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Evaluation of the antibacterial activity of metal impregnated multi-walled carbon nanotubes: impact of domestic wastewater as supporting medium

C. de Klerk, E. Fosso-Kankeu*, F.B. Waanders

School of Chemical and Minerals Engineering, Faculty of Engineering, North-West University, Potchefstroom, South Africa, emails: kaelpfr@yahoo.fr (E. Fosso-Kankeu), Frans.Waanders@nwu.ac.za (F.B. Waanders), corlideklerk@gmail.com (C. de Klerk)

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ABSTRACT

Despite the availability of potential water treatment resources, bacterial infections are still of major concern, especially in developing countries where large numbers of people do not have access to clean and safe water. Nanotechnology offers great possibilities in the field of wastewater treatment for its potency against pathogenic bacteria. This study focuses on the antimicrobial activity of three different nano-metals (silver, copper oxide, and zinc oxide) and the additive effect when they are used in the presence of functionalized multi-walled carbon nanotubes (MWCNTs) against four commonly found domestic wastewater bacterial strains. The bacteria studied included Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative bacteria (*Salmonella typhi* and *Escherichia coli*). Silver nanoparticles (Ag NPs) were the most potent antibacterial agent reducing up to 60.78% ± 8.33% and 63.26% ± 2.12% growth of Gram-positive and Gram-negative bacteria, respectively. The effectiveness increased to 96.66% ± 1.99% against Gram-positive bacteria and 94.59% ± 1.39% against Gram-negative bacteria when the MWCNT was decorated with Ag NPs, due to the improved surface area of the synthesized complex. Additionally, it was found that the inactivation of bacteria by nanomaterials was species-specific, with Gram-negative *E. coli* showing the strongest overall resistance. The overall antibacterial effect of the synthesized compounds was stronger in real wastewater solutions while the nutrients enriched media allowed more prominent growth and bacteria recovery.

Keywords: Antibacterial compounds; Multi-walled carbon nanotubes; Silver nanoparticles; Copper oxide; Zinc oxide; Domestic wastewater treatment

1. Introduction

Significant population growth, urban migration, and extreme weather effects associated with climate change make safe water availability a critical and pressing issue. This has prompted the use of wastewater, a nontraditional water source, in major water-using sectors to benefit agriculture and industrial applications. These applications are, however, hampered by quality discrepancies, requiring high cost for effective treatment and removal of pathogenic microorganisms. Microbial inactivation is commonly the last step in the water treatment process with chlorine treatment being the most popular choice [1,2]. Alternatives include reverse osmosis, ultraviolet light treatment, and distillation [3–5]. All these processes are costly and not possible as point-of-use systems. Noteworthy research is being done to improve nanotechnology in water disinfection for safe and sufficient water supply. The ever-increasing resistance of bacteria towards commercial agents has led to severe health implications. Most infection-causing microorganisms are resistant to one or more commonly used antibiotics that could have been used to eliminate the infections. This has led to the development and expansion of technology to produce agents that can effectively inhibit pathogenic microbial growth [6–8].

Recent advances in nanotechnology have guided the development of unique engineered nanomaterial that is

* Corresponding author.

promising for addressing the challenges related to current water quality. The increased surface areas are ideal for water purification and the enhanced strength to weight properties makes it possible to develop stronger and lighter components, which could be used in current water systems [1]. Metal nanoparticles (NPs) and their derivatives have gained popularity for their antimicrobial potential. Metal NPs such as calcium oxide (CaO), copper oxide (CuO), gold (Au), magnesium oxide (MgO), silver and silver oxide (Ag and Ag_2O), and zinc oxide (ZnO) have exhibited antimicrobial activity [8–10].

Silver nanoparticles (Ag NPs) are the most commonly used nano-metal for antimicrobial studies as it has a broad antibacterial range as well as low toxicity towards humans [11]. Copper oxide nanoparticles have received more attention than any other metal since it is easier to synthesize and economical than silver and gold and it has readily available antimicrobial properties [12]. Zinc oxide (ZnO) nanoparticles have been used in antibacterial cream, ointment and as surface coatings to prevent biofilm formation [13]. ZnO nanoparticles have many attractive features, including chemical and physical stability as well as a strong toxicity towards pathogens [14]. Since ZnO nanoparticles dissolve easily, the application in water treatment is limited.

The antibacterial action of multi-walled carbon nanotubes (MWCNTs) requires direct contact between the nanotubes and the bacteria, while non-functionalized suspensions do not offer satisfactory contact between the microbes and the CNTs for effective disinfection [15].

This study investigates and discusses the antibacterial effect of these nanomaterials on commonly found microbes and the potential of newly synthesized antibacterial compounds to disinfect typical domestic wastewater.

2. Methodology

2.1. Materials, media, and bacterial strains

Copper oxide, zinc oxide, agar powder, ethanol, and N,N-dimethylformamide (DMF) were obtained from Associated Chemical Enterprises (ACE, Johannesburg, South Africa). Luria-Bertani (LB) broth and phosphate buffer tablets were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Sodium borohydride ($NaBH_4$) and silver nitrate ($AgNO_3$) were purchased from Minema Chemicals (Johannesburg, South Africa). MWCNTs were purchased from Graphene Laboratories (USA).

The following bacterial strains were investigated in this study: Gram-negative, *Escherichia coli* and *Salmonella typhi*, and Gram-positive, *Staphylococcus aureus* and *Bacillus subtilis* (Department of Microbiology, North-West University, South Africa). All the tested bacteria were grown aerobically in sterilized LB nutrient broth at a temperature of 37°C. All experiments were conducted with a newly made colloidal solution of particles and MWCNT complex. All analyses were conducted in duplicate to eliminate errors during testing.

2.2. Functionalization of carbon nanotubes

A mass of 1 g MWCNTs was immersed in a volume of 50 mL 30% nitric acid and stirred for 2 h at ambient temperature to remove impurities and increase the functional groups on the nanotube surface. The solution was sonicated for 1 h to disperse the tubes evenly and produce acidified carbon nanotubes. The solution was centrifuged and washed with deionized water until the pH reached a value of 7.0. The product was dried for 24 h at a temperature of 120°C. Similar processes were used by Masipa et al. [16] and Lukhele et al. [17].

2.3. Synthesis of silver nanoparticles

A mass of 10 mg of the acidified nanotubes and 2 mL (0.1 M) silver nitrate solution was added to 6 mL ethanol and sonicated for 1 h to decorate the MWCNTs with silver nanoparticles. The formed product was centrifuged, washed with deionized water and dried at 120°C for 12 h.

2.4. Decoration of metal oxide nanoparticles onto the MWCNTs

The metal NPs were produced by the reduction method in the presence of sodium borohydride as reducing agent. A similar method has been described in a study conducted by Masipa et al. [16]. A concentration of 0.01 M aqueous solution of copper oxide was added to 40 mL ethanol and stirred for 10 min at ambient temperature. A mass of 0.50 g functionalized MWCNTs was then added to the solution, followed by a 1:1 volume ratio of DMF as binding agent. The solution was sonicated for 1 h after which the composite was filtered and washed with deionized water, followed by a drying period of 12 h at 120°C.

2.5. Environmental wastewater sampling

Domestic wastewater was used for the investigation in this study. The rationale was to gain knowledge of the microbial population in the environmental samples using DNA sequencing and plate count techniques, prior to the determination of the performance of antimicrobial compounds in such water. Pre-treated wastewater was collected from the Tlokwe local municipality wastewater treatment facility located in Potchefstroom, South Africa. The water had only undergone primary treatment to remove the large particles in the water. Raw water samples were collected on different occasions and seasons to maximize the chances of identifying a wide variety of microbial species.

After the samples were collected, they were filtered and stored in accordance to the standard procedure for water sample handling for microbial testing as specified by Garcha et al. [18].

2.6. Characterization

2.6.1. Nanoparticle characterization

The decorated nanotubes morphology was characterized by means of scanning electron microscopy (SEM, FEI Quanta 250 FEG ESEM, Ckzech Republic). The MWCNTs supplied had a theoretical diameter span of 50–85 nm and length of 10–15 μm, with a carbon content of >94%.

2.6.2. Wastewater characterization

In order to postulate on the potential of wastewater to support bacterial growth, it was necessary to evaluate the

presence of trace metals and sugars present in the water after pretreatment.

2.6.3. Trace metal characterization

Trace metals were identified with the aid of inductively coupled plasma-optical emission spectroscopy analysis (Agilent Technologies, USA). This analysis method was used because of the advantages of rapid analysis, simplicity, and high extraction efficiency that could selectively analyze samples in the complex wastewater matrix.

2.6.4. Sugar characterization

The sugar content of the wastewater was evaluated by means of high-performance liquid chromatography (HPLC) analysis. The sampled wastewater was diluted to the required dilutions (10 and 50 dilutions) with 0.005 M H_2SO_4. The diluted samples were then filtered through a 0.2 μm micropore syringe filter into clean HPLC vials that were properly sealed before being analyzed.

2.7. Bacterial isolate determination

Filtered wastewater was appropriately diluted and plated onto the Brilliance *E. coli*/coliform (Oxoid, SA) agar plates which were incubated for 24 h at 37°C. Following incubation, the different colonies were carefully detached with a sterile metal loop, sub-cultured onto a fresh Brilliance *E. coli*/coliform (Oxoid, SA) agar plate and incubated for 16 h at 37°C. This process was repeated until only pure colonies were present on each plate. Each plate was sub-cultured onto LB agar plates and sent to Inqaba Biotec (South Africa) for DNA sequencing. This exercise allowed distinguishing the morphology and color of colonies formed by each microorganism.

2.8. DNA sequencing

The wastewater was further characterized by means of DNA sequencing. The genomic DNA extraction was performed using the ZR soil Microbe DNA MiniPrep kitTM according to the manufacturer's instructions. The samples were referred for Sanger DNA Sequencing done in a specific division of Inqaba Biotec (South Africa).

2.9. Preparation of wastewater medium and bacterial inoculum

The collected wastewater samples were sterilized in an autoclave at 121°C for 20 min and cooled to 30°C. All analyses were conducted in aseptic conditions to prevent contamination. Two loops of each bacterium were grown separately in 200 mL sterile nutrient broth at 30°C, at a constant 120 rpm for 24 h. 10 mL of each culture was transferred to 90 mL fresh broth and incubated for 12 h at similar conditions. The cells in the nutrient broth were centrifuged at 2,000 rpm for 15 min and washed with a phosphate buffer solution and centrifuged again to pellet the cells. The cells were harvested and suspended in a 3 mL aliquot of the sterile wastewater solution and vortexed to obtain complete dispersion. The aliquot was added to 97 mL sterile wastewater and grown for 12 h at 30°C. Prior to testing, cell viability was confirmed by culturing the sample on an agar plate. A duplicate plate was used as the control for each sample.

2.10. Antimicrobial test in sterilized wastewater medium

In order to assess the antibacterial efficacy of the metal NPs on the tested microorganisms, controlled amounts of antimicrobial compound were added to the various prepared sterilized wastewater suspensions for a specified period. The optical density (OD_{600}) was assessed to establish microbial cell growth, followed by disc diffusion and broth dilution examinations. The results were compared with that of the nutrient rich agar medium used as the control.

2.10.1. Disc diffusion method

Blank sterile diffusion discs (6 mm, Davies Diagnostics [Pty] Ltd, South Africa) were impregnated with 15 μL of known concentrations of the synthesized compounds in order to obtain discs of 800, 400, 200, 100, 50, 25, and 12.5 μg/disc of each compound. The discs were dried at 30°C and placed on a freshly swabbed agar surface. The plates were left for 10 min to fix the disc in place and incubated in an inverted position for 24 h at a temperature of 37°C. All the disc diffusion evaluations were conducted in double quadruplicate (two sets of experiments, each containing four discs with identical concentration of the same compound), and commercial compounds (carbenicillin 100 μg/disc and vancomycin 30 μg/disc) were used as the positive controls. The clear zone was conveyed as an average of reproduced measurements.

2.10.2. Broth dilution method

The tested solutions were twofold serially diluted in LB nutrient broth in sterile screw cap test tubes that contained experimental concentrations (10–800 μg/mL). 2 mL overnight culture (106 CFU/mL) of each bacterium was added to the test tubes to yield a final volume of 4 mL. The solutions were incubated for 18 h at 37°C. The solution in each tube was serially diluted and spread on LB agar plates to be incubated for 24 h at 37°C. Viable colonies were manually counted and recorded. The minimum inhibitory concentrations (MICs) were determined as the lowest concentration of compound that prevents visible growth and the minimum bactericidal concentrations (MBCs) were determined as the highest dilution of the compound that killed more than 99.9% of the considered bacteria. The experiments were conducted in triplicate and average values were reported.

In order to determine the effect of different ZnO sizes, ZnO powder was crushed to various extents and used for growth inhibition assays on the bacteria. Various concentrations were added to freshly inoculated nutrient broth and wastewater and prepared according to the broth dilution procedure.

3. Results and discussion

3.1. Characterization

3.1.1. Untreated MWCNTs

The untreated MWCNTs initially exhibited the tendency to agglomerate (Fig. 1(A)), which was solved by short periods of sonication (Fig. 1(B)).

The untreated MWCNTs showed diameters in the range of 20–70 nm (Fig. 2(A)). The effect of functionalization is depicted in Fig. 2(B), where the prominent white areas are the active sites where binding on the metal NPs is most likely to occur.

3.2. Functionalization of MWCNTs

The acid treatment removed the impurities in the form of amorphous carbon particles and introduced the –COOH groups to the surface of the nanotubes [19]. The functionalization process increased the solubility of the MWCNTs, indicating the presence of polar functional groups. Without functionalization, MWCNTs are chemically inert and functionalization provides the oxygen containing groups to form active sites to attach the metal nanoparticles as described by Chen et al. [20].

3.3. Decoration of metal nanoparticles on MWCNTs

3.3.1. Silver nanoparticles

The synthesis of Ag NPs decorated onto the MWCNT surface indicated strong morphological alterations at different reaction conditions (Fig. 3(A)). At increased reaction times, the particles were large and insufficient attachment could be observed.

3.3.2. Metal oxide nanoparticles

Successful dispersion of the CuO (Fig. 3(B)) and ZnO nanoparticles in the MWCNT matrix was achieved. The surface morphology and the high availability of contact area of CuO and ZnO make it suitable for effective contact between the bacteria and the NP.

3.4. Wastewater metal trace element characterization

The metal trace elements found in the wastewater are summarized in Table 1. Some of these metals can also act as inhibitors to the growth of bacteria and, in some cases, can be toxic. Magnesium and sodium were found to be the most common elements detected in domestic wastewater. Sodium and sodium containing compounds can have detrimental effects on the bacteria at high concentrations. Magnesium, on the other hand, can be sourced from fertilizer used in agricultural practices that can stimulate potential cell growth and increase oxygen demand [21]. It was also established that the addition of magnesium compounds to wastewater could result in the removal of nutrients by the nitrification–denitrification process where the released ammonium is consumed by anammox bacteria, derived from anaerobic ammonium oxidation bacteria [22].

Arsenic was also present at high concentration and could originate from glass, ceramics, and pesticides. This compound is toxic and would lead to retarded bacterial growth. However,

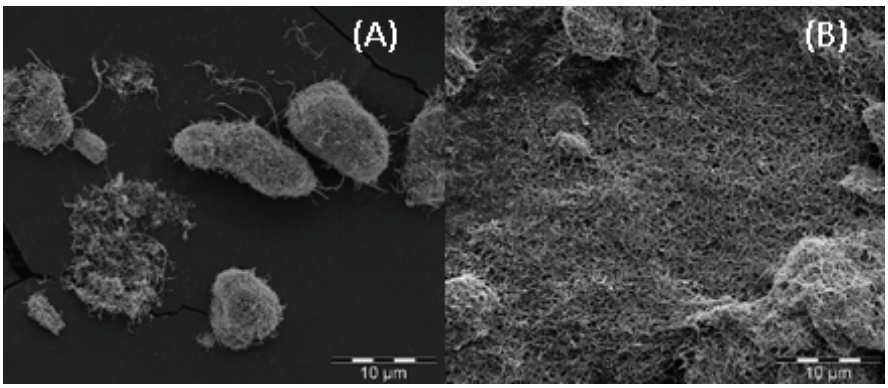

Fig. 1. SEM characterization of MWCNTs. (A) The MWCNTs before treatment exhibited the tendency to agglomerate, decreasing the functionalization capabilities. (B) Effect of 15 min of sonication to separate the nanotubes sufficiently.

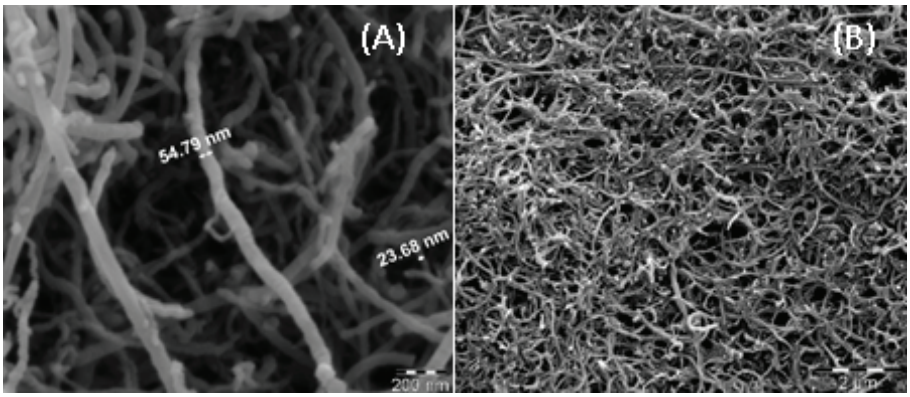

Fig. 2. Size characterization of the MWCNTs at 200 nm magnification (A). (B) The functionalization effect on the MWCNTs after sonication.

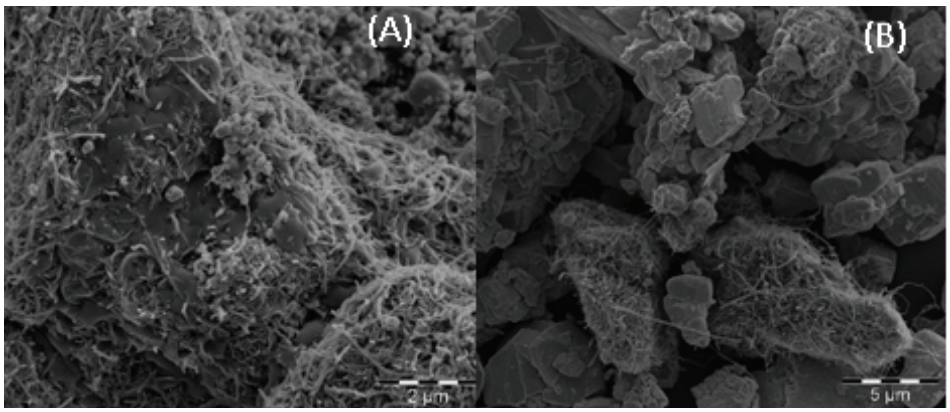

Fig. 3. SEM characterization of (A) silver particles in the presence of the MWCNTs and (B) CuO particles in the presence of MWCNTs.

Table 1
Trace elements' concentrations (parts per million) in wastewater

Element	Concentration	Effect on wastewater bacteria	Reference
Silver	0.048	Cellular damage, growth inhibition and cell lysis. Biofilm is highly resistant to silver NPs.	[24]
Arsenic	9.818	Toxic implications to natural occurring bacteria and reduction in overall water quality. Could lead to an increase of arsenic respiring bacteria.	[25,26]
Calcium	63.513	Increased growth of biofilms and algae settlements. Removal of algae settlements and biofilms can aid bacterial reduction in wastewater systems.	[27]
Cadmium	0.468	Extensive damage and toxicity to natural occurring bacteria, damage to human organs and animal well-being.	[28,29]
Cobalt	0.072	Toxicity towards bacteria at elevated concentrations.	[30–32]
Potassium	11.989	Increased bacterial growth rates at elevated potassium concentrations.	[33]
Magnesium	70.162	Stimulates cell synthesis, growth and cellular division.	[34]
Manganese	0.026	Toxicity towards bacteria at elevated concentrations.	[31,35]
Sodium	69.684	Sodium containing compounds exhibit strong antibacterial effects at variable concentrations.	[36]
Nickel	0.928	Antibacterial activity of metal accumulation that result in the disruption of the cell wall and damage to the cellular constituents.	[32,37]
Lead	2.550	Toxic effect towards plants, humans, animals, and microorganisms.	[38]

it is critical that the wastewater receives extensive treatment to ensure arsenic is not present in the final treated water to maintain the quality standards according the legislation [23].

3.5. Wastewater sugar content characterization

HPLC analysis detected no significant sugar concentrations for both the 50 and 10 times dilutions. The results could only be associated with oligomers or non-sugar components. The component detected may be associated with glucose (0.045 g/L glucose); however, this is within the noise region and might as well be as a result of noise in analysis. The same conclusion could be made for arabinose at a concentration of 0.076 g/L.

3.6. Wastewater bacterial characterization

Table 2 shows the bacteria that were present in the wastewater samples as determined from the heterotrophic plate counts. The table also describes the relevant health risk that each microbe poses in case of human consumption.

The data are presented from an average of duplicate analysis and standard deviation.

From Fig. 4 it can be stated that growth in the pretreated wastewater showed a longer lag phase, minimal overall growth and a faster death phase. This phenomenon was ascribed to the low nutrient environment compared with that of the nutrient broth.

3.7. Antibacterial evaluations

The antibacterial activity of the synthesized complexes was determined to be specific to the bacterial strain analyzed. The bacterial samples showed different levels of sensitivity towards the metal NPs decorated on the MWCNTs. Filtered wastewater samples plated on coliform agar allowed the determination of the microbial ecology of untreated water. Treated wastewater (Table 3) showed significant colony

Table 2
Bacteria found in wastewater determined from DNA sequencing and the health threat it poses to human

Bacteria	Disease/health threat	Reference
Bacillus subtilis	Allergic reactions	[39,40]
Enterobacter cloacae	Bacteremia, endocarditis, lower respiratory tract infections, urinary tract infections, non-traumatic pneumocephalus	[41]
Escherichia coli	Diarrhea, hemorrhagic colitis, neonatal meningitis, urinary tract infections	[42,43]
Enterobacter hormaechei	Nosocomial infections	[44]
Aeromonas piscicola	Septicemia	[45]
Aeromonas salmonicida	Furunculosis	[46]
Aeromonas veronii	Gastroenteritis	[47]
Salmonella spp.	Salmonellosis, septicemia, gastroenteritis	[48]

reductions in the presence of metal impregnated MWCNTs, samples A and B were taken in summer and the temperatures were recorded as 19°C–24°C. Sample C was taken in the winter at a temperature of 12°C.

The functionalized MWCNTs reduced the growth potential (Table 4) of all tested bacterial strains. This was a consequence of the bacterial cells bonding to the MWCNT surface due to the weak electrostatic forces between the bacterial cell wall and the carbon atom surface [49]. However, from the regrowth analysis, it was observed that the cell viability increased over time, therefore, the contact time between the cells and antimicrobial compound was weak and of short duration. *E. coli* showed to be the most susceptible (40.71% ± 5.32% inhibition) and *Salmonella typhi* proved to be the most resilient (25.30% ± 3.87% inhibition). There was a substantial improvement in the performances of the functionalized MWCNTs with the addition of the synthesized metal NPs to the complex.

The nano-silver particles decorated onto the MWCNTs exhibited the strongest antibacterial properties (Tables 3 and 4). The antibacterial mechanism was attributed to the interaction between the bacterial cell membrane and the metal particle surface. The metal NPs make contact with the lipopolysaccharide layer on the exterior of the Gram-negative cell wall [50], resulting in a cell viability reduction of up to 94.59% ± 1.39% and 92.08% ± 2.49% for *E. coli* and *S. typhi* in nutrient rich media. These lipopolysaccharides contain lipids and

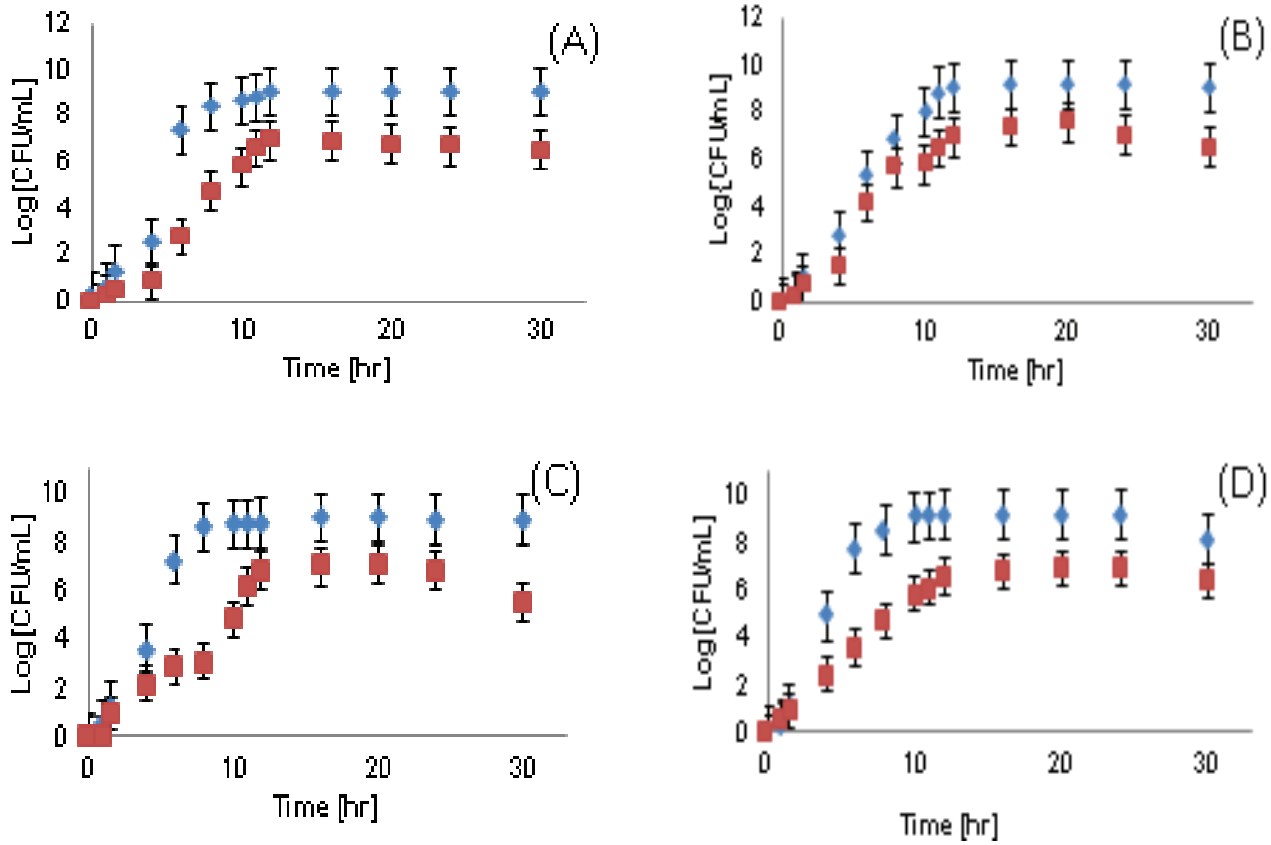

Fig. 4. Growth curves of bacteria in (♦) nutrient broth and (■) in wastewater to estimate the reduction in cell growth and behaviour over time. (A) *Salmonella typhi*, (B) *Escherichia coli*, (C) *Bacillus subtilis*, and (D) *Staphylococcus aureus*.

Table 3
Bacterial cell concentrations in wastewater before and after treatment with the synthesized antimicrobial complexes

Sample name	Control	MWCNT-Ag	MWCNT-CuO	MWCNT-ZnO
Sample A	9.88	1.81	1.36	1.15
Sample B	10.79	1.40	2.08	0.90
Sample C	9.41	0.69	1.93	1.23

Note: Data are presented as log (CFU/mL) to denote microbial concentration. The control sample was prepared without any antimicrobial compound.

Table 4
Concentration of the tested bacteria after 8 h of incubation

Synthesized complex	% Inhibition in nutrient broth	% Inhibition in wastewater
Escherichia coli		
Functionalized MWCNTs	10.59 ± 6.48	40.71 ± 5.32
MWCNT-CuO	51.40 ± 6.07	60.50 ± 3.60
MWCNT-ZnO	62.68 ± 2.12	73.47 ± 4.24
MWCNT-Ag	94.59 ± 1.39	96.23 ± 2.43
Salmonella typhi		
Functionalized MWCNTs	11.14 ± 4.68	25.30 ± 3.87
MWCNT-CuO	18.42 ± 5.65	32.21 ± 4.82
MWCNT-ZnO	35.21 ± 7.87	76.73 ± 9.62
MWCNT-Ag	92.08 ± 2.49	96.43 ± 2.40
Bacillus subtilis		
Functionalized MWCNTs	11.11 ± 8.34	43.65 ± 4.09
MWCNT-CuO	61.76 ± 1.45	76.26 ± 1.52
MWCNT-ZnO	59.48 ± 1.70	92.29 ± 1.26
MWCNT-Ag	96.66 ± 1.99	99.51 ± 0.48
Staphylococcus aureus		
Functionalized MWCNTs	10.89 ± 1.80	37.80 ± 7.96
MWCNT-CuO	35.06 ± 4.07	79.09 ± 1.99
MWCNT-ZnO	63.01 ± 3.92	88.15 ± 2.44
MWCNT-Ag	93.34 ± 1.52	98.29 ± 0.10

Note: The resultant inhibition activities of the synthesized antimicrobial complexes in a nutrient rich environment compared with the pretreated wastewater environment.

polysaccharides that have a shortage of strength and rigidity; therefore, the metal nanoparticle interaction will lead to cell degradation, cellular leakage and cell lysis [51]. The inhibition of bacteria in the pretreated wastewater was found to be higher than in nutrient broth media irrespective of the type of antimicrobial compound used. This showed that the antimicrobial test in the enriched nutrient does not provide a clear estimation of the performance of the tested compound, because the nutrients available in such medium allow the microorganism to gather sufficient energy and recover from the damage caused by the compound; while the environmental water seems not to provide enough support for survival of microorganisms which become more susceptible to the inhibitory effect of the tested compounds. The HPLC analysis (section 2.6.4) on the wastewater revealed the presence of only negligible amount of sugar compounds and would not provide the necessary energy for the bacterial cells to recover after it has been damaged. Furthermore, the presence of trace metals in the water would also inhibit the growth and multiplication of microorganisms, since most of the metals that were identified are toxic and would harm the bacterial cells.

Large inhibition zones could be observed when all the bacteria were exposed to lower concentrations of Ag-MWCNTs (50–100 μg/disc) and maximum clear zones were determined as 19.67 ± 0.16 mm for *B. subtilis*, 18.38 ± 2.38 mm for *Staphylococcus aureus*, 17.79 ± 0.62 mm for *Salmonella typhi*, and 17.38 ± 0.81 mm to the most resistant bacteria, *E. coli* in the nutrient rich growth medium (Table 5).

Even though CuO NPs showed evidence of antibacterial activity, the toxicity was inferior to the other metal NPs tested. Pristine CuO particles demonstrated little antibacterial action against the Gram-positive as well as the Gram-negative bacteria with MBCs ranging from 1,000 μg/mL to more than 2,000 μg/mL, while the MIC was observed at a concentration of 400 μg/disc. The combination of CuO and MWCNTs reduced the MBC value to around 500 μg/mL and larger inhibitory zones were observed, especially in the pretreated wastewater samples (Table 6). It is suggested that the antibacterial effect is due to the accumulation of particles on the bacterial cell surface, depriving the cells of nutrients and damaging the cell membrane. The metal ions in solution can cause cytotoxicity through interaction with the cell membrane or intracellular damage [52–55]. Table 5 indicates CuO produced smaller inhibition zones in a high nutrient environment with stronger cell viability.

Lower concentrations of ZnO had no visible effect from the disc diffusion method. The results indicated that ZnO particles exhibited effective inhibitory action towards both Gram-positive and Gram-negative bacteria at higher concentrations. Additionally, the effect was more prominent towards Gram-positive bacteria (*Staphylococcus aureus* 13.59 ± 0.56 mm and *B. subtilis* 12.92 ± 1.06 mm) while Gram-negative bacteria showed clear zones up to 29%. Pristine ZnO NPs have shown to be bacteriostatic rather than bactericidal. This observation was also reported after a study by Baek and An [56]. The antibacterial mechanism of ZnO is not fully understood, but Dijaz et al. [8] suggested that the antibacterial activity generates hydrogen peroxide that is toxic to bacteria. The synthesized complex of ZnO-MWCNTs showed significant inhibition with *Salmonella typhi* being the most susceptible in the pretreated wastewater milieu (Table 5).

It can, therefore, be concluded that the addition of functionalized MWCNTs enhanced the antibacterial activity of metal oxide nanoparticles against the bacterial cells, resulting in much lower MIC and MBC values, as well as larger inhibitory zones (Tables 5 and 6).

Table 5
MIC and MBC values of the synthesized antimicrobial complexes in the nutrient rich environment

Microorganism	NP complex	MIC (μg/disc)	Maximum inhibition zone (mm)	MBC (μg/mL)
Escherichia coli	Functionalized MWCNT	400	8.25 ± 0.47	>2,000
	Ag NP-MWCNT	50	17.38 ± 0.81	200
	CuO-MWCNT	200	14.54 ± 0.66	400
	ZnO-MWCNT	100	12.96 ± 0.41	50
Salmonella typhi	Functionalized MWCNT	800	9.17 ± 0.66	>2,000
	Ag NP-MWCNT	25	17.79 ± 0.62	100
	CuO-MWCNT	200	14.88 ± 0.54	400
	ZnO-MWCNT	100	14.67 ± 0.39	100
Staphylococcus aureus	Functionalized MWCNT	400	10.13 ± 0.39	>2,000
	Ag NP-MWCNT	25	18.38 ± 2.38	50
	CuO-MWCNT	100	14.79 ± 0.51	200
	ZnO-MWCNT	150	15.38 ± 0.37	25
Bacillus subtilis	Functionalized MWCNT	400	9.13 ± 0.41	>2,000
	Ag NP-MWCNT	12.5	19.67 ± 0.16	100
	CuO-MWCNT	100	16.00 ± 0.81	200
	ZnO-MWCNT	100	13.04 ± 1.04	25

Note: Data are represented as the average from duplicate analysis. Control compound inhibition zones resulted in 18.30 ± 0.83 mm and 24.80 ± 1.09 mm for Gram-positive *Staphylococcus aureus* and *B. subtilis* using vancomycin, and 30.00 ± 1.22 mm and 34.80 ± 1.09 mm for Gram-negative *Salmonella typhi* and *E. coli* using carbenicillin as control.

Table 6
MIC, MBC, and maximum inhibition zone values of the synthesized antimicrobial complexes in the nutrient poor wastewater

Microorganism	NP complex	MIC (μg/disc)	Maximum inhibition zone (mm)	MBC (μg/mL)
Escherichia coli	Functionalized MWCNT	100	13.33 ± 0.62	50
	Ag NP-MWCNT	25	20.83 ± 1.31	12.5
	CuO-MWCNT	100	18.83 ± 0.62	50
	ZnO-MWCNT	50	19.50 ± 0.82	50
Salmonella typhi	Functionalized MWCNT	200	12.25 ± 0.61	50
	Ag NP-MWCNT	25	22.00 ± 1.78	12.5
	CuO-MWCNT	50	18.33 ± 1.03	25
	ZnO-MWCNT	50	21.08 ± 1.48	25
Staphylococcus aureus	Functionalized MWCNT	100	12.58 ± 0.66	100
	Ag NP-MWCNT	12.5	23.25 ± 0.61	6.25
	CuO-MWCNT	100	16.92 ± 0.42	50
	ZnO-MWCNT	100	18.08 ± 1.39	50
Bacillus subtilis	Functionalized MWCNT	200	11.58 ± 1.05	100
	Ag NP-MWCNT	12.5	22.58 ± 0.77	6.25
	CuO-MWCNT	50	17.75 ± 0.54	12.5
	ZnO-MWCNT	50	18.25 ± 1.54	25

Note: Data are represented as the average from duplicate analysis.

4. Conclusion

The order of antibacterial activity of nanomaterials was Ag NP-MWCNT > ZnO MWCNT > CuO MWCNT. It was found that the size and shape of the NPs could also influence the antimicrobial efficacy of the compound, as acid treatment of MWCNT resulted in improved potency of the complex compound. A similar trend was also observed from the single metal compound and has been established by Azam et al. [6] as well as Raghupati et al. [57]. In this study, silver nanoparticles and ZnO exhibited excellent inhibitory efficiencies that increased substantially with the addition of functionalized carbon nanotubes. Enough contact time was also required, as maximum inhibition of bacteria was achieved only after 8 h. The finding in this study suggests that antibacterial test using enriched media tends to underestimate the potency of the examined compounds, which are more likely to present improved results when tested in the natural environment of microorganisms. Therefore, it is important to supplement standard laboratory test methods with direct

applications of antimicrobial compounds to the targeted microbial source for reliable results.

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