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DEPARTMENT OF PHYSICS

Bachelor Thesis

Experimental study into sub-lethal exposure of bacteria to nanoparticles and the possible development of resistance

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Electrical Engineering and Computer Science

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Bachelor’s thesis title in Czech:

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Guidelines:

Bacteria (E. coli) will be exposed to two commercially available ZnO NP of different size and morphology; 50 nm (Sigma Aldrich) and 10 µm (US Nanomaterial research) at 3 different concentrations: 1000, 100 and 10 µg/mL. Optical density of incubated bacteria with different ZnO NP will be measured over 24h using separate bioreactors (BioScan). A sub-sample from each condition after 24 h will be used for the following experiment after cell concentration equilibration by optical density. This process of recovering surviving cells and re-exposure to fresh ZnO NP can be repeated multiple times. Viable cell concentration of all reference and test samples at T0 and at T24 will be assessed by serially diluting the bacteria suspension and inoculation onto agar plates for enumeration after incubation using an automatic colony counter (SphereFlash). By analysing the growth curves of subsequent generations of cells exposed to ZnO NP, it is hoped that one might observe differences in the shape that could be attributed to re-exposure to treatment and relate that to the development of resistance or enhanced sensitivity.

Bibliography / sources:

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I have to thank my thesis supervisor, Mr. David Rutherford, Ph.D. Without his assistance and dedicated involvement, this paper would have never been accomplished. I would like to thank you for your support and understanding over these past challenging months.

Declaration

I hereby declare that I'm the author of this thesis work and that I have not used any sources other than those listed in the bibliography and identified as references. I further declare that I have not submitted this thesis at any other institution in order to obtain a degree.

In Prague 04.01.2021

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Signature…………….

TABLE OF CONTENTS

LIST OF FIGURE 6
ABSTRACT 7
CHAPTER 1 8
1. INTRODUCTION 8
CHAPTER 2 10
2. AIMS AND OBJECTIVES 10
2.1. Goals and Structure of the Thesis 10
2.2. Rationale of the study 10
2.3. Experimental outline 10
CHAPTER 3 12
3. METHODOLOGY 12
3.1. Re-exposure Protocols 12
3.1.1. General info 12
3.1.2. Protocol for Week 1 12
3.1.3. Protocol for Week 2 13
3.2. Tools and devices 13
3.2.1. RTS – 1C: Personal bioreactor 13
3.2.2. Autoclave 14
3.2.3. Elmasonic P bath 14
3.2.4. Advanced vortex mixer 14
3.2.5. Sphere flash 14
3.2.6. Other tools 14
3.3. Materials 15
3.4. Cell viability 15
3.5. Nano – First exposure 15
3.6. Nano – Second Exposure 15
3.7. *E. coli* Growth 16
CHAPTER 4 17
4. RESULTS AND DISCUSSION 17
4.1. Nanoparticle characterization 17

4.2. Cell viability 19
4.3. Micro Exposure 22
4.4. Nano First exposure 22
4.5. Nano second exposure 24
4.6. Micro First Exposure 25
4.7. Micro– Second Exposure 27
CHAPTER 5 29
5. IMPLEMENTATION 29
CHAPTER 6 31
6. CONCLUSION 31
REFERENCES 32

LIST OF FIGURE

Figure no	Content	Pages no
Fig 1	Bacterial growth curve	14
Fig 2	SEM images for nano (SEM Ec NS 11-6um)	18
Fig 3	SEM images for micro ZnO (3-200 um)	20
Fig 4	*E.coli* Growth	20
Fig 5	Nano Second Exposure - Viable concentration against ZnO concentration (T0)	21
Fig 6	Nano Second Exposure - Viable concentration against ZnO concentration (T24)	21
Fig 7	Micro Second Exposure - Viable concentration against ZnO concentration (T0)	21
Fig 8	Micro Second Exposure - Viable concentration against ZnO concentration (T24)	22
Fig 9	Optical density against *E. coli* cell membrane viability in hours	23
Fig 10	Impact of NPs on biofilms of *E. coli* under different concentrations	25
Fig 11	Viable concentration against ZnO concentration	26
Fig 12	Optical density against hours under different ZnO concentrations	28

ABSTRACT

Zinc nanoparticles are the most common nanotechnology NPs that provides a major contribution to the production of large-scale consumer goods. In developed nations, hazardous microbe infections pose a major public health issue, largely due to less awareness of antibiotic usage and vaccination. The creation of compounds to treat infection is essential to handle certain pathogenic bacteria, such as multiple drug resistance lines. To research the resistance to hazardous microbial strains, scientists use nanoparticles (NPs). These NPs are also used to treat diseases like cancer that kill cells with DNA. The thesis aimed to study the effects of nano ZnO and micro ZnO against *E. coli*, by analyzing cell viability. Zinc oxide nanoparticle was characterized and was exposed to the *E. coli*. The cell viability of *E. coli* concentration between 100 to 1000 µg/mL was observed. The growth concentration nano and micro exposure was observed in T0 and T24. The exposure effects indicate that stock solution exhibited a higher impact on its cell membrane viability as the concentration tends towards 0 µg/mL. Viable concentrations are expressed in terms of ZnO concentration and the length of unit time in stock solution. In conclusion, the research findings show the importance of incorporating a wide range of possible endpoints to determine NPs toxicity as it provides an in-depth assessment of discrete impacts, otherwise not revealed by a narrow choice of endpoints.

Keywords: Nanoparticles, toxicity, zinc oxide nanoparticles, sub-lethal exposure.

CHAPTER 1

1. INTRODUCTION

In the recent past, multiple consumer goods have reinvented nanoparticles to enhance consumer experience due to their special properties that provide for an enhancement of the primary functionality of such products. As such, zinc oxide nanoparticles (ZnO NPs) represent some of the most popular NPs in the field of nanotechnology that contributes significantly to the manufacture of a wide-ranging consumer product [1]. For instance, ZnO NP is commonly included in consumer products that include but are not limited to cosmetics and paints, mainly because of its unique blocking characteristics of the UV radiation [2]. Hence, the extensive use of the NP has contributed to its exponential accumulation in the environment, and the growth is not assumed to slow down because of its wide-ranging applications. According to Hou et al. 2018 [3]**,** the toxicity properties of the NP in causing inflammation can increase its chances of negatively impacting the microbes, such as *E. coli*, once they are released into the water bodies. Such microbe's play a critical role in the fixation of nitrogen, as well as reducing the oxidizing capacity of metals in the environment [4]. Hazardous microbe diseases are a significant public health concern in developing countries, mainly because of less understanding of antibiotic usage and vaccination. This has also contributed to the creation of multi-drug resistant strains (MR). The development of compounds to fight many pathogenic bacteria, like MR strains, to cure infection is highly significant. Scientists produced tiny particles known as nanoparticles (NPs), which demonstrate tolerance to hazardous microbial strains. These NPs often are used to cure diseases such as cancer, which either destroy cells individually or in conjunction with DNA [5].

Studies have revealed that NP toxicity towards microbes has been due to oxidative stress when reactive oxygen species are generated. Also, it is argued that the toxic metallic ions play a part in the sub-lethal exposure of bacterial to NPs, such as Zn^{2+} [6]. Also, NPs-microbe's interaction, coupled with respective toxicity, depends on individual physicochemical characteristics of NPs and water aqueous properties [7]. Specifically, multiple studies have

illustrated the impact of aqueous properties such as pH and natural organic matter on NP transformation, as well as subsequent toxicity to microbes such as *E. coli.* Of note, most of the studies focused on the effect of aqueous chemistry on NP transformation together with significant toxicity, evident in synthetic media [8].

The sub-lethal exposure of NPs has been studied at concentrations lower than the modeled levels and recorded NP concentration [9]. As a result, the variations have constrained the ability to obtain an accurate picture of the impacts on aquatic systems. However, a handful of studies have assessed the effect of NPs on microbes at the concentration found within the levels that have been measured in real settings using actual metrics [10,11]. For instance, the impact of Ag NP on other microbes, notably *E. coli,* in a natural aqueous media. *E. coli,* a relatively environmentally ubiquitous microbe, can be identified as a model microorganism because it can endure extreme conditions, as well as a highly stressful environment. The bacterium can also be identified because of its excellent record in nontoxicity studies, on which *E. coli* and *S. aureus*, is more sensitive [12]. Understanding engineered toxicity of NPs on microbes in natural aqueous systems by assessing the endpoints mentioned earlier: ROS, cell viability, ATP concentrations, and membrane viability helps in a quest to develop better possible development of the resistance.

CHAPTER 2

2. AIMS AND OBJECTIVES

2.1. Goals and Structure of the Thesis

In this thesis, the outlines of the methods normally used based on prepared stock solutions, the effects of zinc oxide nanoparticles in an aqueous medium, and in particular, the microorganism in question - *E. coli.* In materials and methods (Chapter 3) of the thesis, mention the cell viability to analysis the behavior of *E. coli* in response to exposure to different concentrations of nanometer-sized ZnO (nZnO)and the possible development of resistance. The results and discussion (Chapter 4) highlight the aforementioned endpoints and the implications of zinc oxide nanoparticles on the microorganisms using the prepared stock solutions. In chapter 5, the effects of the nanoparticle on the environment are discussed, focusing on past literature and policy issues that govern the use of potentially dangerous nanoparticles such as nZnO. Finally, the conclusion in chapter 6 summarizes the thesis by discussing the overarching components of the nanoparticle in question in relation to its effect on the microorganism,

2.2. Rationale of the study

It is well documented that zinc oxide nanoparticles have an antibacterial activity that has the ability to permeate the cell membrane, thus inhibiting its growth. Cellular functions are disrupted major by lipid peroxidation that results from changes in the organism's cell membrane [13]. Such assertions have been echoed by the effects of oxidative stress that involve nZnO in *E. coli* [14]. Thus, the consideration of nZnO toxicity includes toxic ions release owing to the amphoteric nature of the chosen nanoparticles and is known to react with acids and alkalis to yield Zn^{2+} ions. By analyzing the growth curves of subsequent generations of cells exposed to ZnONP, it is hoped that one might observe differences in the shape that could be attributed to re-exposure to treatment and relate that to the development of resistance or enhanced sensitivity.

2.3. Experimental outline

Bacteria (E. coli) will be exposed to two commercially available ZnO NP of different size and morphology, 50 nm (Sigma Aldrich) and 10 µm (US nanomaterial research), at 3 different concentrations: 1000, 100 and 10µg/mL. The optical density of incubated bacteria with different

ZnO NP will be measured over 24h using separate bioreactors (BioScan). A sub-sample from each condition after 24h will be used for the following experiment after cell concentration equilibration by optical density. This process of recovering surviving cells and re-exposure to fresh ZnO NP can be repeated multiple times. Viable cell concentration of all reference and test samples at T0 and T24 will be assessed by serially diluting the bacteria suspension and inoculation onto agar plates for enumeration after incubation using an automatic colony counter.

CHAPTER 3

3. METHODOLOGY

3.1. Re-exposure Protocols

3.1.1. General info

Week 1: *E. coli* + nano ZnO 1st-exposure (bacteria from the freezer - stock)
Week 2: *E. coli* + nano ZnO re-exposure (bacteria from agar plates - survivors from week 1)
Week 3: *E. coli* + micron ZnO 1st exposure (bacteria from freezer - stock)
Week 4: *E. coli* + micron ZnO re-exposure (bacteria from agar plates - survivors from week 3)
Weeks 1/2 and 3/4 are the same procedure, except for the size of ZnO used.

3.1.2. Protocol for Week 1

On day 1 the preparation process was followed. The ZnO stock solution, nanoparticles < 50 nm, add 2 mg/ml in 10ml of water were made. Then 200 mL broth and 500 mL agar were made. The bacteria were cultivated and grown in freezer stock. On day 2, the experiment was carried out. The ZnO solution was sonicated for 30 min at 37 Hz/100 W for better mixing and the two dilutions (1:10 & 1:100) were prepared. This is followed by the removal of bacteria growth from agar plate and added 5 mL broth, which was in dilute of 1:1000. 5mL of bacteria were added to 4 labeled tubes which were labeled as Reference, 1000, 100 and 10 respectively. 5 mL water was added to "reference" tube, 5mL of the 2 mg/mL stock were added to "1000", 5 mL of the 1:10 stock dilution were added to "100" and 5 mL of the 1:100 stock dilution were added to "10". This dilution series was performed on "reference" sample to determine initial bacteria concentration (T_0: -4, -5, -6). The tubes were placed into separate bioreactors and '*E. coli* test' program was started. On day 3, the analysis procedure was carried out. After 24 h, dilution series was performed on all 4 tubes to determine final bacteria concentration (T_{24} Reference: -7, -8, -9; 1000: -1, -2, -3; 100: -4, -5, -6; 10: -7, -8, -9). The images of colonies were taken using a colony counter (Reference: T_0). The data from the colony counter were exported into MS Excel. And the data from bioreactor software were exported into MS Excel. Again on day 4, the images of colonies were taken using colony counter (Reference, 1000, 100 & 10: T_{24}).

3.1.3. Protocol for Week 2

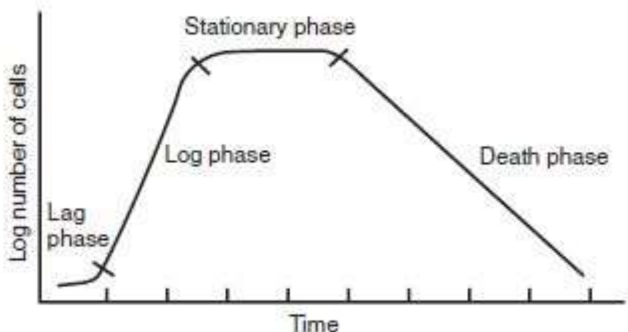
On day 1, preparation and experiment procedure were followed. ZnO stock solution (2 mg/ml) was made, followed by that is autoclave and sonication (30 min @ 37 Hz/100 W), which was then prepared in dilutions (1:10 & 1:100). 200 mL broth and 500 mL agar were prepared. The colonies from T_{24} plates on each sample of week 1 were removed and added into 4 new tubes with new broth; each tube's solution was adjusted to optical density value at 850 nm = 0.07 ± 0.01 (equivalent to 10^7 cfu/mL). The dilution series was performed on each sample tube to determine initial bacteria concentration (T_0: Reference, 1000, 100 & 10: -4, -5, -6). The tubes were placed into separate bioreactors and '*E. coli* test' program was started. On day 2, the analysis procedure was followed. After 24 h, dilution series was performed on all 4 tubes to determine final bacteria concentration (T_{24} Reference: -7, -8, -9; 1000: -1, -2, -3; 100: -4, -5, -6; 10: -7, -8, -9). The images of colonies were captured using a colony counter (Reference, 1000, 100 & 10: T_0). The data from the colony counter were exported into MS Excel. And the data from bioreactor software were exported into MS Excel. On day 3, the analysis procedure was followed. The images of colonies were captured using the colony counter (Reference, 1000, 100 & 10: T_{24}).

3.2. Tools and devices

3.2.1. RTS – 1C: Personal bioreactor

E. coli was mounted on RTS – 1C personal bioreactor for analysis. For cell viability assessment, a colony was inoculated in lysogenic broth at an optimum temperature of 37 °C. The process was accompanied with continuous shaking at an average of 2000 rpm for the entire night until the colony reached a mid-exponential phase of between 0.5 and 0.6 at OD 850nm. The microbi1al cells were obtained through centrifugation for 15 minutes. The bacterial growth curve is represented in Figure 1. The growth curve is hyperbolic as it has an exponential growth pattern that involves Lag Phase, Log Phase or exponential Phase, Stationary Phase and Death Phase or decline Phase. Lag Phase is the initial phase that involves inoculation, adaption to the physical environment and preparation for cell division. In Log Phase, bacteria divide continuously with balanced growth. In the Stationary Phase, there is no increase in the bacterial population, which maintains cell death and cell division. In Death Phase, the bacteria decrease continuously.

Fig 1: Bacterial growth curve



Reference: Onlinebiologynotes.com

3.2.2. Autoclave

The NP solutions with a 2 mg/mL concentration were prepared using an Autoclave to sterilize the tubes from ZnO and bacteria. On the other hand, the control experiment is run with the exclusion of NP suspensions. Finally, both flasks are kept at an optimal temperature of 37°C.

3.2.3. Elmasonic P bath

The solution is sonicated at a frequency of 37 kHz for approximately 30 minutes in an Elmasonic P bath before the sub-lethal exposure. Furthermore, the concentrations are diluted in line with the projected nominal concentration of between 10 and 1000 µg/L in a 250 mL flasks with the microbial cells at OD_{850nm} of 0.5 for the medium to obtain the ultimate volume of 25 mL.

3.2.4. Advanced vortex mixer

An advanced vortex mixer was used to mix the sample after the microbes are exposed to NP. A 100 mL of NP from the matrix is then put in specific wells, containing a 200 µL solution, and mixed proportionately.

3.2.5. Sphere flash

After NP exposure, the microbial viability was determined by colony-forming units using the Sphere flash, where the cell viability percentage is established by the CFU.

3.2.6. Other tools

The other necessary used tools are Scale ABT 320-4M, pipettes and pipette tips.

3.3. Materials

The serial dilution of the aqueous media was done using a 0.9% NaCl. Muller – Hinton broth (CM0405) and Muller – Hinton agar (CM0337) lysogeny broth agar plated with a drop count were used.

3.4. Cell viability

The serial dilution of the aqueous media was done using a NaCl at 0.9% concentration. Thereafter, all viable bacteria are assessed using a lysogeny broth agar plate with a drop count method. The microbial viability after NP exposure was determined by colony-forming units CFU counting from respective agar plates. From there, the cell viability percentage was established by the CFU.

3.5. Nano – First exposure

To determine the cell membrane viability, RTS – 1C: Personal bioreactor kit has been used after the microbes are exposed to NP. The diluted concentrations of NP from the matrix is then put in specific wells, containing a 250 mL Elmasonic P solution, and mixed proportionately. After that, the sample was incubated at temperature of 37°C for 30 minutes in a dark environment. After NP exposure, the microbial viability was determined by colony-forming units using the Sphere flash, where the CFU established the cell viability percentage. From the known proportion of intact, possibly alive cells, the resulting calibration curve was obtained for analysis.

3.6. Nano – Second Exposure

The second nano- exposure as a result of NPs sub-lethal exposure in the sample was assessed to represent the cell viability using RTS – 1C Personal bioreactor after NP-membrane reaction took place. As a result, cells were observed for optical density after the exposure time of approximately 24 hours, under different concentrations at temperature of 37°C.

3.7. *E. coli* Growth

This is attained by measuring the optical density over time in response to NP exposure. Furthermore, a proportionate amount of Elmasonic P - of NP are transferred to respective wells together with a 25 mL Muller – Hinton agar (CM0337) lysogeny broth and after that using appropriately using an advanced vortex mixer. At an approximately 37 °C for up to 24 hours.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1. Nanoparticle characterization

From the observation, zinc oxide nanoparticles have shown regular shapes, no rod-like and no hexagonal-like shape with varying diameter measurements of between 16 to 18 nm, primarily due to morphological asymmetry. Significantly elevated aggregation of NPs demonstrated in some of the freshwater systems is due to low Zeta potential, ranging between −13.3 ± 0.5 and −16.1 ±1.4 mV. The figures are a departure from the regular reading of up to ± 30 mV needed to maintain NPs dispersed as a result of charge stabilization in comparison to aggregation [15]. According to Li et al. 2013 [16], nZnO dissolution is concentration-dependent. At a moderate exposure level of approximately 100 µg/L , up to 15 µg/L dissolved in zinc. However, at an elevated nominal exposure concentration of approximately 1000 µg/L, there can be a notable increase in the dissolution effect of nZnO. Such significant differences are due to the physicochemical characterization, which according to Connolly et al. 2016[17], is known to determine the transformational processes of NPs in water systems. Also, NPs coating, especially the natural organic matter in water systems, can promote or prevent aggregation and stability through known mechanisms such as electrostatic interaction, together with ligand exchange [18].

Furthermore, it is argued that in the absence of Natural organic matter (NOM), cation binding will likely promote NPs aggregation [19]. Noteworthy, freshwater systems demonstrate elevated natural organic matter content of more than 6 mg/L, which is in agreement with the known normal ranges of 0.1 and 32 mg/L in aqueous systems, hence proved the concept of natural organic matter dependent aggregation. Another study demonstrated that the release of Zn2+ diminished with an increase in PO4 because of high metal-complexation between the two metals [20]. The demonstrated ZnO dissolution differential could be due to aggregation differences and aqueous characteristics. Accordingly, another study showed the higher aggregation and minimal dissolution of zinc oxide nanoparticles in freshwater systems such as rivers and lakes, which revealed enhanced aggregation of ionic strength of between 3.3 and 6.5 mM compared to those with low ionic strength characteristics [21]. The results from the study

as mentioned earlier are echoed by other findings where river water demonstrate high ionic strength of approximately 5.2 mM which had an enhanced aggregation and minimal dissolution of zinc oxide nanoparticles as compared to minimal aggregation and enhanced dissolution in the water system with the limited ionic strength of approximately 2.42 mM. Therefore, the results lend credence to natural organic matter coating-controlled NPs ionic release in aquatic systems because Zn^{2+} release is blocked, which as a result, inhibits ionic diffusion from NP. Also, metal ion complexation with natural organic matter and phosphates can contribute to the low dissolution of zinc oxide nanoparticles. The above-outlined developments directly or indirectly impact the bioavailability and sub-lethal exposure of NP in water systems further demonstrated in the subtopics below. Fig 2 represent SEM images for nano (SEM Ec NS 11-6um) and Fig 3 represent SEM images for micro ZnO (3-200 um) show that they are regulrely shaped.[22].

Fig 2: SEM images for nano (SEM Ec NS 11-6um)

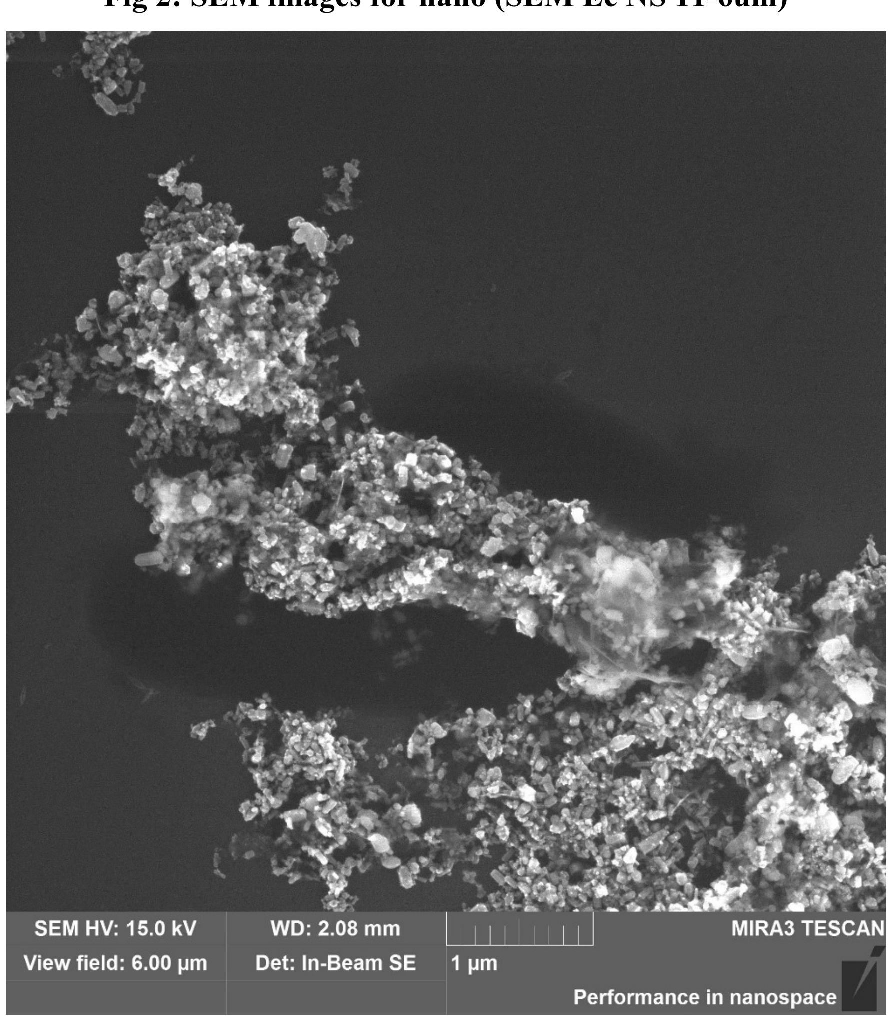
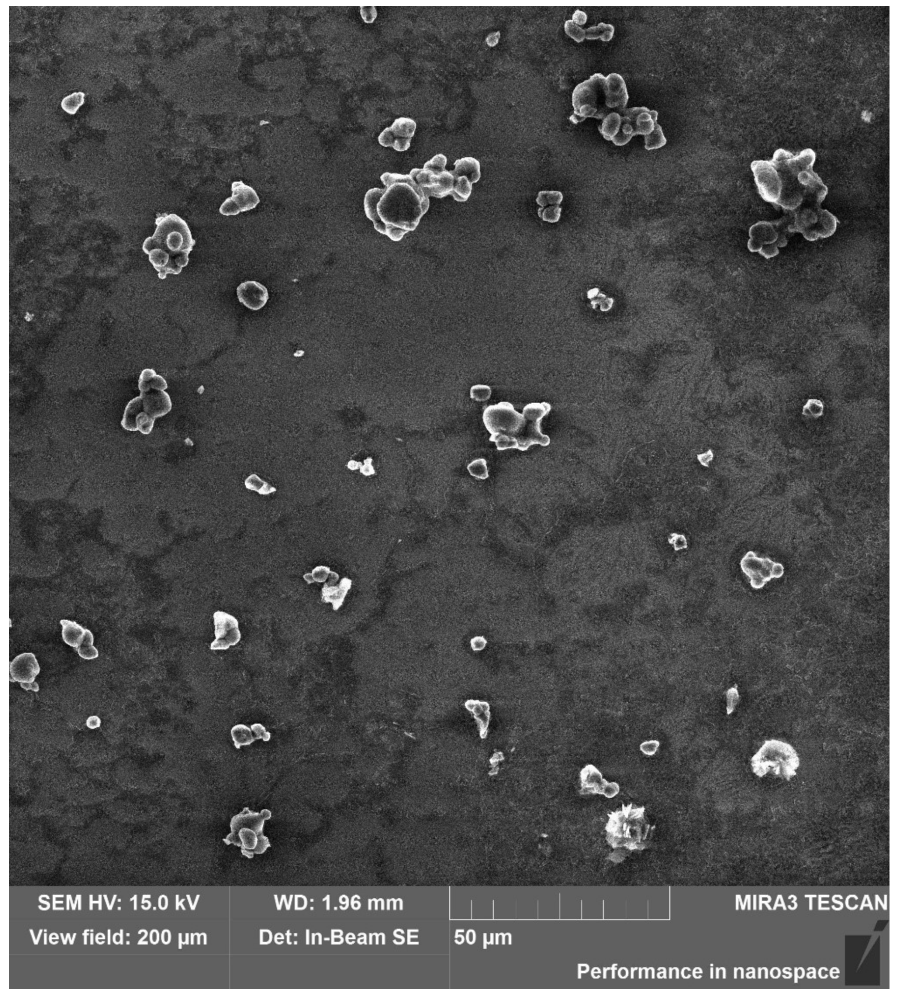


Fig 3: SEM images for micro ZnO (3-200 um)



4.2. Cell viability

Outcomes of *E. coli* exposure to zinc oxide nanoparticles showed distinctive cytotoxic effects (Fig. 4). From the graph, an enhanced concentration, between 100 to 1000 µg/mL*,* there was a notable reduction in *E. coli's* cell viability with more notable effects at 1000 µg/mL. Conversely, the effects are less visible at lower zinc oxide nanoparticles, notably at 10 µg/mL. Also, for other aqueous systems, sub-lethal exposure of zinc oxide nanoparticles did not exhibit adverse effects on cell viability, even at higher concentrations (1000 µg/mL). This is due to less compromise on cell viability caused by larger aggregates that block contact with cells. Fig 5 and Fig 6 represent the Nanosecond exposure of T0 and T24 respectively. Fig 7 and Fig 8 represent the micro-second exposure of T0 and T24 respectively.

Fig 4: *E.coli* Growth

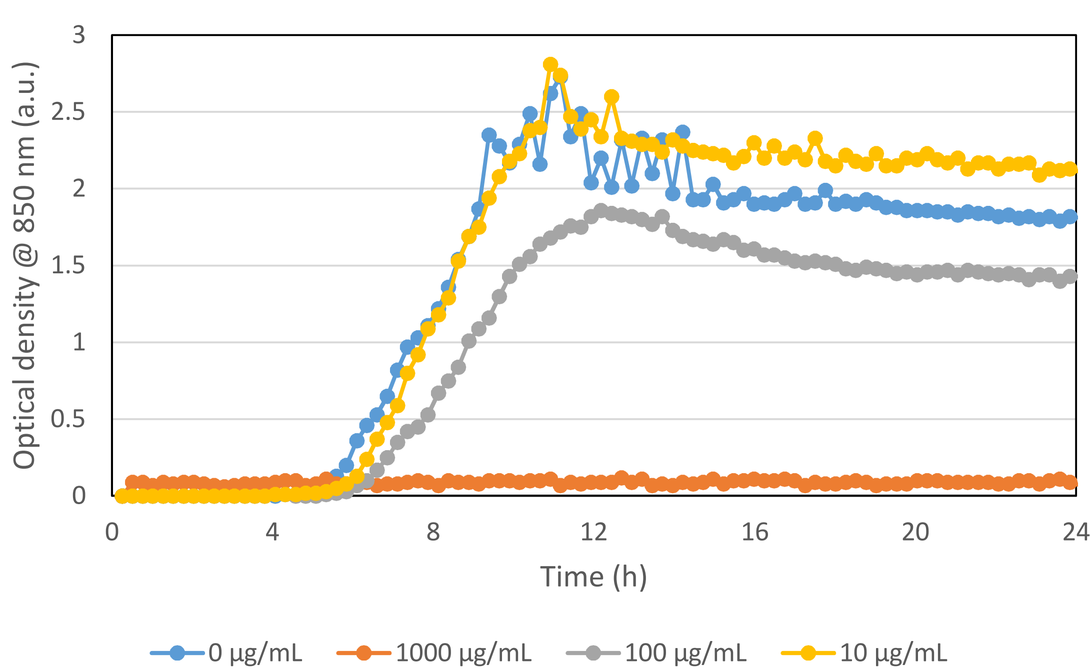


Fig. 5: Nano Second Exposure - Viable concentration against ZnO concentration (T0)

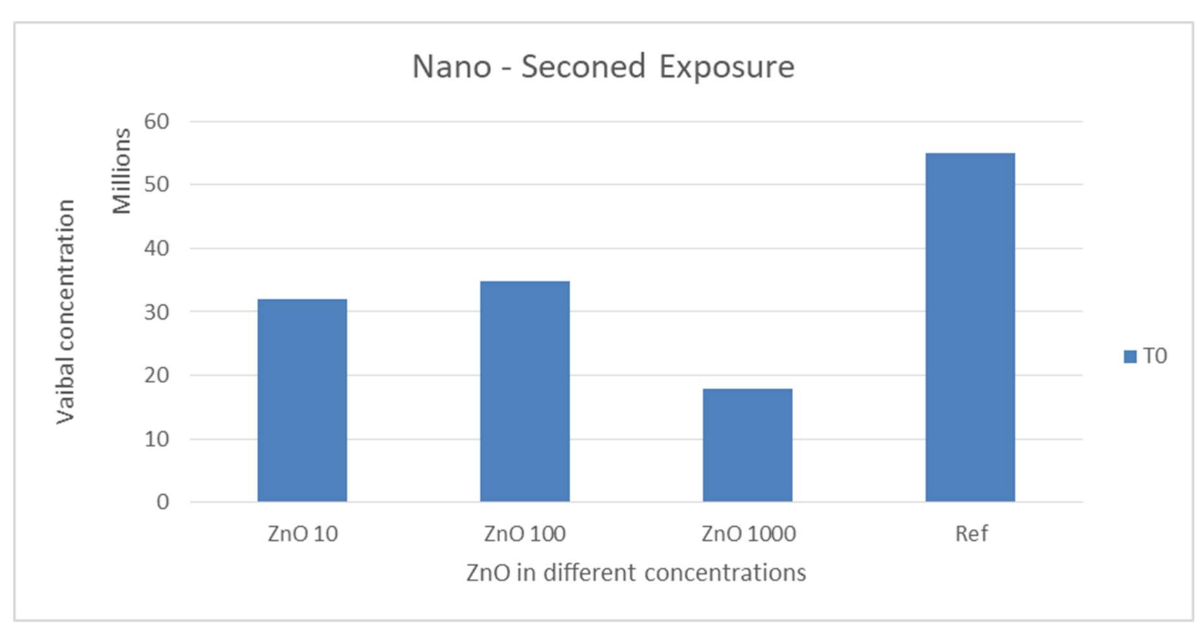


Fig. 6: Nano Second Exposure - Viable concentration against ZnO concentration (T24)

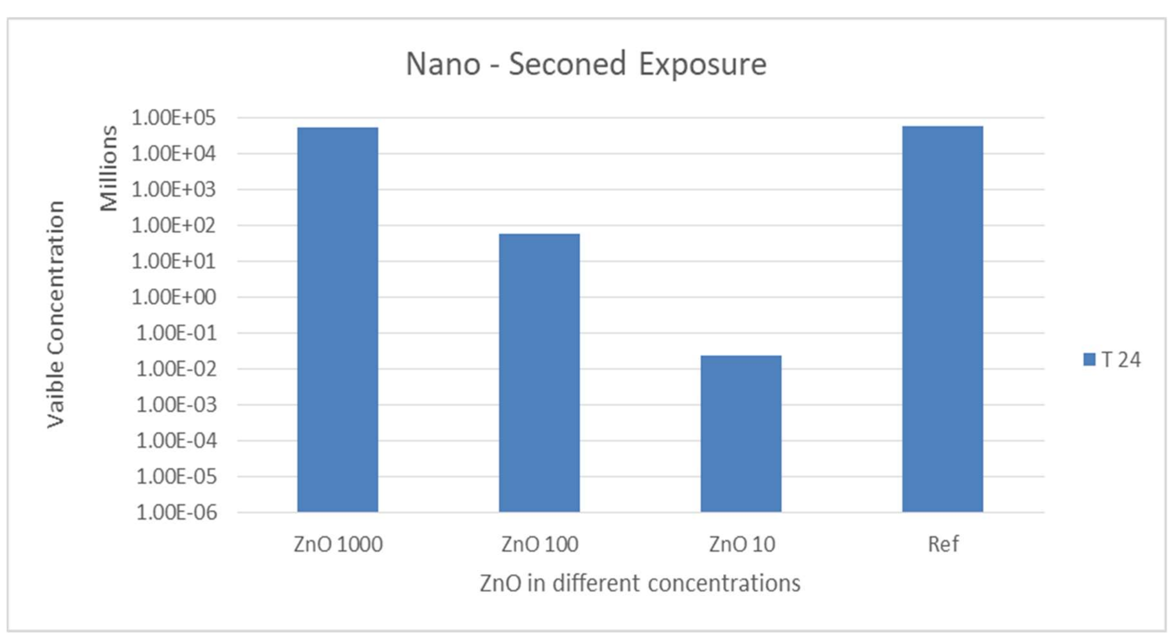


Fig. 7: Micro Second Exposure - Viable concentration against ZnO concentration (T0)

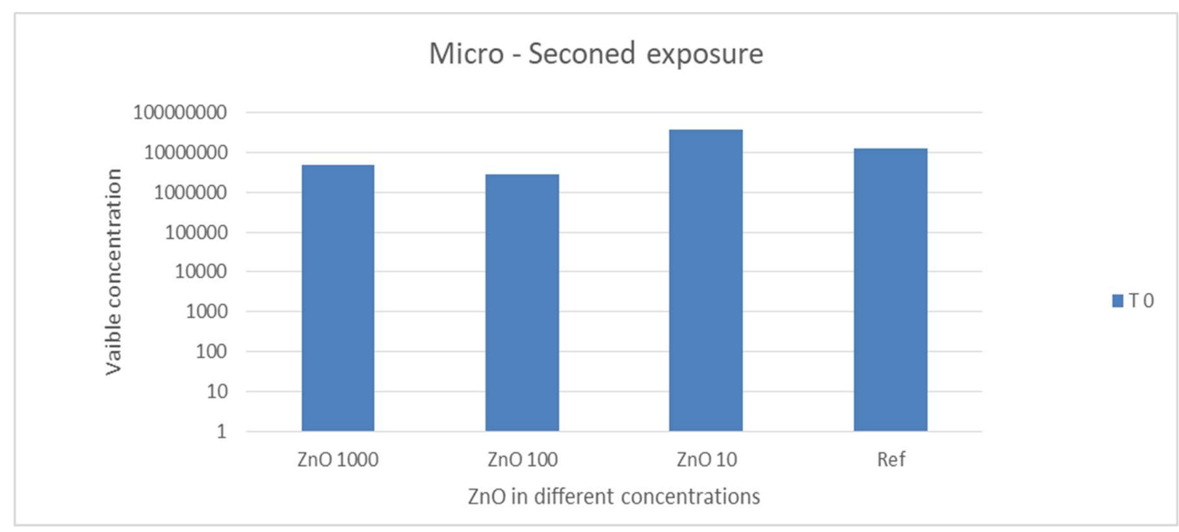
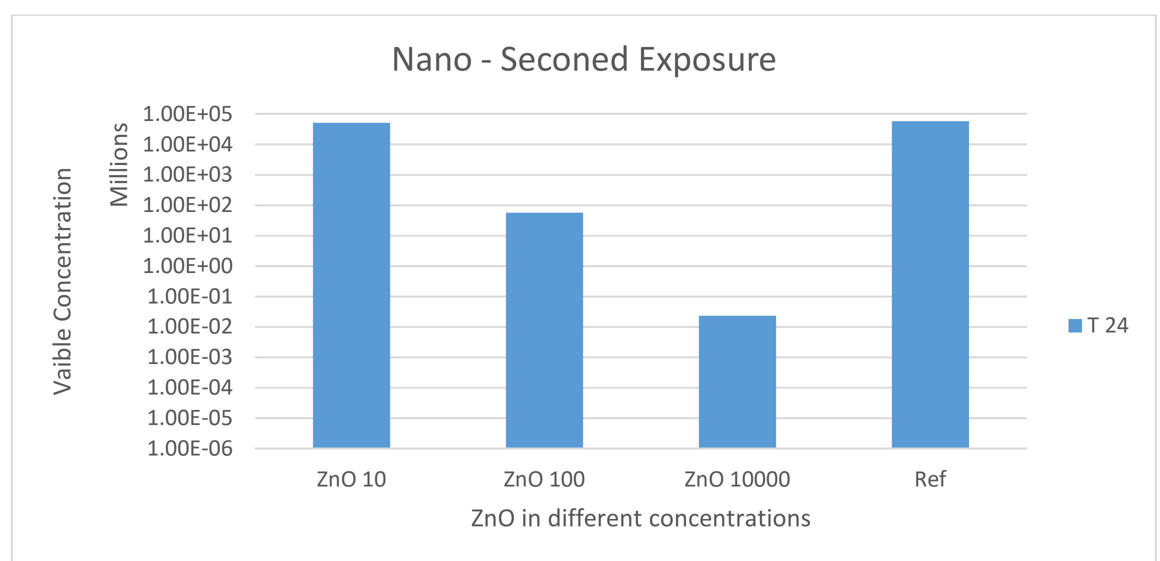


Fig. 8: Micro Second Exposure - Viable concentration against ZnO concentration (T24)



4.3. Micro Exposure

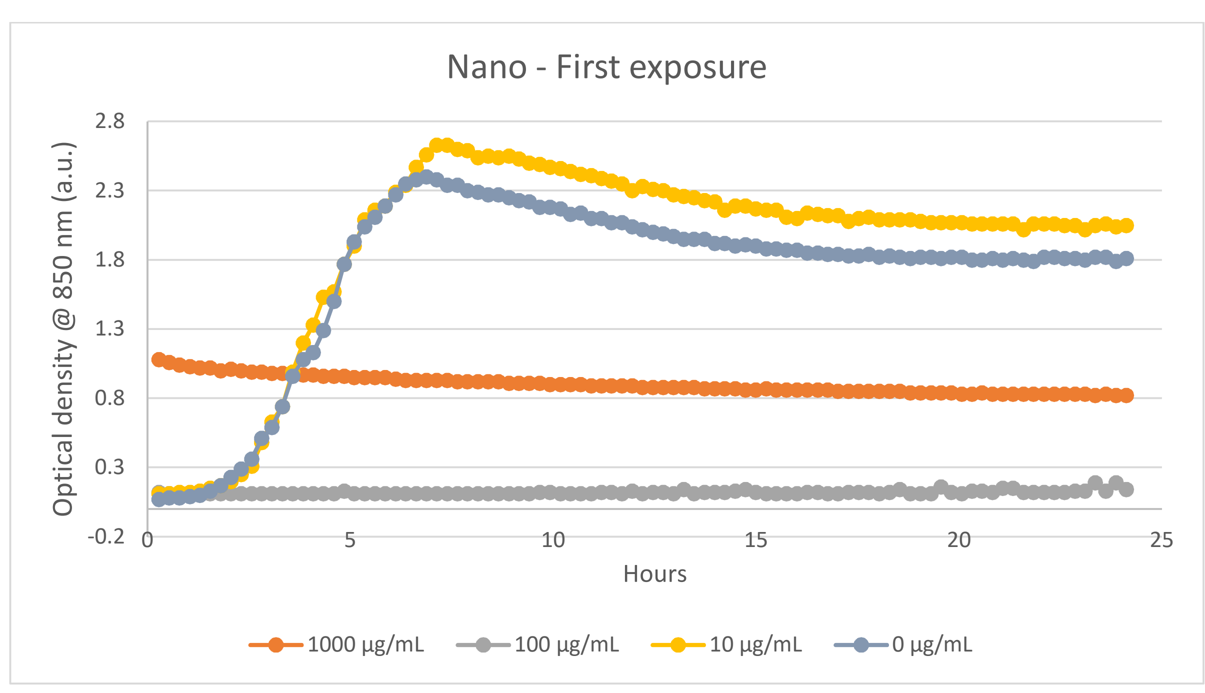
The effect of the typical nuclear morphological changes was analyzed. The changes like nuclear condensation and strong fluorescent spots were observed that show apoptotic nuclei.

4.4. Nano First exposure

To date, the sub-lethal effects of zinc oxide nanoparticles on microbes such as bacteria have been widely researched, with the reported results mainly associated with mechanisms such as the release of ions, reactive agent species production, and NPs surface exchange and uptake. For this study, the effects mentioned above mentioned is evaluated with a target of accounting for existing cytotoxic implications of zinc oxide nanoparticles on *E. coli,* including outcomes summarized in subsequent analyses. The effect of concentration on cell membrane viability was noted in Fig. 9. From the observation, the impact of the NP on the membrane is observed to depend on nZnO concentration as present in the aqueous media. To put it into perspective, Fig. 2 shows a summary of the effects indicate that stock solution exhibited a higher impact on its cell membrane viability as the concentration tends towards 0 µg/mL, and witnessed the lowest

percentage when concentration peaks at 1000 µg/mL. Conversely, the cell membrane viability can be particularly higher at the same concentration at 7 µg/mL. Also, at a lower concentration, the cell membrane viability was barely noticeable and was completely stable at $OD_{0.1}$. Further, the cell membrane viability was comparative the same at different OD, after 7hrs., at 0 and 10 µg/mL. The notable difference of the same degrees of disruption at different concentrations was attributed to a characteristic physicochemical differential, wherein stock solution significant reduction is likely because of minimal aggregation and enhanced dissolution of NPs, especially zinc oxide nanoparticles [23]. Still, OD effects are more evident in-stock solutions as the concentration of nZnO decreased. The findings echo that of other studies by [24], where microbial viability can be discovered to depend on zinc oxide nanoparticle exposure dosage of ranges 3 µg/L and 4000 mg/L.

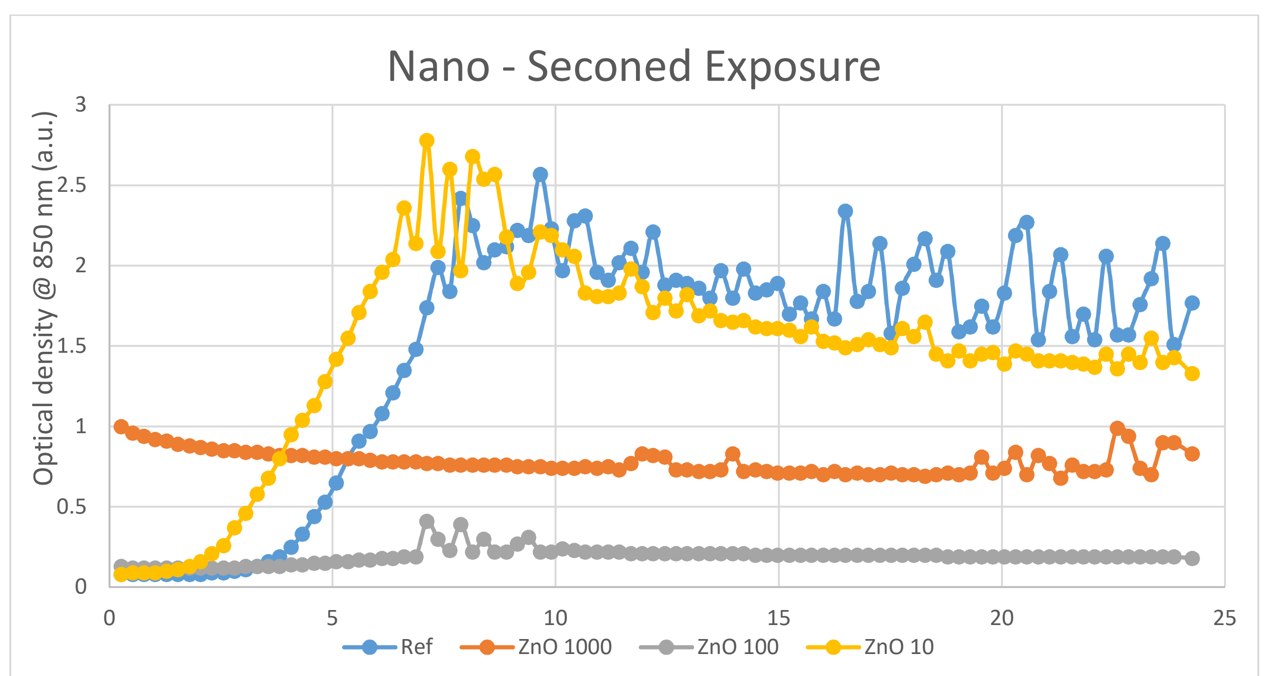
Fig. 9: Optical density against *E. coli* cell membrane viability in hours



4.5. Nano second exposure

As observed in Fig. 10, the effects are likely due to the increased proportion of dissolved zinc concentration, directly linked to the increase in concentration exposure, which depended on stock solution chemistry. Such findings mirror other studies, [25], which argue that dissolved zinc from its constituent nanoparticles plays a role in the evident toxicity to microbes such as *E. coli* by aqueous media chemistry. The findings from other studies on the effects of zinc NPs in water matrix may result from measure concentration of up to 355 μg/L in comparison to 180 μg/L. All measurements are pegged to a 1 μg/L concentration. Moreover, modeled speciation outcomes of dissolved ions in-stock solutions, with Visual MINTEQ, revealed that there are significant dissolved ions combined to form complexes with the dissolved organic stock solution. In contrast, the others developed labile compounds, which according to other findings, could have contributed to toxicity to bacteria [26]. Unlike other metal ions, such as iron oxide nanoparticles, zinc oxide nanoparticles are particularly more damaging to cell membrane viability. This can be attributed to repulsion and limited NP-cell interaction. For example, the hydrodynamic diameter of, say 550 ± 30 nm for zinc nanoparticles as visible in DLS measurement mirrors the size of an average of bigger particles, but this does not rule out the interaction with the bacterial cells as a result of the impact of concentration as posited by other scholars [27]. Such limitations can also be explained by examining other mechanisms such as van der Waals forces, which exhibit weaker electrostatic repulsion. Such can be explained by zinc homeostatic disruption resulting from internalized Zn2+ cell death that occurs due to protein denaturation. In addition, the significant nZnO (1000 μg/L) inhibition, usually between 10 and 20 percent on *E. coli* under, both as well as light conditions without enhanced nano-exposure and limited nZnO-cell interaction. Thus, the outcomes from other studies points to the fact zinc ions could be occurring by cellular functioning limitation as a result of pure chemical impact on the microbial cells.

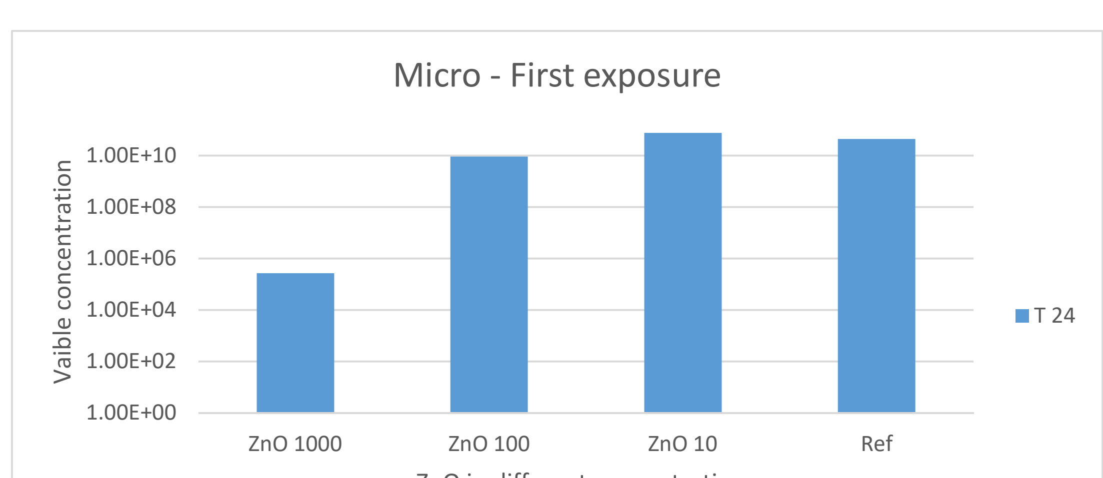
Fig 10: Impact of NPs on *E. coli* under different concentrations



4.6. Micro First Exposure

The evaluation of the capacity of NPs to distort OD was included. The results measured OD after the exposure of the bacterium *E. coli* to ZnO in-stock solutions for up to 24 hours, as summarized in Fig. 11. Viable concentrations are expressed in terms of ZnO concentration and the length of unit time in stock solution. To put it into perspective, zinc oxide nanoparticles exhibited enhanced concentration-dependent on viable concentrations. Noteworthy, there is limited research that examines the possible impacts of nanoparticles on viable concentrations. However, another study finding, that there is an enhanced concentration-dependent limitation in the ZnO concentrations demonstrated using *E. coli* specimen by zinc oxide nanoparticles in lake water at 1000 μg/L for 24 hours incubation. From other studies, the results showed an enhanced reduction in viable concentrations at a lower concentration of less than 20 μg/L of zinc oxide nanoparticles after an hour of incubation in an aqueous medium under visible light [28].

Fig. 11: Micro First Exposure - Viable concentration against ZnO concentration (T0)



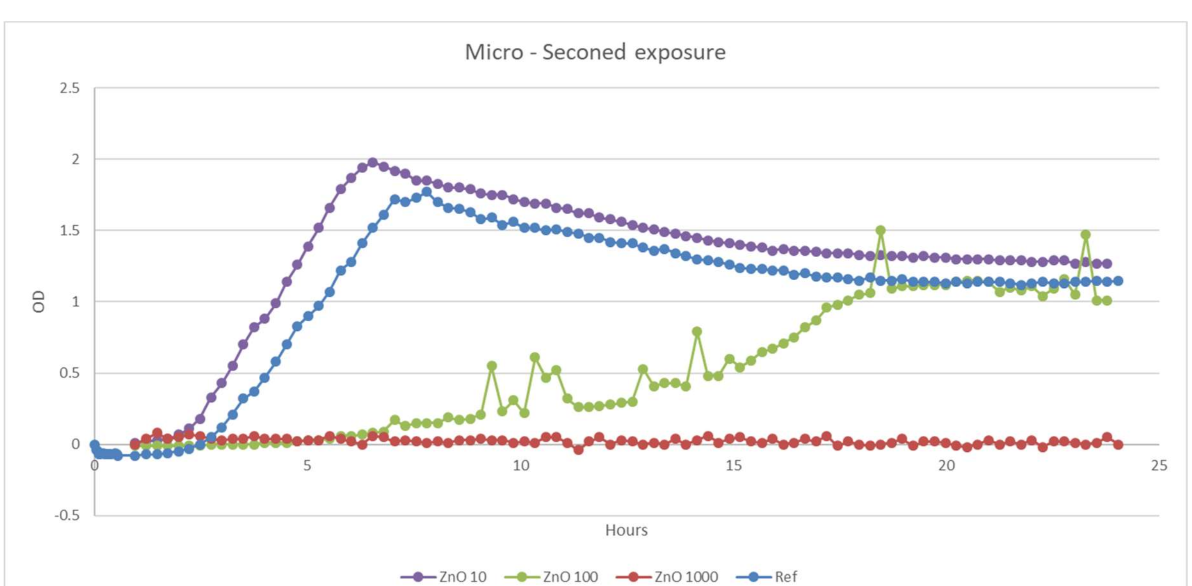
To support the study findings, two possible mechanisms can be attributed to it. First, viable concentrations can be depleted as a result of a cellular membrane distortion that contributed to the loss of homeostatic balance in the bacterial cells. It could also be a result of ion release after the zinc NPs dissolution that may have triggered energy-dependent reactions within the cells. Such phenomena have been reported in other studies using silver and zinc nanoparticles. Zinc is one of the most crucial micronutrients for most organisms, as it is necessary for a majority of biochemical processes. But, when they exist in excess quantities, then they may be detrimental to critical biological pathways. Thus, zinc ions might have been absorbed through transport pathways without inflicting any significant damage to the cell membrane. However, much indirect damage might have occurred in the form of denaturation of ribosomes and enzyme and protein suppression that is responsible for cell viable concentrations – thus leading to the distortion of normal cell functions**.** In conclusion, the results sub-lethal nature of zinc oxide toxicity, as demonstrated by the diminishing of *E. coli* growth, as well as cell viability. The conclusion was attributed to a number of reasons that include less established contact between the cells and the nanoparticles. Also, other findings show that the viable concentrations are in line with dissolution configurations that take part in the releasing of ions, as demonstrated by Wilke et al. Hence, the noted differential in zinc oxide nanoparticles among different stock solutions is a result of dissolution differential caused by aqueous chemistry properties.

The findings illustrated in Fig. 11 demonstrates a nanoparticle-type effect on the production of cell viable concentrations. The outcomes in Fig. 11 reveal that viable concentrations after the exposure of nZnO is significant despite the difference in stock solution concentration. As shown in Fig. 11, nZnO was shown to influence the viable concentration levels after a 24-hour incubation period. Accordingly, the limited effects shown by viable concentrations measurement are significantly low concentration suggest that nZnO, despite its reported high toxicity, may not present a considerable danger to the aquatic microorganisms, particularly with its increased use in the field of nanotechnology.

4.7. Micro– Second Exposure

Demonstrating the impact of NPs on OD, it can be deemed necessary to examine there can be a contribution of ZnO on the cellular membrane of the microorganism under study - *E. coli.* As summarized in Fig. 12, the findings indicate a less significant transformation in *E. coli's* OD. Therefore, the results have shown no direct relation in nZnO toxicity concerning OD. In other studies, it has been stated that OD is a contributing factor to cell damage attributed to sub-lethal mechanisms of NPs resulting from metals such as silver and zinc [29]. For instance, zinc oxide nanoparticles have significantly impacted cell OD from a microorganism such as E. coli compared to control organisms without UV illumination [30]. However, the finding can be limited by lower nanoparticle concentration, particularly zinc oxide, which typically do not exceed 8 mg/L in fresh aquatic systems. In addition**,** in their research, they observed that sub-lethal effects in natural aqueous media after exposure of zinc oxide to microbial groupings at an average of 100 µg/L.

Fig. 12: Optical density against hours under different ZnO concentrations



The above findings echo that of [31], which showed no influence of ZnO on *E. coli* after it is exposed to zinc oxide nanoparticles for concentrations of between 10 and 250,000 µg/mL . Still, the results of the study showed no cytotoxic impact. Also, other findings indicate that cytotoxicity triggered by nZnO as well as damage to the cell membrane of *E. coli* in both aqueous media. However, there is no evidence of the impact of OD. Also, the findings by [32], revealed that cytotoxicity could be present. Hence, more research needs to focus on the possibility of the effects of oxidative stress on NPs toxicity and provide an insight that facilitates understanding of current research findings.

CHAPTER 5

5. IMPLEMENTATION

There is a pressing need to delve deeper into the research on NP toxicity, with interest in their impact on the environment to enable the research world to better understand the effects on microbes that are part of the ecosystem and their role in helping sustain it. The call cannot be underestimated because of the adverse effects of NPs on the environment and especially to bacteria that play a crucial role in the ecological balance. To this effect, the experiment related herein contributes to the understanding of sub-lethal effects of NPs – zinc oxide – to bacteria, particularly *E. coli,* at varying exposure concentrations in two aquatic media. The findings reveal the water chemistry differential is the critical determinant in the observed impacts of NPs on aquatic systems microorganisms because they determine the level of impact of NP change in a particular aqueous medium.

This implies that the findings from this study reveal the overarching extent to which NPs affect bioavailability in freshwater systems and the subsequent effects on microbes available therein. Hence, the role of aquatic properties needs to be keenly considered. The research world has not fully exploited the consequences of NPs to microbes in real environment settings using sufficient exposure concentration, for instance, in freshwater systems. Hence, the in-depth evaluation presented in this study incorporates a wide-ranging assessment of NPs and their impact on the environment, and especially the sub-lethal exposure of zinc oxide nanoparticles and their accompanying endpoints such as ATP production, reactive organic species, and cell viability and viability. Of note, the exhaustive nature of the chosen parameters contributes to an in-depth analysis of the adverse impacts of NPs on microbes. However, the current data insufficiencies that focus on mechanisms that influence NPs toxicity and the levels of sub-lethal exposure on microorganisms in an aqueous media limits the conclusively of the finding from other studies. Hence, the focus needs to focus on expanding the scope of identifying mechanisms that influence NPs and their impact on the environment with a particular emphasis on freshwater systems.

Moreover, the particular areas for considerations entail non-standard techniques of assessing the implications and mechanisms of toxicity at minimal NPs concentration and employing real

environment parameters. Overall, the other findings point to the fact that zinc oxide nanoparticles are increasingly posing considerable risk to bacteria using the toxicity impacts results. Moreover, the impacts of zinc oxide nanoparticles on microorganisms are concentration-dependent, where dissolved zinc ions form a basis for the observed toxicity levels. As such, the subcellular implications need to be included in other widely known endpoints to determine the true extent of NPs toxicity, as demonstrated by nZnO from other studies. However, the study findings are not conclusive or representative of every environmental condition or microorganisms present in freshwater systems. Hence, there is a need to expand the test conditions and media to include other organisms present in various water matrices. Extensive research will also generate more data using freshwater matrices to represent different exposure, taking into account multiple sub-lethal endpoints to enhance NPs risk assessment. Thus, such a comprehensive approach will help obtain exhaustive data and draw more definitive conclusions that will help manage nanoparticles and regulate their release into the environment and everything negative consequences on freshwater microorganisms.

CHAPTER 6

6. CONCLUSION

The sub-lethal exposure of bacteria to nanoparticles – zinc oxide- and the subsequent effects were assessed using the different varied freshwater systems with different physicochemical characteristics. Using the cell viability, it can be revealed that river water matrices have enhanced outcomes from the four parameters as compared to those from other water sources. The findings from other studies also demonstrated the sub-lethal exposure of nZnO to E. coli could be dependent majorly on ionic strength and natural organic matter in the water system. Notably, natural organic matter production demonstrated a diminished level for the NP – zinc oxide. Furthermore, there is no observable NP-bacterial interaction that points to the fact that dissolved ions from the metal influenced the effects of zinc oxide nanoparticles. Also, physicochemical properties greatly influenced the outcomes, as demonstrated by dissimilarities in dissolution levels in both water matrices.

Finally, the research findings show the importance of incorporating a wide range of possible endpoints to determine NPs toxicity as it provides an in-depth assessment of discrete impacts, otherwise not revealed by a narrow choice of endpoints. For instance, cell viability endpoint may not reveal the effects of NP otherwise shown by other endpoints, say ATP production or cell membrane viability. The findings also reveal a possible correlation between the observed effects of zinc oxide nanoparticles and dissolved zinc ions using a different concentration in the two water matrices. Such may mean that engineered adverse interference on metabolic mechanisms and cell membrane frameworks may lead to observed results on the microbe used - *E. coli,* but the contribution of the present nanoparticle may not be entirely excluded. Overall, some of the apparent complexities and physicochemical property differences in freshwater matrices need to be considered when determining NP toxicity to bacteria.

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