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Bacteriostatic impact of nanoscale zero-valent iron against pathogenic bacteria in the municipal wastewater

Ahmed H. Sadek[1,2] · Mohsen S. Asker[3] · Sayeda A. Abdelhamid[3]

Abstract

Nanoscale zero-valent iron particles were investigated as an antibacterial agent against two Gram-positive bacteria; *Staphylococcus aureus* NRRL B-313 (*S. aureus*), *Bacillus subtilus* NRC (*B. subtilus*), and two Gram-negative bacteria; *Escherichia coli* NRC B-3703 (*E. coli*), *Pseudomonas aeruginosa* NRC B-32 (*Ps. aeruginosa*). The characterization of synthesized nZVI particles was obtained by XRD, SEM, EDX, and TG analyses. The results demonstrated that the nZVI particles have a spherical shape, mean crystalline size of 44.43 nm, and exhibited a good chemical and thermal stability performance under different physical conditions. The bacterial suspensions were subjected to the treatment using nZVI particle suspensions with a concentration of 10 mg/mL. The minimum inhibitory concentration of nZVI particles was determined using the well diffusion assay method and found to be 15, 10, 10, and 5 mg for the following four strains; *S. aureus*, *B. subtilus*, *E. coli*, and *Ps. aeruginosa*, respectively. The biological treatment results of municipal wastewater using nZVI particles revealed that the counts of total bacteria, total coliform, fecal coliform, *S. aureus*, *fecal Streptococcus*, and *E. coli* were decreased to 44.29%, 51.76%, 90.95%, 46.67%, 33.33%, and 93.89%, respectively, while the *Ps. aeruginosa* not detected in the wastewater sample. The enhanced inactivation performance of nZVI nanoparticles was mainly attributed to the reactive oxygen species (ROS) production, releasing of iron corrosion products like Fe^{2+}/Fe^{3+} ions, and direct friction of nZVI particles with bacterial cells membranes. In addition, the nZVI particles presented a striking disinfection behavior in comparison with other widespread disinfection technologies such as chlorination. Accordingly, the obtained results introduce the nZVI particles as a promising disinfection technology.

Keywords Antimicrobial activity · Microbial contamination · *Escherichia coli* · *Pseudomonads* · Municipal wastewater · Nanoscale zero-valent iron · Pathogens bacteria

Abbreviations

B. subtilus	Bacillus Subtilus
CFU	Colony Forming Units
DDI	Double-Deionized Water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
EC	*Escherichia coli*
EDX	Energy-Dispersive X-ray Spectroscopy
FAO	Food and Agriculture Organization
FE-SEM	Field-Emission Scanning Electron Microscope
FC	Fecal Coliform
Gram (+)	Gram-Positive Bacteria
Gram (-)	Gram-Negative Bacteria
ISO	International Organization for Standardization
LSB	Lauryl Sulphate Broth
MF	Membrane Filter
MIC	Minimum Inhibitory Concentration
MLS	Membrane Lauryl Sulfate
MPN	Most Probable Number
ND	Not Detected

✉ Ahmed H. Sadek
ahsadek@zewailcity.edu.eg

1 Environmental Engineering Program, Zewail City of Science, Technology and Innovation, 6th October City, Giza 12578, Egypt

2 Sanitary and Environmental Engineering Research Institute, Housing and Building National Research Center (HBRC), Dokki, Giza 11511, Egypt

3 Microbial Biotechnology Department, National Research Centre (NRC), Dokki, Cairo 12622, Egypt

NPs	Nanoparticles
nZVI	Nanoscale Zero-Valent Iron
OD	Optical Density Assay
Ps. aeruginosa	*Pseudomonas aeruginosa*
ROS	Reactive Oxygen Species
S. aureus	*Staphylococcus aureus*
TC	Total Coliform
TG	Thermogravimetric
USEPA	U.S. Environmental Protection Agency
WHO	World Health Organization
XRD	X-ray Diffraction

Symbols

Dp Crystalline diameter of particle, nm

Greek letters

λ CuKα radiation wavelength, Å

β Full width at half maximum (FWHM), radians

θ Bragg angle, degree

Introduction

Various forms of metal and metal oxide nanoparticles have been investigated as antimicrobial agents (Cheeseman et al. 2020; Alt et al. 2004). These compounds have possessed a wide range of applications as disinfectants in the water treatment (Chou et al. 2005), in the biomedical fields (Alt et al. 2004; Furno et al. 2004), the textiles (Jeong et al. 2005; Hamdy et al. 2018), anti-biofilm agents (Sambhy et al. 2006), and as catalysts (Elawwad et al. 2020).

The presence of pathogenic microorganisms in the water sources represents various health risks, where many infectious disease microbes affecting individuals in a community can find their way into municipal sewage. Most of the organisms found in untreated wastewater are known as enteric organisms; they inhabit the intestinal tract where they can cause diseases, such as diarrhea (Al-Gheethi et al. 2018). Table 1 lists the infectious agents potentially present in raw domestic wastewater and the diseases associated with each organism. Montgomery and Elimelech (2007) have indicated that many people die annually from the diseases transmitted over contaminated water. Among these pollutants, those water-borne fungi, bacteria, and viruses showed great danger (Khezerlou et al. 2018; Pandey et al. 2014). Besides, it was found that during the wastewater treatment, the generated biosolids contained a variety of microorganisms (X-q et al. 2007; Paez-Rubio et al. 2007). When these bio-solids have been disposed to the waterways, those microbial species can transport over large distances, thus presenting serious health problems (Paez-Rubio et al. 2007). Whereas the adequate treatment of excreta and wastewater to protect the natural environment besides reducing water wastage and avoiding overexploitation fall among the direct goals of sustainable development according to UNESCO reports. Therefore, in the absence of proper drainage systems, sewage mixes with stormwater causing further pollution. It is estimated that up to 90% of all wastewater in developing countries is discharged untreated directly into rivers, lakes, or the oceans, causing major environmental and health risks (Connor 2015). Consequently, there was a great need for the biological pretreatment for the drinking- and wastewater, which helps in minimizing the associated biological health hazard for these streams.

The metal/metal oxides nanoparticles such as magnesium oxide (Stoimenov et al. 2002), magnetite (Hamdy 2021), copper (Hsiao et al. 2006), zinc oxide (Sánchez-López et al. 2020), and silver (Kaur et al. 2013; Sambhy et al. 2006; Morones et al. 2005) have exhibited remarkable antimicrobial properties. However, the antimicrobial activity mechanism of these compounds was still not clearly understood. Hence, a diversity of hypotheses had been proposed, including cell structures physical disruption (Stoimenov et al. 2002), permeability and respiration disturbance (Morones et al. 2005), and damage of the enzymatic proteins or the DNA by the metal ions that release from the nanoparticles (Gogoi et al. 2006). Among metal nanoparticles, the nZVI nanoparticles had been utilized in many fields such as the permeable reactive barriers for remediation of the groundwater contaminated with halogenated solvents (Farrell et al. 2000), and removal of heavy metals (Hamdy et al. 2021).

when Fe^0 nanoparticles were used, the electron directly transferred from the metallic iron to the contaminants had been recognized as the main pathway of contaminant transformation in the subsurface (Hamdy et al. 2019a). The contaminants could also be oxidized in the presence of oxygen by hydroxyl radical and other oxidants generated during the corrosion process of Fe^0 nanoparticles (Joo et al. 2005; Hamdy et al. 2019b). Accordingly, the nZVI nanoparticles had exhibited that it was a strong substitute for the granular Fe^0 particles (Li et al. 2006). The nZVI nanoparticles have been distinguished from the granular Fe^0 particles due to the improvement in their high mobility and reactivity, because of their higher surface area (Li et al. 2006; Hydutsky et al. 2007). Many studies were interested in the antimicrobial activity of metal nanoparticles and especially the widespread usage of nZVI nanoparticles for environmental remediation. Therefore, the nZVI nanoparticles were subjected to the investigation as an antimicrobial agent (Sun et al. 2019; Chen et al. 2013; Diao and Yao 2009). Furthermore, the previous investigators have mentioned the possibility of using the other kinds of iron-based compounds like microscale iron powder as bacteriophage inactivators (You et al. 2005), or iron oxide-coated sands (Ryan et al. 2002). However, the antimicrobial activity of these compounds demands relatively long treatment periods (for days or weeks), or high doses to be effective. Besides, it has an insignificant impact compared to nZVI particles.

Table 1 Waterborne infectious pathogen bacteria potentially present in untreated domestic wastewater and their health effects in water supplies (EPA 2012; Water and Organization 2006; Division et al. 2004)

Pathogen Bacteria	Health Significance	Persistence in Water Supplies [a]	Resistance to Chlorine [b]	Relative Infectivity [c]	Diseases	Numbers in Raw Wastewater (per liter)
Burkholderia pseudomallei	Low	May multiply	Low	Low	Melioidosis also called Whitmore's disease	
Campylobacter jejuni, C. coli	High	Moderate	Low	Moderate	Gastroenteritis, reactive arthritis	
E. coli –Pathogenic [d]	High	Moderate	Low	Low	Gastroenteritis and septicemia, hemolytic uremic syndrome (HUS)	
E. coli – Enterohaemorrhagic	High	Moderate	Low	High	Bloody diarrhea	
Legionella spp.	High	Multiply	Low	Moderate	Respiratory illness (pneumonia, Pontiac fever)	
Non-tuberculous mycobacteria	Low	Multiply	High	Low	Pulmonary disease resembling tuberculosis, lymphadenitis, skin disease	
Pseudomonas aeruginosa [e]	Moderate	May multiply	Moderate	Low	Skin, eye, ear infections	
Salmonella typhi	High	Moderate	Low	Low	Typhoid fever	Up to 10^5
Other salmonellae	High	May multiply	Low	Low	Salmonellosis	
Shigella spp.	High	Short	Low	Moderate	Shigellosis (bacillary dysentery)	Up to 10^4
Vibrio cholerae	High	Short	Low	Low	Cholera	Up to 10^5
Yersinia enterocolitica	High	Long	Low	Low	Yersiniosis	

[a] Detection period for the infective stage in water at 20 °C: short, up to 1 week; moderate, 1 week to 1 month; long, over 1 month

[b] When the infective stage is freely suspended in water treated at conventional doses and contact times. Resistance moderate, the agent may not be completely destroyed

[c] From experiments with human volunteers or from epidemiological evidence

[d] Includes enteropathogenic, enterotoxigenic, and enteroinvasive

[e] Main route of infection is by skin contact but can infect immunosuppressed or cancer patients orally

Several previous studies report that the magnetic properties of nZVI resulted in significant bacterial removal, and the removal efficiency largely depends on the Fe^0/Fe_3O_4 shell compositions, bacteria type, and bacteria concentrations. ROS generally includes superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) (oxidation potential 1.8 V), and hydroxyl radical ($\cdot OH$) which are the most harmful radicals. Usually, the hydroxyl radicals form in the presence of transition metals such as iron during the redox cycle (Gold et al. 2018; Arakha et al. 2015). To defend against oxidative stress induced by reactive oxygen species (ROS), bacteria have developed their fighting mechanisms. In response to excess iron levels, bacteria not only regulate mineral balance through the use of metalloregulatory proteins, but also by releasing ROS scavengers such as peroxidases, catalase and, superoxide dismutase (SOD), or by complexing Fe^{2+} ions into an inoffensive form (Diao and Yao 2009; Miethke and Marahiel 2007). To regulate iron levels, the bacteria use a highly specific transport system for storage high levels of iron ions through secreting low molecular weight compounds and the siderophores, which bind to Fe^{3+} ions, and the ferric siderophores are then actively transported back into bacterial cells. Whilst, when the iron is in excess, the inactivation of bacteria occurs due to the formation and accumulation of hydroxyl radicals (Xu et al. 2019). Another study indicated that superoxide (produced by aerobic organisms) could accelerate DNA damage by raising levels of free iron in cells indicating that control of iron balance and responses to oxidative stress are actively coordinated by cells (Oprčkal et al. 2017).

In this study, we aimed to examine the synthesized nZVI nanoparticles as a bactericidal agent with various dosages versus the Gram (+) bacteria (*S. aureus* and *B. subtilus*) and the Gram (−) bacteria (*E. coli* and *Ps. aeruginosa*). Synthesis and precise characterization of nZVI nanoparticles using X-ray diffraction (XRD), scanning electron microscope (SEM), energy-dispersive X-ray (EDX), and thermogravimetric (TG) analyzers were reported as well as their chemical and thermal properties. The growth inhibition and the microbial inactivation mechanism of nZVI nanoparticles against different pathogen bacteria which including the release of Fe^{2+} and Fe^{3+} ions, ROS formation, and direct contact of nZVI particles with bacteria membranes were discussed in details. Furthermore, the sterilization effect and bioactivity of nZVI nanoparticles towards pathogenic sewage wastewater-bacteria, and their capability to dispose of these microorganisms from the real samples were studied besides comparing the antibacterial effect of nZVI particles with common wastewater treatment/disinfection technologies. In addition, from a microbiological standpoint, the results of antimicrobial activity of nZVI nanoparticles against water-contamination indicators (coliforms and *E. coil*) were scrutinized and were compared with limits of the international standards that regulate
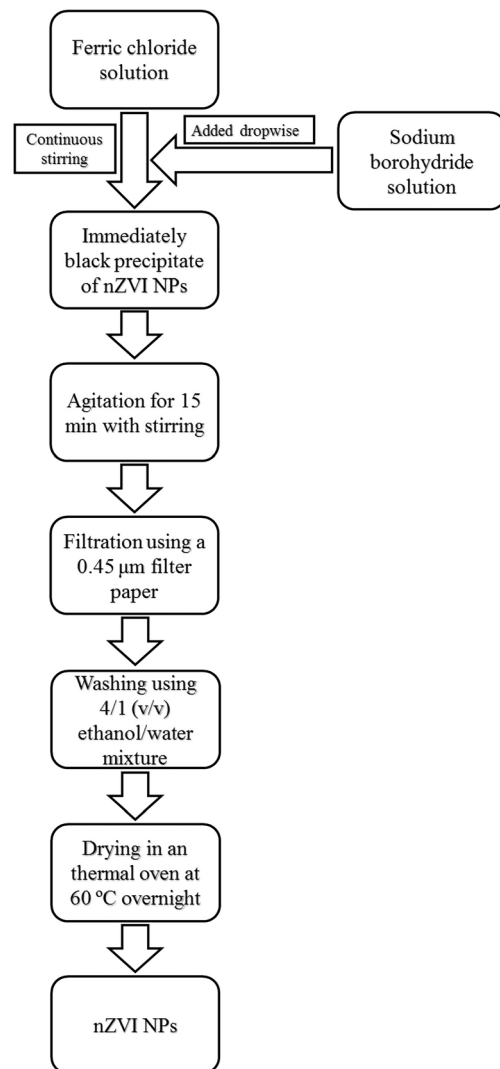

Fig. 1 Flowchart for synthesis process of nZVI nanoparticles

the reuse of reclaimed water in the different purposes for example; the agriculture and irrigation. Finally, the findings from this work provided further evidence that makes the nZVI nanoparticles are a promising antimicrobial agent for water disinfection.

Materials and methods

Synthesis of antimicrobial nZVI nanoparticles

An analytical grade of ferric chloride ($FeCl_3.6H_2O$, Loba Chemie, India), and sodium borohydride ($NaBH_4$, Winlab Co., UK) were used without further purification for the synthesis process of nZVI nanoparticles. All reagents were prepared using double-deionized water (DDI) throughout this study. The nZVI nanoparticles were synthesized by the

chemical reduction method via the reduction of ferric ions using the borohydride agent. 0.5 g of ferric chloride was dissolved in a 4/1 (v/v) ethanol/water mixture. About 0.33 mol/L of sodium borohydride solution was poured into a burette and then added dropwise to the Fe(III) solution with continuous stirring of the mixture. Once drops of the borohydride solution were added to the ferric ions solution, a black precipitate of nZVI nanoparticles appeared. When the borohydride solution was completely added, the prepared nZVI nanoparticles were left for 15 min with stirring. Subsequently, the formed nZVI nanoparticles have separated by vacuum filtration using a 0.45 μm Whatman filter paper. Then, the precipitate had been washed with 4/1 (v/v) ethanol/water mixture followed by washing three times with ethanol only. Finally, the synthesized nZVI nanoparticles were undergone dried in a thermal oven at 60 °C overnight. The redox reaction can be represented by eq. (1):
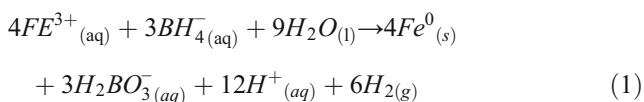
$$4FE^{3+}_{(aq)} + 3BH^-_{4\,(aq)} + 9H_2O_{(l)} \rightarrow 4Fe^0_{(s)}$$
$$+ 3H_2BO^-_{3\,(aq)} + 12H^+_{(aq)} + 6H_{2(g)} \tag{1}$$

Figure 1 represents a flowchart for the synthesis process of nZVI nanoparticles.

Characterization apparatuses

X-ray diffraction instrument (X-Pert – PRO – PANalytical Netherland) was used to perform an XRD analysis of the nZVI particles at 30 mA and 45 kV. CuKα radiation and graphite monochromator were used to yield X-rays with a wavelength of 1.54 Å. The nZVI sample was scanned in the range of 4–80° with a rate of 0.5 s. The estimated value of the crystalline diameter was calculated using the Scherer equation:

$$D_P = \frac{0.9\lambda}{\beta COS\theta} \tag{2}$$

where D_p is the crystalline diameter of particle (nm), λ is the CuKα radiation wavelength (Å), i.e., 0.154 nm, β is the full width at half maximum (FWHM) in radians, and θ is the Bragg angle obtained from 2θ corresponding to maximum peak intensity (°) (Soliman and Vshivkov 2019). For a spherical particle with a diameter of D, the specific surface area (SSA) can be calculated by the following equation:

$$SSA = \frac{Surface\ area}{Mass} = \frac{\pi D^2}{\rho \frac{\pi}{6} D^3} = \frac{6}{\rho D} \tag{3}$$

where ρ is the density of the solid particle (7800 kg/m³ for iron) (Sun et al. 2006).

The morphology, size, and composition of the nZVI nanoparticles were investigated using a scanning electron microscope (FE-SEM, Quanta FEG 250, Philips, USA) equipped

with EDX. The nZVI powder was stacked on gluey carbon tape which is supported on a metallic disk and then has been submitted to the SEM equipment. The elemental analysis of the nZVI sample was assayed using the EDX analyzer to determine the surface structure composition of the individual points or to map out the lateral distribution of the elements from the imaged area, and estimate their proportion at different positions, consequently giving an overall mapping of the sample.

Thermogravimetric analyzer – (TGA-50, Shimadzu, Japan) was used to perform thermal analysis on an nZVI sample weighed 8.678 mg under a nitrogen atmosphere and using a platinum pan in temperature ranges of 0–1100 °C with a heating rate of 10 °C/min compared with alumina (Al_2O_3) as a reference powder.

Stability of nZVI nanoparticles

The chemical and thermal stability of nZVI nanoparticles under different conditions of pH and temperatures were evaluated. Chemical stability was performed by equilibrating nZVI particles with a series of solutions that have pH values from 2 to 12 at 25 °C for 2 h. Several conical flasks contained 100 mL of distilled water adjusted to the selected pH values were shaken together with approximately 0.1 g of powdered nZVI for each flask. The corresponding number of filter papers were dried in an oven at 103 °C for 1–2 h, cooled in a desiccator, and then weighed. At the end of the shaken period, the nZVI nanoparticle residues were filtered using the filter papers that were previously dried and rinsed 3 times with distilled water to remove the excess of acids or alkalis. Finally, the nZVI nanoparticles residues along with filter papers were dried in the oven at 103 °C for 2 h, cooled in a desiccator, and then weighed. The thermal stability of nZVI nanoparticles was determined by weighed 0.1 g of nZVI powder in a beaker and exposed it to different temperatures from 45 to 100 °C for 2 h, cooled, and re-weighed. The weight loss (%) of nZVI was calculated as follows (Nigam Ahuja et al. 2020):

$Wt.of\ nZVI\ residual$

$$= \frac{Wt.of\ residue + Wt\ of\ filter\ paper/beaker}{Wt\ of\ filter\ paper/beaker} \tag{4}$$

$Weight\ loss\ (\%)\ of\ nZVI$

$$= \frac{Wt.of\ nZVI\ taken - Wt.of\ residual\ nZVI}{Wt.of\ nZVI\ taken} x100 \tag{5}$$

Bacterial strains and media

Four bacterial strains were used to test the germicidal activity of the nZVI nanoparticles. Two of the bacterial strains were Gram (+) *Staphylococcus aureus* NRRL B-313 and *Bacillus*

subtilus NRC, while the others were Gram (−) *Escherichia coli* NRC B-3703 and *Pseudomonas aeruginosa* NRC B-32. Nutrient agar media was used for bacterial strain growth. The liquid medium was sterilized using autoclave at 121 °C for 15 min and then sub-cultured. Subsequently, the solid media were used for the agar well diffusion assay (Suganya et al. 2012).

Well diffusion assay method

Determination of the antibacterial activity of nZVI nanoparticles

About 20 mL from the nutrient agar medium was placed into 10 mL Petri dishes, and 0.1 mL of fresh cultures for the four strains; *S. aureus*, *B. subtilus*, *E. coli*, and *Ps. aeruginosa* were spread over the plates using a sterile swap spreader to get a uniform bacterial growth for all plates (Suganya et al. 2012). Thereafter, a well with a 9 mm diameter was created on each agar plate. The wells were filled with 100 μL of the nZVI nanoparticles substance (0.01 g from the nZVI nanoparticles has been dissolved in 1 mL of dimethyl sulfoxide (DMSO)). Then, the plates have left for 120 min in the refrigerator to allow the diffusion of the nZVI substance. After the previous period expiration, the plates of strains were incubated at 37 °C for 24 h. Finally, the inhibition zone was measured with a ruler.

Determination of minimal inhibitory concentration (MIC) of nZVI nanoparticles

MIC is defined as the lowest concentration of the antimicrobial agent that inhibits microbial growth after 24 h of incubation time. Determination of MIC for the *S. aureus*, *B. subtilus*, *E. coli*, and *Ps. aeruginosa* was carried out by the well-diffusion assay method. The nZVI nanoparticles have been tested for determining the MIC according to the method reported by Hammer et al. (1999). For this purpose, the culture medium was poured into the Petri dishes and maintained at 45 °C until the samples were added to the agar. The samples were added using a micropipette by the sequence of 2.25, 2.5, 5.0, 7.5, 10, 15, 20, and 22.5 mg/20 mL agar media for the *S. aureus* and *E. coli*, while 2.5, 5.0, 7.5, and 10 mg/20 mL agar media have been added for the *B. subtilus* and *Ps. aeruginosa*, accompanied with constant stirring to assure the uniform distribution. Afterward, exactly 50 μL of the different bacteria strains were layered using an automatic micropipette over the surface of the solidified medium containing the sample. After the bacteria were absorbed into the agar, the plates were incubated for 24 h at 37 °C. The MIC has been determined as the lowest concentration of the nZVI nanoparticles had caused the inhibition of visible growth of each bacteria on the agar plate.

Potential application of nZVI nanoparticles on sewage water

Real municipal wastewater was collected from the influent of Helwan sewage treatment plant in Arab Abu Said village at latitude 29°44′51″N and longitude 31°19′50″E. Subsequently, the sample was examined for physical and chemical properties according to the standards methods for the examination of water and wastewater 23rd edition for 2017 (Baird and Bridgewater 2017) before undergoes for treatment by nZVI nanoparticles. Table 2 shows the composition of the collected raw sewage sample in addition to the corresponded limitations of reclaimed water quality required to reuse the treated municipal wastewater in agriculture and irrigation according to the recommended concentrations by the different regulatory organizations.

The optical density assay was performed for four trials containing 5 mL of the sewage water mixed with different concentrations (2, 3, 4, and 5 mg/mL) of the nZVI nanoparticles substance (0.01 g from the nZVI nanoparticles in 1 mL DMSO) which compared with the control sample (sewage water without adding the nZVI substance). The tubes were incubated for 48 h at 37 °C, and then the growth inhibition was measured with a spectrophotometer at OD of 620 nm (Shahneh et al. 2013).

Percentage growth inhibition

$$= \frac{OD_{control} - OD_{sample}}{OD_{control}} X 100 \qquad (6)$$

Membrane filter technique

To inspect the microbiological treatment of contaminated sewage water using nZVI nanoparticles, two grams from the nZVI nanoparticles were put into 1 L of the sewage water. After accomplishing the treatment process; the results were compared with the control sample (sewage water without adding the nZVI substance). The growth inhibition was measured by the membrane filter (MF) technique, which is fully accepted as the preferred technique for monitoring the drinking water quality in many countries. In this method, the water sample is filtered on a sterile filter with 0.45 μm pore size which retained the bacteria, then the filter was incubated on a selective medium. Ultimately, the colonies reserved on the filter have been enumerated. Many media and incubation conditions for the membrane filter method had been tested for achieving the optimal recovery of the coliforms from the water samples (Grabow and Du Preez 1979). Using this technique could be determined the total bacteria, total coliform, and fecal coliform. As well, gram (+) *S. aureus* and *fecal streptococci*. Further, gram (−) *E. coli* and *Ps. aeruginosa* in the sewage water samples.

Table 2 Physical and chemical properties of the raw municipal wastewater sample and equivalent recommended requirements for reusing the reclaimed water in the different purposes (Shoushtarian and Negahban-Azar 2020; Division et al. 2004; Blumenthal et al. 2000; Ayers and Westcot 1985)

Items	Unit	Raw Wastewater Results	Reclaimed Water Limitations				
			USEPA (2012) [1]		FAO [2]	WHO [3]	ISO (2015) [4]
			Long-term Use	Short-term Use			
Temperature	°C	22.0	–	–	–	–	–
pH	–	7.16	6	–	–	–	–
Dissolved Oxygen (DO)	mg-O$_2$/L	0.87	–	–	–	–	–
Biological Oxygen Demand (BOD)	mg-O$_2$/L	189	30≥	–	–	–	–
Chemical Oxygen Demand (COD)	mg-O$_2$/L	315	–	–	–	–	–
Total Suspended Solids (TSS)	mg/L	204	30≥	–	–	–	–
Oils & Grease	mg/L	0.29	–	–	–	–	–
Total Nitrogen (TN)	mg-N/L	39.5	–	–	–	–	–
Total Phosphorus (TP)	mg-P/L	2.90	–	–	–	–	–
Sulfides (H$_2$S)	mg-S/L	2.70	–	–	–	–	–
Free Cyanide (CN$^-$)	mg-CN/L	0.02	–	–	–	–	–
Phenol	mg/L	0.002	–	–	–	–	–
Aluminum (Al)	mg/L	0.001	5.0	20	5.0	–	–
Arsenic (As)	mg/L	0.011	0.10	2.0	0.10	–	–
Boron (B)	mg/L	0.008	0.75	2.0	–	–	–
Cadmium (Cd)	mg/L	0.0013	0.01	0.05	0.01	–	–
Chromium (Cr)	mg/L	0.009	0.1	1.0	0.10	–	–
Copper (Cu)	mg/L	0.039	0.2	5.0	0.20	–	–
Iron (Fe)	mg/L	0.109	5.0	20.0	5.0	–	–
Mercury (Hg)	mg/L	0.0058	–	–	–	–	–
Nickel (Ni)	mg/L	0.011	0.2	2.0	0.20	–	–
Silver (Ag)	mg/L	0.01	–	–	–	–	–
Zinc (Zn)	mg/L	0.087	2.0	10.0	2.0	–	–
Lead (Pb)	mg/L	0.11	5.0	10.0	5.0	–	–
Fecal Coliform	CFU/100 mL	2100	≤ 200 [a]	800 [a]	≤ 10^3 [b]	≤ 10^3	≤ 10^3 [c]
E. coli	CFU/100 mL	1800	≤ 200 [d]	≤ 10^4 [d]	–	≤ 10^3 [e]	–

1 Recommended limits for constituents in reclaimed water for agricultural reuse, USEPA (2004)

2 Recommended maximum concentrations according to the FAO guidelines for trace metals in irrigation water (Adapted from National Academy of Sciences (1972) and Pratt (1972)

3 Guidelines for the microbiological quality of treated wastewater used in agriculture: recommendations for revising WHO guidelines

4 ISO 16075-1:2015 Guidelines for treated wastewater use for irrigation projects — Part 1: The basis of a reuse project for irrigation

a Processed food crops/non-food crops

b Geometric mean

c High-quality treated wastewater; restricted urban irrigation and agricultural irrigation of processed food crops

d Global reclaimed water quality standards (non-food crops)

e Unrestricted (Drip irrigated, Low-growing)

Determination of total bacteria

The membrane filter method gives a direct estimation for the count of bacteria in the water-based on the growth of the colonies on the membrane filter modified surface (Dufour et al. 1981). A water sample was filtered through the membrane that retained the bacteria. After filtration, the membrane was placed on a differential and selective medium, mTEC, and then incubated at 35 °C and 22 °C ± 0.5 °C for 24 h. Following the incubation period, the filter was moved to a filter pad saturated with urea as a substrate. After 15 min, the yellow, yellow-green, or yellow-brown colonies were counted using a fluorescent lamp and a magnifying lens.

Determination of fecal coliform and total coliform

The membrane filter technique was used for the identification of fecal coliform (FC) and total coliform (TC) organisms in the two sewage water (before and after treatment) samples according to the APHA method (APHA 1995a). The samples were tested by covering the membrane filter unit with aluminum foil and sterile it subsequently. Then, the membrane was placed on the Membrane lauryl sulfate (MLS) media culture over the Petri dish which incubated at the temperature of 44 °C and 37 °C ± 0.5 °C for the fecal coliform and the total coliform, respectively. After 24 h, the colonies that had yellow colors were counted as a colony-forming unit (CFU) per 100 mL of the sample.

Determination of *S. aureus*

Isolation of the *S. aureus* has been performed according to the APHA method (APHA 1997), which includes the enrichment of one gram of the sample in 10 mL of cooked meat media plus 9% NaCl (*w/v*). Posteriorly, the inoculation process for 24 h for the enrichment culture has performed on Baird-Parker agar that containing potassium tellurite along with egg yolk agar, which had been confirmed via the coagulase test of the lipase-positive jet-black colonies.

Determination of *fecal streptococci*

The *fecal streptococci* characterize by grows on sodium azide medium at 37–44 °C. Where it is detected through reduction of the stain (generally a tetrazolium-containing compound) or the aesculin hydrolysis (APHA 1998).

Determination of *E. coli*

The *E. coli* was considered the most harmful due to its presence is directly related to fecal contamination and its implied enteric disease presence (Rice et al. 1991). Accordingly, to detect *E. coli* strain, tubes are containing gas-positive Lauryl Sulphate Broth (LSB) were undergone to analyzing by the EC broth. Afterward, the EC tubes were incubated for 24 h at 44.5 °C. In this case, the presence of *E. coli* is monitored in the positive tubes by detecting the production of indole with tryptone water. The tubes that give positive results confirm the presence of *E. coli* via indole and gas production (APHA 1995b).

Determination of *Ps. aeruginosa*

Ps. aeruginosa is recognized through the production of fluorescent pigment, which could be disclosed by UV irradiation. According to this method, the number of microbes is the Most Probable Number (MPN) which is a method used to estimate the concentration of viable microorganisms in a sample through replicate liquid broth growth ten-fold dilutions. Thus after each round, the reactor water is cultured on asparagine and set amid broth tubes. By the end of the incubation period that continued for 48 h at 37 °C, the number of cells is counted (APHA 2005).

Results

Structure of nZVI nanoparticles

Crystallinity and specific surface area

The XRD pattern for the nZVI nanoparticles was shown in Fig. 2a. The peak at 2θ of 44.8096° which represent 100% intensity indicates that the sample predominantly of the α-Fe^0 nanoparticles. The peak at $2\theta = 31.8197°$ revealed the existence of a thin layer of iron oxides (Fe_2O_3 or Fe_3O_4). The mean crystalline size of the Fe^0 nanoparticle was found to be 44.43 nm, which was estimated by the Scherer equation. According to eq. 3; the theoretical SSA for 44.43 nm particles is therefore 17.31 m^2/kg, so can be summarized the properties of as-prepared nZVI particles in the following Table 3.

SEM studies

The SEM image for the synthesized nZVI particles as was shown in Fig. 2b has elucidated that the iron particles appear spherical and have a particle size distribution within 20–100 nm, demonstrating the characteristic chain-like morphology. Agglomeration of the nZVI nanoparticles was mentioned to be caused by the magnetic dipole-dipole interactions, and the high surface area of the individual particles. Similar results were observed in previous studies (Peng et al. 2017; Mukherjee et al. 2016). Literature resources indicate that the nZVI nanoparticles possess a core-shell structure, in which the

Table 3 Properties of the synthesized nZVI particles

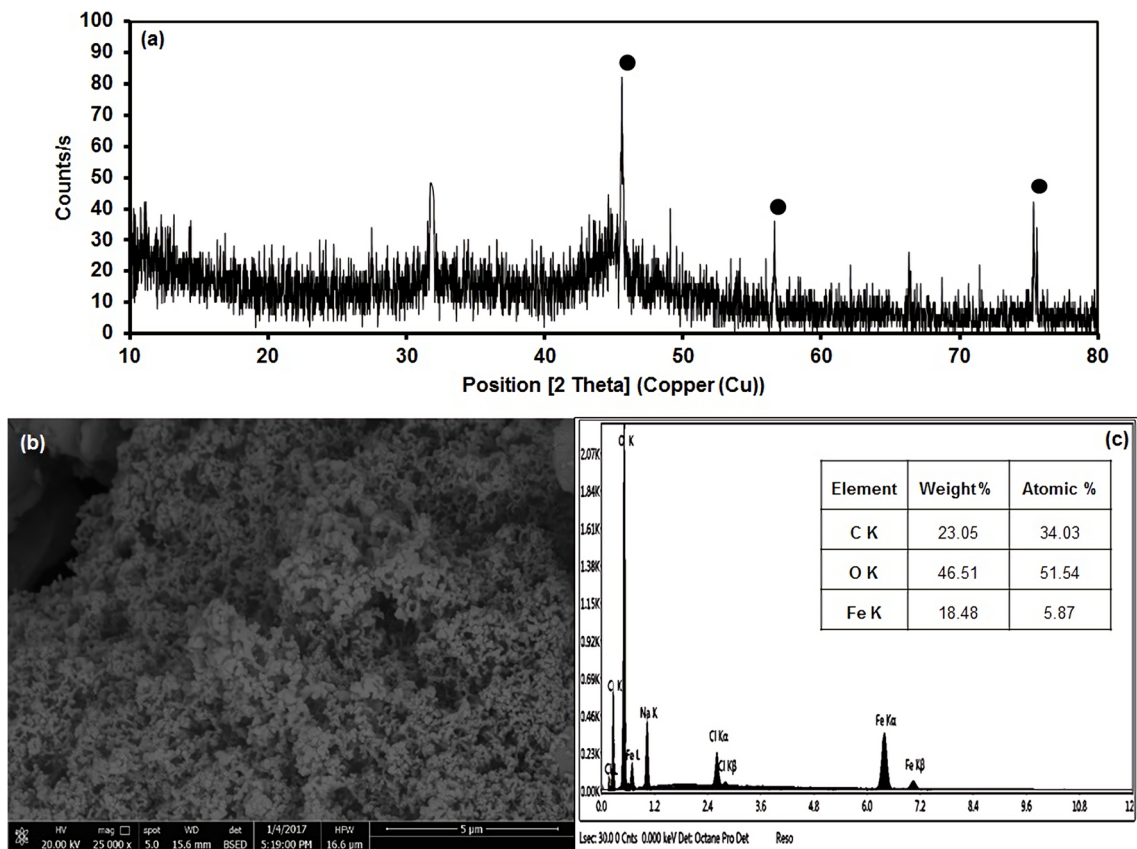| Color	Shape	Average Particle Size (nm)	Theoretical Specific Surface Area (m²/kg)
Black	Spherical	44.43	17.31

Fig. 2 (a) XRD pattern of nZVI particles, Symbol: (•) indicate to Fe^0 peaks, (b) SEM image of nZVI particles, (c) Typical EDX of nZVI particles

shell represents the oxidized part that surrounds the iron core and preserves it against further oxidation. Where Yan et al. mentioned that the analysis provides unambiguous evidence of the structure of the nZVI layers, which consist of a metallic iron core coated with a thin crust of amorphous oxide (Yan et al. 2010).

EDX studies

The data estimated from the EDX spectrum were summarized in the inset Table in Fig. 2c, which illustrated the surface atomic distribution and chemical elemental composition of nZVI particles. As the iron surface layer is so thin, thus the

Fig. 3 Thermogravimetric plot (TGA) of nZVI particles
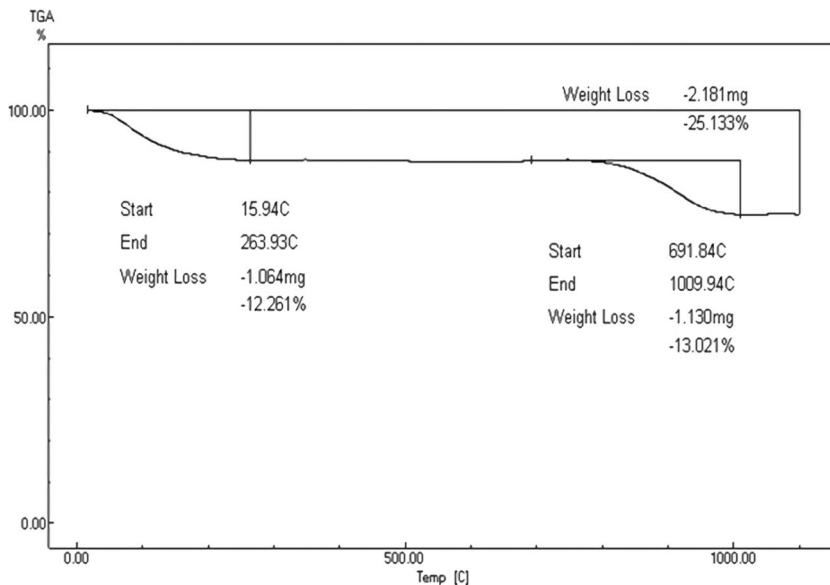
electron beam can penetrate it and arrive at the core of the nZVI particles, and provide detailed information about the composition of the particles. From the Table, it can be observed the presence of high contents of C, O represents 23.05% (w), and 46.51% (w), respectively. Which often adsorbed on the nZVI surface during the washing step using the alcohol through the preparation process, as well maybe resulted due to oxidation of nZVI particles surface within the measurement step on the SEM equipment (Ayob et al. 2016). Additionally, the appearance of sodium and chloride peaks in the spectrum may back to the presence of some residual by-products. Furthermore, the spectrum pattern showed that the energy levels for nZVI were found at 0.8 keV, 6.4 keV, and 7.1 keV, indicating that the main peak at 6.4 keV is the typical characteristic peak of the iron particles as reported in other studies (Prema et al. 2011). Moreover, the iron has represented a %weight of 18.48 in the sample.

TGA studies

Thermogravimetric analysis (TGA) is a method of thermal stability analysis for the samples in which the mass of a sample is measured as the temperature changes over time. This measurement provides information about physical phenomena, such as phase transitions, the absorption-desorption mechanisms; as well as chemical phenomena including chemisorption, thermal decomposition, and solid-gas reactions, e.g.,
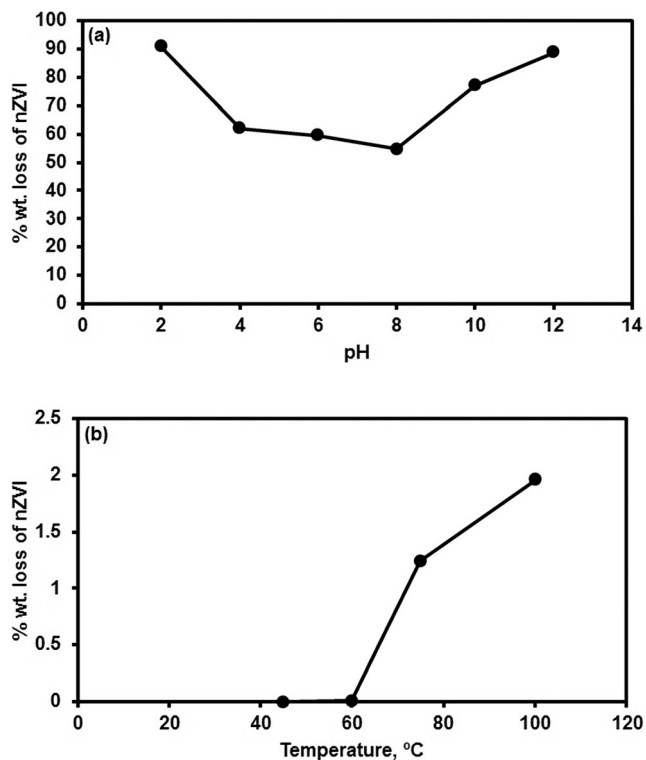

Fig. 4 Weight loss (%) of nZVI particles at; (a) different pH, (b) different temperatures

oxidation or reduction (Prime et al. 2009). According to the thermal analysis results obtained from TGA analysis, the TGA curve of nZVI particles displayed two degradation steps as shown in Fig. 3. The first step was identified in the range of 15–263 °C with a mass loss of 1.064 mg (percent loss = 12.26%) due to possible evaporation of both the adsorbed water that exists as moisture content on the surface of nZVI particles and loss of adsorbed carbon which is originally due to the ethyl alcohol used in wash off the nZVI particles. The second decomposition step at a range of 691–1009 °C with a mass loss of 1.130 mg (percent loss = 13.02%) is mainly resulting due to the decomposition of some nZVI particles, although with the exclusion of the prospective of iron melting entirely at this point. Where the solid iron metal needs a temperature of 1538 °C to reach the melting point. Furthermore, the lately mentioned decay may back to the decomposition of oxide shell surrounds of nZVI particles, which potentially be consists of FeO, and/or FeOOH referring to the breaking of some chemical bonds between iron and oxygen atoms, i.e. reduction of iron oxides through oxygen elimination from the nZVI oxide shell. It is well known that iron may have several crystalline structures. Iron has a body-centered cubic (bcc) lattice known as α-Fe at room temperature. When the temperature is raised above 912 °C, the α-iron transformed into a face-centered cubic (fcc) lattice referred to as γ-Fe; and when the temperature rises above 1394 °C, the γ-iron transformed into a body-centered cubic lattice again, called δ-Fe, which it is the state that the iron remains in it until the melting point (1538 °C). Thus, the second thermal transition in the TGA curve of nZVI particles may be due to the transformation of the crystal lattice of nZVI from a crystalline phase to another phase (Song et al. 2014; Chaung et al. 2014). Generally, nZVI particles were lost almost a weight of 2.181 mg matching a loss percent equal to 25.13% from the original nZVI sample weight, which confirms the high purity of synthesized nZVI particles. Finally, from the previous results, we can conclude that the thermal stability curve exhibited the best thermal performance of nZVI particles at temperatures below 600 °C.

Properties of nZVI nanoparticles

Chemical stability of nZVI nanoparticles

The weight loss (%) of the nZVI sample was used to express the chemical stability of nZVI. Where the weight loss of nZVI samples was calculated for a series of solutions with different pH values as follows 2, 4, 6, 8, 10, and 12. Figure 4a showed that the nZVI particles exposed a considerable weight loss (%) as 90.87 and 88.82% at pH 2 and 12, respectively. This means at very low and high pH values, the nZVI particles were subjected to rapid corrosion and dissolution in the extremely acidic and basic mediums, as a result of the presence of high

concentrations of H^+ and ^-OH, leading to accelerating the redox reactions, and dissolution or precipitation of nZVI particles to Fe(II), Fe(III) and iron hydroxides. This means that the nZVI is unstable in very acidic and basic conditions. On the other hand, the weight loss (%) values of nZVI decreased in the range of pH values from 4 to 8. Where the nZVI exhibited the lowest value of weight loss (%) as 54.75% at pH 8. Thus, it can be concluded that the nZVI is remaining stable and is preferably used in neutral, slightly acidic, and slightly basic mediums.

Thermal stability of nZVI nanoparticles

The thermal stability of nZVI particles was examined under a different temperature range of 45, 60, 75, and 100 °C, and weight loss (%) of nZVI particles was evaluated. Figure 4b showed that the nZVI particles exhibited a negligible weight loss at temperatures till 60 °C, which may be attributed to the removal of moisture content from the nZVI surface. In the contrast, at high temperatures values of 75 and 100 °C, the surface thin layer of nZVI began to corrode and decompose. Where, the nZVI displayed slight values of weight loss (%) equivalent to 1.24 and 1.96%, respectively. Which is still a very low percentage. Consequently, this suggested that the nZVI particles are widely stable under the temperature range of 45–60 °C, and can be used safely at this range.

Discussion

Inactivation mechanisms of nZVI nanoparticles

The nZVI suspension has the iron in zero oxidation state (Fe^0) which loss the electrons causing oxidization of iron metal to soluble ferrous and ferric ions:

$$Fe^0_{(s)} \rightarrow Fe^{+2}_{(l)} + e^- \quad (7)$$

$$Fe^{+2}_{(l)} \rightarrow Fe^{+3}_{(l)} + e^- \quad (8)$$

The free-electron attacks the dissolved oxygen from the atmosphere, and water molecules to yield superoxide anion, and super hydroxides radicals, respectively:

$$e^- + O_{2\ (g)} \rightarrow \cdot O^-_{2(l)} \quad (9)$$

$$e^- + H_2O_{(l)} \rightarrow \cdot OH^-_{2(l)} \quad (10)$$

The HO_2 produces as a result of the reaction of the yielded superoxide anion with H^+:

$$\cdot O^-_{2(l)} + H^+_{(l)} \rightarrow HO_{2(l)} \quad (11)$$

Interfering of HO_2 with electrons leads to the generation of hydrogen peroxide anion subsequently reacts with H^+ to give hydrogen peroxide molecule:

$$HO_{2(l)} + H^+_{(l)} + e^- \rightarrow H_2O_{2\ (l)} \quad (12)$$
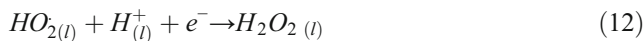
The released iron species oxidizes using dissolved oxygen in redox reactions into many forms of iron oxides:

$$Fe^{+2}_{(l)} + O_{2\ (g)} \rightarrow FeO_{(l)} \quad (13)$$

$$Fe^{+2}_{(l)} + O_{2\ (g)} \rightarrow Fe_2O_{3(l)} \quad (14)$$

$$Fe^{+3}_{(l)} + O_{2\ (g)} \rightarrow Fe_3O_{4(l)} \quad (15)$$

Based on the previous reactions, there are many explanations concerning antimicrobial mechanisms of the nZVI nanoparticles, as follow:

ROS production

- The antimicrobial mechanisms of nZVI included the production of many reactive oxygen species (ROS), such as hydroxyl radical, superoxide radical, active radical species, and hydrogen peroxide molecules. It is known that the generation of ROS inducts oxidative stress of the cellular proteins, cell membranes, and DNA of the bacteria consequently causes fatal damage to the membrane structure of the bacteria cells. Where, the outer bilayer membrane is consists of lipopolysaccharides, proteins, and phospholipids that are destroying by the action of ROS active species. Therefore, most of the hurtful effects on the bacterial cells exceedingly occur due to the high concentrations of ROS, which leads to consecutive Fenton reactions. Thence, the Fe-S groups for the bacterial coenzymes are destructed resulting in bacteria injury and death.
- On the other hand, it can be suggested that the generation of H_2O_2 molecules may be causing the lipid peroxidation of the bacteria cells. Where the H_2O_2 can penetrate the cell membrane causing the degradation of membrane structure and then distorting the cells. Therefore, it can be concluded that increasing the ROS species and metal oxidation are related to the degree of nZVI toxicity.
- The nano-domain size of nZVI particles is recognized by the high specific surface area. This means a high surface activity of nZVI particles which facilitates the entrance of nZVI particles into the bacteria cells. Consequently, causes cell wall decomposition, and then rupturing the cell membrane followed by leakage of cell content. Finally, bacterial cell death occurs.

Liberation of Fe^{2+}, Fe^{3+} ions

- Indeed, the resistance of microorganisms can be declined in front of the ability of nZVI particles on inserting the mechanism of poisoning by the free metal ions. Which this free metal ion concentrations arise as a result of decomposing the surface layers of the nanoparticles. Accordingly, the nZVI nanoparticles have high reactivity. Where the nZVI is localizing in zero-state which tends to oxidize vigorously into divalent and trivalent oxidation states by losing the electrons and releasing Fe^{2+}, and Fe^{3+} ions. Thence promoted the toxicity of nZVI via the high solubility rate of these ions in the microbes-containing medium, and its high capability to penetrate the intracellular contents of the bacteria membranes.
- Furthermore, the antibacterial efficacy of nZVI may be attributed to the strong electrostatic interaction of the liberated positively charged ferrous and ferric ions (Fe^{2+}, Fe^{3+}) with negatively charged bacteria. Therefore, cell death befalls as a result of the destructive attraction between the bacteria wall surface and nZVI particles. Over and above that, these ions could also bind to the outer cell membrane causing bacteria cell disorder.
- Since the presence of atmospheric oxygen in contact with the nZVI particles, it might result in the formation of iron oxides, such as Fe_3O_4, Fe_2O_3, and FeO, which encourage the wide propagation of ROS species to modify the redox status, thus this implies an enhancement of the nZVI ability to killing the microbes.

Contact of nZVI nanoparticles with bacteria

- Sometimes, it is not necessarily an occurrence of cell deterioration due to the diffusion of ions or metals inside the cells. Nevertheless, the direct contact of abrasive nZVI particles with the bacterial membranes may cause many micro-environmental changes encloses the contact areas such as; disorder of bacterial cell wall, likewise increasing of cell membrane permeability, and sequentially leading to a high consecutive cellular internalization of the nZVI particles throughout the cells.
- Therefore, the distinctive capability of nZVI cytotoxicity towards discouragement of the survival of disease-causing microorganisms might be attributed to its ability to doing the changes in the structures of proteins, peptidoglycan, DNA, and lipids structure of the bacteria cell membrane because of the agglomeration of nZVI particles on the bacteria surface. Therefore, these modifications will be causes disruption of the bacterial electron transportation chain by prohibiting metabolic enzyme activities, suppress bacterial duplication, and accordingly prevent the activity of bacteria.

Fig. 5 Schematic drawing representing the antibacterial mechanisms of nZVI particles
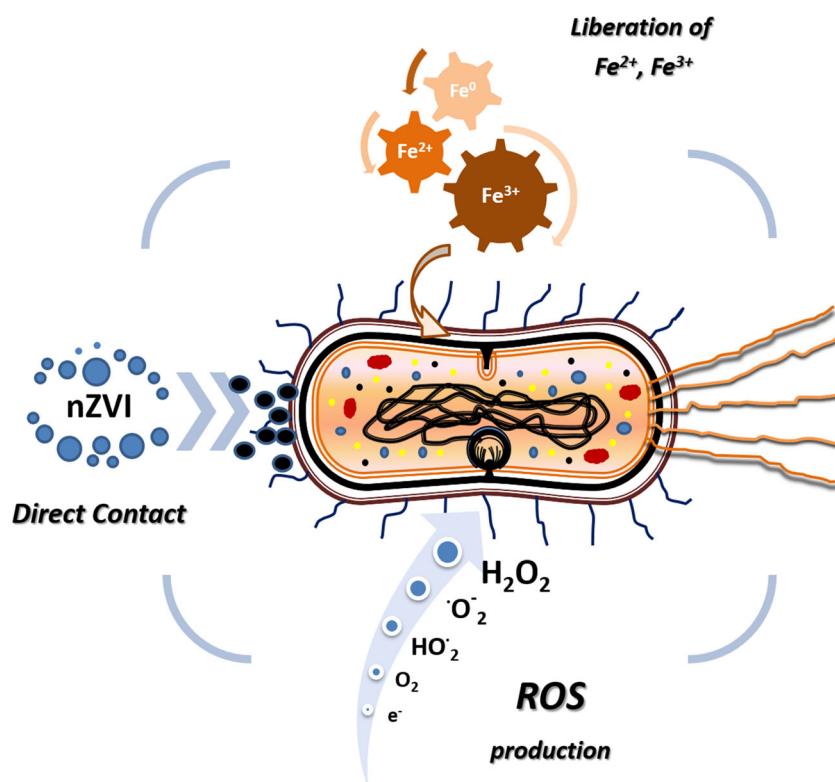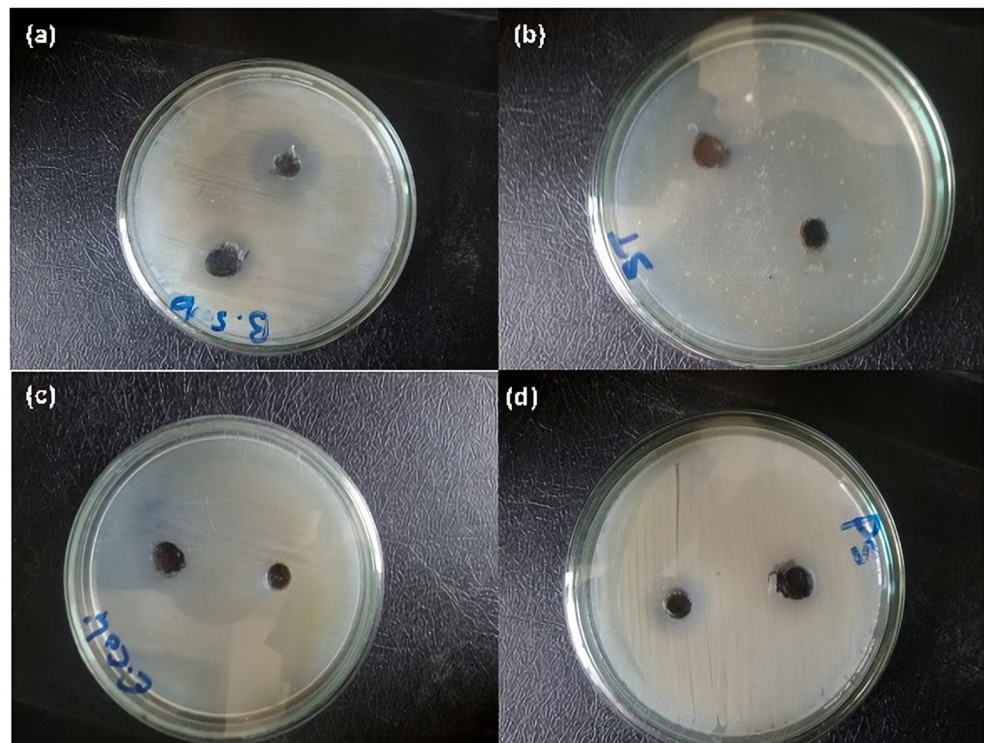
Fig. 6 Inhibition zones of (a) *Bacillus subtilus,* (b) *Staphylococcus aureus*, (c) *Escherichia coli*, (d) *Pseudomonas aeruginosa* as detected by well diffusion assay method

The previous possible mechanisms were represented in Fig. 5 (the figure was designed using Office PowerPoint v. 2016):

Bioactivity of nZVI nanoparticles

Often, the nZVI nanoparticles have shown a varying degree of toxicity against the four strains of the microorganisms namely; *B. subtilus*, *S. aureus*, *Ps. aeruginosa*, and *E. coli* as 14, 9, 18, and 21 mm, respectively. Whereas, the inhibition zone above a diameter of 10 mm has recorded a positive result as shown in Fig. 6. Generally, most of the tested microorganisms displayed a high sensitivity towards the nZVI nanoparticle substance, and these findings have agreed with that previously reported in the literature. Mahdy et al. indicated that the nZVI nanoparticles possess great potential to use as antimicrobials (Mahdy et al. 2012). Besides, they mentioned that the growth of *E. coli* and *S. aureus* was significantly inhibited compared with the control samples using the highest dose of iron oxide which was 30 µg/mL.

Based on the results, the Gram (−) bacteria showed higher sensitivity than Gram (+) bacteria towards the antibacterial activity of the nZVI agent. This may be attributed to the cell wall structure of the Gram (+) bacteria, which consist of a thick layer of lipopolysaccharide, lipid A, and peptidoglycan that have supported it against the nZVI devastating effect. While the cell walls of Gram (−) bacteria contain only a thin layer of peptidoglycan, which allowed the mobility and permeability of nZVI particles into the cells. Therefore, the nanoparticle/bacterial interactions improved due to lacking the ability of a thin peptidoglycan layer to repulsed of nZVI attack. Besides, Gram (−) bacteria have a negatively charged lipopolysaccharide layer, which contributed as a considerable incorporation factor for the positive ions (Fe^{2+}/Fe^{3+}) into the cells causing rapid intracellular damage, and destruction of bacterial DNA. Thus, it could be stated that maintenance of cell integrity, membrane functions, and efficiency of enzymes associated with the membrane depend foremost upon the structure of the cell surface.

The size of the inhibition zone for various group antimicrobial agents previously reported in the literature was compared with the observed zone of the nZVI agent as tabulated in Table 4. It could be concluded that the nZVI agent has achieved resemble results with most of the other antimicrobial materials, which reflects that the inhibition capacity of antimicrobial agents is a function of its particle size, and proportion directly with the concentration.

MIC of nZVI nanoparticles

In general, a higher concentration of the nZVI nanoparticles suspension was observed to have the highest inactivation efficiency versus Gram (+) and Gram (−) bacteria as listed in Table 5. Accordingly, the MIC values determined by the well diffusion assay for the four strains (*S. aureus*, *B. subtilus*,

Table 4 Inhibition zone of nZVI particles and the other antimicrobial agents from the literature

Antimicrobial Agent	Concentrations	Particle Size (nm)	Pathogens Bacteria	Inhibition Zone (mm)	References
Biosynthesized AgNPs synthesized using - Penicillium Citreonigrum Dierck - Scopulaiopsos brumptii Salvanet- Duval	200–300-500 μg/mL	4.24–23.2	S. aureus Ps. aeruginosa E. coli	13–15-25 / 13–15-17 / 13–15-17 / 12–15-17 / 13–15-17 / 13–14-15	Moustafa (2017)
Zinc oxide nanoparticles - ZnO-1, - ZnO-2, - ZnO-3	20–100 μg/mL	41.60–51.43 / 70.27–167.61 / 52.15–76.42	S. aureus / Ps. aeruginosa / E. coli	18–21 / 25–30 / 13–17	Narayanan et al. (2012)
Zinc oxide nanoparticles synthesized using - Mechano-chemical method - Sol-gel method	15–25-50 mM / 50 mM	10–20 / 5–7	E. coli	8–10-12 / 10	Elmi et al. (2014)
Zinc oxide nanoparticles - Bulk ZnO - Chemical ZnO - Green ZnO	2–4-6 mM	25 / 40	S. aureus	14–17-23 / 18–20-27 / 18–22-31	Gunalan et al. (2012)
Bio(AgNPs) produced by Streptacidiphilus durhamensis HGG16n isolate	100 μg/mL	8–48	B. subtilus / S. aureus / Ps. aeruginosa / E. coli	6 / 10 / 10 / 6	Buszewski et al. (2018)
- 4A zeolite, - ZnO/4A zeolite, - TiO2/4A zeolite, - TiO2/ZnO/4A zeolite nanocomposites	1–4 mg/mL	400–600 for 4A zeolite 10–50 for TiO2/ZnO	S. aureus / E. coli	0, 6.21, 7.58, 9.22 / 0, 6.86, 9.13, 10.73	Azizi-Lalabadi et al. (2019)
α-Fe2O3/Co3O4 nanocomposites - FeK4	800 μg/mL	25.34	B. subtilus / S. aureus / E. coli	21 / 24 / 26	Bhushan et al. (2018)
Cerium oxide nanoparticles	100 μg/mL	45	B. subtilus / E. coli	3 / 1	Pelletier et al. (2010)
CuO/C nanocomposites	1 mg/mL	7–11	S. aureus / Ps. aeruginosa / E. coli	11 / 12 / 11	Bhavyasree and Xavier (2020)
nZVI nanoparticles	0.1 g/mL	44.43	B. subtilus / S. aureus / Ps. aeruginosa / E. coli	14 / 9 / 18 / 21	This study

Table 5 Minimal inhibitory concentration of nZVI particles determined by the well diffusion assay with substance concentration in (μL)

Sample	Concentration (mg)	Gram (+ve)		Gram (−ve)	
		S. aureus	*B. subtilus*	*E. coli*	*Ps. aeruginosa*
nZVI nanoparticles	2.5	0	0	0	0
	5.0	0	0	0	11
	7.5	0	0	0	16
	10	0	14	21	18
	15	20	–	23	–
	20	22.5	–	26	–
	22.5	25	–	33	–

E. coli, and *Ps. aeruginosa*) were found to be 15, 10, 10, and 5 mg, respectively.

Table 6 shows the quantitative values of MIC that measured for the proposed nZVI agent and different antimicrobial agents mentioned in some studies to establishing whether the agents are microbistatic or microbicidal towards various pathogens. Proportionately, the relative anti-bacteriophage activity of nZVI suspension in terms of MIC was seemed to be equivalent to other bacteriocins' suspensions. According to this comparison, it can well accommodate the role of agent concentration on the yielded value of MIC. This can be explained that based on the increase of nZVI agent concentrations will result in increasing the specific surface area to the volume and releasing a large number of killer species from the surface of the nanoparticles. Sequentially, this will lead to more inhibitory strength of nanoparticles toward the bacterial growth causing the cells to eventually die.

nZVI nanoparticles for municipal wastewater disinfection

nZVI particles have been examined as a disinfectant agent for municipal wastewater against the pathogen bacteria, and it is found that the nZVI substance had an effective effect on the microbial load in the sewage water. Where Fig. 7 indicated

Table 6 Minimal inhibitory concentration (MIC) of nZVI particles and the other antimicrobial agents from the literature

| Antimicrobial Agent	Pathogens Bacteria	Concentrations	MIC at 24 h	References
ZnO nanoparticles	*S. aureus*	20–100 μg/mL	40 μg/mL	Dimapilis et al. (2018)
Zinc oxide nanoparticles synthesized using - Mechano-chemical method - Sol-gel method	*E. coli*	–	1017 μg/mL	Elmi et al. (2014)
Zinc oxide nanoparticles - Chemical ZnO - Green ZnO	*S. aureus*	0.01–10 mM	0.80 mM 0.40 mM	Gunalan et al. (2012)
Bio(AgNPs) produced by *Streptacidiphilus durhamensis* HGG16n isolate	*S. aureus* *B. subtilus* *E. coli* *Ps. aeruginosa*	100 μg/mL	6.25 μg/mL 6.25 μg/mL 6.25 μg/mL 25 μg/mL	Buszewski et al. (2018)
Biosynthesized of silver and zinc nanoparticles extracellular synthesis using *Pseudomonas hibiscicola* isolate	*S. aureus* *B. subtilus* *E. coli* *Ps. aeruginosa*	10 mg/mL	Ag NPs – Zn NPs 5–2.5 mg/mL >10 - >10 mg/mL 2.5–5 mg/mL 2.5 - >10 mg/mL	Punjabi et al. (2018)
4A zeolite, TiO$_2$/4A zeolite, ZnO/4A zeolite, TiO$_2$/ZnO/4A zeolite nanocomposites	*S. aureus* *E. coli*	1–4 mg/mL	0, 3, 3, 2 mg/mL 0, 2, 2, 1 mg/mL	Azizi-Lalabadi et al. (2019)
Magnesium oxide-germanium oxide nanocomposite	*S. aureus* *E. coli*	50 mg/mL	0.05 mg/mL at 16 h 0.25 mg/mL at 16 h	Avanzato et al. (2009)
nZVI nanoparticles	*S. aureus* *B. subtilus* *E. coli* *Ps. aeruginosa*	2.5–22.5 mg/20 mL	15 mg/mL 10 mg/mL 10 mg/mL 5 mg/mL	This study
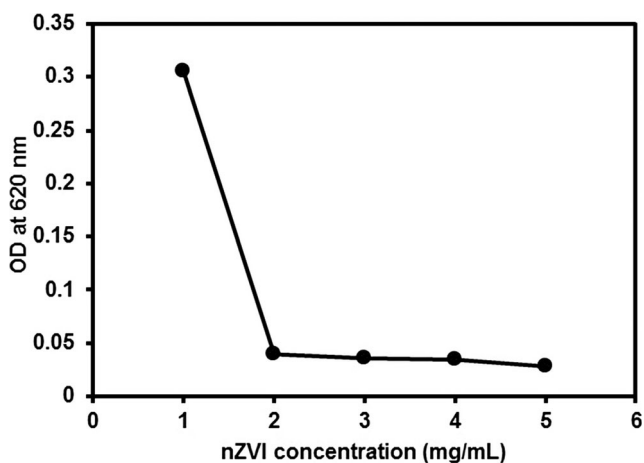
Fig. 7 Effect of nZVI particles concentrations (mg/mL) on bacteria of the wastewater sample as determined by optical density assay

that the high concentration of nZVI nanoparticles suspension was observed to have a high inactivation rate against the microorganisms in the sewage water samples. However, the high concentrations of nZVI suspension might result in movement restriction and high aggregation of nanoparticle causes reducing the activity of their relative surface and inactivation efficiency. in this context, Nurmi et al. reported the difficulty to avoid such aggregations (Nurmi et al. 2005). Where nature of the active sites on particle surface was observed to be affected by the surrounding environment in addition to the particle size (Kuhn et al. 2002; Signorini et al. 2003). Generally, under normal environmental conditions, the nZVI particles are covered with passivation layers that form a shell of oxides and giving nZVI particles the properties of the core-shell structure (Nurmi et al. 2005; Mulvaney 2001). Both the Gram (+) and Gram (−) bacteria extremely exist in municipal wastewater. Thus, an investigation survey has been procced to examine the inactivation proficiency of nZVI particles for these bacteria. From Fig. 8, and Table 7, it could be concluded the antimicrobial effect of nZVI particles on the bacteria of the wastewater sample. Where the counts of total bacteria, total coliform, an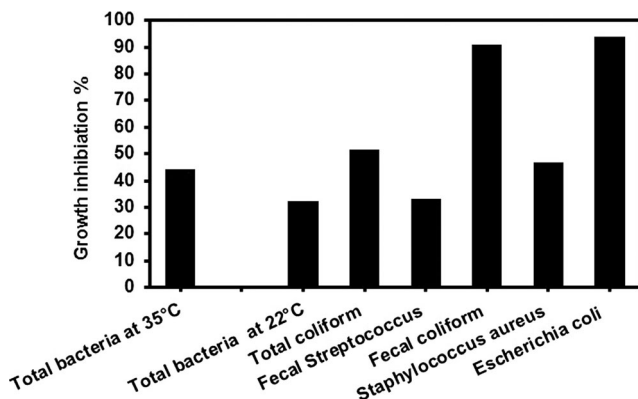d fecal coliform had decreased to 44.29%, 51.76%, and 90.95% respectively. Likewise, the counts of Gram (+) *S. aureus*, and *fecal Streptococcus* had decreased to 46.67%, and 33.33%, respectively. Furthermore, the count of Gram (−) *E. coli* had decreased to 93.89%. While the *Ps. aeruginosa* was not detected in the wastewater sample. Nevertheless, many previous studies have reported similar results, for example; Diao and Yao (2009) mentioned that the nZVI nanoparticles had accomplished a complete inactivation for Gram (−) *Ps. fluorescent*, whilst the inactivation efficiency reached 95% and 80% for Gram (+) *B. subtilis* and *Aspergillus Versicolor* fungus, respectively. Also, Lee et al. (2008) indicated that the nZVI nanoparticles in the aqueous solution rapidly inactivate the *E. coli*. Besides, George et al. (2013) reported that the maximum inhibition percentages of the nZVI nanoparticles have been recorded against *Ps. aeruginosa* with a value of 92%, followed by *E. coli* with more than 91%, then pursued by the *S. aureus* with inhibition percentage reached 89%. While, Hsueh et al. (2017) demonstrated that the nZVI nanoparticles with a concentration of 1000 mg/L have high toxicity against the Gram (+) *B. thuringiensis* and *B. subtilis*, but didn't affect the Gram (−) *E. coli* strains.

Generally, the treatment processes efficacies for microbial reduction differ among the microbial groups due to the inherently different properties of the microbes (e.g., size, nature of protective outer layers, physicochemical surface properties, etc.). However, the differences in treatment process efficiencies are smaller among the specific species, types, or strains of microbes. Table 8 provides a summary of treatment processes that are commonly used individually or integrally to achieve microbial reductions. The table presents conservative estimates of microbial reductions based on the more resistant or persistent pathogenic members of the microbial group. Besides, the different treatment processes were compared with the effect of nZVI particles against the total bacteria count in the sewage sample to demonstrate their efficiency as a disinfector agent.

Sun et al. (2019) reported that in the comparison of nZVI nanoparticles with chlorine, it was found that the nZVI particles are safer as a disinfector agent due to the absence of carcinogenic disinfection byproducts (DBPs) such as trihalomethanes (THMs), haloacetic acids (HAAs), chlorite, and bromate. Where the United States Environmental Protection Agency (USEPA), European Union, and World Health Organization (WHO) classify these compounds as major pollutants in drinking water. At the same time, disinfection using nZVI particles is advantageous over chlorination especially in the disinfection of wastewater where large amounts of pollutants are present. This advantage is due to the ability of nZVI particles to remove coexisting contaminants like organic pollutants and heavy metals simultaneously. In the case of chlorination, these pollutants must be removed because they might act as precursors of DBPs, and therefore increase the operation cost. The inactivation through magnetic separation along with

Fig. 8 Impact of nZVI particles on growth inhibition of Gram (+) and Gram (−) bacteria in the municipal wastewater sample

Table 7 Antibacterial activity of nZVI particles against microorganisms of the sewage water

Tested Bacteria	Before Treatment by nZVI Nanoparticles	After Treatment by nZVI Nanoparticles	Growth Inhibition (%)
Total bacteria CFU/mL at 35 °C	420	243	44.29
Total bacteria CFU/mL at 22 °C	463	314	32.18
Total coliform CFU/100 mL	34,000	16,400	51.76
Fecal streptococcus CFU/100 mL	14,100	9400	33.33
Fecal coliform CFU/100 mL	2100	190	90.95
S. aureus CFU/100 mL	1200	640	46.67
E. coli CFU/100 mL	1800	110	93.89
Ps. aeruginosa	ND*	ND	ND

* ND: Not detected

accompanying physical removal of bacteria from water bodies using nZVI particles was remarkably beneficial for practical disinfection. Whereas this physical treatment process reducing the chemical oxygen demand (COD) by eliminating the risks of the potential spread of antibiotic resistance genes through horizontal gene transfer and regrowth of inactivated bacteria by removing the debris of inactivated microbes. Besides, the nZVI particles are more applicable for handling, transport, storage, and deployment than chlorine, which enables their uses in more valuable applications such as point-of-use water purification, and disinfection of the noncentral water supply.

Microbiological aspect for wastewater reuse

Wastewater treatment is the most effective way to reduce the health, environmental, and other risks associated with the use of reclaimed water. Choosing the most appropriate treatment technology for water reuse is a complex procedure that must take into consideration various criteria, including technical and regulatory requirements, as well as social, political, and economic considerations specific to the local conditions. It is important to stress that economic and financial constraints have to be taken into account in countries where reclaimed water is a vital water resource for sustainable development. Some developing countries advocate another strategy of controlling health risks by adopting a low technology/low-cost approach based on the WHO recommendations (Al-Gheethi et al. 2018; Division et al. 2004).

Efforts to rehabilitate urban water resources include the increasing use of wastewater for peri-urban agriculture and energy production. An increasing number of initiatives are now looking at opportunities to integrate water management with urban needs in energy, green spaces, and food security. It is a matter of distinguishing the right kind of treatment for the right kind of use. There are a growing number of examples of reclaimed wastewater being used in agriculture, for irrigating municipal parks and fields, in industrial cooling systems, and even in some cases, mixed in with drinking water (2030 WRG, 2013) (Jaramillo and Restrepo 2017; Connor 2015). The parameters recommended for the minimum monitoring of community supplies are those that best establish the hygienic state of the water and thus the risk of waterborne disease. The essential parameters of water quality and common indicator bacteria are *E. coli* and thermotolerant (fecal) coliforms which are accepted as suitable substitutes – and chlorine residual (if chlorination is practiced) (Water and Organization 2006).

The Guidelines suggest that, regardless of the type of reclaimed water use, some level of disinfection should be provided to avoid adverse health consequences from inadvertent contact or accidental or intentional misuse of a water reuse system. For nonpotable uses of reclaimed water, two levels of disinfection are recommended. Reclaimed water used for applications where no direct public or worker contact with the water is expected should be disinfected to achieve an average fecal coliform concentration not exceeding 200/100 mL (Division et al. 2004). Moreover, the health-based targets in WHO guidelines (2006) are: 1. determine the realistic human exposure scenarios and the number of pathogens that could be ingested under different irrigation regimes for different crop types; 2. calculate the required reduction of pathogen numbers that need to be achieved depending on the initial wastewater quality and the crop type (Mara and Kramer

Table 8 Reductions of bacteria achieved by typical and enhanced water treatment processes compared with nZVI as a disinfecting agent (Water and Organization 2006)

Treatment Process	Baseline Removal	Maximum Removal Possible
Pretreatment		
Roughing filters	50%	Up to 95% if protected from turbidity spikes by dynamic filter or if used only when ripened
Microstraining	Zero	Generally ineffective
Off-stream/bankside storage	Zero (assumes short-circuiting)	90% removal in 10–40 days actual detention time
Bankside infiltration	99.9% after 2 m 99.99% after 4 m (minimum based on virus removal)	
Coagulation/flocculation/sedimentation		
Conventional clarification	30%	90% (depending on the coagulant, pH, temperature, alkalinity, turbidity)
High-rate clarification	At least 30%	
Dissolved air flotation	No data available	
Lime softening	20% at pH 9.5 for 6 h at 2–8 °C	99% at pH 11.5 for 6 h at 2–8 °C
Ion exchange	Zero	
Filtration		
Granular high-rate filtration	No data available	99% under optimum coagulation conditions
Slow sand filtration	50%	99.5% under optimum ripening, cleaning and refilling and in the absence of short-circuiting
Precoat filtration, including diatomaceous earth and perlite	30–50%	96–99.9% using chemical pretreatment with coagulants or polymers
Membrane filtration – microfiltration	99.9–99.99%, providing adequate pretreatment and membrane integrity conserved	
Membrane filtration – ultrafiltration, nanofiltration and reverse osmosis	Complete removal, providing adequate pretreatment and membrane integrity conserved	
Disinfection		
Chlorine	Ct_{99}: 0.08 mg·min/l at 1–2 °C, pH 7; 3.3 mg·min/l at 1–2 °C, pH 8.5	
Monochloramine	Ct_{99}: 94 mg·min/l at 1–2 °C, pH 7; 278 mg·min/l at 1–2 °C, pH 8.5	
Chlorine dioxide	Ct_{99}: 0.13 mg·min/l at 1–2 °C, pH 7; 0.19 mg·min/l at 1–2 °C, pH 8.5	
Ozone	Ct_{99}: 0.02 mg·min/l at 5 °C, pH 6–7	
UV irradiation	99% inactivation: 7 mJ/cm^2	
nZVI nanoparticles *	44.29% at 35 °C for 24 h; 32.18% at 22 °C for 24 h	

Note: Ct and UV apply to microorganisms in suspension, not embedded in particles or in biofilm

*This study

Table 9 Suggested considerations for reclaimed water reuse focusing on microbiological aspect according to the WHO recommendations and the EPA regulatory guidelines comparing with results obtained by nZVI nanoparticles (EPA 2012; Blumenthal et al. 2000)

Reuse Category	Treatment	Microbiological Reclaimed Water Quality [2]			
		EPA Guidelines	Treatment Goal in Reclaimed Water [13]	WHO & FAO [14]	nZVI Nanoparticles *
Urban Reuse Unrestricted	•Secondary [3] •Filtration [4] •Disinfection [5]	•No detectable fecal coliform /100 mL [6, 7] •1 mg/L Cl_2 residual (min.) [8]	•Total coliforms: <1 CFU/100 mL - 200 CFU/100 mL •Fecal coliforms: <1 CFU/100 mL - 103 CFU/100 mL	•Fecal Coliforms (CFU/100 mL): <200 (g) <1000 (m) [15]	•16,400 total coliform CFU/100 mL •190 fecal coliform CFU/100 mL •No Cl_2 residual
Restricted	•Secondary [3] •Disinfection [5]	•≤200 fecal coliform /100 mL [6, 9, 10] •1 mg/L Cl_2 residual (min.) [8]			
Agricultural Reuse Food Crops [11]	•Secondary [3] •Filtration [4] •Disinfection [5]	•No detectable fecal coliform/100 mL [6, 7] •1 mg/L Cl_2 residual (min.) [8]			
Processed Food Crops [11]	•Secondary [3] •Disinfection [5]	•≤200 fecal coliform /100 mL [6, 9, 10] •1 mg/L Cl_2 residual (min.) [8]			
Non-Food Crops	•Secondary [3] •Disinfection [5]	•≤200 fecal coliform /100 mL [6, 9, 10] •1 mg/L Cl_2 residual (min.) [8]			
Impoundments Unrestricted	•Secondary [3] •Filtration [4] •Disinfection [5]	•No detectable fecal coliform/100 mL [6, 7] •1 mg/L Cl_2 residual (min.) [8]			
Restricted	•Secondary [3] •Disinfection [5]	•≤200 fecal coliform/100 mL [6, 9, 10] •1 mg/L Cl_2 residual (min.) [8]			
Environmental Reuse Environmental Reuse	•Variable •Secondary [3] and disinfection [5] (min.)	•Site-specific and use-dependent			
Industrial Reuse Once-through Cooling	•Secondary [3]	•≤200 fecal coliform/100 mL [6, 9, 10] •1 mg/L Cl_2 residual (min.) [8]			
Recirculating Cooling Towers	•Secondary [3] •Disinfection [5] (chemical coagulation and filtration [4] may be needed)	•≤200 fecal coliform/100 mL [6, 9, 10] •1 mg/L Cl_2 residual (min.) [8]			
Groundwater Recharge – Nonpotable Reuse The use of reclaimed water to recharge aquifers which are not used as a potable drinking water source	•Site-specific and use-dependent •Primary (min.) for spreading •Secondary [3] (min.) for injection	•Site-specific and use-dependent			
Indirect Potable Reuse Groundwater Recharge by Spreading into Potable Aquifers	•Secondary [3] •Filtration [4] •Disinfection [5] •Soil aquifer treatment	•No detectable total coliform/100 mL [6, 7] •1 mg/L Cl_2 residual (min.) [8]			
Groundwater Recharge by Injection into Potable Aquifers	•Secondary [3] •Filtration [4] •Disinfection [5] •Advanced wastewater treatment [12]	•No detectable total coliform/100 mL [6, 7] •1 mg/L Cl_2 residual (min.) [8]			
Augmentation of Surface Water Supply Reservoirs	•Secondary [3] •Filtration [4] •Disinfection [5] •Advanced wastewater treatment [12]	•No detectable total coliform/100 mL [6, 7] •1 mg/L Cl_2 residual (min.) [8]			

(1) These guidelines are based on water reclamation and reuse practices in the U.S. and are specifically directed at states that have not developed their own regulations or guidelines. While the guidelines should be useful in many areas outside the U.S., local conditions may limit the applicability of the guidelines in some countries. It is explicitly stated that the direct application of these suggested guidelines will not be used by USAID as strict criteria for funding

(2) Unless otherwise noted, recommended quality limits apply to the reclaimed water at the point of discharge from the treatment facility

(3) Secondary treatment process includes activated sludge processes, trickling filters, rotating biological contractors, and may stabilization pond systems. Secondary treatment should produce effluent in which both the BOD and SS do not exceed 30 mg/l.

(4) Filtration means; the passing of wastewater through natural undisturbed soils or filter media such as sand and/or anthracite; or the passing of wastewater through microfilters or other membrane processes

(5) Disinfection means the destruction, inactivation, or removal of pathogenic microorganisms by chemical, physical, or biological means. Disinfection may be accomplished by chlorination, ozonation, other chemical disinfectants, UV, membrane processes, or other processes

(6) Unless otherwise noted, recommended coliform limits are median values determined from the bacteriological results of the last 7 days for which analyses have been completed. Either the membrane filter or fermentation tube technique may be used

(7) The number of total or fecal coliform organisms (whichever one is recommended for monitoring in the table) should not exceed 14/100 ml in any sample

(8) This recommendation applies only when chlorine is used as the primary disinfectant. The total chlorine residual should be met after a minimum actual modal contact time of at least 90 min unless a lesser contact time has been demonstrated to provide indicator organism and pathogen reduction equivalent to those suggested in these guidelines. In no case should the actual contact time be less than 30 min

(9) The number of fecal coliform organisms should not exceed 800/100 ml in any sample

(10) Some stabilization pond systems may be able to meet this coliform limit without disinfection

(11) Commercially processed food crops are those that, prior to sale to the public or others, have undergone chemical or physical processing sufficient to destroy pathogens

(12) Advanced wastewater treatment processes include chemical clarification, carbon adsorption, reverse osmosis, and other membrane processes, advanced oxidation, air stripping, ultrafiltration, and ion exchange

(13) Adapted from Lazarova, 2001; Metcalf and Eddy, 1991; Pettygrove and Asano, 1985 (EPA 2004) (Division et al. 2004)

(14) WHO microbiological quality guidelines for wastewater use in agriculture (Organization WH 2006), report of the WHO/AFESD regional consultation to review national priorities and action plans for wastewater reuse and management in the Eastern Mediterranean Region (WHO-EM/CEH/106/E) (Organization WH 2004), and FAO recommended microbiological quality guidelines for wastewater use in agriculture (Pescod 1992)

(15) (g) signifies that the standard is a guideline and the water is appropriate for public lawns, such as hotel lawns, with which the public may come into direct contact, while (m) signifies that the standard is a mandatory regulation

*This study

Table 10 Comparison of growth inhibition efficiency of nZVI particles and the other antimicrobial agents from the literature against different pathogenic bacterial strains

Antimicrobial Agent	Pathogens Bacteria	Concentrations	Growth Inhibition, %	References
Silver nanoparticles coated with polyurethane foam	Total coliform Fecal coliform *S. aureus* *Fecal Streptococcus*	1118.6 mg/L	98.5% at 48 h 100% at 48 h 99.9% at 24 h 99.9% at 24 h	Moustafa (2017)
Magnesium oxide-germanium oxide nanocomposite	*S. aureus* *E. coli*	>5 mg/mL	>95% at 16 h	Avanzato et al. (2009)
MgO nanoparticles	*E. coli*	3 mg/mL	100% at 24 h	Tang and Lv (2014)
Novel nystatin conjugated iron oxide nanocomposite - Nys-CS-MNP	*S. aureus* *E. coli* *Ps. aeruginosa*	2 mg/mL	1.312% at 24 h 35.08% at 24 h 99.09% at 24 h	Hussein-Al-Ali et al. (2014)
nZVI nanoparticles	Total coliform Fecal coliform *S. aureus* *Fecal streptococcus* *E. coli* *Ps. aeruginosa*	2–5 mg/mL	51.76% at 48 h 90.95% at 48 h 46.67% at 48 h 33.33% at 48 h 93.89% at 48 h ND	This study

2008). Table 9 presents suggested treatment processes, reclaimed water quality, and monitoring frequency for water reuses in various categories. These guidelines apply to domestic wastewater from municipal or other wastewater treatment facilities having a limited input of industrial waste. The suggested regulatory guidelines are based on microbiological quality represented by the coliforms that are reduced principally for water reclamation with highlighting the role of nZVI particles as an effective disinfectant against these indicators.

Comparison with other antimicrobial agents

The antibacterial behavior of the nZVI particles was compared with other antimicrobial agents in the literature based on the eligibility of these agents on hindering bacterial growth. As shown in Table 10, the nZVI exhibited a satisfactory growth inhibition effect towards the majority of tested bacteria, and its efficacy consistent with that for the other antimicrobial agents. Furthermore, the collected results from the literature survey provided evidence for the important considerations, which highlighted the effective factors for the microbiological treatment of municipal wastewater using various antimicrobial agents against numerous types of pathogenic bacteria. In addition to introducing the restriction mechanisms of its harmful existence in the treated water as a necessary step for the toxicity risk assessment of these pathogenic bacteria in the aquatic environment and its implications for the common health.

Conclusion

In this study, the nZVI nanoparticles were prepared via the reduction of ferric iron by a borohydride agent. The XRD analysis demonstrated that the mean crystalline dimension of the Fe^0 nanoparticle was found to be 44.43 nm with an appearance of low-intensity peaks an indication of the formation of thin oxide layers on the nZVI surface. The SEM image showed that the nZVI particles have a spherical shape and were formed in chain-like aggregates, where tend to accumulate in larger conglomerates. Also, the EDX spectrum was confirmed the chemical structure of the nZVI particles. Moreover, the thermal properties of synthesized nZVI particles were studied using the TGA technique. The nZVI particles had been shown a synergistic enhancement of the antimicrobial activity towards both Gram (+) and Gram (−) bacteria. Where, the minimum inhibitory concentration of nZVI particles was estimated by the well diffusion assay method, and was found to equivalent to 15, 10, 10, and 5 mg for the following four bacteria strain *S. aureus*, *B. subtilus*, *E. coli*, and *Ps. aeruginosa*, respectively. In general, the use of nZVI nanoparticles as a pre-treatment and disinfection technique for the municipal wastewater were helped in minimizing the associated biological health hazard for both the Gram (+) and Gram (−) bacteria, which probably highly exist in the polluted water.

Code availability Not applicable.

Author contributions Ahmed H. Sadek performed research, Conceptualization, Methodology, Software, and Writing-Original draft preparation. Mohsen S. Asker performed Data curation, Investigation, Resources, reviewed and edited the paper. Sayeda A. Abdelhamid performed analysis, Validation, Writing-Reviewing, and Editing.

Data availability All data generated or analyzed during this study are included in this manuscript.

Declarations

Ethics approval Not applicable.

Consent to participate All the authors listed have approved the manuscript.

Consent for publication All the authors listed have approved the publication.

Conflict of interest The authors declare that they have no conflict of interest.

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