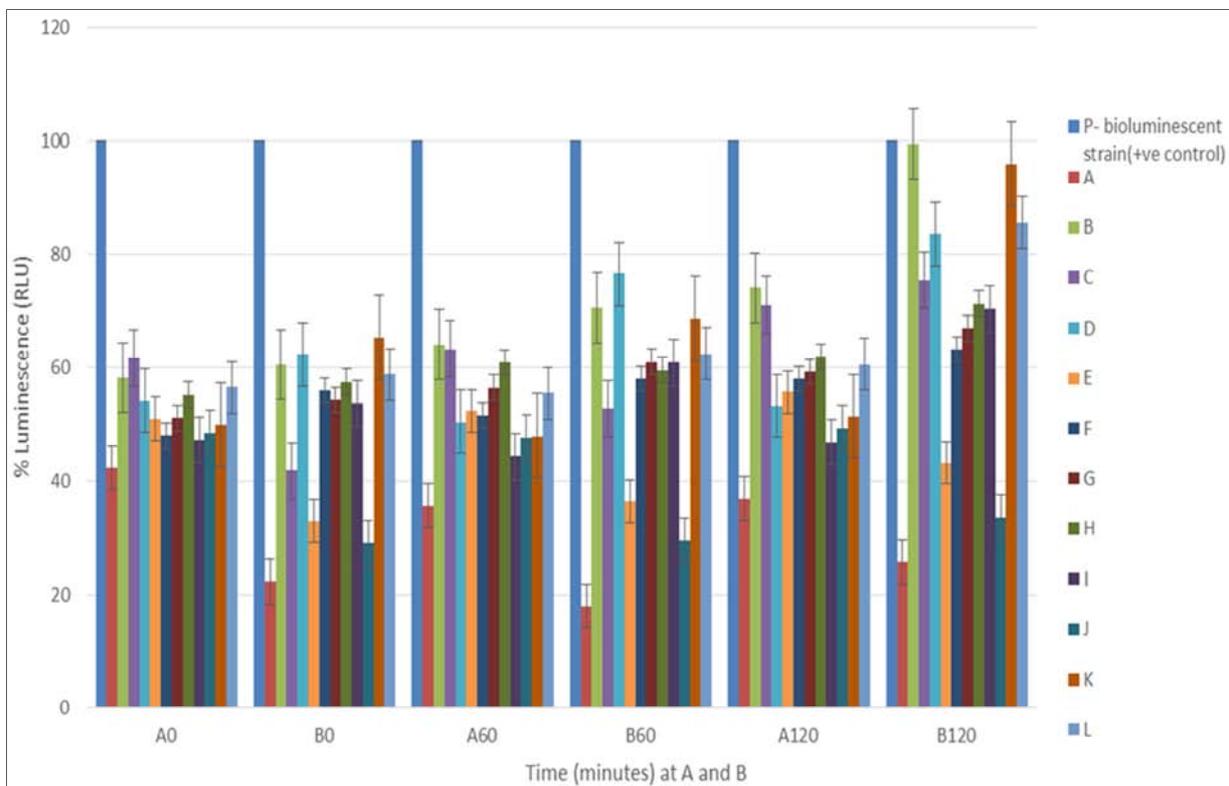
other concentrations (Figure 3d).

Silver nanoparticles appeared to elicit a larger response from the two transconjugants, E and D than bulk silver; silver nanoparticles at the concentrations of 625, 1250, 2500 $\mu\text{g/ml}$  caused a 100% reduction of luminescence output of both E and D (Figure 2 a and c) at time 0 to 300 minutes with even 312 $\mu\text{g/ml}$  causing 100% luminescence reduction in D (Figure 2c) while bulk silver at various concentrations did not lead to 100% reduction of light output from transconjugant E (Figure 2b) but caused complete bioluminescence reduction with transconjugant D at a concentration of 2500 $\mu\text{g/ml}$  at time 0 to 300minutes (Figure 2d).

After 120 minutes, bioluminescence output from transconjugant E increased above the level of the untreated sample's 100% light emission at lower concentration range of 9 to 39 $\mu\text{g/ml}$  of AgNPs and bulk silver (Figure 2a and b respectively). This was not the case for transconjugant D (Figure 2c and d).

**Table 1.** Twelve bioluminescent transconjugant *Pseudomonas* spp. subcultured from overnight cultures of bacterial strains stored at 4°C for 72 hours, incubated at 27°C for 24 hours, grouped based on their level of light output according to figure 1; weak (05 – 39) %, medium (40 – 69) % and strong (70 – 100)%.

Bioluminescence output intensities	Transconjugant codes
Weak (05 - 39%)	A, E and J
Medium (40 – 69%)	C, D, F, G, H, I and L
Strong (70 – 100%)	B and K



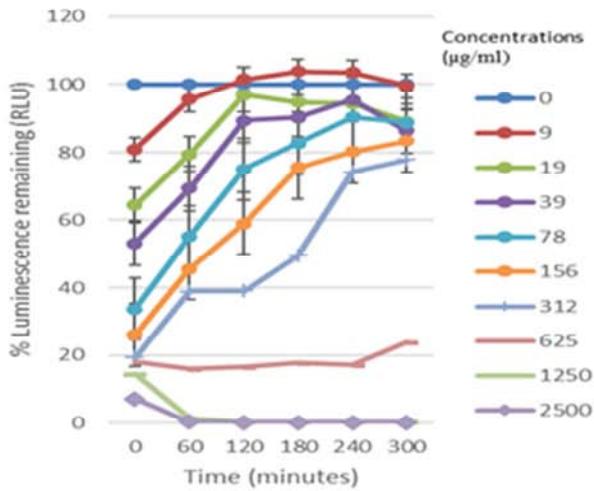
**Figure 1.** Bioluminescence output readings from twelve transconjugant *Pseudomonas* isolates (A, B, C, D, E, F, G, H, I, J, K and L) grown in LB broth, incubated overnight at 27°C for 24 hours, measured using a microtitre-plate luminometer (FLUOstar Optima, BMG Labtech, UK) at time after transconjugants were freshly subcultured from glycerol stock (A) and at time after they were subcultured from overnight LB bacterial cultures stored at 4°C for 72 hours (B). Mean luminescence values of four replicates plotted against time. Error bars represent standard deviation among replicates.

**Table 2.** EC<sub>50</sub> (µgml<sup>-1</sup>) values for AgNPs or bulk silver tested against the bioluminescence output of transconjugant *Pseudomonas D*, calculated using priprobit software at different time periods during 5-hour toxicity tests.

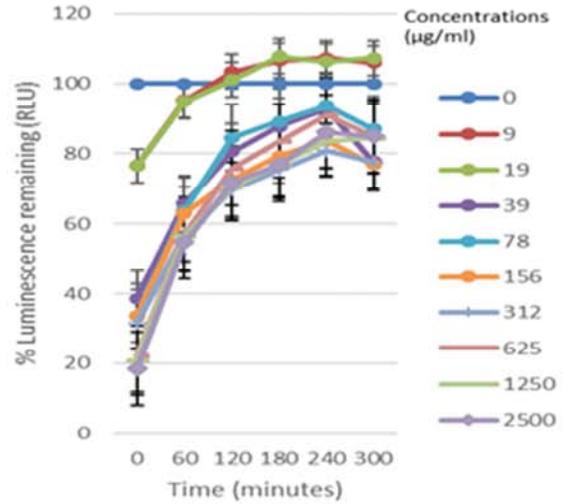
	EC <sub>50</sub> values		(µgml <sup>-1</sup> )			
Time (minutes)	0	60	120	180	240	300
AgNPs	158	280	537.5	615	620.5	629
Bulk Ag	618	750	1012.5	1137	1700.25	1900

\* Calculated using PriProbit (v 1.62) software.

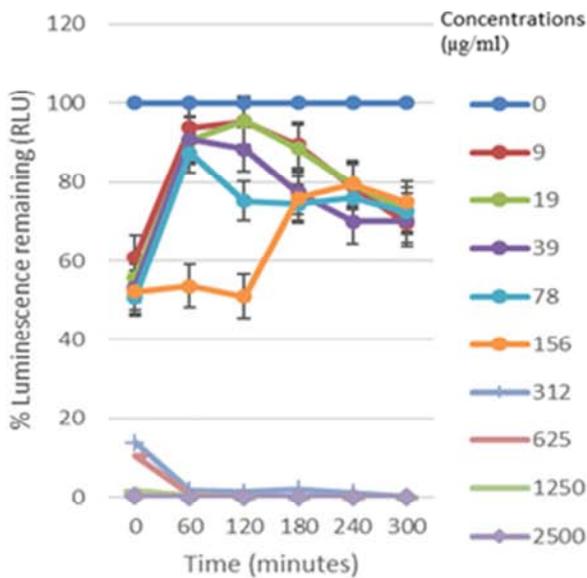
a(E/Nano)



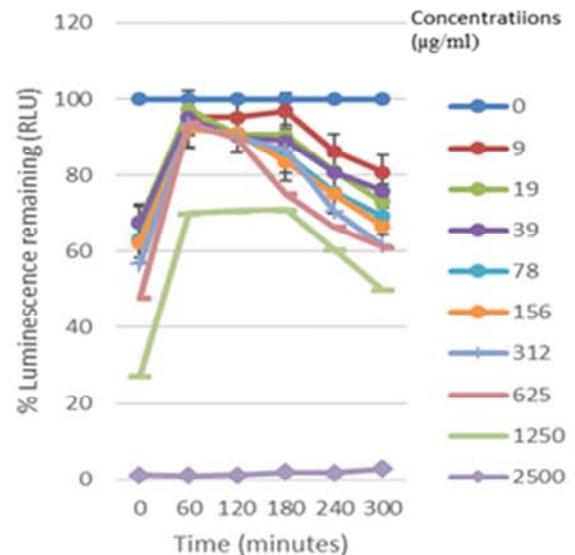
b(E/Bulk)



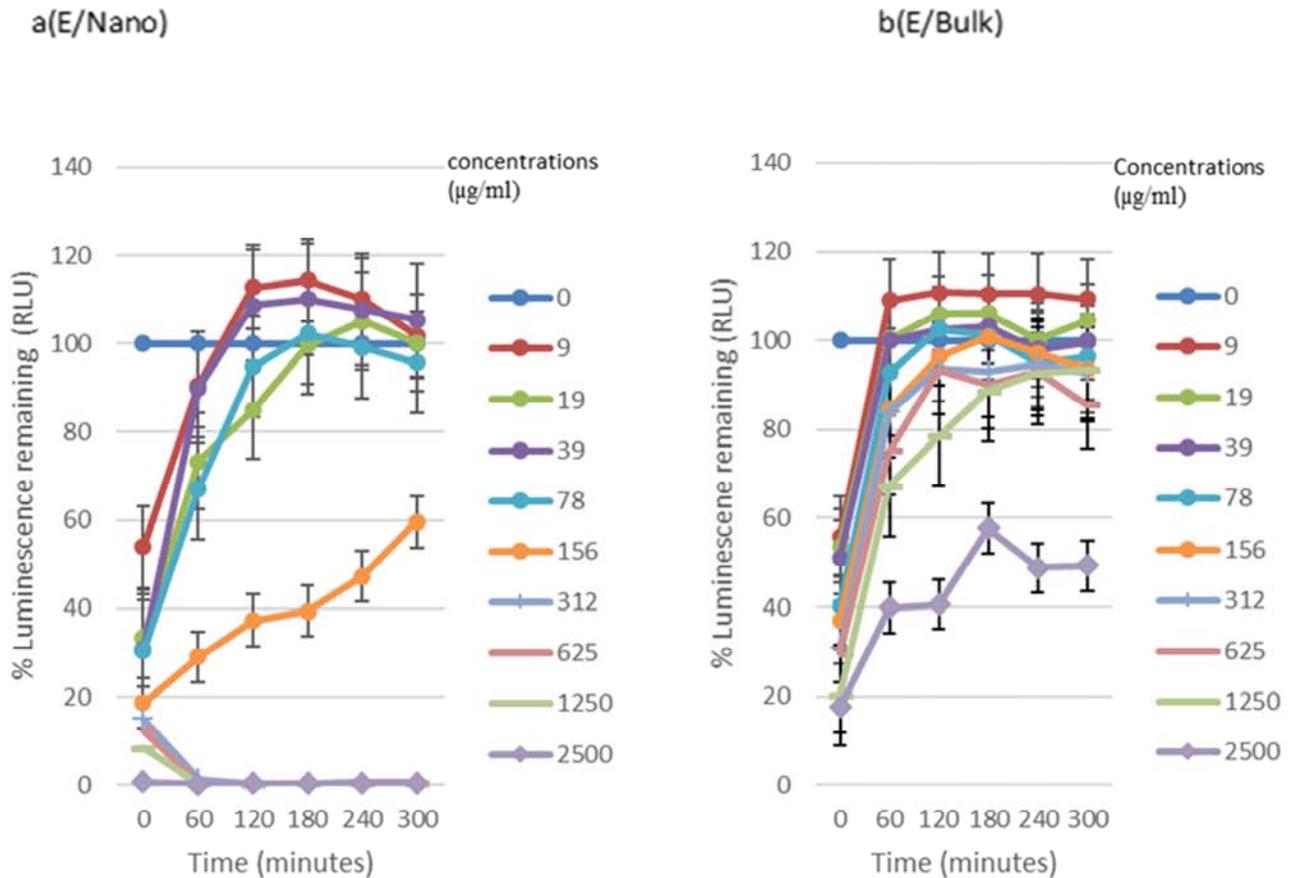
c(D/Nano)



d(D/Bulk)



**Figure 2.** Reduction in bioluminescence output of transconjugant E (a and b) and transconjugant D (c and d) grown in LB broth to an OD<sub>600</sub> of 1.3 (end of exponential phase), when challenged with various concentrations of silver nanoparticles solution (a and c) and bulk silver solution (b and d) ranging from 9µg/l to 2500µg/l at various time periods within 5 hours of incubation. Bioluminescence output measured using the luminometre (FLUOstar OPTIMA, BMG LABTECH, Germany). Average luminescence output of four replicates measured as relative light units (RLU) plotted against concentrations of silver materials. RLU was normalized and converted to the percentage of the bioluminescence output (at 100%) of untreated biosensor sample (at 0µg/ml). Error bars represent standard deviations.



**Figure 3.** Reduction in bioluminescence output of transconjugant E grown to an OD600 of 0.8 (mid-exponential phase), when challenged with various concentrations of silver nanoparticles solution (a) and bulk silver solution (b) ranging from 9 µg/ml to 2500 µg/ml at various time periods within 5 hours of incubation in the luminometer. Bioluminescence output measured using the luminometer (FLUOstar OPTIMA, BMG LABTECH, Germany). Average luminescence output of four replicates measured as relative light units (RLU) plotted against concentrations of silver materials. RLU was normalized and converted to the percentage of the bioluminescence output (at 100%) of untreated biosensor sample. Error bars represent standard deviations among replicates.

## 4. Discussion

All the twelve transconjugants emitted stable light even after storage at 4°C for 72 hours and when freshly subcultured from glycerol stock indicating that these transconjugants *Pseudomonas spp.* are tolerant to a wide variety of physical conditions, including temperature (4°C). [13] reported that *Pseudomonas aeruginosa* is resistant to high and low temperatures, high salt and dyes concentrations, weak antiseptics and some antibiotics. Nevertheless, there was a slight reduction in light emission from transconjugant A, E and J upon storage at 4°C compared to those of fresh subculture from glycerol stock. This could be due to less tolerance of these strains to varying conditions; the transconjugants are of different species or strains of the genus, *Pseudomonas*. Hence, the different species may vary slightly in features [13]. Some of the transconjugants, B, C, I, K and L increased in the level of RLU upon storage at 4°C compared to the first subculture assay. Since growth experiment (not shown this article) indicate that increase in cellular densities increases light output of the cells, it could be supposed that these transconjugants were slowly growing

at 4°C for luminescence output to have increased. However, [14] reported that most *Pseudomonas aeruginosa* strains are able to grow at 42°C but not at 4°C. Again, this may be due to difference among strains as [15] also reported that the Gram-negative psychrotrophic bacterium (*Pseudomonas fluorescens*) has the ability to grow at temperatures ranging from 0 to 32°C.

The high settling properties of the silver nanoparticles, AgNPs resulted in an incomplete interaction of the particles with the putative biosensors in the microtitre plate over time; therefore, toxicity levels of AgNPs to the bacterial cells may be much higher than observed in this study. This was also the same for bulk silver but to a lower extent. These settling properties that do not allow for even dispersion of particles in solution were issues in a study of silver particles toxicity by [10] where he used bovine serum albumin (BSA) solution to disperse and dissolve silver particles prior to toxicity testing. He reported that a higher toxicity was observed upon using BSA as stabilizer. Though, BSA was also used in this study with little or no effect on the dispersal of the particles in solution (particles still remained undispersed). [10] observed that solubility strongly affects the toxicity level and without stabilizer for solubility, toxicity was reduced; this may

probably indicate that compounds that were not soluble were not available to the bacterial cells (no interaction). In addition, [16] showed that nanoparticles like titanium oxide with low solubility showed no toxicity to mammalian cells while nanoparticles such as Zinc oxide with higher solubility showed a higher toxicity. [17] reviewed some other stabilizers that could be used for the synthesis process of AgNPs so as to control the size, dissolution and speciation of particles, to be amines (8%), sodium citrate (27%), polyvinyl chloride (18%) and citric acid. [10] reported citric acid as an effective chelating and complexing agent in preventing agglomeration of metals in solution. The effects of using different stabilisers for toxicity assay should be assessed most especially if bioassay methods developed in the laboratory are to be used to derive consents of WWTP [18]. Nevertheless, this settling issue of silver particles provides a clue as to the behaviour of these particles in natural environments like water bodies; rapidly settling to the bottom of the system, thereby avoiding cellular interactions.

A limitation is that the settling property (unless optimized) might not be beneficial for an efficient assay for toxicity of these particles using the recombinant bacterial organisms where a much better cellular interaction with particles is needed. For effectiveness, nanoparticles need to be in constant contact with the bacterial cells [19]. It was observed in this study that even shaking by vortexing, pipette and rotary shaker did not seem to provide an even dispersion of the silver particles in the solution. Immediately after shaking and dispersion is obvious, particles quickly (within 5 seconds) settled again to the bottom of the container, this also indicates how they settled really fast in the micro-plates. To determine the effective concentrations of silver particles available for interactions with microbial cells in WWTP is currently challenging and can extensively vary from the overall silver concentration. This implicates that most silver particles concentrations present in WWTP may not be effective for microbial contact and hence, toxicity assays [20]. In addition, some silver are good absorbent particles due to their unique structure and electronic properties and therefore, could precipitate and lead to reduced bioavailability [21].

An additional method that could be used for improving cellular contact with the particles is to immobilize cells in an artificial matrix as described by [22, 23]. This method has been proven to improve the specificity, sensitivity and stability of the biosensor cells and allows for a longer time of cellular activity because available nutrients are maintained in this method. [8, 24] have used the method successfully for various analyses in locally increased concentrations of the toxicants. Generally, it is difficult to perform ecotoxicity assays of these particles due to their characteristics because their toxicity is dependent on many factors such as presence of stabilizers which indicates that if such stabilizing agent is present in WWTP, the toxicity of the particles will be altered. [25] reported that the presence of sulfhydryl-containing ligands altered the reactivity and bioavailability of silver nanoparticles thereby giving positive but false toxicity assessments.

The putative biosensors E and D responded immediately at time 0 (as seen in Figure 2) to the addition of AgNPs and bulk silver as reduction in their light output. Although, the 'time 0' is the time at which the luminometer read the first cycle of the bioluminescence output which was immediately after setting the inoculated microplate into it. Therefore, it could be assumed that the cellular response took place within the short period (few seconds) of getting the microplates from the bench into the luminometer. Hence, these strains could be used for rapid, immediate and sensitive detection of toxicants like silver nanoparticles. This light emission reduction may be due to cellular death and / or an extreme cellular metabolic burden as a result of the toxicity of the silver particles. The light reduction observed indicates that there was cellular contact to an extent.

The toxicity of silver particles to the microbial cells seems to be time-dependent. Over the period, the EC<sub>50</sub> values obtained at time 60minutes for AgNPs and bulk Ag was about two times lower than those of 300 minutes which indicates that toxicity of the silver particles reduced with time. Again, monitoring or assessing WWTP for silver particles toxicity can be done immediately or within 60 minutes because highest toxicity was observed at this stage. The addition of BSA to the silver particles in this study might have influenced the toxicity of the particles as reported by [10] that addition of BSA resulted in higher toxicity of silver nanoparticles through good dispersion of the particles providing more surface area for silver materials to be effective. The highest AgNPs toxicity in his work was observed at 90 minutes with EC<sub>50</sub> value of 35µg/ml, however, [10] did not read luminescence output at time 0 prior to taken readings every 30 minutes. He would probably have observed the highest reduction in light emission at time 0 before the subsequent reduction which could have suggested an immediate assessment of toxicants in WWTP. Also, it could also suggest that treatment of wastewater polluted with silver particles can be more efficiently treated with microorganisms at late hours when toxicity to microbes is lower than at early hours when toxicity is higher because results of this study suggests that silver wastes been washed into WWTP will be toxic immediately to microbes than when it has been allowed to settle resulting in less interactions with microbes present in WWTP.

AgNPs appeared to elicit a larger response from the two transconjugants, E and D than bulk silver; silver nanoparticles at the concentrations of 625, 1250, 2500µg/ml caused a 100% reduction of luminescence output from both E and D (Figure 2 a and c) at time 0 to 300 minutes with even 312 µg/ml causing 100% luminescence reduction indicating that D is more susceptible to the toxicant than E (Figure 2c) whereas this is much less the case for bulk silver; bulk silver at various concentrations did not lead to 100% reduction of light output from transconjugant E (Figure 2b) but caused complete bioluminescence reduction with transconjugant D at a concentration of 2500µg/ml at time 0 to 300minutes (Figure 2d). Therefore, the *Pseudomonas* transconjugants E and D (putative 'general' biosensors) isolated from

wastewater are differently responsive to both AgNPs and bulk silver in a concentration-dependent way. Variability in response among species could be due to difference in strains of these species because the exact strains of these microorganisms are not known. In an experiment carried out by [10], 35nm-sized AgNPs were tested against a recombinant *Pseudomonas putida* isolated from activated wastewater sludge. Their study showed that AgNPs were of higher toxicity than bulk silver and inhibits light output by 50% at a starting concentration of  $90\mu\text{g l}^{-1}$ . This EC 50 value is more effective than the values generated from this study. Though, [10] used an ultrasonic bath to improve dissolution of the AgNPs which probably explains the elevated toxicity.

As observed in Figure 2 that at lower concentrations of AgNPs the transconjugants E and D seem to adapt slowly to the toxicity over time; first undergoing a sharp reduction in luminescence output and with time, they began to increase in their luminescence output especially at lower concentrations of toxicants. This might be that cells got adapted to cellular damage over time and eventually overcome the damage or It may be that silver particles settled finally in the wells and stopped interacting with the cells because prior to setting the inoculated microplate into the luminometer for light output readings, the suspensions in the well were evenly mixed by pipetting; this may be the reason for the immediate response of the cells to the toxicant as the mixing allowed them efficient interactions with the cells; they therefore gradually or rapidly settled in the microplate over time while in the luminometer thereby, stopping contact with the cells. Another reason may be that the cells continued to grow during the assays without increase in toxicant concentration resulting in an overall increase in light emission. It is said that several toxic substances can adsorb onto the bacterial cells surface. Therefore more cells would result in a greater total surface area for toxic substances to adsorb thereby, leading to a reduction in the observed toxicity (equivalent to increase in light output overtime despite presence of toxicant in this study) as demonstrated in the experiment performed by [26] who examined the impacts of algal cell optical densities on toxicity and bioavailability of toxicants and reported that a low ratio of cells to toxicant concentration mixture would be advantageous because the dilution effect of the cell samples will be relatively small for the toxicant suspension thereby, preserving the original chemical properties in the toxicant suspension to a large extent even after the cell suspension and constituents have been introduced.

At lower concentrations ( $0.9$  to  $19\mu\text{g ml}^{-1}$ ) of AgNPs and bulk silver, an increase in luminescence compared to the untreated sample could be observed (Figure 2 a and b). This might be due to 'hormesis', a term described by [27] to be the 'overcompensation' in cells metabolic activities; experiencing little stress at low concentration of an administered toxicant which could result in an increase in metabolic activity and thereby, increase in light output. Hence the toxicity of silver particles at these low concentrations could be negligible because over time, they

stimulated the light output metabolism of the strains. [18] reported that Ag NPs at moderate concentrations,  $40\text{ mg/L}$  had negligible effects on anaerobic digestion and assemblages of methanogenic.

## 5. Conclusions

Dose-dependent light outputs were obvious despite the high settling properties of the silver particles. Nevertheless, it is advisable to develop an assay environment (WWTP) that encourages microbial interactions of silver particles over time. This study reports on silver-microbial cell interactions and toxicity restricted only to effects of various concentrations of silver in their pristine forms whereas, silver particles that have aged in the environment (such as WWTP) and wastewater microbial interaction is at best hypothetical with contradictory variables [28].

It is obvious that the EC data generated from this study cannot be compared to the concentrations in the predicted environment, such as WWTP for a significant silver particles toxicity assessment [29] because the behaviour of these particles and their potential toxicity to microbial cells in the presence of variable characteristics of wastewater may be determined by their nature, size and shapes [2]. As a result of these uncertain issues, the method presented in this study may be limited except if these challenges are further researched to satisfactorily predict uncertainties in silver nanoparticles toxicity estimations which will create a robust and logical model for probabilistic evaluations of relationships among varying complexes. An excellent interpretation of silver particles toxic effects would therefore include silver particles characterisation and framework that compares in-vitro, in-vivo, chronic and acute, predictive and formalized bioassay data with those from aged-silver particles present in relevant environment, in this case, WWTP [28]. Insights into toxic effects of silver nanoparticles on wastewater microbial population will therefore need step-by-step assessments to comprehend their behaviour and how to manage nanowaste in an environment-friendly manner.

This study suggests further research for more convincing evidences, into the effects of pristine silver nanoparticles as assessed in this work compared to environmentally relevant aged-silver particles on wastewater microbial community in treatment processes. This knowledge gap is greatly owed to the lack of suitable analytical tools and models to clarify factors that positively stimulate or weaken silver nanoparticle toxic effects. Therefore, research into developing such analytical tools is encouraged. This assay method, upon the afore mentioned further research is a rapid, reliable, cost-efficient and greatly beneficial method that can be applied to assay for toxicity and implications of toxicology of both silver nanoparticles and bulk silver on dynamics of microbial population during the treatment of wastewater.

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